BBA 12162

THE KINETICS OF ENZYME-CATALYZED REACTIONS WITH TWO OR MORE SUBSTRATES OR PRODUCTS

II. INHIBITION: NOMENCLATURE AND THEORY*

W. W. CLELAND

Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison, Wisc. (U.S.A.)

(Received May 22nd, 1962)

SUMMARY

A nomenclature is proposed to describe different types of inhibitions of enzyme-catalyzed reactions, particularly for reactions with more than one substrate and product. The rate equations for such inhibitions are discussed, as are methods for distinguishing between the various types of inhibition and obtaining inhibition and kinetic constants from experimental data. Several examples are given of the type of information that can be obtained from inhibition studies.

INTRODUCTION

If the concentrations of all substrates but one are held constant, the initial velocities observed for an enzyme-catalyzed reaction will usually vary with the concentration of the varied substrate according to the equation:

$$\frac{1}{v} = \frac{K}{V}\left(\frac{1}{A}\right) + \frac{1}{V} \qquad (1)$$

where v is initial velocity, A is variable substrate concentration, V is the velocity when A is infinite, and K is the concentration of A giving a velocity equal to half of V. V is customarily called the maximum velocity, and K the Michaelis constant. V and K in this equation are only apparent constants unless there is only one substrate for the reaction, and both may vary as the concentrations of the other substrates in the reaction are changed.

Compounds which slow down enzyme-catalyzed reactions are called inhibitors. In the presence of a constant concentration of an inhibitor, the slope, the vertical intercept, or both in Eqn. 1 may be increased (or in certain cases a straight line may no longer be obtained when $1/v$ is plotted against $1/A$). Many workers have used terms such as competitive, uncompetitive, or non-competitive for the different types of inhibition, although there has not been universal agreement on the definitions of these terms. SEGAL has reviewed the historical development of enzyme kinetics, including the use of these terms[1]. Recently there has been some recognition of the

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

necessity for more precise terminology, and REINER has avoided the use of these terms completely[2]. In this paper a nomenclature for the different types of enzyme inhibition will be presented which can be used to describe not only the simple cases usually met, but also more complex types of inhibition.

In discussing the theory of enzyme inhibitions, most authors have considered inhibitory effects by assuming a simple mechanism with only one substrate and one product[2,3]. Even though Eqn. 1 is followed in practice by most enzymes, it is not valid to extrapolate from a theoretical treatment of this simple mechanism where the constants in Eqn. 1 are really constant to more complex cases where there are larger numbers of substrates and products. Some work has been done on product inhibition in mechanisms with several substrates and products[4–6], and HEARON *et al.*[7] recently have treated in a very general way the inhibitory effects to be expected in mechanisms with obligatory reaction order, but no general approach applicable to both product and dead end inhibition has been developed. In this paper a general method applicable to mechanisms of any reactancy will be presented and several examples of its use will be given.

NOMENCLATURE

Enzymic mechanisms will be described by using the nomenclature presented in the previous paper[8], and rate equations will be written when possible in terms of kinetic constants as described there. Where Eqn. 1 is followed for any inhibition, the inhibition will be called competitive, uncompetitive, or non-competitive, respectively, when the slope, vertical intercept, or both are a function of inhibitor concentration. If the slope or intercept is a linear function of inhibitor concentration, the inhibition will be called linear, and the slope or intercept term will include a factor $(1 + I/K_i)$ where I is inhibitor concentration and K_i is an inhibition constant. (In linear inhibitions, K_i represents the value of I that doubles the slope or intercept term, but this is not the value of I that halves the initial velocity.) Thus Eqn. 2 describes a linear competitive inhibition.

$$\frac{1}{v} = \frac{K}{V}\left(1 + \frac{I}{K_i}\right)\left(\frac{1}{A}\right) + \frac{1}{V} \tag{2}$$

Reciprocal plots of $1/v$ *versus* $1/A$ at various I concentrations form a family of lines intersecting on the vertical axis. Eqn. 3 represents linear uncompetitive inhibition.

$$\frac{1}{v} = \frac{K}{V}\left(\frac{1}{A}\right) + \frac{1}{V}\left(1 + \frac{I}{K_i}\right) \tag{3}$$

where the family of reciprocal plots is a series of parallel lines. Eqn. 4 represents linear non-competitive inhibition.

$$\frac{1}{v} = \frac{K}{V}\left(1 + \frac{I}{K_{i\ \text{slope}}}\right)\left(\frac{1}{A}\right) + \frac{1}{V}\left(1 + \frac{I}{K_{i\ \text{intercept}}}\right) \tag{4}$$

A family of reciprocal plots represented by this equation always intersects at one point to the left of the vertical axis, and the lines cross above, below, or on the horizontal axis when $K_{i\ \text{slope}}$ is smaller than, greater than, or equal to $K_{i\ \text{intercept}}$. The coordinates of the intersection point are:

$$-\frac{1}{K}\left(\frac{K_{i\ slope}}{K_{i\ intercept}}\right) \quad \text{and} \quad \frac{1}{V}\left(1-\frac{K_{i\ slope}}{K_{i\ intercept}}\right).$$

When the lines cross on the axis and the two K_i's are the same we have simple linear non-competitive inhibition*.

The K_i's for linear inhibitions are easily obtained by replotting slopes or intercepts *versus* I. The horizontal intercept of such a replot gives K_i, which can also be obtained from the ratio of vertical intercept to slope if the horizontal intercept cannot be accurately determined.

