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Determination of Inhibition Constants, I_{50} Values and the Type of Inhibition for Enzyme-Catalyzed Reactions

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The therapeutic effect of most drugs depends on their ability to interact with particular biomolecules and in many cases the biomolecule is an enzyme for which the drug acts as a specific inhibitor. A commonly used measure of the potency of an enzyme inhibitor is its I_{50} which is defined as the concentration required to produce 50% inhibition. However, the I_{50} is a relatively uninformative quantity since it depends on the type of inhibition (competitive, noncompetitive, or uncompetitive), the inhibition constant(s), and the experimental conditions under which it is determined.

The type of inhibition may be characterized by observing the effect of an inhibitor on a Lineweaver-Burk plot, in which the reciprocal of the rate of the enzyme-catalyzed reaction is plotted against the reciprocal of the substrate concentration. A competitive inhibitor affects only the slope of this plot giving a series of lines which intersect on the ordinate. An uncompetitive inhibitor affects only the intercept resulting in a series of parallel lines. When both the slope and the intercept are affected most enzyme kineticists would describe this as noncompetitive inhibition, irrespective of the position of the point where the lines intersect. However, the term noncompetitive is sometimes used in a narrower sense to indicate the situation where the slope and intercept on the ordinate are equally affected, giving lines which meet on the abscissa. When the lines intersect elsewhere, this is then described as "mixed" inhibition. While these different usages of the term noncompetitive is basically a semantic problem, the more narrow definition can sometimes be misleading.

Brandt *et al.* (1) have recently described a method for determining the type of inhibition and the inhibition constant for an enzyme inhibitor. The method is based upon measurements of I_{50} and the manner in which it changes when the substrate concentration is varied. While their method is theoretically sound as far as it goes, it is flawed by the interpretation of noncompetitive inhibition in the narrow sense described above. As a result, their method could easily lead to false conclusions. In this report the basic flaw in their approach is exposed and an alternative method is described.

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The new method described here has several advantages. First, it yields not only inhibition constants but their standard errors as well. This information is vital for an assessment of whether inhibition constants determined by other laboratories, for related enzymes, or using analogous inhibitors, are significantly different from one another. Second, the method also yields the Michaelis–Menten parameters (maximum velocity and Michaelis constant) and their standard errors. This information may be combined with the knowledge of inhibition constants to predict the I_{50} (and its standard error) at any chosen substrate concentration. Finally, the method offers an objective, statistical criterion for deciding the type of inhibition. The present approach is illustrated with experimental data.

Some years ago, Cheng and Prusoff (2) gave a thorough account of the relationship between I_{50}, inhibition constants, and the type of inhibition. While the work presented here differs in several important respects from theirs, a statement they made is pertinent here. "Although what is presented is no doubt readily apparent to the enzyme kineticist, those who are less familiar with enzyme kinetics and yet concerned with studying the effect of drugs on enzymes may find this communication useful."

THEORY

The method of Brandt *et al.* is most easily considered in relation to the general model shown as Scheme 1.

$$\begin{array}{ccc} E + A \rightleftharpoons EA & \longrightarrow & E + \text{product} \\ +I \qquad\quad +I & & \\ \downarrow\uparrow K_{is} \qquad \downarrow\uparrow K_{ii} & & \\ EI \qquad\quad EAI & & \end{array}$$

In competitive inhibition, the inhibitor does not bind to the enzyme–substrate complex (EA) which is equivalent to saying that K_{ii} is infinite. The inhibition constant (K_i) corresponds to K_{is} and, as Brandt *et al.* have shown, I_{50} increases as the substrate concentration ([A]) increases and is equal to $2K_i$ when [A] is exactly equal to the Michaelis constant (K_a). For uncompetitive inhibition ($K_i = K_{ii}$; $K_{is} = \infty$), I_{50} decreases as [A] increases but is again equal to $2K_i$ when [A] equals K_a.

The third case they consider is for what they term "noncompetitive" inhibition, which is identified as occurring when the two inhibition constants are equal ($K_i = K_{is} = K_{ii}$). Under these conditions, I_{50} equals K_i irrespective of the substrate concentration. It is evident from Scheme 1 that the situation where K_{is} equals K_{ii} is nothing more than a coincidence since the two inhibition constants correspond to binding of the inhibitor to different molecular species. Cheng and Prusoff, in their analysis of the relationship between I_{50} and inhibition constants, made it clear that the equality of K_{is} and K_{ii} is not a general rule for noncompetitive inhibition. As will be shown shortly, the analysis of Brandt *et al.* fails when this fact is recognized.

