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COMMENTARY

ENZYME KINETICS IN RELATION TO ENZYME INHIBITORS

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A LARGE number of pharmacologically important compounds have been found to act as enzyme inhibitors and a kinetic study of the inhibitory process can provide important information on the potency of the compound and on its mode of interaction with the enzyme. Kinetic studies are generally simple to perform and interpret but their misuse can easily result in erroneous and misleading conclusions. The purpose of this paper is to emphasize the points of kinetic theory that are most frequently misapplied. Since any study of the kinetics of enzyme inhibition will necessarily involve a study of the kinetics of the enzyme in the absence of inhibitor, the first part of this paper deals with such systems. The material discussed in this paper is in no way original and neither does it represent a complete survey of the kinetics of simple enzyme systems; such treatments may be found in a number of textbooks.[1–4] I have chosen to take specific cases in which the enzyme kinetic studies have been frequently misapplied and to illustrate these with reference to the simplest single substrate reaction system. I do not intend to list or give examples of specific errors that have appeared in the literature since the purpose of this paper is not to criticize but to provide information which I hope will lead to a more constructive use of enzyme kinetic studies.

THE TIME-COURSES OF ENZYME-CATALYSED REACTIONS

The time-course of an enzyme-catalysed reaction can be followed by determining the rate of substrate disappearance or product appearance. The curve obtained is usually linear at first but falls off at greater time intervals. There are a number of possible causes for such a decline in rate and these have been discussed by Dixon and Webb (Chapter 4).[1] Some of the most common causes are listed below.

(i) A fall in the substrate concentration may cause the velocity to decrease as the enzyme becomes progressively less saturated. This effect will be particularly pronounced if the initial substrate concentration is not sufficient to saturate the enzyme.

(ii) The approach to the equilibrium position of a reversible reaction will cause a decrease in velocity as the rate of the forward reaction becomes balanced by the increasing rate of the backward reaction.

(iii) The products of the reaction may be inhibitors of the enzyme so that the reaction may be inhibited as their concentration rises.

(iv) A component of the reaction mixture may be unstable under the assay conditions used and may be progressively decaying. If, for example, this component is

the enzyme itself the progressive decline in enzyme concentration, possibly by denaturation, will cause a progressive decrease in velocity.

(v) The initial assay conditions may change during the reaction. Thus if an enzyme reaction which produces H^+ or OH^- ions is assayed in a poorly buffered medium the pH of the assay medium could change during the reaction and if this change were away from the pH optimum a decline in velocity would be seen.

A decline in velocity could of course, be due to more than one of these factors operating simultaneously. Whatever the cause of this fall-off it is important to measure the velocity before it is appreciable. Such conditions should, of course, apply at zero time and therefore the velocity of an enzyme reaction should be measured by drawing a tangent to the initial linear portion of the progress curve. A useful "rule of thumb" procedure is to keep measurements down to a time period in which less than 20 per cent of the substrate has been used (or product has been formed) although this may be too great a change in some cases.

Occasionally the progress curve of the reaction may show an initial lag followed by a phase of acceleration. This can occur with some complex enzymes (see e.g. Ref. 5) but it is more frequently an artefact of the assay system used. Accelerating progress curves are frequently observed when a second enzyme is used as part of the assay system[6] and often can be minimized by increasing the concentration of the second enzyme or by adjusting the assay conditions. If such an acceleratory phase is incomplete before the reaction velocity begins to fall off it will, of course, be impossible to obtain a meaningful measure of the true initial rate.

A number of enzyme-catalysed reactions are difficult to assay continuously and "stopped assays" are commonly used in these cases. In such assay systems a mixture containing the enzyme and substrates is incubated and samples are withdrawn after different time-intervals for the determination of substrate or product concentration after stopping the reaction in some way. Such assays are frequently tedious and many workers use a determination made after a single time interval as a measure of enzyme activity. If the rate of reaction is not constant for this time such results will, of course, have no validity and it is essential that time-courses are determined under all the conditions used so that the period for which the appearance of product is linear with time can be established.