Inhibitions may occur in which the slopes, intercepts, or both are non-linear functions of inhibitor concentrations. Inhibitions will be called hyperbolic where a replot is a hyperbola of the type represented by Eqn. 5, which gives the intercepts of a hyperbolic uncompetitive inhibition**.

$$\text{Intercept} = \frac{1}{V}\left(\frac{1+\dfrac{I}{K_{i\ num}}}{1+\dfrac{I}{K_{i\ denom}}}\right) \tag{5}$$

Both the slopes and intercepts of reciprocal plots for non-competitive inhibitions may be hyperbolic functions, giving hyperbolic non-competitive inhibition, or one can be linear. In the latter case both functions will be specified and the letters S and I will be used to indicate slope and intercept. For example: S-linear I-hyperbolic non-competitive inhibition. If both slope and intercept are hyperbolic functions, the lines will cross at one point if the two $K_{i\ denom}$'s are the same, giving intersecting hyperbolic non-competitive inhibition. The coordinates of the intersection point are:

$$-\frac{1}{K}\left(\frac{\dfrac{1}{K_{i\ num\ intercept}}-\dfrac{1}{K_{i\ denom}}}{\dfrac{1}{K_{i\ num\ slope}}-\dfrac{1}{K_{i\ denom}}}\right), \quad \frac{1}{V}\left(1-\frac{\dfrac{1}{K_{i\ num\ intercept}}-\dfrac{1}{K_{i\ denom}}}{\dfrac{1}{K_{i\ num\ slope}}-\dfrac{1}{K_{i\ denom}}}\right)$$

and the lines cross above or below the axis when $K_{i\ num\ slope}$ is, respectively, less than or greater than $K_{i\ num\ intercept}$. If these $K_{i\ num}$'s are equal, the lines cross on the horizontal axis, and we have simple intersecting hyperbolic non-competitive inhibition. The lines never cross at one point in mixed linear and hyperbolic non-competitive inhibition.

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* Most authors have restricted the term non-competitive to this type of inhibition, and some methods for distinguishing between competitive and non-competitive types of inhibition depend on the assumption that slope and intercept K_i's are the same[9]. These methods can easily be misused when applied to experimental data, however, and the standard reciprocal plots are to be preferred for analysis of inhibitory effects (particularly if the points are fitted to the line by proper statistical methods[10]). For instance, RICHARDSON AND TOLBERT[11] have recently observed inhibition by oxalate of glyoxylate oxidation by glycolic acid oxidase. In Fig. 3 of their paper they present a plot of $1/v$ *versus* I at three different substrate levels, and because the lines cross above the horizontal axis, they call the inhibition competitive. Careful analysis of the figure shows, however, that the inhibition is linear non-competitive and that $K_{i\ intercept}$ is about four times the value of $K_{i\ slope}$. This type of plot makes it very difficult to distinguish competitive from non-competitive inhibition if $K_{i\ slope}$ is smaller than $K_{i\ intercept}$.

** $K_{i\ denom}$ must be greater than $K_{i\ num}$ to have inhibition. If the reverse is true we have hyperbolic activation.

The K_i's for hyperbolic inhibition can be easily determined by making a reciprocal plot of

$$\left(\frac{1}{\text{slope}_I - \text{slope}_0}\right) \text{ or } \left(\frac{1}{\text{intercept}_I - \text{intercept}_0}\right)$$

versus $1/I$ (where slope_I is the slope at inhibitor concentration I, and slope_0 is the slope when $I = 0$, and the same for the intercepts); since the hyperbola is identical in form to the one describing the initial rate of an enzyme-catalyzed reaction if the horizontal axis is raised so that the curve goes through the origin.

When slope or intercept is a function of I of the type represented by Eqn. 6,

$$\text{slope} = \frac{K}{V}(1 + aI + bI^2) \tag{6}$$

where a and b are constants, a replot of slope or intercept *versus* I is a parabola and we have parabolic inhibition. This can be competitive, uncompetitive, or non-competitive, and in non-competitive cases, either or both terms can be parabolic. For example: S-parabolic I-linear non-competitive inhibition. In such mixed non-competitive cases the lines do not cross at one point, but in parabolic non-competitive cases, the lines will intersect at one point, giving intersecting parabolic non-competitive inhibition, if the ratio of constants a to b is the same in slope and intercept terms. The intersection point has coordinates

$$-\frac{1}{K}\left(\frac{(a_{\text{intercept}})}{(a_{\text{slope}})}\right), \quad \frac{1}{V}\left(1 - \frac{(a_{\text{intercept}})}{(a_{\text{slope}})}\right)$$

and the lines cross above, below, or on the horizontal axis depending on whether $a_{\text{intercept}}$ is less than, greater than, or equal to a_{slope}. If the lines cross on the axis we have simple intersecting parabolic non-competitive inhibition.

The constants a and b for a parabolic inhibition can be determined by fitting a parabola to the replot using the least-squares method, or by replotting the slopes of the parabola against I. The former method is more accurate, while obtaining a straight line from the latter method proves that the curve is really a parabola.