While noncompetitive inhibition in Scheme 1 depends on the inhibitor binding to two different enzyme forms, it can also arise when the inhibitor binds to a

single enzyme form (3). For example, consider the two-substrate reaction shown as Scheme 2.

$$\begin{array}{l} E + A \rightleftharpoons EA + B \rightleftharpoons EAB \longrightarrow E + \text{product(s)} \\ +I \\ \downarrow\uparrow K_i \\ EI \end{array}$$

The inhibitor will be competitive with substrate A but noncompetitive with substrate B. In the latter case, inhibition depends on two inhibition constants which are expected to be unequal since they represent different combinations of rate constants. In general neither of them will be numerically equal to the dissociation constant, K_i. The two inhibition constants can be equal to one another, but this is purely fortuitous.

It may be said that all types of inhibition form part of a continuum which will depend on an inhibition parameter (C_i) defined by Eq. [1].

$$C_i = K_{is}/(K_{is} + K_{ii}). \qquad [1]$$

It is also useful to define an overall inhibition constant (K_{io}) as the harmonic sum of K_{is} and K_{ii} (Eq. [2]).

$$K_{io} = 1/(1/K_{is} + 1/K_{ii}). \qquad [2]$$

Competitive inhibition ($K_{ii} = \infty$) corresponds to $C_i = 0$ and $K_{io} = K_{is}$, while uncompetitive inhibition ($K_{is} = \infty$) corresponds to $C_i = 1$ and $K_{io} = K_{ii}$. A fractional C_i indicates noncompetitive inhibition and the special case considered by Brandt *et al.* occurs when $C_i = 0.5$.

The relationship between I_{50}, K_{io}, and C_i is defined by Eq. [3] and is illustrated in Fig. 1.

$$I_{50} = \frac{K_{io}(K_a + [A])}{K_a + C_i([A] - K_a)}. \qquad [3]$$

First it should be noted from Fig. 1 that when $[A] = K_a$, I_{50} is equal to twice K_{io}, irrespective of the type of inhibition. This is in full agreement with Brandt *et al.*, except that for their "noncompetitive" case, the overall inhibition constant as defined by Eq. [2] is twice their K_i. Further, the dependence of I_{50} on [A] is as they describe; increasing for competitive inhibition, decreasing for uncompetitive inhibition and constant when $K_{is} = K_{ii}$. However, it is clear from Fig. 1 that the effect on I_{50} of varying [A] is not sufficient to establish the type of inhibition since I_{50} increases with [A] whenever C_i is less than 0.5. It follows that such a result is not diagnostic of competitive inhibition and means only that $K_{is} < K_{ii}$. Similarly, if I_{50} is observed to decrease with increasing [A] this means only that $K_{is} > K_{ii}$ and it would be wrong to deduce that the inhibition is uncompetitive.

The suggestion of Brandt *et al.* that inhibition constants and the type of inhibition should be determined by studying the effect of the inhibitor over a range of substrate concentrations is a good one. The problem is in the analysis and arises from interpreting noncompetitive inhibition as occurring only when

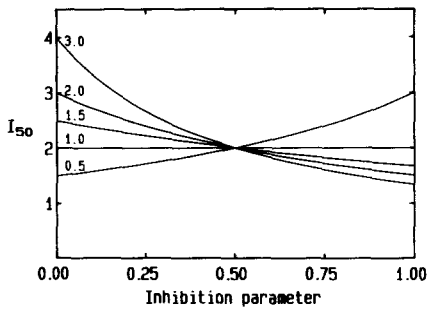

FIG. 1. Dependence of I_{50} on the inhibition parameter at several substrate concentrations. The I_{50} was calculated over the range of values of the inhibition parameter (C_i; Eq. [1]) from 0 to 1 using Eq. [3]. To emphasise the generality of results obtained at different combinations of K_{is} and K_{ii}, I_{50} was expressed relative to the overall inhibition constant (K_{io}) as defined by Eq. [2]. The calculation was performed at substrate concentrations ranging from one-half to three times K_a and the label associated with each of the lines corresponds to the ratio $[A]/K_a$ used in the calculation.

