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Steady-State Enzyme Kinetics with High-Affinity Substrates or Inhibitors

A STATISTICAL TREATMENT OF DOSE–RESPONSE CURVES

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A statistical treatment of steady-state enzyme kinetic measurements is described that allows for depletion of free substrate or free inhibitor concentrations owing to significant binding to the enzyme. $V_{max.}$, K_m or K_i, enzyme concentration, the concentration of substrate or inhibitor required for a half-maximal effect and standard errors of these parameters can be calculated from dose–response measurements; the concentration of each component of the system may be estimated also. The statistically best values of the parameters are used to convert dose–response curves into convenient linear forms. The method is applied to dose–response measurements of hydroxyquinoline *N*-oxide inhibition of bacterial respiration and aminopterin inhibition of dihydrofolate reductase. Two FORTRAN programs for this method have been deposited as Supplementary Publication no. SUP 50019 at the National Lending Library for Science and Technology, Boston Spa, Yorks. LS23 7BQ, U.K., from whom copies may be obtained on the terms indicated in *Biochem. J.* (1973) **131**, 5.

In measurements of enzyme steady-state reaction velocities, it is normally assumed that formation of enzyme–substrate or enzyme–inhibitor complexes does not diminish significantly the concentration of substrate or inhibitor in solution (Dixon & Webb, 1964; Webb, 1963). However, it is recognized that the relatively high concentration of some enzymes *in vivo* (Srere, 1967; Sols & Marco, 1970), or a high affinity of an enzyme for a substrate or inhibitor (Goldstein, 1944; Morrison, 1969; Cha, 1970), may lead to binding of a significant proportion of substrate or inhibitor molecules to the enzyme. The effect is significant when the ratio E_t*$/K_m$ or E_t/K_i is greater than 0.01 (Goldstein, 1944; Webb, 1963; Henderson, 1972); as the ratio increases over 0.01, so an analysis based on the Michaelis–Menten equation becomes increasingly invalid (Morrison, 1969; Cha, 1970; Khoo & Russell, 1970).

Equations have been developed that allow for such 'tight binding' in relating steady-state velocity to the concentrations of enzyme, substrate and inhibitor (Goldstein, 1944; Krupka & Laidler, 1959; Morrison, 1969; Henderson, 1972; Dixon, 1972). However, a statistical method of calculating the most-probable values of the parameters ($V_{max.}$, K_m, K_i, E_t) from experimental results has not been described, probably because the parameters are grouped in a non-linear form intractable to the normal methods of least-squares analysis. The present paper describes the use of linear forms of two such equations to analyse dose–response measurements. The statistical method is based on that of Hoare (1972) and requires a small computer. Two FORTRAN programs have been developed and tested, and copies deposited as Supplementary Publication no. SUP 50019 with the National Lending Library for Science and Technology, Boston Spa, Yorks. LS23 7BQ, U.K., or they may be obtained from the author. The first program analyses substrate binding and requires an estimate of $V_{max.}$ and a set of velocities measured at different substrate concentrations; by using the method described below, the program calculates and prints out $V_{max.}$, E_t, K_m, $S_{0.5}$ and standard errors of the values. The second program analyses inhibitor binding, and requires a series of velocity values at different inhibitor concentrations, including at least one velocity measured without inhibitor; by a similar procedure, the program calculates v_0, E_t, $K_{i,app.}$, $I_{0.5}$ and standard errors. Each program also computes the proportion of substrate or inhibitor bound to the enzyme at each of the experimental concentrations.

* Abbreviations: E_t = total concentration of enzyme; S_t = total concentration of substrate; I_t = total concentration of inhibitor; v_i = velocity in the presence of inhibitor; v_0 = velocity obtained at zero inhibitor concentration; $I_{0.5}$ = inhibitor concentration at which $v_i = v_0/2$; $S_{0.5}$ = substrate concentration at which $v = V_{max.}/2$; K_i = dissociation constant for inhibitor; $K_{i,app.}$ = apparent dissociation constant for inhibitor; K_m = Michaelis constant for substrate; D = denominator of enzyme rate equation; N_i = term in the denominator representing the enzyme form that combines with an inhibitor; $V_{max.}$ = enzyme reaction velocity when substrate is saturating.

