# Relationships between inhibition constants, inhibitor concentrations for 50% inhibition and types of inhibition: new ways of analysing data

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The concentration of an inhibitor that decreases the rate of an enzyme-catalysed reaction by 50%, symbolized $i_{0.5}$, is often used in pharmacological studies to characterize inhibitors. It can be estimated from the common inhibition plots used in biochemistry by means of the fact that the extrapolated inhibitor concentration at which the rate becomes infinite is equal to $-i_{0.5}$. This method is, in principle, more accurate than comparing the rates at various different inhibitor concentrations, and inferring the value of $i_{0.5}$ by interpolation. Its reciprocal, $1/i_{0.5}$, is linearly dependent on $v_0/V$, the uninhibited rate divided by the limiting rate, and the extrapolated value of $v_0/V$ at which $1/i_{0.5}$ is zero allows the type of inhibition to be characterized: this value is 1 if the inhibition is strictly competitive; greater than 1 if the inhibition is mixed with a predominantly competitive component; infinite (i.e. $1/i_{0.5}$ does not vary with $v_0/V$) if the inhibition is pure non-competitive (i.e. mixed with competitive and uncompetitive components equal); negative if the inhibition is mixed with a predominantly uncompetitive component; and zero if it is strictly uncompetitive. The type of analysis proposed has been tested experimentally by examining inhibition of lactate dehydrogenase by oxalate (an uncompetitive inhibitor with respect to pyruvate) and oxamate (a competitive inhibitor with respect to pyruvate), and of cytosolic malate dehydrogenase by hydroxymalonate (a mixed inhibitor with respect to oxaloacetate). In all cases there is excellent agreement between theory and experiment.

Key words: agonist–antagonist relationships, half-inhibition concentration, inhibition kinetics.

## INTRODUCTION

Many drugs exert their biological effects through the metabolic consequences of enzyme inhibition, and inhibition studies have become an essential component of investigations of the mechanisms of action of drugs. However, biochemical and pharmacological practices have developed along somewhat different lines: biochemists usually express the results of inhibition studies in terms of rate equations, and hence in terms of inhibition constants, whereas pharmacologists normally use the concentration of inhibitor that produces 50% inhibition under standard conditions, symbolizing this as $i_{0.5}$ or in some similar way (e.g. [1–5]). This practice is also found in strictly biochemical investigations, and may be especially useful when the objective is to compare the inhibition properties of a series of mutant forms of an enzyme (e.g. [6]).

It is well known that $i_{0.5}$ is not an inhibition constant except in special circumstances (e.g. pp. 110–111 of [7]), and there have been detailed reports on the relationships between $i_{0.5}$ and the corresponding inhibition constants (e.g. [8, 9]). However, it appears not to have been noticed previously that there is a very simple relationship between $i_{0.5}$ and the plots often used to characterize enzyme inhibition and to obtain preliminary estimates of inhibition constants. Specifically, when a straight line is obtained by plotting a function of the rate $v$ against the inhibitor concentration $i$, whether this is the reciprocal rate $1/v$ [10], or the reciprocal rate multiplied by the substrate concentration $a$, i.e. $a/v$ [11], the intercept of the extrapolated line on the $i$ axis is $-i_{0.5}$. This provides a simple and accurate way of estimating $i_{0.5}$, important if it is to be used to calculate the inhibition constant (e.g. [12]).

This relationship has several uses that we shall develop in the present paper: it provides a simple and direct correspondence between the quantities used in biochemistry and pharmacology; it permits $i_{0.5}$ to be estimated from observations at other inhibitor concentrations more simply and accurately than can be done by interpolation (e.g. [3]); it illustrates straightforwardly how the variation of $i_{0.5}$ with the substrate concentration is related to the type of inhibition (competitive, uncompetitive, etc.); and finally, it provides a novel way of analysing data to characterize inhibition, without the statistical problems associated with plotting reciprocal rates on the ordinate.

## EXPERIMENTAL METHODS

### Reagents

Hydroxymalonic acid, oxaloacetic acid, sodium oxalate, sodium oxamate, sodium pyruvate, pig muscle L-lactate dehydrogenase ($M_4$ isoenzyme) and pig heart cytosolic malate dehydrogenase were purchased from Sigma. NADH, disodium salt (grade I) was supplied by Boehringer Mannheim. All other chemicals used were of standard reagent grade.

