Combination plots as graphical tools in the study of enzyme inhibition

William W.-C. CHAN
Department of Biochemistry, McMaster University, Hamilton, Ontario, Canada L8N 3Z5

Although statistical regression has become the method of choice in the analysis of enzyme kinetics, graphical methods continue to be useful on account of their illustrative capabilities. It is pointed out in this paper that enzyme inhibition data may be presented more efficiently as a single linear plot than the traditional way as a family of lines. This approach has been taken previously by Hunter and Downs [Hunter and Downs (1945) J. Biol. Chem. **157**, 427–446] but has remained neglected. A new version of this type of plot (combination plot) has been devised which is linear for competitive, non-competitive, uncompetitive and linear mixed inhibition and has a characteristic appearance for each type of inhibition behaviour. The slopes and intercepts not only indicate directly the dissociation constant but also provide quantitative criteria for the nature of inhibition. This plot should serve as a useful graphical tool in enzyme research as well as in biochemical education.

INTRODUCTION

Graphical analysis has traditionally occupied an important position in enzyme kinetics. In particular, linear transformations of the rate equations (e.g. [1]) have found widespread application. With the general availability of microcomputers, many experts now recommend non-linear regression as a more rigorous and precise approach [2]. Nevertheless, biochemists have continued to a large extent to use graphical methods, perhaps partly because they consider the benefit of visual inspection of the data to outweigh any loss of precision. Even advocates of non-linear regression (e.g. [3]) regard graphical display to be indispensable for assessing the goodness of fit. The illustrative capabilities of graphical analysis may explain why these methods are still given prominence in standard textbooks of biochemistry as well as in advanced monographs on enzyme kinetics [4,5]. Not all graphical procedures are equally suitable for various purposes and so improvements may sometimes be made to existing methods.

In studying enzyme inhibition, the inhibitor concentration represents an additional experimental parameter which may be varied independently. In the most commonly employed procedures (e.g. [6]), the concentrations of substrate and inhibitor are varied separately to create a family of lines. Interpretation of the results then relies on judging the intersecting tendencies of the above lines and often requires a secondary plot of slopes or intercepts. It is not widely appreciated that these cumbersome aspects of the above graphical methods can be avoided by suitable transformation of the rate equation so that a single line accommodates all data points regardless of the inhibitor and substrate concentrations. A plot of this type was in fact published many years ago [7] but has been largely neglected (I am indebted to Dr. A. Cornish-Bowden who pointed out that reference to this previous work was omitted in an earlier draft of this manuscript). In this paper, it is shown that different versions of these plots may be devised. To draw attention to their special character, it is proposed to introduce the new term 'combination plots' which emphasizes their ability to combine several lines into one. A new combination plot is described below which appears to have optimal properties as a graphical tool in studying enzyme inhibition.

METHODS

Rate equations

The following equations and the symbols employed are all described in standard textbooks (e.g. [5]) and should require no further explanation.

In the absence of inhibitors and under steady-state conditions:

$$v_0 = V_{max.}[S]/(K_m + [S]) \quad (1)$$

For competitive inhibition,

$$v_i = V_{max.}[S]/\{[S] + K_m(1 + [I]/K_i)\} \quad (2)$$

For non-competitive inhibition,

$$v_i = V_{max.}[S]/(1 + [I]/K_i)(K_m + [S]) \quad (3)$$

For uncompetitive inhibition,

$$v_i = V_{max.}[S]/\{K_m + [S](1 + [I]/K_i)\} \quad (4)$$

For linear mixed inhibition (as defined later in Scheme 1),

$$v_i = V_{max.}[S]/\{K_m(1 + [I]/K_i) + [S](1 + [I]/\alpha K_i)\} \quad (5)$$

From the above equations, it is a simple matter to derive the required expression in the Results section [eqns. (9)–(15)].