Theory

Assumptions

The following assumptions operate in the statistical treatment of kinetic data when binding to the enzyme does not significantly decrease the concentration of free ligand (Cleland, 1967), and apply to the following treatment also. Reaction velocities are measured in the steady state; velocities are subject to errors of experimental measurement, but the total concentration of substrate or inhibitor is known exactly; only one molecule of substrate or inhibitor interacts with a single site on the enzyme; and there are no co-operative effects between different enzyme sites. Inhibitors are assumed to bind reversibly, and to be of the 'dead-end' type (Cleland, 1963). Reaction velocities should be measured over a range of substrate or inhibitor concentrations to obtain a 'dose–response' curve.

Calculation of parameters

Enzyme reaction velocities very often follow the Michaelis–Menten relationship (1) when depletion of S_t from solution is insignificant (Cleland, 1970):

$$v = \frac{V_{max.}\,S_t}{S_t + K_m} \tag{1}$$

Under circumstances where binding becomes appreciable eqn. (1) is modified to eqn. (2):

$$v = \frac{V_{max.}}{2E_t}\{E_t + K_m + S_t - \sqrt{[(E_t + K_m + S_t)^2 - 4S_t E_t]}\} \tag{2}$$

(Goldstein, 1944; Reiner, 1969; Cha, 1970). Eqn. (2) can be rearranged to the quadratic form and further rearranged to eqn. (3):

$$S_t\frac{V_{max.}}{v} = K_m\frac{V_{max.}}{V_{max.} - v} + E_t \tag{3}$$

Morrison (1969) and Henderson (1972) have derived the analogous equations, (4) and (5), for the case when an inhibitor is tightly bound:

$$v = \frac{v_0}{2E_t}\left\{E_t - \frac{D}{\sum\frac{N_i}{K_i}} - I_t + \sqrt{\left[\left(I_t + \frac{D}{\sum\frac{N_i}{K_i}} - E_t\right)^2 + 4\frac{D}{\sum\frac{N_i}{K_i}}E_t\right]}\right\} \tag{4}$$

$$I_t\frac{v_0}{v_0 - v_i} = \frac{D}{\sum\frac{N_i}{K_i}}\frac{v_0}{v_i} + E_t \tag{5}$$

$D\Big/\sum\frac{N_i}{K_i}$ (Henderson, 1972) may be treated as a single parameter, $K_{i,app.}$, which will be discussed in more detail below.

It can be seen that eqns. (2) and (3) contain the parameters $V_{max.}$, K_m and E_t, and eqns. (4) and (5) contain v_0, $K_{i,app.}$ and E_t. If a value for $V_{max.}$ is available, then a plot of S_t $V_{max.}/v$ against $V_{max.}$ $(V_{max.} - v)$ [or $1/(1 - v/V_{max.})$] is linear and yields K_m from the slope and E_t from the intercept (Fig. 1*a*); similarly, if v_0 is known, $K_{i,app.}$ and E_t may be obtained from plots of I_t $v_0/(v_0 - v_i)$ [or $I_t/(1 - v_i/v_0)$] against v_0/v_i (Fig. 1*b*). Two problems arise when this is attempted. First, the value of $V_{max.}$ is subject to uncertainty, because it is normally obtained by extrapolation to infinite substrate concentration; also, v_0 is not necessarily the correct value because it is subject to the errors of experimental measurements; use of incorrect values of $V_{max.}$ or v_0 leads to non-linear plots and erroneous calculations of slopes and intercepts as illustrated in Figs. 1(*a*) and 1(*b*). Secondly, the x and y errors of such plots are not independent; hence a least-squares estimation of the slope and intercept is complicated by the difficulty of assigning a correct weighting factor to each point (Johansen & Lumry, 1961). Fitting data to the original equations (2) or (4), would eliminate the weighting problem, but the parameters are in a complex non-linear form not susceptible to the normal methods of least-squares analysis. Hoare (1972) has described how the availability of a linear form of such complex equations allows the generation of sets of values of the parameters; the least-squares criterion may be applied to find the 'most-probable' set of parameter values, i.e. the set that gives the best fit to the experimental measurements (Hoare, 1972). The following iterative procedure is based on this method.