### Enzyme activity and inhibition experiments

Lactate dehydrogenase and malate dehydrogenase activities were measured in 50 mM sodium phosphate buffer, pH 7.4, with 0.14 mM NADH and variable concentrations of the other substrate, pyruvate or oxaloacetate, in the presence and absence of variable concentrations of inhibitors: oxalate and oxamate for lactate dehydrogenase and hydroxymalonate for malate dehydrogenase. Initial reaction rates were determined by monitoring the change in absorbance at 340 nm due to NADH

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oxidation in a Unicam UV3 spectrophotometer, in cells of path length 1 cm, thermostatically controlled at 30±0.1 °C. A typical kinetic experiment consisted of 20–25 steady-state rates at different combinations of substrate and inhibitor concentrations, as indicated in the Figures, which were measured in quintuplicate.

## THEORY

### Determining $i_{0.5}$ from standard inhibition plots

The common types of inhibition are all special cases of linear mixed inhibition, in which the rate $v$ depends on the concentrations $a$ of substrate and $i$ of inhibitor, in accordance with the following equation:

$$v = \frac{Va}{K_m\left(1+\frac{i}{K_{ic}}\right)+a\left(1+\frac{i}{K_{iu}}\right)} \quad (1)$$

in which $V$ is the limiting rate, $K_m$ is the Michaelis constant, $K_{ic}$ is the competitive inhibition constant and $K_{iu}$ is the uncompetitive inhibition constant. In competitive inhibition $i/K_{iu}$ is negligible, in uncompetitive inhibition $i/K_{ic}$ is negligible, and in pure non-competitive inhibition the two inhibition constants are equal, $K_{ic} = K_{iu}$.

As noted by Dixon in 1953 [10], plots of $1/v$ against $i$ at different $a$ values yield a series of straight lines that intersect at a unique point whose $i$ coordinate is $-K_{ic}$, and hence the competitive inhibition constant can be read directly off such a plot. The plot of $a/v$ against $i$ [11] has a similar appearance, but the $i$ coordinate of the common intersection point yields $-K_{iu}$ instead. In both cases any individual line intersects the $i$ axis at a value $-i_{0.5}$ that directly supplies the value $i_{0.5}$ of the inhibitor concentration that inhibits the enzyme by 50% at the particular $a$ value considered, i.e. it directly provides the parameter of interest to pharmacologists.

This characteristic appears not to have been reported previously, but it is easily demonstrated by reference to eqn (1). To define any line in a plot of $a/v$ against $i$, this equation needs to be transformed as follows:

$$\frac{a}{v} = \frac{K_m}{V}\left(1+\frac{i}{K_{ic}}\right)+\frac{a}{V}\left(1+\frac{i}{K_{iu}}\right) \quad (2)$$

from which it follows that $a/v = 0$ when

$$K_m\left(1+\frac{i}{K_{ic}}\right)+a\left(1+\frac{i}{K_{iu}}\right) = 0 \quad (3)$$

i.e. when

$$i = -\frac{K_m+a}{\frac{K_m}{K_{ic}}+\frac{a}{K_{iu}}} \quad (4)$$

However, if the minus sign is omitted and the resulting positive value of $i$ is substituted into eqn (1) the corresponding rate is seen to be equal to half the uninhibited rate $v_0$:

$$v = \frac{Va}{2(K_m+a)} = 0.5v_0 \quad (5)$$

An alternative way of arriving at the same result is to solve for $i$ in eqn (1) after replacing the left-hand side with the expression in eqn (5): this yields eqn (4) with the minus sign replaced by a plus sign.

As this derivation is based on the equation for mixed inhibition, it also applies to all the special cases of mixed inhibition, i.e. to competitive, pure non-competitive and uncompetitive inhibition. In fact, the simple geometrical considerations illustrated in Figure 1 show that corresponding relationships apply much more generally, to any straight-line plot that yields a positive intercept on the ordinate and a negative intercept on the abscissa. Not only does it apply equally well when $a/v$ is replaced throughout by $1/v$ [10], but it also applies to kinetic behaviour other than inhibition. For example, if the abscissa variable is $1/x$, the reciprocal of an activator concentration $x$, then the intercept is $-1/x_{0.5}$, where $x_{0.5}$ is the activator concentration that gives a rate equal to half that at a saturating concentration of activator. However, the most useful applications of such relationships are likely to lie in the area of inhibition studies, and so we shall only consider these in the present paper.