Inhibitions where slopes or intercepts are more complicated functions of inhibitor concentration are not uncommon, especially if there are alternate reaction pathways in the mechanism. The most common function, which will be referred to as a 2/1 function in this paper*, is the type represented by Eqn. 7 and plots as

$$\text{slope} = \frac{K}{V}\frac{(1 + aI + bI^2)}{(1 + cI)} \tag{7}$$

a curved line near the vertical axis which becomes linear with a slope of $\left(\frac{K}{V}\right)\left(\frac{b}{c}\right)$ as I is increased. The slope of the curve near the vertical axis, which is $(K/V)(a - c)$

* This particular function is actually a hyperbola with non-horizontal asymptotes. The expression 2/1 refers to the highest power of inhibitor concentration occurring in numerator and denominator. When the rate equations for complex inhibitions with alternate reaction sequences are inverted to put them in reciprocal form, the expressions for slopes and intercepts will be the ratio of two functions of inhibitor concentration. The author has encountered 3/1, 3/2, 4/2 and higher degree functions in analyzing mechanisms. What has been called hyperbolic inhibition in this paper is a 1/1 function.

when $I = 0$, can either be greater or less than the limiting slope reached at high I values, depending on whether $(a - c)$ is greater or less than (b/c). Good data over a sufficient range of I concentrations and careful analysis would be necessary to distinguish this type of inhibition from linear or, depending on the direction of curvature, from either hyperbolic or parabolic inhibition*.

Inhibitors may inhibit by combining with substrates, activators, or with various enzyme forms so that these either cannot function in the mechanism, or function at reduced rates. In this paper only combinations between inhibitor and various enzyme forms will be considered, since this covers most of the cases met in practice.

A compound that is a product of a reaction and inhibits by combining only with the enzyme form it would react with as a substrate in the reverse reaction will be called a product inhibitor. A compound which could have been a normal product produced at a reasonable rate had an alternate substrate been used, and which combines only with the enzyme form it would combine with as a substrate in a reverse reaction, will be called an alternate product inhibitor. The enzyme–inhibitor complexes formed by reaction of product or alternate product inhibitors are normal intermediates in the reaction sequence (or in a similar sequence with an alternate substrate) and can be further transformed into other enzyme forms occurring earlier in the reaction sequence.

A compound which is never a normal product but which can combine with one or more enzyme forms so that the enzyme–inhibitor complex cannot undergo any further conversion to other enzyme forms in the reaction sequence is a dead end inhibitor. If the enzyme–inhibitor complex can undergo the same reaction it would have undergone in the absence of inhibitor but at a lowered rate, and the inhibitor can later dissociate, there are alternate reaction sequences in the mechanism and the inhibitor is a partial inhibitor.

It is possible for product or alternate product inhibitors to combine with enzyme forms other than the ones they normally react with and give mixed dead end and product (or alternate product) inhibition. Substrates may also react with an enzyme form other than the one they are supposed to react with and give dead end substrate inhibition; DALZIEL has treated several such cases[12]. Such situations are usually more easily treated experimentally by studying the reverse reaction with this substrate as a mixed product and dead end inhibitor, so inhibition by substrates will not be considered in this paper.

RATE EQUATIONS FOR INHIBITED REACTIONS

Product inhibition

The rate equation for any product inhibition is obtained from the full rate equation for the entire mechanism by setting the concentrations of all other products equal to zero. In mechanisms without alternate reaction sequences product inhibitions are always linear**, and may be competitive, uncompetitive, or non-competitive.

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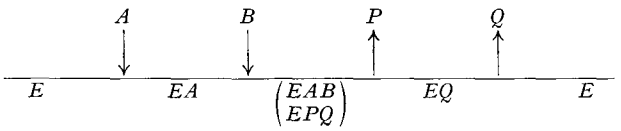
* An apparent example of S-2/1 I-linear non-competitive inhibition has recently been found in this laboratory for product inhibition by ethanol with acetaldehyde as variable substrate, using liver alcohol dehydrogenase (WRATTEN AND CLELAND, unpublished experiments).

** This assumes that all products are different. If two molecules of one product are released, non-linear inhibitions by this product may occur. For example, if myokinase is assumed to be

However, the apparent K_i's calculated from replots of slopes or intercepts *versus* I may or may not be identical with the K_i's defined as kinetic constants for the mechanism[8] or with the dissociation constants of EI complexes. As an example, consider the product inhibition equations for Ordered Bi Bi (Mechanism 8), which are obtained by setting either Q or P equal to zero in the rate equation[8], and rearranging



(8)

into the form of Eqn. 2 or 4:

P as inhibitor, A varied,

$$\frac{1}{v} = \frac{K_a}{V_1}\left(1 + \frac{K_{ia}K_b}{K_a B}\right)\left(1 + \frac{P}{\frac{K_p K_{iq}}{K_q}\left(1 + \frac{K_a B}{K_{ia}K_b}\right)}\right)\frac{1}{A} + \frac{1}{V_1}\left(1 + \frac{K_b}{B}\right)\left(1 + \frac{P}{\left(\frac{1 + \frac{K_b}{B}}{\frac{1}{K_{ip}} + \frac{K_q K_b}{K_p K_{iq} B}}\right)}\right) \quad (9)$$

B varied,

$$\frac{1}{v} = \frac{K_b}{V_1}\left(1 + \frac{K_{ia}}{A}\right)\left(1 + \frac{P}{\left(\frac{K_p K_{iq}}{K_q}\right)}\right)\frac{1}{B} + \frac{1}{V_1}\left(1 + \frac{K_a}{A}\right)\left(1 + \frac{P}{K_{ip}\left(1 + \frac{K_a}{A}\right)}\right) \quad (10)$$

Q as inhibitor, A varied,

$$\frac{1}{v} = \frac{K_a}{V_1}\left(1 + \frac{K_{ia}K_b}{K_a B}\right)\left(1 + \frac{Q}{K_{iq}}\right)\frac{1}{A} + \frac{1}{V_1}\left(1 + \frac{K_b}{B}\right) \quad (11)$$