THE EFFECT OF ENZYME CONCENTRATION

Since the enzyme is acting as a catalyst and is normally present at a considerably lower concentration than that of the substrate one would expect that the initial velocity of an enzyme-catalysed reaction would be proportional to the enzyme concentration and this is indeed so in the vast majority of cases. Exceptions which are not artefacts of the assay system can occur if there are reversible inhibitors or activators in the enzyme solution or irreversible inhibitors in the assay medium (see Ref. 1, Chapter 4). Since the enzyme concentration may be varied during kinetic studies or when comparisons are made between different sources it is essential that linearity be established experimentally.

It is a fundamental assumption of Michaelis kinetic theory (either equilibrium or steady-state) that the concentration of free substrate is unaffected by the formation of the enzyme–substrate complex (i.e. total substrate (s) = free substrate). This is

usually the case in *in vitro* studies since the enzyme concentration (e) is considerably less than the total substrate concentration. And the assumption that $e \ll s$ is frequently considered to be an essential postulate of Michaelis theory.

Under conditions where the substrate concentration is not much greater than the enzyme concentration the equation for the variation of initial velocity with total substrate concentration is considerably more complicated than the Michaelis equation (see Ref. 3, Chapter 3), although the variation of initial velocity with the concentration of free (i.e. unbound) substrate will still obey the simple Michaelis equation under these conditions. It has been shown that for several enzymes their concentrations within the cell are of the same order of magnitude as that of their substrates[7] and therefore kinetic measurements made *in vitro* when $e \ll s$ will be inapplicable. The Michaelis kinetic theory can still be applied if the free substrate concentration can be estimated. If the enzyme under study is part of a metabolic pathway which is in an equilibrium or a steady-state such that the concentrations of all the intermediates remain constant the activities of the enzymes which operate before the one under study may maintain the concentration of the free substrate for this enzyme essentially constant despite its binding to and removal by the enzyme under study.

K_m AND AFFINITY

The steady-state kinetic equation for the simple one substrate enzyme reaction:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} E + \text{Products}$$

was shown by Briggs and Haldane (see any textbook of biochemistry) to take the following form

$$v = \frac{V.s}{K_m + s}$$

where v is the initial velocity of the reaction, s is the substrate concentration, $V = k_{+2}e$ and represents the maximum velocity that the reaction can reach at that fixed concentration of enzyme (e) and K_m (the Michaelis constant) $= (k_{-1} + k_{+2})/k_{+1}$.

Under the limiting conditions when $k_{-1} \gg k_{+2}$, that is, when the breakdown of the *ES* complex to give products is relatively so slow that the free enzyme and substrate remain in equilibrium with the *ES* complex, the expression for K_m simplifies to k_{-1}/k_{+1}. This is the dissociation constant of the enzyme–substrate complex and is sometimes designated K_s. Under these conditions K_m is an inverse measure of the affinity of the enzyme for its substrate. The form of the Michaelis equation is the same regardless of whether or not this important simplification occurs and, unless it can be shown that these equilibrium conditions exist, any discussion of K_m values in terms of affinities will be valueless.

Since the K_m value represents the substrate concentration which will give rise to half maximum velocity, this constant can provide important information on the efficiency with which an enzyme will function with the steady-state substrate concentrations which it normally encounters in the cell. In the case of enzyme reactions that have two or more substrates the K_m value for one substrate is frequently dependent on the

concentration of the other substrate (or substrates). The apparent K_m value for one substrate may either increase or decrease with increasing concentrations of the other substrate. This may have profound effects on the efficiency with which an enzyme will utilize a given substrate within the cell[8] and because of this the concentrations of all the other substrates must be stated when the K_m value for one substrate is given. The true K_m value of a substrate for such a system is the value determined when all other substrates are present in saturating concentrations.