Use of $V_{max.}$ in place of v_0

The mathematical functions used for the Hunter and Downs plot and the 'optimized' plot described in the Results section both involve the parameter v_0. Although v_0 values are easy to determine experimentally, it becomes a practical burden if values must be obtained for every substrate concentration used. Another and perhaps more important consideration is that significant scatter may be introduced into the data points due to the statistical fluctuations of individual v_0 and v_i values associated with errors in measurements. In fact, it is likely that this property of combination plots may have previously hampered the acceptance of the Hunter and Downs plot. In this respect, it has been pointed out before that reciprocal plots such as the Lineweaver–Burk appear to give deceptively good fits [8].

The above drawbacks of combination plots may be avoided by using expressions containing $V_{max.}$ instead of v_0. Using eqn. (1), it can be shown that the function in the Hunter and Downs plots [eqns. (9)–(11)], namely: $[I]v_i/(v_0 - v_i)$, is completely equivalent to

$$[I]v_i(K_m + [S])/\{V_{max.}[S] - v_i(K_m + [S])\}$$

and the function in the 'optimized' plot [eqns. (12)–(15)], namely $(1 + [S]/K_m)(v_0 - v_i)/v_i[I]$, is completely equivalent to

$$(V_{max.}[S] - v_i[S] - v_iK_m)/v_i[I]K_m$$

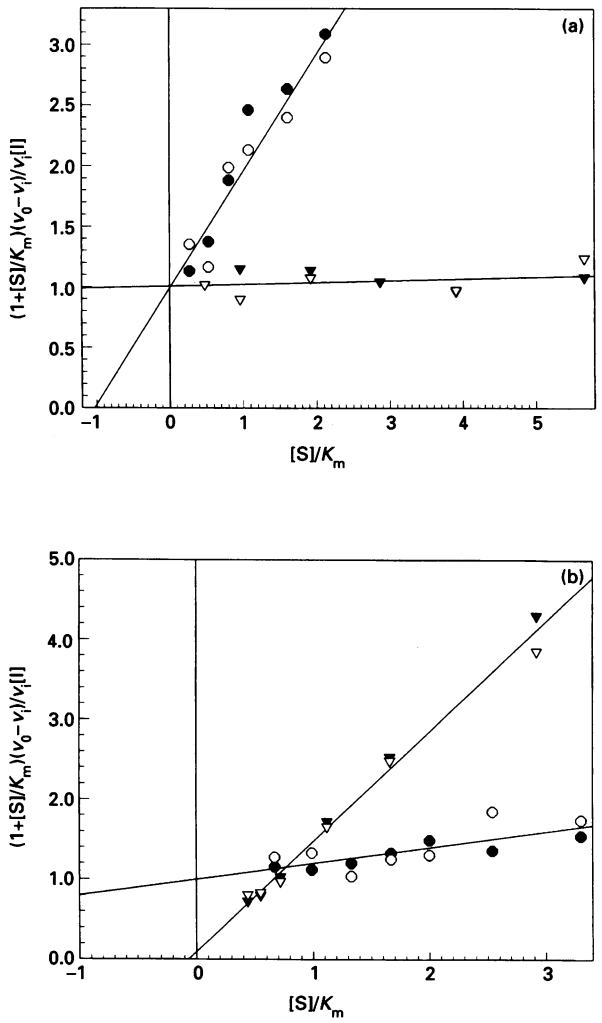

Figure 1 Combination plot of the function $(1+[S]/K_m)(v_0-v_i)/v_i[I]$ versus $[S]/K_m$

The data are taken from the literature. (**a**) ADP (product) inhibition of creatine kinase [10]. The inhibition is competitive with respect to the substrate ATP (triangles) and non-competitive with respect to creatine, the other substrate (circles). (**b**) Inhibition of alanopine dehydrogenase [11] by oxamate (circles, mixed inhibition) and 2-oxoglutarate (triangles, uncompetitive). The pattern of inhibition was determined with respect to pyruvate as substrate in both cases. The inhibitor concentrations for both (**a**) and (**b**) were normalized to their K_i values in order to present two plots conveniently in each diagram. In all cases, the closed symbols indicate data points obtained with inhibitor concentrations twofold higher than those used for the corresponding open symbols.