It is apparent that eqn. (3) can be divided through by $V_{max.}$, and eqn. (5) by v_0, to obtain the simpler linear forms eqns. (6) and (7):

$$\frac{S_t}{v} = K_m\frac{1}{V_{max.} - v} + \frac{E_t}{V_{max.}} \tag{6}$$

$$\frac{I_t}{v_0 - v_i} = K_{i,app.}\frac{1}{v_i} + \frac{E_t}{v_0} \tag{7}$$

Employment of eqns. (6) and (7), rather than eqns. (3) and (5), in the programs economizes on calculation time. An estimate of the correct value of $V_{max.}$ is made by extrapolation from the velocity at the highest S_t used, or an estimate of the correct v_0 by experimental measurement. The value is supplied to the appropriate program, which generates ten more values ranging ±20% of the estimate; the range may be widened or narrowed according to the reliability of the initial estimate. All the pairs of experimental measurements

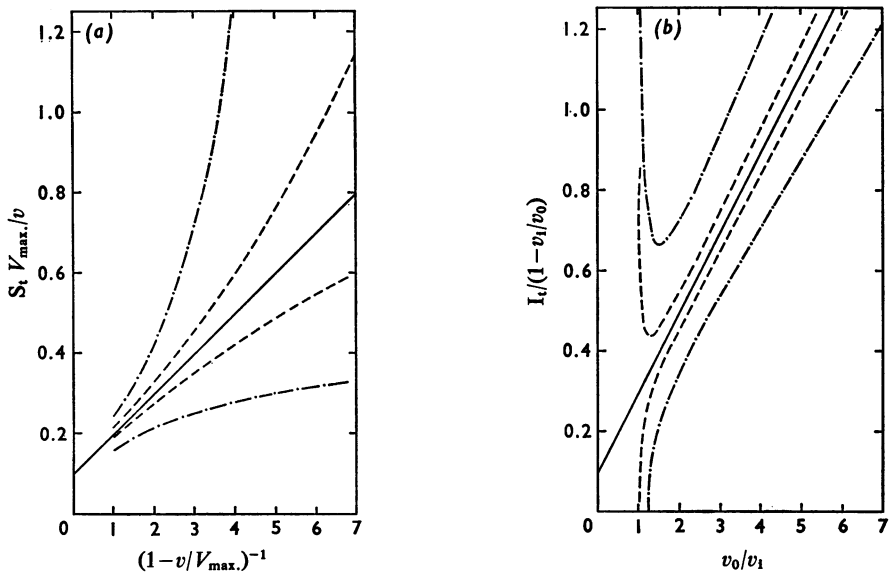

Fig. 1. *Effects of an incorrect $V_{max.}$ or v_0 value on linear replots*

(*a*) Eqn. (2) was used to calculate velocities for a range of S_t values from 0.025 to 1.000 for $E_t = 0.1$, $K_m = 0.1$ and $V_{max.} = 100.0$. (*b*) Velocities were calculated with eqn. (4) for a range of I_t values from 0.02 to 0.80 for $E_t = 0.1$, $K_{i,app.} = 0.2$ and $v_0 = 50.0$. The transformed variables shown were calculated for the correct values of $V_{max.}$ and v_0 (——), for values of $V_{max.}$ and v_0 in error by ±20% (—·—·) and by ±5% (----). If the estimate of $V_{max.}$ is lower than the correct value, the curve is below the correct line (*a*); if the estimate of v_0 is low, the curve is above the correct line (*b*).

(v, S_t) or (v_i, I_t) are then read into the program. For each of the 11 values of $V_{max.}$ the first program constructs a plot of S_t/v against $1/(V_{max.}-v)$, examples of which are shown in Fig. 2; similarly the second program constructs a plot of $I_t/(v_0-v_i)$ against $1/v_i$ for each v_0 value. Although each plot only approximates to a straight line, a normal least-squares calculation of the slope (K_m or $K_{i,app.}$) and intercept ($E_t/V_{max.}$ or E_t/v_0) is carried out. Thus for each value of $V_{max.}$ corresponding values of K_m and E_t are found; similarly, sets of parameter values (v_0, $K_{i,app.}$, E_t) are generated for inhibition plots. By using the non-linear eqn. (2), a parameter set ($V_{max.}$, K_m, E_t) may be used to calculate a reaction velocity at each substrate concentration. That is, for one parameter set a series of predicted velocity values are obtained, each one corresponding to an experimentally measured velocity. The sum of the squares of the differences between the predicted and observed values (SS) may now be calculated, as suggested by Hoare (1972):