B varied,

$$\frac{1}{v} = \frac{K_b}{V_1}\left(1 + \frac{K_{ia}}{A}\right)\left(1 + \frac{Q}{K_{iq}\left(1 + \frac{A}{K_{ia}}\right)}\right)\frac{1}{B} + \frac{1}{V_1}\left(1 + \frac{K_a}{A}\right)\left(1 + \frac{Q}{K_{iq}\left(1 + \frac{A}{K_a}\right)}\right) \quad (12)$$

When P is the inhibitor a constant inhibition constant ($\frac{K_p K_{iq}}{K_q}$, which is not necessarily equal to K_{ip}) is observed only for the slopes when B is varied; otherwise the observed inhibition constants are functions of the fixed substrate concentration. As long as B is very small, $K_{i\ slope}$ when A is varied is also $\frac{K_p K_{iq}}{K_q}$ but increases to infinity as B becomes saturating. $K_{i\ intercept}$ when A is varied also equals $\frac{K_p K_{iq}}{K_q}$ when B is very small, but changes to K_{ip} when B is saturating. This may involve an increase, a decrease, or no change, depending on whether K_{ip} is greater than,

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Ordered Bi Bi with $P = Q =$ ADP, then ADP will give S-parabolic I-linear non-competitive inhibition against the first substrate to react with free enzyme, and parabolic non-competitive, changing to linear non-competitive when the first substrate is saturating, against the second substrate in the reaction. If the mechanism were Rapid Equilibrium Random with a dead end E–AMP–ADP complex, ADP would give parabolic competitive inhibition *versus* ATP, and S-parabolic I-linear inhibition against AMP.

less than, or equal to $\frac{K_p K_{iq}}{K_q}$. $K_{i\ intercept}$ when B is varied equals K_{ip} when A is saturating, but increases towards infinity as A decreases to zero. When Q is inhibitor, a constant $K_{i\ slope}$ equal to K_{iq} is obtained when A is varied. When B is varied, the K_i's equal K_{iq} only when A is very small and increase to infinity as A becomes saturating.

The differences in product inhibition patterns may obviously be used to distinguish mechanisms; for example, the Theorell–Chance mechanism does not give the term containing K_{ip} in Eqns. 9 and 10. As a result inhibition by P when B is varied appears competitive, regardless of the concentration of A, and inhibition by P when A is varied is overcome by saturation with B, instead of becoming uncompetitive. The variation of the observed K_i's with the concentration of the fixed substrate may also be used to distinguish between mechanisms, particularly when it is experimentally difficult to use the other product as an inhibitor. For example, in the Rapid Equilibrium Random Bi Bi mechanism, Q also gives competitive inhibition when A is varied, but because there is no BQ term in the rate equation, $K_{i\ slope}$ is now

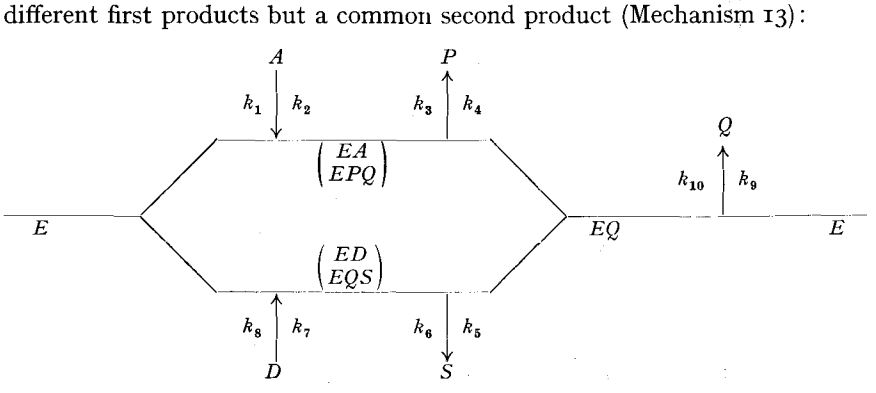
$$K_{iq}\left(1 + \frac{K_a B}{K_{ia} K_b}\right), \quad \text{or} \quad K_{iq}\left(1 + \frac{B}{K_{ib}}\right)$$

since $K_{ia}K_b = K_a K_{ib}$ in this mechanism. Thus a constant $K_{i\ slope}$ at two levels of B would identify the mechanism as Ordered, and a changing $K_{i\ slope}$ as Rapid Equilibrium Random.

If there are alternate reaction sequences involving a product inhibitor in a mechanism, there will be terms including the inhibitor concentration in both numerator and denominator of the rate equation, and the resulting product inhibition will be hyperbolic or of greater complexity. Inhibition by either product in Random Uni Bi is S-2/1 I-hyperbolic non-competitive where 2/1 identifies the function given in Eqn. 7. Inhibition by either product in Random Bi Bi when the non-varied substrate is saturating is intersecting hyperbolic non-competitive.

Alternate product inhibition

In alternate product inhibitions, alternate reaction sequences are set up and non-linear inhibitions may be observed. The rate equations for these inhibitions are obtained by using the method of KING AND ALTMAN[13] on the entire mechanism including the alternate reaction sequences. The rate of the reaction will vary, depending on which product or substrate concentration is followed as a measure of velocity. For example, consider a Uni Bi mechanism where two possible substrates give different first products but a common second product (Mechanism 13):



(13)

The velocities of appearance of P and Q in a system initially containing only A and S are given by Eqns. 14 and 15:

$$v_{pA} = \frac{dP}{dt} = \frac{(k_1k_3\,(k_6 + k_7)\,k_{10}\,A + k_1k_3k_5k_7\,AS)\,E_t}{\text{denominator}} \tag{14}$$

$$v_{qA} = \frac{dQ}{dt} = \frac{(k_1k_3\,(k_6 + k_7)\,k_{10}\,A)\,E_t}{\text{denominator}} \tag{15}$$

where the denominator is: $(k_2 + k_3)\,(k_6 + k_7)\,k_{10} + k_1(k_3 + k_{10})\,(k_6 + k_7)\,A + (k_2 + k_3)\,k_5k_7\,S + k_1k_5(k_3 + k_7)\,AS$. Eqn. 14 has two terms in the numerator, since there are two pathways by which P may be liberated with A as substrate: by breakdown of EQ to E and Q and by addition of S to EQ followed by the release of D to give E. Only the breakdown of EQ liberates Q, however, so Eqn. 15 has only one term in the numerator.

