Biochem. J. (1989) 257, 419–424 (Printed in Great Britain)

Analysis of kinetic data for irreversible enzyme inhibition

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Many organophosphorus compounds are irreversible inhibitors of acetylcholinesterase. The methods used in the literature to determine the inhibition kinetic constants usually involve either manual determination of the slope at various points along the inhibition progress curve or fitting polynomials to the curve. The present study investigates the use of non-linear-regression analysis to determine the various parameters. A method is suggested that yields accurate values for the inhibition constants under a range of circumstances.

INTRODUCTION

The organophosphorus compounds constitute a group of which many are of economic or military significance (Heath, 1961; Hart & O'Brien, 1974). The usefulness of these compounds as insecticides and their threat as chemical weapons arises from their ability to irreversibly inhibit acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7). The inhibition is an example of complexing competitive inhibition (Lin & Tsou, 1986) and is described as follows:

$$EH + IX \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EH \cdot IX \xrightarrow{k_{+2}} EI + HX \xrightarrow[H_2O]{k_{+3}} EH + IOH + HX \quad (1)$$

where EH is the enzyme and IX the inhibitor. Inhibition proceeds by the reversible formation of an enzyme–inhibitor complex (EH·IX) followed by formation of the enzyme–phosphorus bond with displacement of the leaving group (X). The reaction may be described by the dissociation constant ($K_d = k_{-1}/k_{+1}$), the unimolecular rate constant k_{+2} and the re-activation rate constant k_{+3}. For many inhibitors k_{+3} is much smaller than k_{+2} and the reaction is observed to be irreversible over the time course of most experimental studies. The overall inhibitory power is usually expressed as $k_i = k_{+2}/K_d$. When the inhibition is studied by adding the enzyme to a mixture of substrate and inhibitor and monitoring product (P) formation with time, the progressive inhibition curve is described by the following equations (Tsou, 1965*a*,*b*; Duggleby *et al.*, 1982):

$$v = v_0 \cdot e^{-k't} \quad (2)$$

$$[P] = v_0 \cdot (1 - e^{-k't})/k' \quad (3)$$

$$v_0 = \frac{V_{max.}[S]}{K_m\left(1 + \frac{[S]}{K_m} + \frac{[IX]}{K_d}\right)} \quad (4)$$

$$k' = \frac{k_{+2}[IX]}{K_d\left(1 + \frac{[S]}{K_m} + \frac{[IX]}{K_d}\right)} \quad (5)$$

In these equations, v_0 represents the initial velocity, v is the velocity at time t, $V_{max.}$ is the maximum velocity and K_m is the Michaelis constant, and [S] represents the substrate concentration, which is assumed to be unchanged for the duration of the experiment. No allowance for possible inhibition by accumulated product is made.

In recent years there have been many studies of the inhibitory process with a wide range of organophosphorus and carbamate inhibitors of acetylcholinesterase. There are three main methods that have been used to extract the values of k_{+2} and K_d from the experimental data, as follows.

1. Double-Reciprocal Method. This method involves determining the slope (v) of tangents to the progressive inhibition curve at various times and plotting the natural logarithm of v against time. The slope of the resulting straight line $[\Delta(\ln v)/\Delta t]$ is related to the inhibitor concentration by the following equation (Hart & O'Brien, 1974):

$$\frac{\Delta t}{\Delta(\ln v)} = \frac{K_d}{k_{+2}}\left(\frac{1}{[IX](1-\alpha)}\right) + \frac{1}{k_{+2}} \quad (6)$$

where $\alpha = [S]/(K_m + [S])$. A plot of $\Delta t/\Delta(\ln v)$ versus $1/[IX](1-\alpha)$ yields K_d as the reciprocal of the intercept on abscissa, and k_{+2} is obtained from the reciprocal of the intercept on the ordinate.