The use of these alternative expressions therefore obviates the need to determine v_0 values at every substrate concentration. The data points should also be subjected to less scatter because $V_{max.}$ represents a parameter which has been averaged over a number of v_0 values.

Illustration of combination plots using published data

In order to illustrate the newly derived plots, a standard handbook [9] was consulted to locate suitable data for the various types of inhibition behaviour. Only studies using many different substrate concentrations were suitable because the plots (Figures 1 and 2) both involve this parameter as a variable. Furthermore, in order to show that the plots accommodated all results on the same line regardless of their [I] and [S] values, it

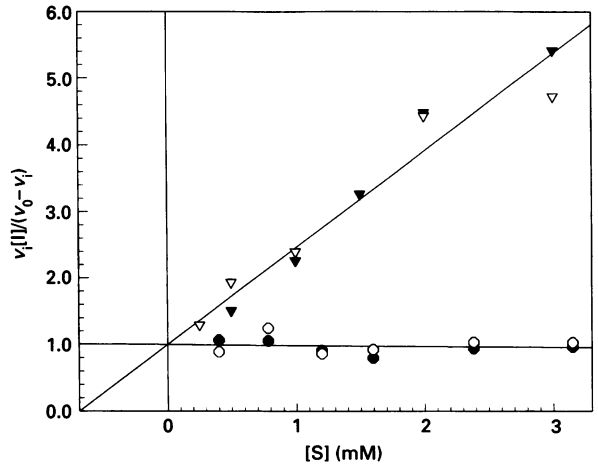

Figure 2 Combination plot of $[I]v_i/(v_0-v_i)$ versus [S]

The data and symbols used are identical to those in Figure 1(a). The general appearance of the plots is exactly the reverse of that in Figure 1(a) with non-competitive inhibition as a horizontal line in this case. The substrate concentration was divided by 10 in the case of creatine (circles) in order to use the same scale for ATP. As in Figure 1, the inhibitor concentration values were normalized with respect to their K_i values for convenient presentation.

was necessary to have at least two series of data with different inhibitor concentrations. Finally, for convenience it was advantageous to use only two enzymes to illustrate all four patterns of simple inhibition.

The data were obtained directly from the Figures in the published papers [10,11] by careful measurement. After plotting to determine K_i, the inhibitor concentrations were divided by K_i in order to normalize their values so that two plots could be accommodated in one diagram. (This procedure is of course unnecessary in normal application). In the revised plots, the vertical intercepts are therefore always equal to 1 (except in the case of uncompetitive behaviour). The lines were drawn according to linear regression and the intercepts and slopes have the expected values within experimental error. In the case of mixed inhibition of alanopine dehydrogenase by oxamate (Figure 1b, circles), the horizontal intercept yields a value of 5.0 for α (see Scheme 1) which was not calculated by the previous authors. In the plot of $[I]v_i/(v_0-v_i)$ versus [S] (Figure 2), the horizontal intercept gives a K_m value of 0.69 mM for ATP, which does not differ significantly from the previously determined value [10].

Physical basis of the plot

The following equations [5] may be used to relate the kinetic expressions to the concentrations of various molecular species.

$$v_i = [ES]k_{cat.} \quad (6)$$

$$V_{max.} = [E]_t k_{cat.} \quad (7)$$

$$K_m = [E][S]/[ES] \quad (8)$$

where $[E]_t$ is the total concentration of all enzyme-containing species. As pointed out earlier, the expression used for the optimized plot may be rearranged as three separate terms:

$$(V_{max.}[S]-v_i[S]-v_iK_m)/v_i[I]K_m$$

Using eqns. (6)–(8), each of these terms can be expressed as concentrations of the appropriate molecular species and rearranged to give $([E]_t-[ES]-[E])/[E][I]$. The physical significance of this expression for different inhibition behaviour is explained in the Results section.