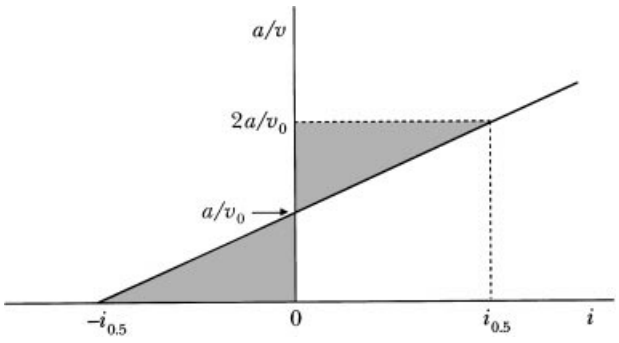


**Figure 1 Simple geometrical proof**

If a plot of $a/v$ against $i$ gives a straight line, and $i_{0.5}$ is the value of $i$ that gives a value of $a/v$ that is double the value $a/v_0$ at $i = 0$ (i.e. $v = 0.5v_0$), then the intercept of the line on the abscissa is equal to $-i_{0.5}$. This follows in an obvious way from the fact that the two shaded triangles in the Figure must be congruent, which itself follows from elementary principles of geometry.

### Cooperativity

In practice, binding of an inhibitor is often cooperative, with a dependence that can be expressed with adequate accuracy by the Hill equation (e.g. [4, 5]). In this case, the inhibitor concentration and the inhibition constants in eqn (1) can be raised to the power $h$, the Hill coefficient, which is approximately constant and typically has a value in the range between 1 and 4 for cooperative binding of the inhibitor:

$$v = \frac{Va}{K_m\left(1+\frac{i^h}{K_{ic}^h}\right)+a\left(1+\frac{i^h}{K_{iu}^h}\right)} \quad (6)$$

As the same replacements can be done in all of the algebra that follows from eqn (1), all of the same results apply provided that $i$ is also replaced by $i^h$ when the plots are made. In particular, as eqn (5) does not contain $i$, $K_{ic}$ or $K_{iu}$, it is unaffected by cooperativity; so when $a/v$ is plotted against $i^h$ the value of $-i_{0.5}^h$ is given by the intercept on the abscissa of the resulting straight line.

### Variation of $i_{0.5}$ with substrate concentration for different types of inhibition

Once it is established that the abscissa intercepts in plots of $a/v$ or $1/v$ against $i$ provide the values of $i_{0.5}$ at the different values of $a$, it becomes clear that the same plots provide a simple qualitative rationale for the way $i_{0.5}$ varies with the substrate