SPECIFIC ACTIVITIES

Since the velocity of an enzyme reaction is usually proportional to the enzyme concentration the expression v/e should be a constant and should provide a strictly quantative measure of the enzyme activity. The most commonly used method of expressing enzyme activity is the specific activity and one unit is defined as the amount of enzyme in mg which will catalyse the transformation of 1 μmole of substrate/min and, thus, the specific activity of an enzyme sample will be the number of these units present in 1 mg of the preparation (see Ref. 1, Chapter 2). The specific activity provides a useful means of comparing the activities of different enzymes and of different preparations of the same enzyme perhaps from different organs and species. It is only a constant, however, under constant conditions of substrate concentration, pH and temperature. The standard conditions recommended by the Enzyme Commission of the International Union of Biochemistry are saturing concentrations of substrate and a temperature of 30°. The pH at which the specific activity was determined should always be stated. When the molecular weight of the enzyme is known the activity can be expressed as the *molecular activity* which is defined as the number of molecules of substrate transformed per minute per molecule of enzyme.

TABLE 1. SPECIFIC ACTIVITIES OF AN ENZYME DETERMINED WITH FOUR SUBSTRATES AT TWO FIXED CONCENTRATIONS

Substrate	K_m (mM)	Sp. act. (units/mg) At 1·0 mM s	Sp. act. (units/mg) At 0·1 mM s
S_1	1·0	5·0 (55)	0·91 (18)
S_2	0·5	6·67 (73)	1·67 (33)
S_3	0·2	8·33 (92)	3·3 (66)
S_4	0·1	9·1 (100)	5·0 (100)

The maximum velocity of the enzyme is 10 units/mg for all substrates. The figures in brackets are the activities expressed as a percentage of that towards S_4 in each case.

In many cases workers have compared the specificities of enzymes by determining the specific activities of the enzyme toward a number of substrates at an arbitrarily fixed constant concentration of each of these substrates. Such values can be very confusing as is shown in Table 1 in which the specific activities of an enzyme with four substrates which have different K_m values but the same V are given as measured at

two different fixed substrate concentrations. The variations of activities towards the different substrates can be seen to have little similarity beyond the fact that they increase from S_1 to S_4 in both cases. If, on the other hand, the specific activities were all determined at their K_m concentrations (or some fixed fraction or multiple of the K_m) in each case all the substrates would be transformed at the same rate since the maximum velocity is the same for each substrate in this example. Thus specific activities determined for a fixed concentration of several substrates can provide little useful evidence on the specificity of an enzyme.

REVERSIBLE INHIBITION

In the simplest case the reaction between the inhibitor and the enzyme can be represented by the scheme:

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EI.$$

The dissociation constant for this reaction (k_{-1}/k_{+1}) is designated the inhibitor constant, K_i. It is usually characteristic of such reactions that inhibition shows no time-dependence, being a reaction in which no stable bond is formed between enzyme and inhibitor, and in which the enzyme and inhibitor react rapidly. The inhibition can be reversed by dialysis or gel-filtration or simply by dilution of the enzyme–inhibitor mixture.

Simple reversible inhibitors are classified into a number of types according to the effects that they have on the Michaelis parameters for the reaction. An inhibitor that increases K_m without affecting V is termed a competitive inhibitor whilst one which decreases V without affecting K_m is termed a noncompetitive inhibitor. In addition there are two classes of inhibition in which both K_m and V are affected: uncompetitive inhibitors cause an equal decrease in K_m and V which will give rise to a family of parallel lines when reciprocal plots ($^1/v$ against $^1/s$) are constructed at a series of fixed concentrations of the inhibitor, mixed inhibition occurs when K_m and V are affected to different extents. Mixed inhibition will lead to a family of lines in reciprocal plots that intersect above or below the $^{-1}/s$ axis. Some workers do not make any distinction between noncompetitive and mixed inhibition and use the former term to cover both types. It should be emphasized that a specific type of inhibition is defined solely by the effect that the inhibitor has on the Michaelis parameters of the reaction without any reference to the mode of interaction of the inhibitor with the enzyme. Indeed even in a simple single substrate reaction there may be more than one mechanism that can give rise to a given type of inhibition.[9,10]