Practical considerations

In contrast to other commonly used graphical procedures, the above approach allows substrate and inhibitor concentrations to be varied in any desired manner. In general, it is expected that the maximal accuracy will be achieved when $(v_0-v_i)/v_i$ is kept close to unity. Under such conditions, both the inhibitory effect and the residual activity are substantial enough to be measured accurately. For competitive inhibitors, it is clear from eqn. (14) (see the Results section) that [I] and [S] must be increased together in order that $(v_0-v_i)/v_i$ remains relatively unchanged. It should also be pointed out that in the optimized version the inhibition data are plotted against $[S]/K_m$ and not $1/[S]$. Thus there will be minimal statistical bias associated with the linear regression analysis of this plot if the above precaution is taken to maintain $(v_0-v_i)/v_i$ near unity.

An unusual aspect of the above optimized plot is that the expressions used contain the term K_m. The requirement for the value of K_m should pose no problems for most applications because inhibition would normally be studied under standard assay conditions for which K_m would most likely have been determined previously. Frequently, a series of inhibitors will be compared under identical assay conditions and clearly the same value of K_m applies. It was pointed out above that the expression containing v_0 could be transformed into an alternative one containing $V_{max.}$ instead. Thus a reasonably reliable study of an inhibitor might require only eight enzyme assays if K_m is already known. Three of these assays would be performed without inhibitor to yield a value for $V_{max.}$. The other five would be conducted in the presence of inhibitor at different substrate concentrations (with equal increments between [S] values) to provide a linear plot with five equally spaced data points. From this simple set of experiments, both K_i and the pattern of inhibition behaviour are readily determined. If a series of inhibitors are studied at the same time so that a single $V_{max.}$ value could be used, each inhibitor requires only five additional assays for the above analysis. In comparable studies using the Dixon or Lineweaver–Burk plots, some 20 assays would be required to produce a secondary replot with five data points.

RESULTS AND DISCUSSION

Rationale for combination plots

In the graphical procedures currently in use for enzyme kinetics [1,6,12–14], the mathematical functions chosen for the plots are either the variable parameters themselves (v, [S] or [I]), or simple derivatives thereof (e.g. reciprocals). In principle, however, there is no reason why more complicated functions should not be selected. The individual values for such functions can be easily calculated from experimental data using computers or electronic calculators. Therefore the choice of the ideal function should be governed by the following considerations regarding characteristics which are most desirable in the resulting plots. First, in order to be generally useful, the plot should be applicable to as many different types of inhibition behaviour as possible. Secondly, the nature of inhibition should be visually evident and preferably supported by quantitative criteria. Thirdly, in a linear plot the desired constants should be directly represented by the slope or intercepts. In this respect, it is well to remember that the plot of a single line may be used to display two separate constants. This is sufficient in simple cases of inhibition because the behaviour is specified completely by the dissociation constant (K_i) and a further coefficient indicating the nature of inhibition (as shown below). Graphical methods involving a family of lines should only be necessary for more complex inhibition behaviour.

Table 1 Summary of slopes and intercepts for various combination plots

Nature of inhibition	Intercepts		Slope
	Vertical	Horizontal	
For $(1+[S]/K_m)(v_0-v_i)/v_i$ [I] versus $[S]/K_m$			
Competitive	$1/K_i$	$-\infty$	0
Non-competitive	$1/K_i$	-1	$1/K_i$
Uncompetitive	0	0	$1/\alpha K_i$*
Linear mixed	$1/K_i$	$-\alpha$	$1/\alpha K_i$
For $[I]v_i/(v_0-v_i)$ versus [S]			
Competitive	K_i	K_m	K_i/K_m
Non-competitive	K_i	$-\infty$	0
For $[S](V_{max.}-v_i)/v_i$ versus [I]			
Competitive	K_m	K_i	K_m/K_i

*The quantity usually referred to as the inhibition constant in this case is actually αK_i (see Scheme 1).