In a system with D as substrate and S as product inhibitor, the rate of appearance of Q (or S) is given by Eqn. 16:

$$v_D = \frac{((k_2 + k_3)\,k_6k_8k_{10}\,D)\,E_t}{\text{denominator}} \tag{16}$$

The denominator here lacks A and AS terms, but contains D and DS terms: $(k_2 + k_3)k_8(k_6 + k_{10})D + (k_2 + k_3)k_5k_8\,DS$. If these equations are written in coefficient form[8], the following kinetic constants can be defined as they would be for a normal Uni Bi mechanism:

$$V_{1A} = \frac{(\text{numerator}_A)}{(\text{coef } A)} \qquad V_{1D} = \frac{(\text{numerator}_D)}{(\text{coef } D)} \qquad K_{1d} = \frac{(\text{coef } S)}{(\text{coef } DS)}$$

$$K_a = \frac{(\text{constant})}{(\text{coef } A)} \qquad K_d = \frac{(\text{constant})}{(\text{coef } D)} \qquad K_{1s} = \frac{(\text{coef } D)}{(\text{coef } DS)}$$

If the numerators and denominators of Eqns. 14 and 15 in coefficient form are now divided by (coef A), and those of Eqn. 16 by (coef D), the equations become:

$$v_{pA} = \frac{V_{1A}\left(1 + \dfrac{(\text{numerator}_{AS})\,S}{(\text{numerator}_A)}\right)A}{K_a\left(1 + \dfrac{(\text{coef } S)\,S}{(\text{constant})}\right) + A\left(1 + \dfrac{(\text{coef } AS)\,S}{(\text{coef } A)}\right)}$$

$$v_{qA} = \frac{V_{1A}\,A}{(\text{same denominator})}$$

$$v_D = \frac{V_{1D}\,D}{K_d\left(1 + \dfrac{(\text{coef } S)\,S}{(\text{constant})}\right) + D\left(1 + \dfrac{(\text{coef } DS)\,S}{(\text{coef } D)}\right)}$$

But close examination shows that:

$$\frac{(\text{numerator}_{AS})}{(\text{numerator}_A)} = \frac{(\text{coef } S)}{(\text{constant})} = \frac{k_5k_7}{(k_6 + k_7)k_{10}} = \frac{K_{1d}}{K_d\,K_{1s}}$$

Defining a new constant:

$$K_{iss} = \frac{(\text{coef } A)}{(\text{coef } AS)}$$

and inverting the equations, we now obtain:

$$\frac{1}{v_{pA}} = \frac{K_a}{V_{1A}}\left(\frac{1}{A}\right) + \left(\frac{1}{V_{1A}}\right)\frac{\left(1 + \frac{S}{K_{iss}}\right)}{\left(1 + \frac{K_{id}\,S}{K_d\,K_{is}}\right)} \tag{17}$$

$$\frac{1}{v_{qA}} = \frac{K_a}{V_{1A}}\left(1 + \frac{K_{id}\,S}{K_d\,K_{is}}\right)\left(\frac{1}{A}\right) + \frac{1}{V_{1A}}\left(1 + \frac{S}{K_{iss}}\right) \tag{18}$$

$$\frac{1}{v_D} = \frac{K_d}{V_{1D}}\left(1 + \frac{K_{id}\,S}{K_d\,K_{is}}\right)\left(\frac{1}{D}\right) + \frac{1}{V_{1D}}\left(1 + \frac{S}{K_{is}}\right) \tag{19}$$

The inhibition constant K_dK_{is}/K_{id} is found in all three equations, while the other inhibition constant is K_{is} for the product and K_{iss} for the alternate product inhibition. Although the same inhibition constants are involved in both Eqns. 17 and 18, they are arranged differently, so that measurement of Q formation gives linear non-competitive inhibition, while measurement of P gives hyperbolic uncompetitive inhibition.

Actual observation of hyperbolic uncompetitive inhibition by such an alternate product inhibitor demonstrates that this compound does not combine with free enzyme (this would add slope variation and produce S-linear I-hyperbolic non-competitive inhibition). If this compound in the same concentration range gives linear non-competitive inhibition as a product inhibitor (Eqn. 19), one has proven that this latter inhibition is really product inhibition, and that the $K_{i\ slope}$ obtained is that predicted by the rate equation, and not partly the result of dead end inhibition by combination with free enzyme*. HSU, CLELAND AND ANDERSON[14] have recently used this approach with success in studying the inhibition of *p*-nitrophenol against the hydrolysis of *p*-nitrophenyl phosphate and β-glycerophosphate by potato acid phosphatase.