$$SS = \sum_{j=1}^{n} w_j [v_j(\text{predicted}) - v_j(\text{observed})]^2 \quad (8)$$

w_j is the weighting factor that may be assigned to each point. The parameter set that yields the minimum value of SS contains the most-probable value of each parameter (Hoare, 1972). An almost identical procedure is followed for sets of inhibition parameters (v_0, $K_{i,app.}$, E_t), eqn. (4) being used to generate predicted velocities. Since eqns. (2) and (4) describe simple dose–response curves, an appropriate weighting factor may be assigned to each point exactly as described for the analysis of normal steady-state kinetic measurements (Cleland, 1967). Thus for the common case where the absolute error of a measured velocity is constant throughout the range of values, w_j is 1.0 (Cleland, 1967). The best value of $V_{max.}$ or v_0 now replaces the initial estimate, and a new series of $V_{max.}$ or v_0 values either side of the new estimate are generated; these can cover a much narrower range of values than used in the first calculation. The set of parameters giving the minimum value of SS within the narrower range is calculated, and the process repeated until the calculated best parameter values cease to change by a significant amount. It is important to note that the least-squares criterion is being obtained from the original non-linear forms of the equations rather than from one of the linear transformations.

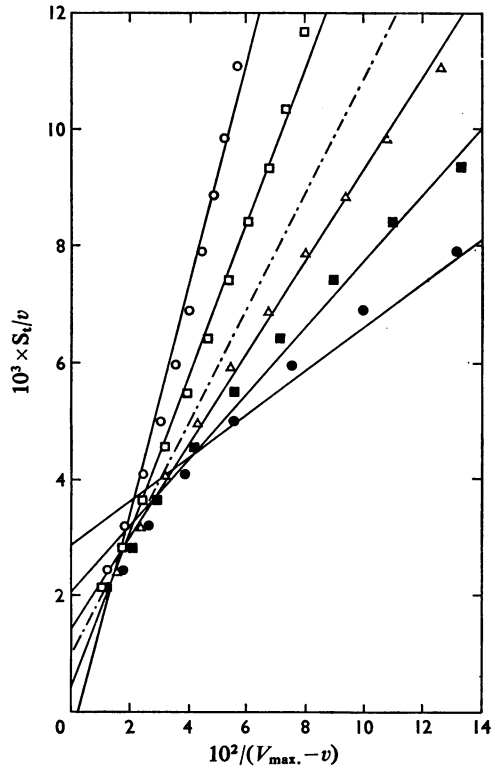

Fig. 2. *Computer-generated approximations to fit dose–response measurements*

The substrate-binding program analysed pairs of (v, S_t) values that fit eqn. (2) exactly when $E_t = 0.1$, $K_m = 0.1$ and $V_{max.} = 100.0$. An initial estimate of $V_{max.} = 98.0$ was also provided, and the program selected $V_{max.}$ values of 107.8 (○), 102.9 (□), 98.0 (△), 95.6 (■) and 88.2 (●) to generate the linear plots shown. In this case the line with a minimum SS value is △, and the program converged successfully on the correct line (—·—·) in a further four iterations.

Estimation of error limits

Once the minimum SS value is established, the values of SS at 75%, 95% and 99% confidence levels are calculated from eqn. (9) (Hoare, 1972):

$$SS_{(x\%)} = SS_{(minimum)}\left(1+\frac{p}{n-p}\cdot F(p, n-p, x\%)\right) \quad (9)$$

where $x\%$ = confidence level, p = number of constants to be evaluated (three in this case), n = number of data points, and $F(p, n-p, x\%)$ is derived from the F test for equality of variance; appropriate values of F for 4–100 data points are stored within the program. It should be noted that eqn. (9) obtains an approximate confidence level for non-linear equations (Draper & Smith, 1966). The iterative generation of the parameter sets is then repeated, but each iteration is made to converge towards the value of $SS_{(x\%)}$ rather than $SS_{(minimum)}$. Values of parameters in the set that yields the exact value of $SS_{(x\%)}$ represent the standard error limits. As predicted by Hoare (1972), the limits are not necessarily symmetrical about the best value of a calculated parameter. If required, the program will print out values of SS corresponding to a series of values of one parameter; by plotting one against the other, the extent of the assymetry may be easily visualized (Hoare, 1972).