2. Apparent-Rate-Constant Method. The progressive inhibition curve may be described by rearranging eqns. (3) and (5) to give (Lin & Tsou, 1986):

$$[P] = [P]_\infty(1 - e^{-A[IX]t}) \quad (7)$$

where [P] and $[P]_\infty$ are the product concentrations at t and $t = \infty$ respectively, and A is the apparent rate constant for the formation of the inhibited enzyme, which is determined from the slope of a plot of $\ln([P]_\infty - [P])$ against time. For complexing competitive inhibitors (Tsou, 1965*a*,*b*):

$$A = \frac{k_{+2}K_a}{1 + \frac{[S]}{K_m} + K_a[IX]} \quad (8)$$

where K_a is the inhibitor association constant ($1/K_d$). Therefore a plot of $1/A[IX]$ versus $1/[IX]$ enables the determination of k_{+2} as the reciprocal of the intercept on the ordinate and K_a from the slope $\{(1/K_a k_{+2})(1 + [S]/K_m)\}$.

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3. Zero-Time Method. In contrast with the previous two methods, this procedure allows calculation of both K_d and k_{+2} from a single progress curve, in conjunction with a control curve (Hart & O'Brien, 1973; Horton *et al.*, 1977). First K_d is determined from the velocity of a control reaction in the absence of inhibitor (v_c) and the rate at $t = 0$ in the presence of inhibitor (v_0), by using the equation:

$$K_d = \frac{K_m[IX]}{(K_m + [S])\left(\frac{v_c}{v_0} - 1\right)} \quad (9)$$

Then k_{+2} may be calculated from either of the following two equations:

$$k_{+2} = \frac{\Delta(\ln v)}{\Delta t}\left(\frac{K_d}{[IX](1-\alpha)} + 1\right) \quad (10)$$

or

$$k_{+2} = \frac{\Delta(\ln v)}{\Delta t}\left(\frac{v_c}{v_c - v_0}\right) \quad (11)$$

The Zero-Time Method offers the advantage of allowing the determination of K_d and k_{+2} from a single kinetic experiment rather than requiring measurements to be made at a number of inhibitor concentrations. However, the nature of the expressions $v_c/v_0 - 1$ and $v_c/(v_c - v_0)$ makes the calculations prone to error. For example, Horton *et al.* (1977) obtained values of 1.86×10^{-3} A unit/s for v_c and 1.59×10^{-3} *A* unit/s for v_0 for the inhibition of acetylcholinesterase by carbaryl. If v_0 were underestimated by 5%, this would result in an error of 27% in $v_c/v_0 - 1$.

Both the Zero-Time Method and the Double-Reciprocal Method require the determination of $\Delta(\ln v)/\Delta t$. Hart & O'Brien (1973) and Forsberg & Puu (1984) used tangents drawn by hand to calculate v. This is an inaccurate method of obtaining the reaction velocity. Horton *et al.* (1977) used polynomial fitting to obtain the velocities. However, although this method is less subjective than drawing tangents by hand, polynomial fitting is also subject to error, especially at $t = 0$, where the polynomial is ill-defined (Cornish-Bowden, 1975). After obtaining the value of v, a secondary plot of $\ln v$ versus t is required to obtain $\Delta(\ln v)/\Delta t$ and subsequent calculations or plots to obtain k_{+2} and K_d (Hart & O'Brien, 1973, 1974; Horton *et al.*, 1977; Forsberg & Puu, 1984).

The Apparent-Rate-Constant Method also requires two manipulations of the data to obtain the inhibition constants (Lin & Tsou, 1986). A plot of $\ln([P]_\infty - [P])$ versus t gives a straight line with a slope of $A[IX]$. A secondary plot of $1/A[X]$ versus $1/[IX]$ yields K_d and k_{+2}. The method, as used by Lin & Tsou (1986), imposes the inconvenience of having to allow the reaction to proceed until $[P]_\infty$ can be determined accurately.