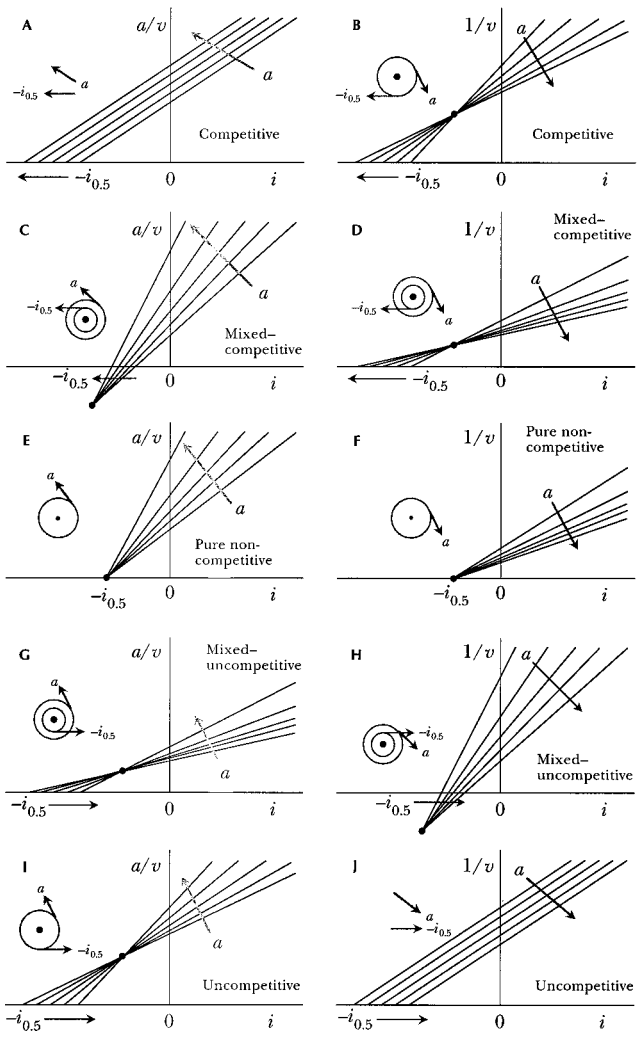


**Figure 2 Dependence of $i_{0.5}$ on substrate concentration for different kinds of inhibition**

The left-hand column (**A**, **C**, **E**, **G**, **I**) shows plots of $a/v$ against $i$, and the right-hand column (**B**, **D**, **F**, **H**, **J**) shows plots of $1/v$ against $i$ for (**A**, **B**) competitive, (**C**, **D**) mixed (predominantly competitive), (**E**, **F**) pure non-competitive, (**G**, **H**) mixed (predominantly uncompetitive) and (**I**, **J**) uncompetitive inhibition. In every case, the intercept of an individual line on the abscissa provides the value of $-i_{0.5}$ for the particular $a$ value corresponding to the line plotted. The direction in which $a$ increases is shown for each set of lines by a grey arrow, and a corresponding grey arrow along the abscissa axis shows whether $-i_{0.5}$ increases or decreases as $a$ increases. Note that because of the minus sign, $i_{0.5}$ itself changes in the opposite direction from that indicated by the arrow. These directions of change can be rationalized by regarding a change in $a$ as a rotation of the plot around the common intersection point, as indicated by the symbols in the second quadrant of each plot.

concentration in the different kinds of inhibition. As illustrated in Figure 2, whenever an inhibition plot consists of a set of lines intersecting at a unique point, the effect of changing the substrate concentration is roughly equivalent to rotating the line about the common intersection point, and the direction in which $i_{0.5}$ changes is determined by the sense of the rotation. In all cases, the rotation is anticlockwise for increasing $a$ in the plot of $a/v$ against $i$ and clockwise in the plot of $1/v$ against $i$, and the direction in which $i_{0.5}$ changes is determined by whether the centre of rotation is above or below the abscissa axis: in competitive inhibition or mixed inhibition with a predominantly competitive component it is below the axis in the plot of $a/v$ against $i$ and above it in the plot of $1/v$ against $i$, so $i_{0.5}$ increases with $a$; in uncompetitive inhibition or mixed inhibition with a predominantly uncompetitive component it is above the axis in the plot of $a/v$ against $i$ and below it in the plot of $1/v$ against $i$, so $i_{0.5}$ decreases when $a$ increases; in pure non-competitive inhibition it is on the axis in both plots, and so $i_{0.5}$ is then independent of $a$. Note that this last case, the least common in studies of real enzymes (contrary to the impression given by some textbooks), is the only one in which $i_{0.5}$ is equal to the inhibition constant.

### Assessing the competitive component from plots of *a/v* against *i*

The plot of $a/v$ against $i$ provides a simple way of estimating $K_{iu}$ (as minus the $i$ coordinate of the common intersection point), but no correspondingly simple way of estimating $K_{ic}$. Moreover, although it discriminates clearly between most types of inhibition, the plots for uncompetitive inhibition and mixed inhibition with a predominant uncompetitive component are not immediately distinguishable (Figures 2G and 2I). This might appear to be of no importance, given that the complementary plot of $1/v$ against $i$ does provide this information (Figures 2H and 2J). However, as in the better known case of the double-reciprocal plot, using $1/v$ as ordinate variable distorts the appearance of the experimental error to such a degree that the plot gives a quite false impression of the best-fit model unless experimental error is essentially absent, but the corresponding distortion implied by use of $a/v$ as ordinate variable is much less [13,14]. The value of $K_{ic}$ is in principle calculable from the $a/v$ coordinate of the common intersection point in the plot of $a/v$ against $i$, which is $K_m(1-K_{iu}/K_{ic})/V$, but in practice the $a/v$ coordinate of the common intersection point often cannot be estimated accurately enough for this to be useful. This is illustrated in Figure 3 for two values of $K_{iu}/K_{ic}$.