Conversion of dose–response curves into linear forms

Plots of v versus S_t or v_t versus I_t are normally curved, although they tend to linearity when binding is very tight (Morrison, 1969; Henderson, 1972). In computing $V_{max.}$ or v_0 the programs also obtain the best straight lines of S_t $V_{max.}/v$ versus $V_{max.}/(V_{max.}-v)$ or I_t $v_0/(v_0-v_i)$ versus v_0/v_i replots. Such transformations afford the same convenience as the Lineweaver–Burk transformation of v versus S_t curves to $1/v$ versus $1/S_t$ straight lines; the goodness of fit is easily apparent and the slope and intercepts have useful physical significance. Accordingly both programs print out experimental points and best fits of linear replots; Fig. 3 shows two examples, which will be discussed further below.

Calculation of bound and free ligand

Eqns. (3) and (5) may be rearranged to give the following relationships (Goldstein, 1944; Henderson, 1972):

$$S\ (bound) = E_t\frac{v}{V_{max.}} \quad (10)$$

$$S\ (free) = K_m\frac{v}{V_{max.}-v} \quad (11)$$

$$I\ (bound) = E_t\frac{v_0-v_i}{v_0} \quad (12)$$

$$I\ (free) = K_{i,app.}\frac{v_0-v_i}{v_i} \quad (13)$$

For each S_t or I_t concentration, the program uses the best values of ($V_{max.}$, K_m, E_t) or (v_0, $K_{i,app.}$, E_t) with eqns. (10)–(13) to predict the proportion of S or I bound to the enzyme. The values may be printed out directly or transformed into the variables of linear Scatchard or Klotz plots (Klotz & Hunston, 1971).

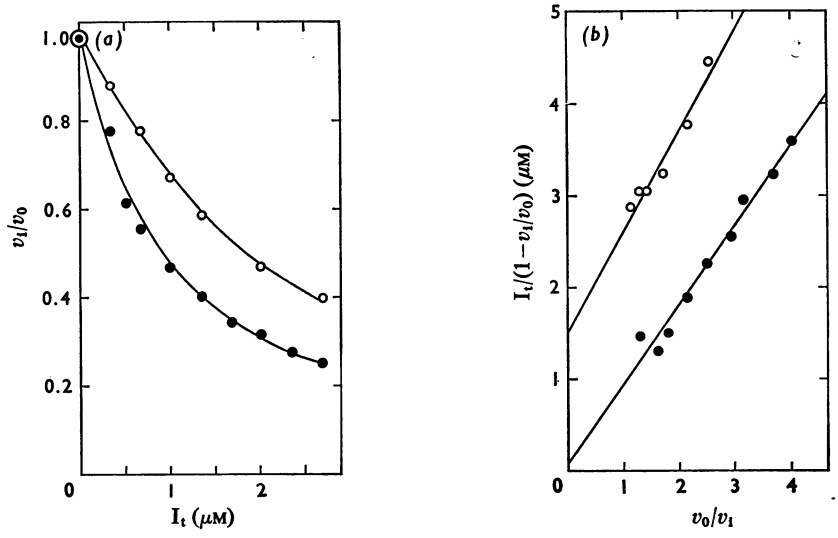

Fig. 3. *Inhibition of Azotobacter vinelandii particle respiration by hydroxyquinoline N-oxide*

(*a*) Respiration was measured at 2°C with 3.33mg of particle protein, $v_0 = 0.0835$ μg-atom of $O \cdot min^{-1} \cdot mg^{-1}$ (○), and at 30°C with 0.555mg of protein, $v_0 = 1.42$μg-atoms of $O \cdot min^{-1} \cdot mg^{-1}$ (●). (*b*) Computer-generated replots of the data in (*a*) with calculated best values of $v_0 = 0.0844$μg-atom of $O \cdot min^{-1} \cdot mg^{-1}$ (○) and 1.429μg-atoms of $O \cdot min^{-1} \cdot mg^{-1}$ (●). In (*a*) and (*b*) the continuous lines are the computer-calculated least-square fits. Experimental details are given in Ackrell & Jones (1971).