The three methods described above all suffer from some deficiencies. The double handling of the data is time-consuming and may introduce errors. Several of the formulae are error-prone, as are the use of double-reciprocal plots and polynomial fitting (Cornish-Bowden, 1975, 1976). Further, Brooks & Suelter (1986) have described graphical analysis as giving a false impression of the accuracy of the data, providing no information about the precision of the estimated parameters and allowing bias in the weighting of data points.

It is important, especially if results from different laboratories are to be compared (Horton *et al.*, 1977; Forsberg & Puu, 1984; Gray & Dawson, 1987), that the most reliable method be used. The availability of cheap computers and reliable software may provide such a method.

In the present paper the method of non-linear-regression analysis is applied to the determination of K_d and k_{+2} from data for irreversible enzyme inhibition. The three methods described above and an additional method that enables the calculation of K_d and k_{+2} directly from the data are compared. The results suggest an optimal method for the determination of those parameters.

METHODS

Generation of progress curves

Theoretical progressive inhibition curves were calculated by using eqns. (7) and (8). $[P]_\infty$ was calculated as follows (Tsou, 1965*a*,*b*; Tian & Tsou, 1982):

$$[P]_\infty = \frac{V_{max.}[S]K_d}{k_{+2}[IX]K_m} \quad (12)$$

The value of [P] was determined at equally spaced values of t. The total reaction time was the time required for the reaction to reach a specified degree of completion (fraction of $[P]_\infty$). The true values of A and $\Delta(\ln v)/\Delta t$ were calculated by using the appropriate equations.

Simulated experimental errors proportional to the value of [P] were incorporated by multiplication by a number chosen at random from a normal distribution with a mean of 1 and a specified standard deviation. The random numbers were generated by computer using a routine from the NAG library based on the algorithm of Brent (1974). Ten replicates were calculated for each condition examined.

A family of progress curves was calculated by using the inhibition constants determined by Forsberg & Puu (1984) for soman. The inhibitor concentration ranged from $0.01\,K_d$ to $1.0\,K_d$. $V_{max.}$ (2.5×10^{-6} M·s^{-1}) was chosen so that when the inhibition reaction was allowed to proceed to 95% completion with the lowest inhibitor concentration no more than 10% of the substrate was hydrolysed. The K_m for the substrate *p*-nitrophenyl acetate (4.52×10^{-3} M) was that determined by Horton *et al.* (1977) and the substrate concentration (1 mM) is the concentration commonly used (Horton *et al.*, 1977; Forsberg & Puu, 1984).

Product concentrations were determined at equally spaced times and rounded to three significant places. The following assumptions were made: (1) the independent variable (time) was error-free; (2) the residual rate at infinite time was zero; (3) the product concentration at $t = 0$ was zero.

Analysis of inhibition data

Analysis of the data was performed by using the non-linear-regression program of Duggleby (1984). This is a general non-linear-regression program capable of fitting any equation to a set of experimental data. The program is based on a modification of Marquardt's (1963) algorithm, and is written in BASIC. A copy of the program and a user's manual are available on request from R.G.D.

The apparent rate constant (A) was determined by fitting eqn. (7) to the data, and v_0 and $\Delta(\ln v)/\Delta t$ were determined by fitting eqn. (3). $\Delta(\ln v)/\Delta t$ is equivalent to k'. K_d and k_{+2} were then calculated by using the appropriate equations described in the Introduction.

In addition to these methods, two additional procedures were used, as follows.

Direct Method 1. This method involved directly fitting eqns. (7) and (8) to each individual progress curve. In this way the values of K_d and k_{+2} were determined without any secondary calculations or plots. There was one independent variable (time) and one dependent variable ([P]).

Direct Method 2. Alternatively, eqns. (7) and (8) may be fitted to a family of progress curves simultaneously. In this case two independent variables (time and [IX]) and one dependent variable ([P]) were used.

The results are described in terms of their 'accuracy' and 'variability'. Accuracy is measured by the difference between the true value of a parameter and the mean value determined from the simulations. Variability is expressed as the standard deviation.