A simple construction in the plot allows the value of $K_{ic}$ to be projected into the $i$ dimension and made more visible. The first step is to draw a straight line that intersects the axes at values given by the common intersection point of the primary line, i.e. it cuts the $i$ axis at $-K_{iu}$ and the $a/v$ axis at $K_m(1-K_{iu}/K_{ic})/V$. This line is given by the following equation:

$$\frac{a}{v}=\frac{K_m}{V}\left(1-\frac{K_{iu}}{K_{ic}}\right)\left(1+\frac{i}{K_{iu}}\right) \quad (7)$$

The next step is to determine where this line intersects the primary line that corresponds to $a=K_m$ (shown in black in Figure 3A, the other primary lines being shown in grey), which is given by the following equation:

$$\frac{a}{v}=\frac{K_m}{V}\left(2+\frac{i}{K_{ic}}+\frac{i}{K_{iu}}\right) \quad (8)$$

Equating the expressions in eqns (7–8) and solving for $i$ shows that this secondary intersection point occurs at an $i$ value of minus the mean inhibition constant, i.e. at $i=-\bar{K}_i=-(K_{iu}+K_{ic})/2$. Reflecting the value of $K_{iu}$ about this value then gives $K_{ic}$.

Figure 3(A) illustrates this construction in the case where the inhibition is predominantly competitive ($K_{ic}<K_{iu}$), and Figure 3(B) shows the case where it is predominantly uncompetitive ($K_{iu}<K_{ic}$).

### Use of $1/i_{0.5}$ as the plotted variable in secondary plots

The quantitative expressions for $i_{0.5}$ as functions of $a$ were derived by Cheng and Prusoff [8] for competitive, uncompetitive

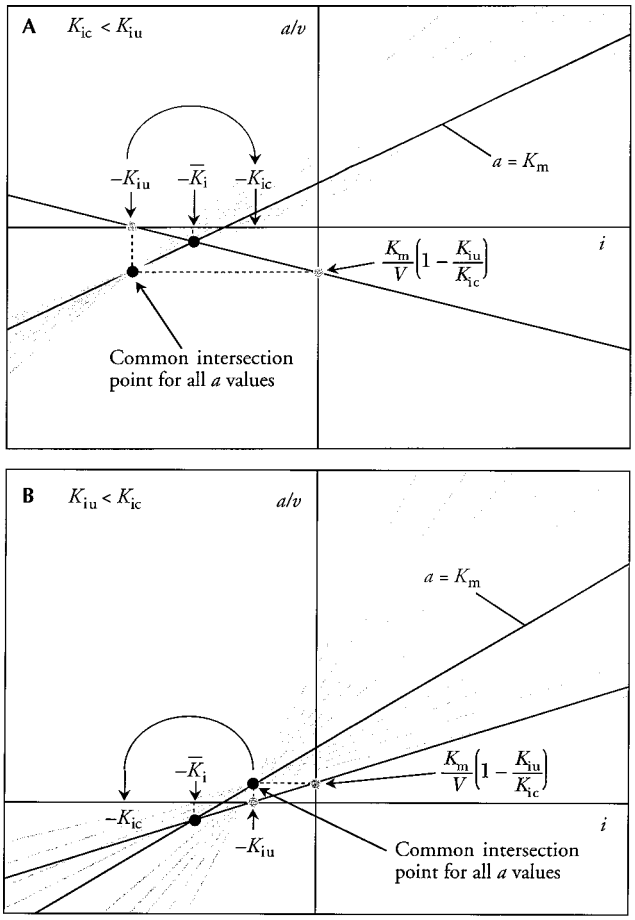


**Figure 3 Determination of the competitive inhibition constant from plots of *a*/*v* against *i***

(**A**) When $K_{ic} < K_{iu}$, (**B**) when $K_{iu} < K_{ic}$. The *a*/*v* coordinate of the common intersection point of the primary lines contains the value of $K_{ic}$, but this point usually lies too close to the *i* axis for this to provide a useful way of determining it. However, if a line is drawn that intersects the axes at values corresponding to the two coordinates of the common intersection point, then this line intersects the primary line corresponding to $a = K_m$ (shown in black, the other primary lines being drawn in grey) at a point with $i = -(K_{ic} + K_{iu})/2$, from which the value of $K_{ic}$ may readily be found by reflecting the value of $K_{iu}$ around it.

and mixed inhibition. These can be used directly for measuring inhibition constants (e.g. [12]), though often they serve just to emphasize that $i_{0.5}$ values are not themselves inhibition constants (e.g. [15]). From eqns (4–5) it follows that the general expression for $i_{0.5}$ in mixed inhibition is as follows:

$$i_{0.5} = \frac{K_m + a}{\frac{K_m}{K_{ic}} + \frac{a}{K_{iu}}} = \frac{1 + \frac{a}{K_m}}{\frac{1}{K_{ic}} + \frac{1}{K_{iu}} \cdot \frac{a}{K_m}} \quad (9)$$