Calculation of $I_{0.5}$ or $S_{0.5}$ and significance of the slope term

When the concentration of S_t or I_t is such that the reaction velocity is exactly one-half the maximal value, eqns. (3) and (5) simplify to:

$$S_{0.5} \text{ or } I_{0.5} = \text{Slope} + \frac{E_t}{2} \qquad (14)$$

where the slope is that of the appropriate linear form. Further, it is easily shown that when E_t/K_m or E_t/K_i are less than 0.01, the slope = $S_{0.5}$ or $I_{0.5}$ respectively. For substrate plots this is not surprising because eqn. (3) shows that slope = K_m, and K_m is $S_{0.5}$ by definition when the Michaelis–Menten treatment is valid (Cleland, 1970). It does mean that $D \Big/ \sum \frac{N_i}{K_i}$ in eqns. (4) and (5) is the I_t concentration that decreases the velocity to $v_0/2$ in the absence of significant depletion of I_t from solution. Hence the use of $K_{i,app.}$ instead of $D \Big/ \sum \frac{N_i}{K_i}$ in the above treatment. To calculate $I_{0.5}$ or $S_{0.5}$ when depletion is significant, a factor of $E_t/2$ must be added to the slope. It is apparent from Henderson (1972) that a low ratio of slope/E_t indicates a high degree of binding, and vice versa. Since $I_{0.5}$ and $S_{0.5}$ are very useful parameters, e.g. $I_{0.5}$ for assessing toxicity of drugs, their values with standard errors are calculated by the programs.

For inhibitors dose–response plots may be repeated at different fixed concentrations of substrate; the variation of $K_{i,app.}$ with S_t is diagnostic of the mechanism; a graph of $K_{i,app.}$ versus S_t is linear for competitive inhibition and of $K_{i,app.}$ versus $1/S_t$ is linear for uncompetitive inhibition (Henderson, 1972). The vertical intercept of such secondary replots yields the true K_i value (Henderson, 1972). Although such secondary replots are not analysed by the computer program, their reliability is greatly enhanced by the availability of the most-probable values of the slopes from the program, and by the use of the program-calculated standard error limits as weighting factors (Cleland, 1967).

It is noteworthy that both eqns. (2) and (4) simplify to the normal Michaelis–Menten forms when E_t/K_m or E_t/K_i is less than 0.01 (Morrison, 1969; Cha, 1970; Henderson, 1972). Thus, the computer programs are competent to fit data obtained when it is valid to make the Michaelis–Menten assumptions; the efficiency of program operation can then be enhanced by weighting the linear replots to pass through or near the origin.

Table 1. *Analysis of aminopterin inhibition of dihydrofolate reductase (Nixon, 1967)*

The two values given are the upper and lower limits above and below the most-probable value.

pH 5.5; $E_t = 443$ nM*; measured $v_0 = 160.0$ nmol·min^{-1}·ml^{-1}.

	Calculated values	75% confidence limits		95% confidence limits	
E_t (nM)	455.3	437.1	473.7	427.0	483.9
v_0 (nmol·min^{-1})	156.2	154.8	157.8	154.2	158.8
$I_{0.5}$ (nM)	230.4	221.7	239.1	216.9	243.9
$K_{i,app.}$ (nM)	2.70	2.23	3.150	1.970	3.390

pH 5.5; $E_t = 15.77$ nM*; measured $v_0 = 7.92$ nmol·min^{-1}·ml^{-1}.

	Calculated values	75% confidence limits		95% confidence limits	
E_t (nM)	15.55	14.90	16.28	14.32	17.08
v_0 (nmol·min^{-1})	8.026	7.841	8.226	7.676	8.436
$I_{0.5}$ (nM)	7.947	7.641	8.289	7.370	8.662
$K_{i,app.}$ (nM)	0.172	0.148	0.191	0.119	0.206

pH 5.5; $E_t = 1.83$ nM*; measured $v_0 = 61.25$ nmol·min^{-1}·ml^{-1}.

	Calculated values	75% confidence limits		95% confidence limits	
E_t (nM)	1.115	0.927	1.474	0.816	2.034
v_0 (nmol·min^{-1})	60.54	54.82	66.75	51.24	72.90
$I_{0.5}$ (nM)	0.665	0.578	0.813	0.521	1.022
$K_{i,app.}$ (nM)	0.107	0.076	0.114	†	†

pH 8.0; $E_t = 15.77$ nM*; measured $v_0 = 14.42$ nmol·min^{-1}·ml^{-1}.