RESULTS

Fig. 1 shows the family of progress curves used in the study. For the purpose of illustration, the curves are truncated at the time for which the inhibition reaction for an inhibitor concentration of $1.00K_d$ is 95% complete, although the actual data used covered 95% of the reaction for all inhibitor concentrations.

Preliminary studies were carried out with perfect data rounded to three significant places. In order to avoid the errors inherent in manual manipulation of the data and polynomial curve-fitting, the parameters required for the Double-Reciprocal Method, the Apparent-Rate-Constant Method and the Zero-Time Method (k', v_0, A and $[P]_\infty$) were determined by non-linear-regression analysis. K_d and k_{+2} were determined by Direct Method 1. With the use of 50 data points, the differences between the true and calculated values of k', v_0, A and $[P]_\infty$ were very small

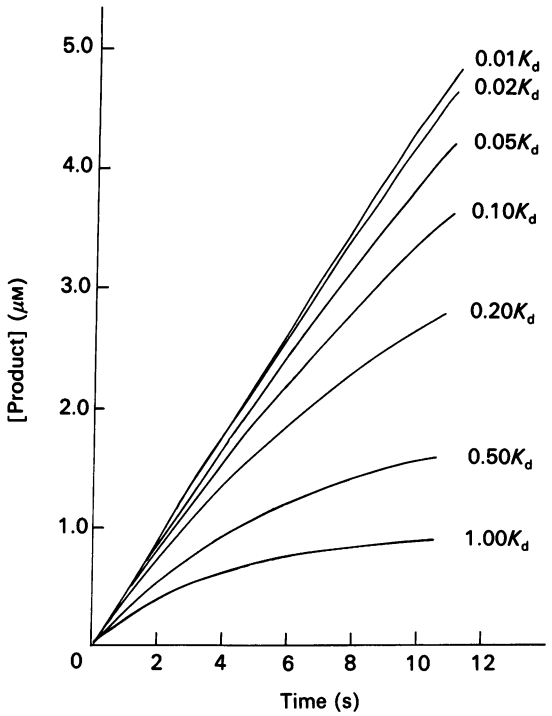

Fig. 1. Theoretical progress curves for the inhibition of acetylcholinesterase by soman

$K_d = 6.1 \times 10^{-7}$ M; $k_{+2} = 0.57\ s^{-1}$. The inhibitor concentration ranged from $0.01K_d$ to $1.0K_d$.

(< 0.5%) at all the inhibitor concentrations shown in Fig. 1. In contrast, the differences between the true and calculated values of K_d and k_{+2} calculated by Direct Method 1 increased rapidly from < 0.1% at $1.0K_d$ to 13.7% and 12.1% respectively at $0.02K_d$. At $0.01K_d$ the software failed to converge to a solution. Inhibitor concentrations of $0.1K_d$ or above were required for the differences to fall below 1%.

Simulated experimental errors with a standard

Table 1. Effects of added error and degree of completion of the reaction on the parameters

k' and v_0 were determined by fitting eqn. (3) to the data, and A and $[P]_\infty$ by fitting eqn. (7). k_{+2} and K_d were determined by Direct Method 1. The results are shown as the means and standard deviations of ten replicates with obvious outliers ($k_{+2} > 1.2$) removed. The asterisks (*) indicate those conditions under which outliers occurred. The inhibitor concentration was 0.061 μM and 50 data points were collected.