The right-hand form of this expression emphasizes that in a series of experiments at a constant value of $a/K_m$ there is a fixed relationship between $i_{0.5}$ and the inhibition constants. This is useful for studying inhibition of variants of an enzyme that differ in their saturation characteristics for the substrate; for example, Moukil et al. [6] studied inhibition of a series of mutant forms of hexokinase D by various competitive inhibitors, and eliminated effects due to different kinetics with respect to glucose by studying each enzyme/inhibitor combination at the glucose concentration that gave half-maximal activity in the absence of inhibitor.

In the case of competitive inhibition eqn (9) assumes a particularly useful and simple form:

$$i_{0.5} = K_{ic}\left(1 + \frac{a}{K_m}\right) \quad (10)$$

from which it follows that a plot of $i_{0.5}$ against $a$ is a straight line with slope $K_{ic}/K_m$ and intercept $-K_{ic}$ on the abscissa. However, although this is useful, as $K_{ic}$ is the constant that does not emerge in a simple and direct way from the plot of $a/v$ against $i$, it is limited by the fact that other types of inhibition (other than the trivial case of pure non-competitive inhibition, with no dependence at all) do not give straight lines when $i_{0.5}$ is plotted against $a$.

We have therefore examined the relationship between $i_{0.5}$ and the normalized rate of the uninhibited reaction, $v_0/V$, the "relative velocity" in the terminology of Segel ([7], pp. 37–38) and of Dixon and Webb [16]. This quantity is normally known by direct measurement, but in any case it can readily be calculated from the Michaelis–Menten equation, which is easily transformed to show $a$ as a function of $v_0$:

$$a = \frac{K_m v_0}{V - v_0} \quad (11)$$

After substitution of this expression for $a$ into eqn (9), rearrangement of the resulting equation provides the following expression for $1/i_{0.5}$ in terms of the relative velocity $v_0/V$:

$$\frac{1}{i_{0.5}} = \frac{1}{K_{ic}} + \frac{v_0}{V}\left(\frac{1}{K_{iu}} - \frac{1}{K_{ic}}\right) \quad (12)$$

Thus a plot of $1/i_{0.5}$ against $v_0/V$ is a straight line with slope $(1/K_{iu} - 1/K_{ic})$ and intercept $1/K_{ic}$ on the ordinate. In combination with the plot of $a/v$ against $i$ (left-hand column of Figure 2), such a plot discriminates clearly between all the different types of linear inhibition, and supplies the values of both inhibition constants, as illustrated in Figure 4. Furthermore, it provides a direct link between the quantities normally measured in pharmacology and the biochemical characteristics of the inhibition.

## EXPERIMENTAL EXAMPLES

### Inhibition of lactate dehydrogenase

The practical application of the linear dependence of $1/i_{0.5}$ on $v_0/V$ may be illustrated with some results for the inhibition of lactate dehydrogenase by oxalate (Figure 5A), an uncompetitive inhibitor with respect to pyruvate [17], and oxamate (Figure 5B), a competitive inhibitor with respect to pyruvate [18]. In both cases, the plots in the insets agree closely with the corresponding theoretical cases shown in Figure 4. For oxamate, the parallel lines in the primary plot indicate without additional information that the inhibition is competitive, but for oxalate the primary plot fails to distinguish between mixed inhibition with a predominant uncompetitive component and pure uncompetitive inhibition. However, the secondary plot shows clearly that it is uncompetitive, as the straight line goes through the origin, indicating that the intercept $1/K_{ic}$ is zero.