Similar experiments can be carried out with reactions that have an Ordered Bi Bi mechanism if suitable alternate second substrates that give alternate first products can be found. With the concentration of the second substrate varied and an alternate first product as inhibitor, hyperbolic uncompetitive inhibition should be observed if release of the first product is measured. Any variation in slope would show dead end combination of the inhibitor with the EA complex (dead end combination with E would also affect the slope, but can be eliminated by keeping the concentration of A high).

Dead end inhibition

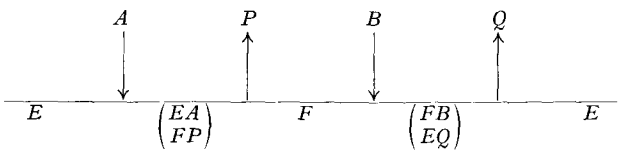
The effect of a dead end inhibitor that combines with only one enzyme form is to multiply certain terms or parts of terms in the denominator of the rate equation

* Dead end combination with E in addition to the normal non-competitive product inhibition should give S-parabolic I-linear non-competitive inhibition, but if the $K_{i\ slope}$ for the product inhibition were much larger than either $K_{i\ intercept}$ or the K_i for the dead end inhibition, the parabolic variation of the slopes with inhibitor concentration would be difficult to see. The alternate product inhibition study can rule out this possibility.

by the factor $(1 + I/K_i)$ where K_i is the dissociation constant of the complex containing inhibitor. Such inhibition is always linear and can be competitive, uncompetitive, or non-competitive. The terms multiplied by this factor are those appearing in the numerator of the distribution equation which describes the proportion of the enzyme in the form which reacts with the inhibitor*. If the combination is with a stable enzyme form, entire terms in the rate equation are multiplied by the factor, and the value of K_i is easily obtained. (An example is given later under DISCUSSION). If the combination is with an enzyme form for which the distribution equation can be written in terms of kinetic constants[8], K_i likewise can be evaluated, but if the distribution equation cannot be written in terms of kinetic constants, K_i cannot be evaluated kinetically, since only part of certain denominator terms will be multiplied by the factor, and without knowing the rate constants there is no way to express how large a part. The apparent K_i that is observed will be a function of the true K_i and of certain rate constants in the mechanism.

If a dead end inhibitor reacts with more than one enzyme form there will be more than one $(1 + I/K_i)$ factor introduced into the denominator of the rate equation, but the inhibitions will still be linear since the different factors are multiplying different portions of the denominator. If the inhibitor reacts with the enzyme–inhibitor complex to form an EI_2 complex, however, two such factors are multiplied together and the inhibition is parabolic.

As an example, consider dead end inhibition in the Ping Pong Bi Bi mechanism (Mechanism 20). If one assumes that the inhibitor



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can only be adsorbed on a vacant portion of the active site, then we need only consider dead end combinations with the two stable forms E and F, since the entire active site is presumably filled with reactants in the central complexes. The appropriate distribution equations are[8]:

$$\frac{E}{E_t} = \frac{K_a B}{K_b A + K_a B + AB} \tag{21}$$

$$\frac{F}{E_t} = \frac{K_b A}{K_b A + K_a B + AB} \tag{22}$$

and the initial velocity equation for the mechanism in the absence of inhibitor is:

$$v = \frac{V_1 AB}{K_b A + K_a B + AB} \tag{23}$$

* Such distribution equations are given for several mechanisms in an earlier paper[8]. What happens here can be understood readily by remembering that the denominator of the rate equation is obtained from the sum of the numerators of all of the distribution equations. If I combines as a dead end inhibitor with E, the concentration of the EI complex will be $(E)\,(I/K_i)$ in the steady state, and the proportion of the enzyme in this complex will be $(E/E_t)\,(I/K_i)$. Thus in the denominator of the rate equation those terms from the numerator of the equation for (E/E_t) are all multiplied by the factor $(1 + I/K_i)$.

Thus if the inhibitor I reacts with E to form an EI complex with a dissociation constant K_i, the K_aB term is multiplied by $\left(1 + \frac{I}{K_i}\right)$ and the rate equation becomes:

$$v = \frac{V_1 AB}{K_b A + K_a \left(1 + \frac{I}{K_i}\right) B + AB} \tag{24}$$

When Eqn. 24 is inverted, we obtain Eqn. 25 (which shows competitive inhibition) when A is varied, and Eqn. 26 (which shows uncompetitive inhibition) when B is varied:

$$\frac{1}{v} = \frac{K_a}{V_1}\left(1 + \frac{I}{K_i}\right)\left(\frac{1}{A}\right) + \frac{1}{V_1}\left(1 + \frac{K_b}{B}\right) \tag{25}$$

$$\frac{1}{v} = \frac{K_b}{V_1}\left(\frac{1}{B}\right) + \frac{1}{V_1}\left(1 + \frac{K_a}{A}\right)\left(1 + \frac{I}{K_i\left(1 + \frac{A}{K_a}\right)}\right) \tag{26}$$

If I also reacts with F to give an FI complex with a dissociation constant K_{i2}, then the K_bA term of Eqn. 24 is multiplied by $\left(1 + \frac{I}{K_{i2}}\right)$, and the equations corresponding to Eqns. 25 and 26 will both show linear non-competitive inhibition.