The inhibitor constant, which is the dissociation constant of the enzyme species to which it binds, is independent of the substrate concentration. It may be determined from reciprocal plots carried out at a series of fixed concentrations of the inhibitor[1,11] or from Dixon plots (see Ref. 1, Chapter 8). In pharmacological work I_{50} values have all too frequently been used. These values represent the inhibitor concentration which is necessary to reduce the measured activity by one half at some fixed substrate concentration. I_{50} values will therefore depend on the nature of the substrate used and on its concentration whereas K_i values should be independent of these. The I_{50} values which would be found for an inhibitor when several substrates with different K_m values are

used at fixed concentrations are shown for the simple case of a competitive inhibitor in Table 2 from which it can be seen that although K_i is an absolute entity, being independent of the concentration and K_m value of the substrate the I_{50} value is dependent on both of these.

TABLE 2. CALCULATED I_{50} VALUES FOR A COMPETITIVE INHIBITOR

Substrate	K_m (mM)	I_{50} at $s = 1{\cdot}0$ mM (mM)	I_{50} at $s = 0{\cdot}1$ mM (mM)
S_1	1·0	2·0	1·1
S_2	0·5	3·0	1·1
S_3	0·2	6·0	1·5
S_4	0·1	10·9	2·0

The maximum velocity of the enzyme is 10 units/mg and the K_i value for the competitive inhibitor is 1·0 mM.

The effects of an inhibitor are sometimes expressed simply in terms of the amount of inhibition that is obtained with a fixed concentration of both substrate and inhibitor. Such values are, of course, of little significance but a number of workers have interpreted the observation that different degrees of inhibition are obtained when different substrates are used as consituting evidence for the existence of more than one enzyme acting on these substrates. This of course is not the case and a variation of the degree of inhibition with substrates having different K_m values would be expected. For the system shown in Table 2 the amounts of inhibition obtained at 1·0 mM substrate and 1·0 mM inhibitor concentrations would be 33 per cent for S_1, 25 per cent for S_2, 14 per cent for S_3 and 8·5 per cent for S_4.

IRREVERSIBLE INHIBITION

The reaction of an irreversible inhibitor with an enzyme can be represented by

$$E + I \xrightarrow{k} E{-}I.$$

This is a second-order reaction and will obey the second-order rate equation

$$\frac{\mathrm{d}[E{-}I]}{\mathrm{d}t} = k(e - [E{-}I])\,(i - [E{-}I])$$

where e, i and $[E{-}I]$ represent the total concentrations of enzyme, inhibitor and the $E{-}I$ compound respectively. This expression integrates to give

$$k = \frac{1}{t([E{-}I])} \ln \frac{i(e - [E{-}I])}{e(i - [E{-}I])}.$$

There are a number of ways in which the rate constant may be determined by using either the integrated or differential form of this equation (see Ref. 2, Chapter 2).

There are two important simplifying conditions which can be used to aid the analysis of second-order reactions (see Ref. 2, Chapter 2; and Ref. 12,).

(i) If the initial concentrations of the two reactants are equal ($e = i$) the second-order rate equation simplifies to give

$$\frac{d[E{-}I]}{dt} = k(e - [E{-}I])^2$$

which integrates to give

$$k = \frac{1}{t} \frac{[E{-}I]}{e(e - [E{-}I])}$$

which can be rearranged to give

$$kt = \frac{1}{(e - [E{-}I])} - \frac{1}{e}$$

and thus a graph of $1/(e - [E{-}I])$ against time will be linear and will have a slope of k.

(ii) Under conditions in which the inhibitor is present in a large enough excess over that of the enzyme for its free concentration to be essentially unchanged during the course of the reaction the system can be written as

$$E \xrightarrow{k.i} E{-}I$$

which is a *pseudo* first-order reaction which will obey the equation

$$\frac{d[E{-}I]}{dt} = k'(e - [E{-}I])$$

where $k' = k.i.$

Integration yields

$$k' = \frac{1}{t} \ln \frac{e}{(e - [E{-}I])}$$

which can be rearranged to give

$$k't = \ln e - \ln (e - [E{-}I])$$

Thus a graph of $\ln (e - [E{-}I])$ against t will be linear and will have a slope of k'. *Pseudo* first-order conditions frequently apply in the study of irreversible inhibition of enzymes.