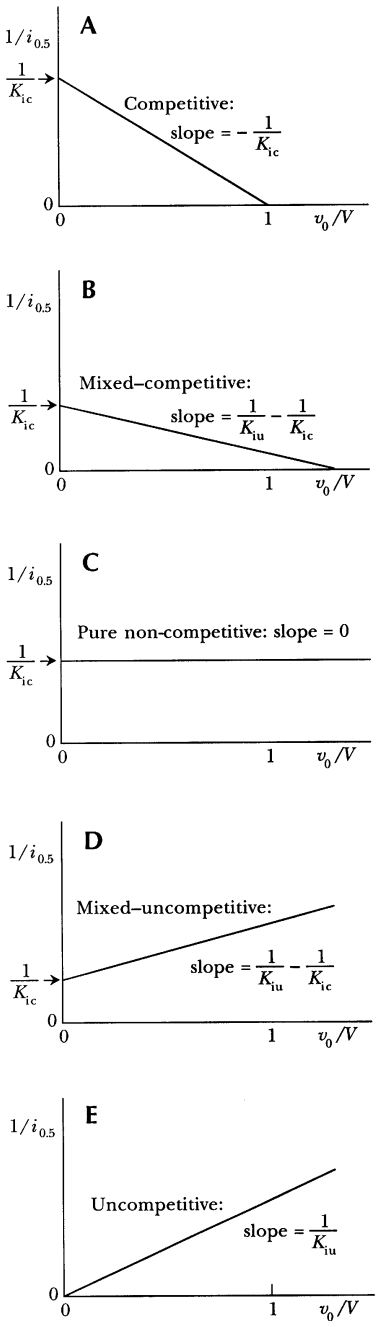


**Figure 4 Determination of the inhibition type and the inhibition constants from the dependence of $1/i_{0.5}$ on $v_0/V$**

If $1/i_{0.5}$, which may be obtained from the $i$ intercepts in plots of $a/v$ or $1/v$ against $i$ (see Figure 2), is plotted against the relative rate $v_0/V$ of the uninhibited reaction, the result is a straight line, regardless of the type of inhibition, but the appearance of the plot is different in the five cases, and thus allows the type of inhibition to be identified immediately. (**A**) When the inhibition is competitive there is an intercept of 1 on the abscissa; (**B**) when it is mixed, but predominantly competitive, this intercept is greater than 1; (**C**) when it is pure non-competitive, the line is horizontal and there is no intercept on the abscissa; (**D**) when it is mixed but predominantly uncompetitive there is a negative intercept; and (**E**) when it is uncompetitive the line passes through the origin. The intercept on the ordinate provides $1/K_{ic}$ in all cases, and $1/K_{iu}$ can be calculated in all cases by subtracting $1/K_{ic}$ from the slope, which is always $1/K_{iu} - 1/K_{ic}$.

### Inhibition of malate dehydrogenase

An example of a different kind is provided by the inhibition by hydroxymalonate of the reduction of oxaloacetate catalysed by the cytosolic isoenzyme of malate dehydrogenase. The results, shown in Figure 6, indicate clearly that this inhibition is mixed with the competitive component predominant, as expected from other experiments (A. Cortés, unpublished work).