Percentage reaction	Error	$10^2 \times k'$ (s^{-1})	$10^7 \times v_0$ ($M \cdot s^{-1}$)	$10^{-5} \times A$ ($M^{-1} \cdot s^{-1}$)	$[P]_\infty$ (μM)	k_{+2} (s^{-1})	$10^7 \times K_d$ (M)
95	0	4.31 (< 0.01)	4.19 (< 0.01)	7.07 (< 0.01)	9.71 (< 0.01)	0.58 (< 0.01)	6.15 (0.02)
	1.0	4.31 (0.04)	4.18 (0.02)	7.07 (0.07)	9.70 (0.05)	0.57 (0.04)	6.11 (0.40)
	2.5	4.31 (0.11)	4.18 (0.05)	7.06 (0.17)	9.70 (0.12)	0.57 (0.12)	6.12 (1.20)
	5.0	4.30 (0.22)	4.16 (0.11)	7.06 (0.36)	9.67 (0.35)	0.51 (0.11)*	5.41 (1.09)*
75	0	4.31 (< 0.01)	4.18 (< 0.01)	7.06 (0.01)	9.71 (0.01)	0.57 (< 0.01)	6.13 (0.05)
	1.0	4.31 (0.07)	4.18 (0.02)	7.06 (0.11)	9.71 (0.11)	0.57 (0.04)	6.10 (0.37)
	2.5	4.30 (0.16)	4.18 (0.05)	7.06 (0.26)	9.71 (0.26)	0.57 (0.11)	6.08 (0.98)
	5.0	4.30 (0.34)	4.16 (0.10)	7.05 (0.55)	9.71 (0.54)	0.51 (0.12)*	5.45 (1.07)*
50	0	4.32 (0.01)	4.19 (< 0.01)	7.07 (0.02)	9.70 (0.02)	0.58 (0.01)	6.18 (0.06)
	1.0	4.30 (0.11)	4.19 (0.02)	7.04 (0.18)	9.74 (0.21)	0.57 (0.04)	6.09 (0.34)
	2.5	4.26 (0.28)	4.18 (0.04)	7.04 (0.46)	9.75 (0.53)	0.57 (0.12)	6.06 (0.91)
	5.0	4.29 (0.58)	4.16 (0.09)	7.03 (0.96)	9.83 (1.12)	0.50 (0.13)*	5.47 (1.01)*
True value		4.31	4.19	7.07	9.70	0.57	6.10

deviation of 1%, 2% or 5% were incorporated into the data as described in the Methods section. An inhibitor concentration of $0.1K_d$ (0.061 μM) was used since this was the lowest concentration for which Direct Method 1 gave acceptable results with error-free data.

In Table 1 is shown the influence of both random errors and the degree of completion of the reaction on the accuracy and variability in the determination of the parameters. The results calculated for data with no added error are shown with the standard errors in parentheses. The other results are shown as the means and standard deviations of ten replicates. As expected, increasing the simulated experimental error decreased the accuracy and increased the variability of the calculated results. This was reflected as an increase in the deviation of the calculated value from the true value and an increase in the standard deviation. However, k_{+2} and K_d were determined with lower accuracy and higher variability than the other parameters. Irrespective of the degree of completion of the reaction or the magnitude of the stimulated error, the difference between the true and calculated values of k', v_0, A and $[P]_\infty$ remained quite small (< 1% with two exceptions). In contrast, the differences between the true and calculated values of k_{+2} and K_d rose rapidly to greater than 10% with a simulated error of 5% and the standard deviations exceeded 20%. With this simulated error, one particular data set yielded an obvious outlier ($k_{+2} > 1.2$) at all degrees of completion of the reaction. These values were omitted from the calculation of the mean and standard deviation. As for the other parameters, variation in the degree of completion of the reaction between 50% and 95% did not affect greatly the accuracy of the final result.

In Table 2 are shown the effects of variation in the number of data points and the inhibitor concentration on the accuracy and variability of the results. A simulated error with a standard deviation of 2.5% was used. The reaction was 95% complete. Mean values for k', v_0, A and $[P]_\infty$ remained close to the true values even if the number of data points was decreased from 50 to ten. With ten data points the difference between the true and calculated values increased to about 1% with a concomitant increase in the standard deviation from ~2% to ~4%. Both the accuracy and variability were independent of the inhibitor concentration. However, there was an inhibitor-concentration-dependent decrease in accuracy and increase in variability in the values of k_{+2} and K_d as the number of data points was lowered from 50 to 10. At the lowest concentration, the differences between the true and calculated values of k_{+2} and K_d increased from < 0.1% to 14.0% and 12.8% respectively. As the inhibitor concentration was raised to $0.5K_d$ both these differences and the standard deviation decreased.