$K_{is} = K_{ii}$. Fortunately, the series of experiments that they suggest can be used to determine the type of inhibition and the inhibition constant or constants if the analysis is structured differently.

An experiment to determine I_{50} will involve measuring the rate of an enzyme-catalyzed reaction over a range of inhibitor concentrations under otherwise fixed reaction conditions. If the dependence of I_{50} upon [A] is to be examined, the above experiment must be repeated at a series of substrate concentrations. Thus, the analysis suggested by Brandt *et al.* requires rate measurements over a series of [I] at each of several [A] and the data will be described by the general inhibition Eq. [4].

$$v = V_m[A]/\{[A](1 + [I]/K_{ii}) + K_a(1 + [I]/K_{is})\}. \quad [4]$$

While this equation does not involve the I_{50}, the relationship between the four kinetic parameters (V_m, K_a, K_{is}, and K_{ii}) and the I_{50} will be discussed later. Equation [4] can be fitted to the data and the four kinetic parameters can be evaluated. This fitting will involve finding a set of values for the parameters which minimizes the sum of the squares of the residuals (i.e., the differences between the experimentally determined rates, and those predicted by Eq. [4]). Cleland (4) has described this general approach in detail and nonlinear regression computer programs have been published (e.g., Duggleby (5)) for this purpose.

The estimated values for the two inhibition constants may be interpreted in the following manner. With perfect data for competitive inhibition, K_{ii} would be infinite but for real data a better fit (a lower residual sum of squares) will always be obtained with a finite value which may be either positive or negative. Nevertheless, K_{ii} is likely to be much larger (neglecting the sign) than both K_{is} and the maximum inhibitor concentration used in the experiment. When the fit indicates this situation, the data can be reanalyzed by fitting the equation for competitive inhibition which is identical to Eq. [4] except that it lacks the $(1 + [I]/K_{ii})$ term. This second fit will be worse, as judged by a higher residual sum of squares and

what must be ascertained is whether this second fit is so much worse that it may be rejected on statistical grounds. A variance ratio (F) test, as described by Ellis and Duggleby (6), may be used for this purpose and examples of this test will be shown later.

A similar procedure may be applied to uncompetitive inhibition, except that it is the analyses with and without the $(1 + [I]/K_{is})$ term of Eq. [4] which are compared. Finally, inhibition which is really noncompetitive will give values for K_{is} and K_{ii} neither of which will be negative, excessively large, or able to be omitted on statistical grounds. Thus the procedure advocated here applies objective criteria to the determination of the type of inhibition.

Fitting Eq. [4] to experimental data can provide estimates of the four parameters and their standard errors and this information is valuable for several reasons. First, the Michaelis–Menten parameters are derived from the analysis which is always useful information about an enzyme. Second, having values for the inhibition constants and their standard errors permits comparison with related inhibitors, homologous enzymes, or with results obtained in other laboratories. However, it is a third aspect which will be focused upon here: the prediction of I_{50} values.

Cheng and Prusoff (2) gave an expression for I_{50} in terms of K_a, K_{is}, K_{ii}, and [A] which, after a minor rearrangement, is given as Eq. [5].

$$I_{50} = \frac{K_{is}K_{ii}(K_a + [A])}{K_aK_{ii} + [A]K_{is}}. \qquad [5]$$

Thus, when K_a, K_{is}, and K_{ii} have been determined as described above, I_{50} may be calculated at any substrate concentration. However, it should be remembered that these kinetic parameters have associated errors of estimation, and these errors will contribute some uncertainty to the calculated value of I_{50}. To determine the error in I_{50} it is necessary to take account of the variances of the kinetic parameters as well as their mutual covariances. Formulae for the propagation of these errors are available (e.g., Cleland (4)) so it is possible to calculate a standard error for the I_{50}; again, examples will be given later. This information is essential for any comparison between I_{50} values obtained independently.

METHODS

Potato acid phosphatase was obtained from Calbiochem and was assayed at 25° and pH 7.2, using p-nitrophenyl phosphate as substrate, in 0.1 M sodium MOPS buffer which contained 0.1 M NaCl. Rates were determined, in duplicate, by following the absorbance increase at 410 nm for 1–5 min. Rates are expressed as the change in absorbance/100 min.

Hog muscle lactate dehydrogenase (M_4 isoenzyme) was obtained from Boehringer and was assayed at 30° and pH 7.5 in 0.1 M sodium TES buffer. Pyruvate concentrations were varied while NADH was added at a constant concentration of 0.1 mM. Rates were determined by following the absorbance decrease at 340 nm and were expressed as the change in absorbance/100 min.