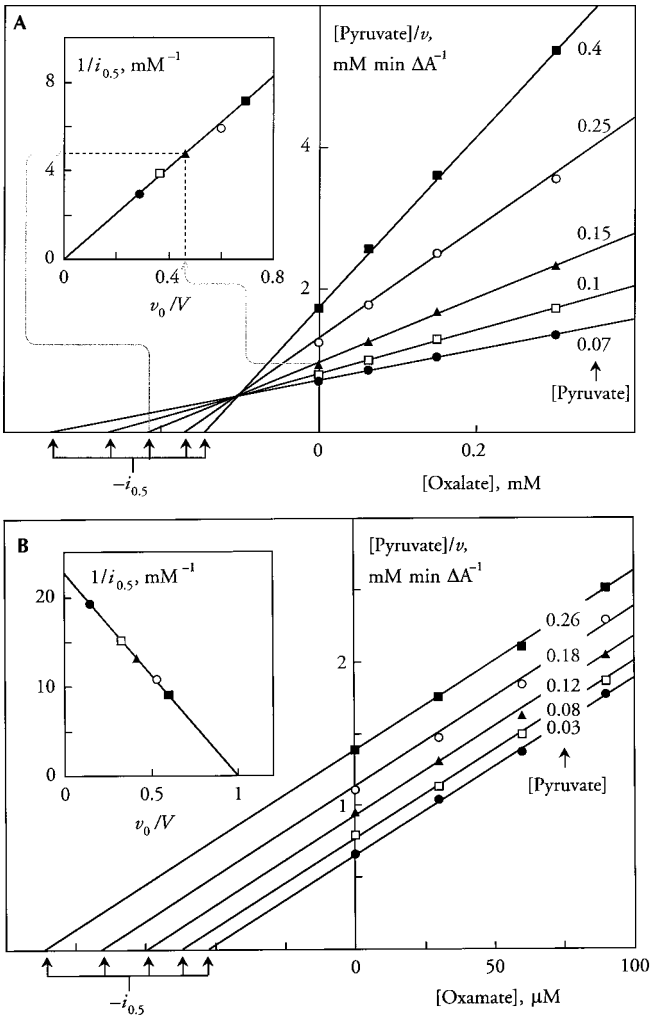


**Figure 5 Inhibition of lactate dehydrogenase by (A) oxalate and (B) oxamate**

In each case, the intercepts on the abscissa of the primary plots of [pyruvate]/$v$ against inhibitor concentration provide the values of $-i_{0.5}$ used to obtain the plots of $1/i_{0.5}$ against $v_0/V$ shown in the insets; this relationship is shown more explicitly for the case of 0.15 mM pyruvate with inhibition by oxalate. In all cases, the symbols in the secondary plot are the same as those used in the corresponding primary plot, which are labelled with the pyruvate concentrations used (in mM). The line plotted in the inset to (**A**) passes through the origin and shows that oxalate acts as an uncompetitive inhibitor with respect to pyruvate (cf. Figure 4E), whereas the corresponding line in the inset to (**B**) intersects the abscissa axis at a value of 1, showing that oxamate acts as a competitive inhibitor (cf. Figure 4A).

## DISCUSSION

Although the most usual biochemical practice is to characterize enzyme inhibition in terms of inhibition constants, the use of $i_{0.5}$ values is by no means rare, and in pharmacological practice may even predominate. It is important, therefore, to recognize that inhibition constants and $i_{0.5}$ values can be interconverted in a very simple way, using the fact that the extrapolated inhibitor concentration at which the rate becomes infinite is equal to minus the value of $i_{0.5}$ for all of the common linear inhibition

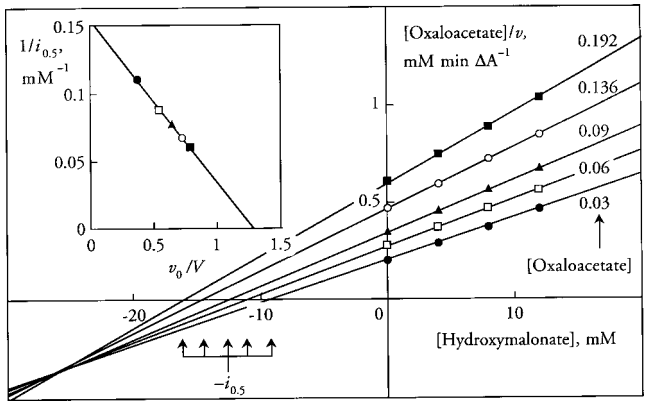


**Figure 6 Inhibition of cytosolic malate dehydrogenase by hydroxymalonate**

The plots are constructed in the same way as in Figure 5, the lines of the primary plots being labelled with the concentrations of the substrate oxaloacetate (in mM). As the line in the secondary plot shown in the inset intersects the abscissa axis at a value greater than 1, it shows that hydroxymalonate acts as a mixed inhibitor with a predominantly competitive component (cf. Figure 4B).

types. These types of inhibition yield straight-line plots of $a/v$ against $i$, as illustrated theoretically in Figures 1 and 2 and experimentally in Figures 5 and 6. In all of these common cases, $1/i_{0.5}$ varies linearly with the relative rate $v_0/V$ of the uninhibited reaction, the exact nature of the dependence defining the type of inhibition unambiguously, as illustrated theoretically in Figure 4 and experimentally in Figures 5 and 6.

In a valuable discussion of how effects of antagonists are analysed, Barlow et al. [19] pointed out that antagonists can only be studied in the presence of agonists, and argued that this made it natural to express an antagonist effect as a percentage reduction in the corresponding agonist effect. There is an obvious parallel here with the effects of enzyme inhibitors, which are likewise manifest only in the presence of substrates, and much of the analysis is likewise parallel. It follows therefore that methods developed for studying inhibitors should be equally applicable to the study of antagonists (e.g. [20,21]).

The analysis we have presented shows first how $i_{0.5}$ can be determined without the need for interpolation, and secondly how the resulting estimate can be used to characterize the inhibition. In this way we hope to have brought biochemical and pharmacological practice closer together, and to have made it easier to relate the results obtained in the different ways.

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