The data so far indicate that the best results were obtained for the direct calculation of k_{+2} and K_d if the inhibitor concentration was greater than $0.1K_d$, that the reaction was allowed to proceed to over 50% completion and at least 50 data points were collected. With these conditions, inhibition curves were calculated at five equally spaced concentrations (on the 1/[IX] scale) of inhibitor between $0.1K_d$ and $0.5K_d$ and the values of k_{+2} and K_d were determined by each of the procedures described in the Methods section. The range of concentrations is approximately that used by others (Forsberg & Puu, 1984). Simulated experimental errors with

Table 2. Effects of inhibitor concentration and number of data points on the parameters

The reaction was 95% complete and 2.5% error was incorporated. The asterisks (*) indicate those conditions for which two convergence failures occurred. The inhibitor concentration is expressed as a fraction of the K_d. The other details are as for Table 1.

Concn. of inhibitor	No. of data points	$10^2 \times k'$ (s^{-1})		$10^7 \times v_0$ ($M \cdot s^{-1}$)		$10^{-5} \times A$ ($M^{-1} \cdot s^{-1}$)		$[P]_\infty$ (μM)		k_{+2} (s^{-1})		$10^7 \times K_d$ (M)	
		True	Calc.	True	Calc.	True	Calc.	True	Calc.	True	Calc.	True	Calc.
$0.1K_d$	10	4.31	4.26 (0.18)	4.19	4.17 (0.10)	7.07	6.99 (0.30)	9.70	9.79 (0.22)	0.57	0.49 (0.08)*	6.10	5.32 (0.76)*
	25		4.36 (0.14)		4.19 (0.08)		7.11 (0.24)		9.67 (0.15)		0.63 (0.20)		6.69 (1.92)
	50		4.31 (0.11)		4.18 (0.05)		7.06 (0.17)		9.70 (0.12)		0.57 (0.12)		6.12 (1.20)
$0.2K_d$	10	8.02	7.93 (0.35)	3.89	3.88 (0.09)	6.57	6.50 (0.28)	4.85	4.90 (0.11)	0.57	0.57 (0.12)	6.10	6.14 (1.13)
	25		8.07 (0.27)		3.90 (0.08)		6.62 (0.22)		4.84 (0.08)		0.59 (0.09)		6.32 (0.91)
	50		8.01 (0.19)		3.88 (0.05)		6.57 (0.16)		4.85 (0.06)		0.57 (0.06)		6.09 (0.57)
$0.5K_d$	10	16.6	16.4 (0.01)	3.21	3.20 (0.08)	5.43	5.36 (0.23)	1.94	1.96 (0.04)	0.57	0.56 (0.06)	6.10	6.05 (0.05)
	25		16.7 (0.01)		3.22 (0.06)		5.46 (0.18)		1.93 (0.03)		0.58 (0.05)		6.17 (0.42)
	50		16.5 (0.01)		3.20 (0.04)		5.42 (0.13)		1.94 (0.03)		0.57 (0.03)		6.07 (0.03)

Table 3.

K_d and k_{+2} were determined by each method. The reaction was 95% complete, 50 data points were collected and 2.5% error was incorporated. The control velocity, v_c, for the Zero-Time Method was determined from ten replicates and was found to be 4.41 ($\pm$0.016) $\times 10^{-7}$ M·s^{-1}. The true value was 4.52×10^{-7} M·s^{-1}.