Choice of mathematical functions

In the search for suitable functions, an important objective is to simplify the rate equations (for details of the equations see the Methods section). This was achieved previously [7] by using the expression $(v_0-v_i)/v_i$. The rate equations can then be transformed as follows for competitive inhibition:

$$[I]v_i/(v_0-v_i) = K_i(1+[S]/K_m) \quad (9)$$

For non-competitive inhibition:

$$[I]v_i/(v_0-v_i) = K_i \quad (10)$$

Therefore plotting the expression on the left versus [S] gives a straight line. This plot of Hunter and Downs, however, is not linear for uncompetitive inhibition because in this case:

$$[I]v_i/(v_0-v_i) = K_i(1+K_m/[S]) \quad (11)$$

As already pointed out by Cornish-Bowden [4] it is possible to plot the same function against $1/[S]$ to give an alternative version which is linear for uncompetitive and non-competitive inhibition but is now curved for the more common competitive behaviour.

The 'optimized' plot

In order to improve the above situation, the equations need to be further transformed to obtain, for competitive inhibition:

$$(1+[S]/K_m)(v_0-v_i)/v_i[I] = 1/K_i \quad (12)$$

The corresponding equation for non-competitive inhibition is:

$$(1+[S]/K_m)(v_0-v_i)/v_i[I] = (1+[S]/K_m)/K_i \quad (13)$$

Similarly, for uncompetitive inhibition:

$$(1+[S]/K_m)(v_0-v_i)/v_i[I] = [S]/K_mK_i \quad (14)$$

From these equations, it is readily apparent that a plot of the composite function on the left-hand side against $[S]/K_m$ should give linear plots in all these cases of simple inhibition behaviour. The nature of inhibition should be easily discernible because each type of behaviour gives a highly characteristic plot. Thus competitive inhibition is represented by a horizontal line with the vertical intercept of $1/K_i$. Non-competitive inhibition has the same intercept but the slope is also $1/K_i$. Interestingly, the plot for uncompetitive inhibition intersects the axes at the origin. It can be seen from Table 1 that the slopes and intercepts not only indicate the desired constants directly but also provide quan-

titative criteria for the nature of inhibition. To illustrate the above combination plot, data have been taken from the literature for different types of inhibition and recalculated accordingly. As shown in Figure 1, the resulting plots behave in the expected manner with all data points falling on a single line regardless of [I] and [S] values (within experimental error).

Mixed inhibition

The favourable properties of the above 'optimized' plot may be more fully appreciated by considering the case of linear mixed inhibition. Such a system is defined by Scheme 1:

$$\begin{array}{ccc} E+S & \overset{K_s}{\rightleftarrows} & ES \rightarrow E+P \\ +I \updownarrow K_i & & +I \updownarrow \alpha K_i \\ EI+S & \underset{\alpha K_s}{\rightleftarrows} & EIS \end{array}$$

Scheme 1

As in the case of non-competitive inhibition, the ternary complex EIS forms and remains unproductive. However, the binding of the substrate is assumed to affect the K_i value for the inhibitor to an extent determined by the factor α. Under steady-state conditions, the transformation of the rate equation for this system (see the Methods section) yields:

$$(1+[S]/K_m)(v_0-v_i)/v_i[I] = (1+[S]/\alpha K_m)/K_i \quad (15)$$

Thus, the above plot is also linear for the system under consideration, making it decidedly superior to the Hunter and Downs plot. Remarkably, the horizontal intercept is simply $-\alpha$ (Table 1). Thus this important constant is also represented directly. As shown in Figure 1(b) (circles), application of this plot to some previously published data yields an α value of about 5 in that particular case. In addition, this plot represents a generalized description of inhibition in which the relationship between the different kinds of behaviour is clearly illustrated. In this description, competitive and non-competitive inhibition are simply special situations in which α takes on the value of ∞ and 1, respectively. In the case of uncompetitive inhibition, both α and $1/K_i$ approach zero while the apparent inhibition constant (which is actually αK_i; Scheme 1) has a finite value. This plot may therefore be useful also as an instructional tool for explaining the concept of inhibition in biochemical education.