Fitting the appropriate equation to experimental data was done by nonlinear regression using the DNRP53 computer program (5), assuming that the experimental

error in determination of the rate is proportional to the rate. The equations fitted were Eq. [4] and variants of this lacking either the $(1 + [I]/K_{is})$ or $(1 + [I]/K_{ii})$ terms. In addition, to estimate I_{50} from a series of rate measurements obtained while varying the concentration of the inhibitor at a constant substrate concentration, Eq. [6] was fitted to the data. This fit also yields a value for v_o, the uninhibited rate.

$$v = v_o/(1 + [I]/I_{50}). \qquad [6]$$

The DNRP53 program, and programs to predict I_{50} values, are available on request from the author.

RESULTS AND DISCUSSION

The first set of data which was analyzed concerned the inhibition of acid phosphatase by inorganic phosphate, which was expected (7) to show competitive inhibition. The upper section of Table 1 shows values of I_{50} determined at several substrate concentrations.

It is clear that I_{50} rises as the substrate concentration is increased but, as noted above, this is not sufficient to establish that the inhibition is competitive. The lower part of Table 1 shows the result of fitting Eq. [4] to the combined data at all substrate concentrations and these results strongly suggest competitive inhibition. If the results from the fit to noncompetitive inhibition are considered first, it is seen that K_{ii} is negative. While this does not establish competitive inhibition, it is certainly indicative that the data are inconsistent with noncompetitive inhibition. A more definitive conclusion can be drawn from the absolute magnitudes of K_{ii}

TABLE 1
Inhibition of Acid Phosphatase by Phosphate[a]

Substrate concentration	Uninhibited rate	I_{50} (mM)	
		Fitted	Predicted[b]
1.00 mM	2.548 ± 0.039	0.373 ± 0.013	0.381 ± 0.011
1.30 mM	3.045 ± 0.052	0.399 ± 0.015	0.417 ± 0.012
1.81 mM	3.456 ± 0.069	0.494 ± 0.022	0.479 ± 0.013
3.05 mM	4.282 ± 0.055	0.663 ± 0.023	0.628 ± 0.016
10.96 mM	6.622 ± 0.052	1.682 ± 0.062	1.582 ± 0.042
Parameter	Competitive		Noncompetitive
V_m	7.799 ± 0.170		7.570 ± 0.227
K_a	2.161 ± 0.098		2.043 ± 0.123
K_{is}	0.261 ± 0.010		0.247 ± 0.013
K_{ii}	—		−9.833 ± 6.759
SSQ	0.051409		0.048780
		$F = 1.89$	

[a] Phosphate concentrations used were 0, 0.268, 0.535, and 0.803 mM.
[b] Calculated from the competitive inhibition fit.

and K_{is} where the former is nearly 40 times the latter. Inhibition is dominated by the K_{is} term since the maximum concentration of inhibitor used in these experiments (0.803 mM) was less than one-tenth of K_{ii}.

Reanalysis of these data by omitting the $(1 + [I]/K_{ii})$ term gave the fit to competitive inhibition which is also shown in Table 1. From a comparison of the residual sum of squares of the two fits it is possible to make a statistical judgment as to whether the data are best described by competitive or noncompetitive inhibition. This sum of squares is smaller for the noncompetitive fit which is as expected since the additional term permits more flexibility; the question is whether this reduction in the sum of squares is statistically significant. This question may be answered using an F test (6) which gave a value of 1.89 corresponding to a probability between 0.1 and 0.2. In other words, there is a 10–20% probability that the reduction in the sum of squares has arisen by chance and a substantially greater reduction in the sum of squares (and a much larger F value) would be required to reject the fit to competitive inhibition. It has been this author's experience that probabilities of 1% or less are an appropriate rejection criterion; values in the range 1–5% are marginal and it is safest to repeat the experiment using higher concentrations of inhibitor to obtain a clearer result. It may be noted in passing that this same statistic, in conjunction with residual plots (6), may be used to identify partial (hyperbolic) and parabolic inhibition (3).