Mixed dead end and product inhibition

If a product or alternate product inhibitor combines with an enzyme form other than the one it reacts with as a substrate in the reverse reaction, and gives dead end inhibition in addition to the regular product or alternate product inhibition, certain portions of the denominator of the rate equation are again multiplied by $\left(1 + \frac{I}{K_i}\right)$. If these terms already include I, however, the resulting inhibition will be parabolic. Examination of the appropriate distribution equation will indicate the type of inhibition. Consider what happens in Ordered Bi Bi (Mechanism 8), for instance, if P reacts with EA in dead end fashion as well as with EQ. The distribution equation for EA can be written[8]:

$$\frac{EA}{E_t} = \frac{K_b A + \frac{K_b K_q AP}{K_p K_{iq}} + \frac{K_a PQ}{K_{ia} K_{eq}}}{\text{denominator of rate equation divided by } V_2} \tag{27}$$

The denominator of the initial velocity equation with P as product inhibitor will thus have the A and AP terms multiplied by $\left(1 + \frac{P}{K_i}\right)$ where K_i is the dissociation constant of the dead end EAP complex. (The PQ term drops out since $Q = 0$.) When the equation is put into reciprocal form, and the denominator terms are divided by AB, these terms become

$$\frac{K_b}{B}\left(1 + \frac{P}{K_i}\right) \quad \text{and} \quad \frac{K_b K_q P}{B K_p K_{iq}}\left(1 + \frac{P}{K_i}\right)$$

and occur in the intercept term of Eqn. 9 (A varied), and the slope term of Eqn. 10 (B varied). Thus when A is the variable substrate, P will give S-linear I-parabolic non-competitive inhibition, and when B is varied, S-parabolic I-linear non-competitive inhibition. FROMM AND NELSON[6] have recently shown this type of parabolic inhibition for ribitol dehydrogenase.

Partial inhibition

Partial inhibitors, like alternate product inhibitors, introduce alternate reaction pathways into the mechanism. The method of KING AND ALTMAN[13] should be used on the entire mechanism to obtain the rate equation. In general, the resulting inhibition will be hyperbolic or of greater complexity. In Ordered Uni Bi, for instance, partial inhibition results from combination of an inhibitor with EQ and E (but not with the central complex) and conversion of EQI into EI at a reasonable velocity. The inhibition is S-2/1 I-hyperbolic non-competitive.

If the alternate pathways involve substrate additions, the kinetics in the presence of inhibitor will no longer follow Eqn. 1. Reciprocal plots would be straight in the absence of inhibitor, but would become more curved as inhibitor was added. In mechanisms with two or more substrates, partial inhibition is very likely to produce such effects. The fact that partial inhibitions are not more common indicates that most inhibitors combine with the active sites of enzymes in such a manner that addition of substrates is totally blocked.

DISCUSSION

The general method of approach outlined in this paper makes it possible to study in precise fashion the inhibition of enzyme-catalyzed reactions with more than one substrate and product, and greatly widens the possibilities for determination of enzymic mechanisms by kinetic studies. Product inhibition studies can be used to distinguish all mechanisms with different rate equations, and will detect also any dead end inhibitions by the products involved. Alternate product inhibition experiments provide additional checks on the assumed mechanism and on the validity of experimentally determined inhibition constants. These types of experiments can also be used to determine kinetic constants other than inhibition constants, as shown by the following example. KUBY, NODA AND LARDY[15] in their careful and elegant work on ATP–creatine transphosphorylase found that ADP was a competitive inhibitor when ATP was varied, but a non-competitive inhibitor when creatine was varied. REINER[16] has attempted to analyze these data in terms of the Ordered Bi Bi mechanism, but made the error of assuming that ATP was saturating at a level of 0.004 M when creatine was the variable substrate*. As will be shown, $K_a =$

* REINER's assumption of saturation was no doubt based on the apparent K for ATP of $5 \cdot 10^{-4}$ M which was obtained at a creatine concentration only slightly above its K_m level, and on the statement of the authors that this was a maximal value. This statement is not true, since as has been discussed in the previous paper[8], the apparent K can increase, decrease, or stay the same as the concentration of the other substrate is raised, and in this case the K obtained was minimal. This situation points out the necessity of being very cautious in assuming saturation by any reactant. An ATP concentration ten times K_a would still leave considerable inhibition.

$9.5 \cdot 10^{-4}$ M and $K_{ia} = 5.7 \cdot 10^{-4}$ M (A is ATP), and the non-competitive inhibition (which indeed would disappear if ATP were saturating) is still very evident. If we assume an Ordered Bi Bi mechanism for this enzyme* the competitive and non-competitive inhibitions are described by Eqns. 11 and 12. From Eqn. 11 it can be seen that a replot of slopes *versus* Q will give a straight line with a horizontal intercept of $-K_{iq}$. From the data in Fig. 9 of KUBY, NODA AND LARDY's paper[15], $K_{iq} = 2.5 \cdot 10^{-4}$ M. For data plotted according to Eqn. 12, a replot of slopes *versus* Q gives a line with a horizontal intercept of

$$-K_{iq}\left(1 + \frac{A}{K_{ia}}\right)$$

while a similar replot of vertical intercepts gives

$$-K_{iq}\left(1 + \frac{A}{K_a}\right)$$

Replots of the experimental data (Fig. 10, ref. 15) yield horizontal intercepts of -0.002 M and -0.0013 M from slopes and intercepts, respectively. In combination with the known concentration of A (0.004 M) and the previously determined value of K_{iq}, these data permit calculation of K_a and K_{ia} as given above**. V_1 can now be calculated from the vertical intercept of the line representing Eqn. 12 with $Q = 0$. The value of 0.22 obtained is somewhat higher than 0.18, the highest apparent V observed by the authors. K_b can be obtained from the slope of the same plot and is 0.0165 M in this case. Calculation from the vertical intercepts of the competitive inhibition plots gives 0.020 M, so an average value of K_b is about 0.018 M. Analysis of one set of product inhibition experiments may thus give values for all of the kinetic constants that could be obtained by initial velocity analysis in addition to evidence concerning the type of mechanism and the order of addition of substrates and release of products. The experiments are often simpler to run in the laboratory, since it is often desirable to saturate as nearly as possible with the non-varied substrate in a product inhibition experiment, while it is not permissible to do so with either substrate in initial velocity analysis. If the Michaelis constants are low, the equilibrium constant not too favorable, and the method of measurement not too sensitive, the reaction velocity may drop off very rapidly due to approach to equilibrium in the initial velocity analysis, while this is no problem with the high levels of the non-varied substrate in the product inhibition study.