Concn. of inhibitor	Zero-Time Method		Direct Method 1	
	k_{+2} (s^{-1})	$10^7 \times K_d$ (M)	k_2 (s^{-1})	$10^7 \times K_d$ (M)
$0.1K_d$	0.63 (0.08)	6.80 (0.85)	0.57 (0.12)	6.13 (1.20)
$0.125K_d$	0.62 (0.07)	6.71 (0.67)	0.57 (0.09)	6.13 (0.92)
$0.167K_d$	0.62 (0.09)	6.73 (0.86)	0.56 (0.07)	6.08 (0.70)
$0.250K_d$	0.61 (0.06)	6.54 (0.55)	0.57 (0.05)	6.09 (0.48)
$0.5K_d$	0.58 (0.03)	6.33 (0.29)	0.57 (0.03)	6.07 (0.27)
Double-Reciprocal Method			0.56 (<0.01)	6.04 (0.05)
Apparent-Rate-Constant Method			0.57 (< 0.01)	6.08 (0.02)
Direct Method 2			0.58 (< 0.01)	6.17 (0.03)
True value			0.57	6.10

a standard deviation of 2.5% were incorporated into the data and the results are shown in Table 3. For the Zero-Time Method the differences between the true and calculated values of k_{+2} and K_d fell from 10.5% to 1.8% and from 11.5% to 3.8% respectively as the inhibitor concentration was increased from $0.1K_d$ to $0.5K_d$. The standard deviation fell in a similar manner. For Direct Method 1 the errors in the mean were much lower at each concentration and did not change appreciably as the concentration increased. However, below $0.167K_d$ the standard deviations were much higher. The standard deviations also fell as the inhibitor concentration increased.

Both the errors in the mean and the standard errors, shown in parentheses, of k_{+2} and K_d determined by the Apparent-Rate-Constant Method, Double-Reciprocal Method and Direct Method 2 procedures were approx. 1% or less. Direct Method 2 produced similar results with ten, 25 or 50 data points and 50%, 75% or 95% completion of the reaction (results not shown).

DISCUSSION

The results show that, by using non-linear-regression analysis, k', A, $[P]_\infty$ and v_0 can be calculated very accurately from progressive inhibition curves that contain a substantial degree of error. Furthermore, these parameters can be calculated accurately for a broad range of inhibitor concentrations, with as few as ten data points and with as little as 50% of the inhibition reaction completed. This method should obviate the need to draw tangents by hand or to wait until the reaction is almost complete to obtain accurate values for $[P]_\infty$.

The standard deviation of the stimulated experimental error chosen for the final comparison of methods was 2.5%, i.e. 99.7% of the values fell within $\pm$7.5% of the theoretical progress curves. Although this error is higher than we have observed experimentally, it was chosen to provide a reasonably rigorous test of the calculation methods.

The first comparison to be made is that between the Zero-Time Method and Direct Method 1. Although the parameters used in the Zero-Time Method (v_0, v_c and k') were calculated with high accuracy, the nature of the expressions used to calculate k_{+2} and K_d decreased the accuracy. For example, at $0.1K_d$, although the parameters were determined to within > 99.0% of the true value, the difference between the true and calculated values of k_{+2} and K_d was greater than 10%. The Zero-Time Method results were worse than those determined by Direct method 1 at all inhibitor concentrations used. In addition, the Zero-Time Method required two manipulations of the data. This procedure, then, was the less useful of the methods.

The Apparent-Rate-Constant Method, Double-Reciprocal Method and Direct Method 2 all provided accurate estimates of k_{+2} and K_d with substantially less variability than Direct Method 1. This occurred under conditions optimized for Direct Method 1. Both the Apparent-Rate-Constant Method and the Double-Reciprocal Method required double handling of the data. The extra time required makes these methods less useful than Direct Method 2.

Direct Method 2 therefore produced the best estimates of k_{+2} and K_d, required the least manipulation of the data and performed well with as few as ten data points and as little as 50% of the reaction complete.

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Received 26 May 1988/2 August 1988; accepted 5 August 1988