Having established the type of inhibition, it is now possible to calculate the expected I_{50} at any substrate concentration and the results of these calculations are also shown in Table 1. There is excellent agreement between the observed and predicted values of I_{50} at each of the substrate concentrations used in this experiment. While the predicted I_{50} values given in Table 1 relate only to the substrate concentrations used in this experiment, it is equally easy to calculate values at any desired substrate concentration. Thus, the predicted I_{50} at 5 mM substrate is 0.864 ± 0.022 mM which could be compared with a value obtained in another laboratory.

The second example concerns the inhibition of lactate dehydrogenase by NAD^+ and the results are given in Table 2. The fit to noncompetitive inhibition gives K_{is} and K_{ii} values which are both positive, are similar in magnitude (6.6 and 11.9 mM), and are each likely to contribute significantly to the observed inhibition since NAD^+ concentrations ranged from 0 to 12 mM. To determine whether the inhibition is really noncompetitive, the data were reanalyzed as both competitive and uncompetitive inhibition. In both cases, the sum of squares was considerably higher and the F values were very large. Even for the smaller of the two F values (competitive inhibition; $F = 144$), there is less than a 0.1% probability that such results could have arisen by chance and it is safe to conclude that the inhibition is truly noncompetitive.

Although the results shown in Table 2 are somewhat less dramatic than those shown in Table 1, it is clear that I_{50} rises with the substrate concentration. According to Brandt *et al.*, this would be interpreted as indicating competitive inhibition, a conclusion which turns out to be quite incorrect. The inhibition is noncompetitive, although the two inhibition constants are not identical and cor-

TABLE 2
Inhibition of Lactate Dehydrogenase by NAD^+[a]

Substrate concentration	Uninhibited rate	I_{50} (mM)	
		Fitted	Predicted[b]
0.08 mM	1.647 ± 0.066	7.62 ± 0.70	7.60 ± 0.48
0.16 mM	2.557 ± 0.136	7.87 ± 0.98	8.28 ± 0.36
0.25 mM	3.004 ± 0.078	9.44 ± 0.63	8.83 ± 0.34
0.50 mM	3.915 ± 0.107	9.94 ± 0.72	9.74 ± 0.49
1.00 mM	4.795 ± 0.120	10.10 ± 0.67	10.54 ± 0.76

Parameter	Competitive	Noncompetitive	Uncompetitive
V_m	3.918 ± 0.225	5.528 ± 0.208	8.315 ± 1.236
K_a	0.090 ± 0.017	0.192 ± 0.016	0.503 ± 0.104
K_{is}	2.84 ± 0.56	6.61 ± 0.68	—
K_{ii}	—	11.90 ± 1.34	3.97 ± 0.84
SSQ	0.25137	0.03196	0.41921
	$F = 144$		$F = 254$

[a] NAD^+ concentrations used were 0, 3, 6, 9, and 12 mM.
[b] Calculated from the noncompetitive inhibition fit.

respond to a C_i (Eq. [1]) of 0.357. The fact that I_{50} rises with the substrate concentration is a consequence of the fact that C_i is less than 0.5 and not, as would be concluded by Brandt *et al.*, that the inhibition is competitive.

The procedures described in this report have several advantages over that described by Brandt *et al.* The most important of these is that the present methodology may be applied to competitive, noncompetitive, and uncompetitive inhibition. While Brandt *et al.* concede that their approach cannot be applied to "mixed" (i.e., noncompetitive) inhibition, in fact the whole procedure becomes invalid if the possibility of "mixed" inhibition is admitted. Their method is supposed to pinpoint the type of inhibition and in this aim it fails. By contrast, the present method identifies the type of inhibition and it does so by an objective, statistical criterion.

SUMMARY

A procedure is proposed for determining whether an inhibitor of an enzyme-catalyzed reaction is competitive, noncompetitive, or uncompetitive with respect to the substrate. The method is based on fitting the equation for noncompetitive inhibition to data obtained by measuring the rate of the reaction over a range of substrate and inhibitor concentrations. The results of this fit may suggest that the inhibition may be either competitive or uncompetitive, whereupon the data are reanalyzed using the appropriate equation. Comparison of this second fit with the first using an F test permits a statistical decision to be made on the type of inhibition. The chosen fit yields values and standard errors for the Michaelis–Menten parameters (maximum velocity and Michaelis constant), as well as the inhibition constant(s). From these values it is then possible to predict

the I_{50}, and its standard error, at any chosen substrate concentration, thereby facilitating comparison with results obtained with similar inhibitors, for homologous enzymes, or in other laboratories.

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