Physical basis of the 'optimized' plot

The virtually ideal properties of the above plot have a relatively simple physical basis. As shown in the Methods section, the mathematical function used can be expressed in terms of the concentrations of the various molecular species as follows:

$$(1+[S]/K_m)(v_0-v_i)/v_i[I] = ([E]_t-[ES]-[E])/[I][E] \quad (16)$$

In essence, this expression is a device for considering the combined concentration of all inhibitor-containing complexes because the numerator of this term ($[E]_t-[ES]-[E]$) is clearly equal to $[EI]+[EIS]$. By plotting this term against $[S]/K_m$, we are exploring how this combined concentration is affected by the substrate concentration. The vertical intercept represents extrapolation to a situation where [EIS] must be zero (because [S] is zero) so that the expression becomes $[EI]/[I][E]$ (which is equal to $1/K_i$). The various types of inhibition are clearly distinguished depending on whether EI and EIS can form or not. The ability of this plot to illustrate the physical basis of different inhibition behaviour further enhances its value for instructional purposes.

Alternative combination plots

Apart from the Hunter and Downs plot and the 'optimized' plot presented above, a further combination plot may be worth mentioning. This plot is based on the following equation for competitive inhibition:

$$[S](V_{max.}-v_i)/v_i = K_m(1+[I]/K_i) \quad (17)$$

It can be seen that a linear plot is obtained when the composite function on the left is plotted against [I]. Because this plot is linear only for competitive inhibition it is clearly inferior to the other combination plots discussed above. Consequently, the illustration of the plot has been omitted in this paper but the slope and intercepts are listed in Table 1. However, this plot has some practical advantages because it uses $V_{max.}$ instead of v_0 (for more details see the Methods section). It may be found frequently that the use of $V_{max.}$ is preferable and in such cases one should also consider alternative versions of the Hunter and Downs plot and the optimized plot formulated in terms of $V_{max.}$ (see the Methods section).

General discussion

If the aim of analysing kinetic data is simply to determine the inhibition constant with maximal accuracy, the more rigorous method is undoubtedly computer curve-fitting using statistical regression analysis. However, as explained in the Methods section, statistical bias should not be a severe problem for the optimized plot presented here if attention is given to the choice of inhibition concentrations. Thus this graphical approach offers a simple alternative especially for situations where high precision is not critical. For example, in studying structure–activity relationships in a series of compounds, the difference in K_i between any two inhibitors is usually large enough (from severalfold to many thousandfold) for minor deviations in their values to be fairly insignificant. On the other hand, the number of assays used to estimate each K_i value becomes an important consideration if many compounds (often 20 or more) need to be surveyed. As graphical procedures, the combination plots presented above are superior in many ways to the alternative Dixon or Lineweaver–Burk plots. First, there is greater freedom in the design of the experiment because it is not necessary to keep either the inhibitor or the substrate concentration constant for any group of assays. Instead, the only consideration in selecting the above parameters is to maximize accuracy. Secondly, combination plots represent a more efficient way of analysis because all data points are directly compared. In essence, one proceeds in this approach to make a secondary replot without the need for a primary plot. Thus each point of the graph derives from a single enzyme assay rather than a series of assays. As explained in the Methods section, the greater efficiency of these plots should result in a significant reduction in the number of assays required. Thirdly, in the optimized plot, the horizontal intercept gives the important coefficient α (as defined in Scheme 1), which is the most useful criterion for determining inhibition behaviour. This approach is arguably more rational and systematic than the interpretation of intersecting behaviour in a family of lines. Finally, because the single linear plot achieves a more compact

presentation, the data from two or more related inhibition studies may often be placed in the same diagram for comparison. Because of the above advantages, the concept of combination plots is being applied to the analysis of other enzyme–ligand interactions in this laboratory.

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