	Calculated values	75% confidence limits		95% confidence limits	
E_t (nM)	32.68	15.60	49.07	10.05	†
v_0 (nmol·min^{-1})	13.71	12.36	15.52	†	17.28
$I_{0.5}$ (nM)	24.18	17.21	30.41	14.04	†
$K_{i,app.}$ (nM)	7.840	5.872	9.406	†	†

* Obtained by protein determination; experimental details in Nixon (1967).
† Not calculable.

Results

The correct functioning of the computer programs has been tested in two ways. First, sets of numbers that fit eqns. (2) or (4) exactly were generated by using a wide range of enzyme, substrate and inhibitor concentrations and K_i or K_m values, so that E_t/K_m or E_t/K_i varied from 0.001 to 1000.0. Each set of data was analysed and in every case the program converged successfully on the exact parameter values used to generate a particular data set; an example is shown in Fig. (2). This method does not serve to test the accuracy of the computed error limits.

Secondly, experimental values obtained by Nixon (1967) from dose–response measurements of the inhibition of dihydrofolate reductase by aminopterin were analysed; in Table 1 the concentration of enzyme calculated by the program is compared with that obtained independently by assay of protein concentration. Very precise agreement is obtained when the conditions are such that nearly all the inhibitor is bound (high E_t, low pH), and in every case the values agree within the computed 95% confidence limits. With this data the range of values is not ideal for assessing the linearity of replots I_t $v_0/(v_0-v_i)$ versus v_0/v_i. Fig. 3, however, depicts such plots obtained from dose–response analysis of inhibition of bacterial respiration by hydroxyquinoline *N*-oxide (C. W. Jones, unpublished work); a linearity is apparent. From these and other analyses it appears that about ten points are required to yield reasonably precise estimates of the parameters when velocities are measured to an accuracy of ±5%. It may be predicted theoretically that the precision of a determination of E_t decreases as E_t/K_i or E_t/K_m decrease towards 0.01, and conversely the precision of the slope determination increases.

Discussion

The computerized numerical method described above allows values and errors of kinetic parameters

to be calculated when a significant proportion of substrate or inhibitor is bound to the enzyme. If a computer is not available the non-linear graphical methods of Dixon (1972) or Morrison (1969) may be used to the same end. These methods, however, do not yield the statistically most-probable parameter values (by the least-squares criterion) or their standard errors. Knowledge of the error limits is invaluable when comparing parameters obtained under different conditions to elucidate the kinetic mechanism of an enzyme (Cleland, 1967). An important difference between the numerical method and the graphical method of Dixon (1972) is that the latter requires a knowledge of the exact $V_{max.}$ or v_0 value (there are internal checks to ensure that the right value is selected), whereas the former treats $V_{max.}$ or v_0 as values to be calculated.

Although the consideration of substrate binding has been based on the normal Michaelis–Menten equation (1), it is not difficult to treat more complex rate equations that describe multi-substrate reactions. At present, experimental evidence is not available to substantiate the linearity of the plots of S_t $V_{max.}/v$ against $V_{max.}/(V_{max.}-v)$. The inhibitor data depicted in Fig. 3 does support the prediction of linearity for plots of $I_t/(1-v_i/v_0)$ against v_0/v_i.

For tightly bound, non-metabolizable inhibitors, a rigorous method of analysing dose–response data may be particularly useful. It is not necessary to know the mechanism of interaction (competitive, uncompetitive etc.) between the inhibitor and the enzyme to undertake the analysis. Hence, an experimentally induced change in $I_{0.5}$ for a drug, for example, may be attributed directly to a change in enzyme concentration, an altered affinity, or both. Inhibitor titrations can be used to obtain reliable estimates of enzyme concentrations, and a more comprehensive investigation of the behaviour of the slope term can reveal the mechanism of interaction (Henderson, 1972). Finally, results obtained with the tight-binding inhibitors hydroxyquinoline *N*-oxide and aminopterin (the present paper) and oligomycin and bongkrekic acid (Henderson, 1972), support the application of the theoretical equations of Goldstein (1944), Morrison (1969) and Henderson (1972) to practical enzyme kinetics.

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