The rate constant for the reaction is the only kinetic constant which can be obtained from an irreversible reaction.

All too frequently authors attempt to calculate K_i values from data obtained with an irreversible inhibitor. Such calculations are meaningless since the value obtained will obviously depend on the amount of enzyme present and the time period for which the enzyme and inhibitor are incubated together. Unfortunately if reciprocal plots are constructed of rates obtained in the presence of a series of fixed concentrations of the inhibitor after the mixture has been incubated for a sufficient time for complete reaction in each case the inhibition pattern obtained will frequently appear to be non-competitive; that is, the maximum velocity will be decreased but the K_m will be unchanged. This "non-competitive" inhibition bears no relationship to true

non-competitive inhibition but reflects the situation in which the amounts of inhibitor added have each simply removed a proportion of the enzyme from the system by inactivating it. Thus at increasing inhibitor concentrations the amount of enzyme in solution will progressively decrease giving a decrease in V but the active enzyme that remains will be unaffected so that K_m will remain constant. Similar "non-competitive" plots would be obtained if one constructed reciprocal plots at a series of different enzyme concentrations. In the simplest case in which the inhibitor was completely stable and did not react with any other component of the solution save a single site on the enzyme a K_i calculated from such a plot would correspond to the amount of irreversible inhibitor necessary to reduce the velocity by one half which would correspond to half the amount of enzyme originally present.

Since any kinetic treatment of irreversible inhibitors in this way will give rise to misleading results it is obviously necessary to check whether an inhibitor is reversible or irreversible before going any further. The simplest way to check for irreversibility is obviously to see that activity is not regained following dialysis or gel filtration to remove the excess (unbound) inhibitor or by dilution (*vide supra*).

If the inhibitor is reacting with a group at or near the active site it may happen that the presence of one of the substrates protects the enzyme against inhibition. Since the substrate will be combining reversibly with the enzyme the effect will be to slow down the rate of reaction of the enzyme with the inhibitor. Such a protective effect has sometimes been referred to as being a "competitive" effect of the substrate but this term is misleading and bears no simple relationship to the competitive inhibition seen with reversible inhibitors. If reciprocal plots are constructed at various concentrations of the inhibitor under conditions in which the incubation of enzyme plus inhibitor was not carried out for a sufficient time for the reaction to go to completion, this "competitive" effect which will result in a slowing down of the rate of inhibition at higher substrate concentrations will be superimposed on the "non-competitive" effect giving rise to inhibition that may appear to be "mixed".

In some cases the reaction of an inhibitor with the enzyme may be a two step reaction in which the inhibitor first forms a noncovalent (EI complex):

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EI \overset{k_{+2}}{\rightarrow} E{-}I.$$

In a case like this it may be possible to treat the two steps separately.[13] The reversible combination of enzyme and inhibitor can be determined by measuring the effect of inhibitor concentration on the rate of irreversible inhibition. The rate of the formation of $E{-}I$ will depend on the concentration of the EI complex

$$\frac{\mathrm{d}[E{-}I]}{\mathrm{d}t} = k_{+2}[EI]$$

and if the formation of EI represents a simple equilibrium which is unperturbed by the reaction to produce $E{-}I$

$$K_i = \frac{(e - [EI])i}{[EI]}$$

$$[EI] = \frac{e}{\frac{K_i}{i} + 1}$$

and therefore

$$\frac{d[E-I]}{dt} = \frac{k_{+2}e}{\frac{K_I}{i} + 1}.$$

This equation is similar to the simple Michaelis equation for a single substrate reaction and predicts that the dependence of the initial rate of irreversible inhibition on inhibitor concentration will obey saturation kinetics in the same way as a normal enzyme reaction so that the dissociation constant of the initial non-covalent enzyme inhibitor complex may be obtained from a reciprocal plot.

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