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* The Theorell–Chance mechanism does not represent the data for this enzyme since the relationship

$$\frac{V_2 K_a}{V_1 K_{ia}} = 1$$

is not satisfied. Rapid Equilibrium Random Bi Bi with a dead end E–ADP–Creatine complex cannot be ruled out on the basis of present knowledge and is in fact a very likely mechanism. However, the calculations are given here for purposes of illustration only, and to correct the impression that data of this pattern are inconsistent with the Ordered mechanism[16].

** Although $K_{ia} = 0.6\, K_a$ and the family of lines for the non-competitive inhibition cross slightly below the axis, the authors in analyzing their data originally assumed the lines to cross on the axis, and thus obtained an average inhibition constant. The importance of replotting intercepts and slopes separately for determination of K_i's cannot be overemphasized, since crossing of the lines on the axis in any non-competitive inhibition is purely fortuitous.

Studies on dead end inhibitions may also give much useful information, as exemplified by the thorough study by NOVOA and coworkers of oxalate and oxamate inhibition of lactic dehydrogenase[17]. Another application can be seen from the following analysis of the phosphate inhibition of transaldolase observed by BONSIGNORE *et al.*[18]. This reaction has a Ping Pong Bi Bi mechanism with free enzyme and a dihydroxyacetone-enzyme complex as the two stable enzyme forms E and F (Mechanism 20). When A and B are sedoheptulose-7-P and D-glyceraldehyde-3-P, respectively, inorganic phosphate is a non-competitive inhibitor of either substrate (Figs. 3 and 4, ref. 18), but when A and B are fructose-6-P and D-glyceraldehyde, phosphate gives competitive inhibition when A is varied and uncompetitive inhibition when B is varied (Figs. 1 and 2, ref. 18; the data in Fig. 2 of this paper plot as two parallel lines in a reciprocal plot). Comparison of these data with our earlier analysis of dead end inhibition for this mechanism shows that when sedoheptulose-7-P and D-glyceraldehyde-3-P are the substrates, phosphate appears to react with both E and F, while when fructose-6-P and free glyceraldehyde are the substrates phosphate appears to react only with E. The simplest explanation for these data is that the active site of the enzyme includes an adsorption site for a phosphate group of the substrate, which is exposed on both E and F. Adsorption of phosphate on this site of E or F would block adsorption of phosphorylated substrates, but not the adsorption of unphosphorylated substrates. Thus when glyceraldehyde was the second substrate, there would be no apparent inhibition due to combination of phosphate with F even though phosphate were adsorbed on F, and kinetically it would appear that phosphate did not combine with F. A kinetic study of this type thus helps map the active site of the enzyme.

The experimental data can further be analyzed quantitatively to give information on the kinetic constants and the dissociation constants of the enzyme–phosphate complexes.

When fructose-6-P and glyceraldehyde are A and B, replots give $K_{i\ slope} = 0.05$ M and $K_{i\ intercept} = 0.09$ M for the competitive and uncompetitive inhibitions, respectively. According to Eqn. 25, K_i should equal $K_{i\ slope}$ or 0.05 M. We can also see from Eqn. 26, that $K_{i\ intercept} = K_i(1 + A/K_a)$. Since A (fructose-6-P) was $9 \cdot 10^{-4}$ M in these experiments, $K_a = 1.1 \cdot 10^{-3}$ M. When A was varied in the absence of inhibitor, the slope of the line, which from Eqn. 25 is K_a/V_1, was $4.3 \cdot 10^{-3}$. This gives a value of $1/V_1$ of 3.9. The intercept of this line, which should be $(1/V_1)(1 + K_b/B)$ is 21 and B (glyceraldehyde) was $3 \cdot 10^{-3}$ M, so K_b is $1.3 \cdot 10^{-2}$ M. The inhibition study thus yields both of the limiting Michaelis constants, which could be obtained in the absence of inhibitor only by varying the fixed substrate concentration. The values obtained here should not be considered precise, since the original data were derived from published figures, but the calculation serves to illustrate the methods involved in such an analysis. A similar analysis could be made for the non-competitive inhibitions when sedoheptulose-7-P and glyceraldehyde-3-P were substrates, but replots of slopes and particularly intercepts measured from the published figures do not give straight enough lines to determine the K_i's accurately, and the generation of glyceraldehyde-3-P from fructose diphosphate and aldolase with an unknown amount of triose phosphate isomerase present makes calculation of the concentrations of this substrate uncertain. Approximately the same K_i as the one given above is obtained for the dissociation constants of the EI and FI complexes, however.

The theoretical analysis and examples given in this paper should make clear the importance of separately replotting slopes and intercepts from reciprocal plots in inhibition studies, and of determining whether the observed K_i's vary with the concentrations of the fixed substrates. It is hoped that this paper will stimulate more thorough inhibition studies and result in the discovery of much interesting information which until now has remained hidden in experimental data because methods were not available to extract it.

ACKNOWLEDGEMENT

Supported by a grant from the National Science Foundation (G 14388).

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