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

III. PREDICTION OF INITIAL VELOCITY AND INHIBITION PATTERNS BY INSPECTION*

W. W. CLELAND

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

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SUMMARY

A general method for predicting initial velocity and dead end and product inhibition patterns by inspection of the mechanism is presented. This method is applicable to any non-random mechanism without alternate reaction sequences. Several examples of the application of the method are given.

INTRODUCTION

The previous two papers[1,2] have described a nomenclature and general theory for handling the kinetics of enzymic reactions with more than one substrate or product. With the approach outlined there, one can use kinetic studies as a tool for determining enzymic mechanisms and for studying the properties of active sites on enzymes. Many of these studies involve determining initial velocity, or product or dead end inhibition patterns. While these are easily deduced for a given mechanism from the overall rate equation by simplification and rearrangement, this takes considerable time, particularly if the rate equation is not available and must be derived. Furthermore, kinetic studies can only show consistency or inconsistency of the experimental data with possible mechanisms. In order to be sure that one has correctly determined the mechanism it is necessary to examine every possible mechanism that is at all plausible, and reject those that do not fit. If one is thorough about this, one ends up analyzing a very large number of mechanisms and deriving a very large number of rate equations. All of this labor would be tremendously reduced if one could look at a mechanism and tell by inspection what the initial velocity or the various inhibition patterns were. In this paper, rules will be given for this type of analysis, together with examples and several applications. The notation and nomenclature used will be those described in the previous papers[1,2].

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THEORY

It will be assumed that experimental data are to be plotted as reciprocal plots according to the general equation:*

$$\frac{1}{v} = (\text{slope})\left(\frac{1}{A}\right) + (\text{intercept})$$

where v is initial velocity, A is substrate concentration, and the slope and intercept may be functions of the concentrations of other substrates, activators, products, dead end inhibitors, etc., but are constant for any particular reciprocal plot. When the concentration of any of these other variables is changed, the slope of the line, the intercept, or both may be changed, and the change may be linear, parabolic, hyperbolic, or of greater complexity. The type of variation of slope and intercept with the concentration of the other variable constitutes the desired initial velocity or inhibition pattern.

The intercept of a reciprocal plot represents the reciprocal velocity at infinite substrate concentration. Thus, if changing the concentration of the other variable affects the rate even at saturation, the intercepts will vary as a function of this variable; if the variable no longer can influence the rate when the substrate is saturating, the intercepts do not vary.

The slope of a reciprocal plot, on the other hand, is a measure of how fast the reaction slows down as the substrate concentration decreases from infinity to zero. The net rate of the step where the substrate adds to the enzyme, which in a steady-state mechanism is equal to the overall rate, can be expressed:

$$v = k_1 A (E) - k_2 (EA)$$

where E represents the enzyme form that reacts with A. When the concentration of A is infinite the steady-state concentration of E is zero, and the actual rate of reaction is determined only by how fast EA is converted back to E by the rest of the reaction sequence. As the concentration of A is decreased, the steady-state concentration of E rises and that of EA drops, finally becoming zero when A becomes zero; so that when A is very small, the rate of reaction is represented only by $k_1 A(E)$. If changing the concentration of a variable other than the substrate specifically tends to increase the steady-state concentration of E, or decrease that of EA, then the rate of reaction decreases less rapidly as A is lowered, and the slope of the reciprocal plot is decreased. If changing the concentration of the other variable specifically decreases the concentration of E or increases that of EA, then the slope of the reciprocal plot is increased.

With these considerations in mind, we can now state two fundamental rules:

1. A compound affects the intercept of a reciprocal plot when it combines reversibly with an enzyme form other than the one the variable substrate combines

* If A/v is plotted *versus* A, the slopes become intercepts and *vice versa*, and the rules given in this paper yield the proper patterns if this is kept in mind. Because of the fundamental nature of slope and intercept variation, data should be plotted only by one of these two equations. Since in practice one uses statistical methods to fit the experimental points to the actual hyperbola $v = VA/(K + A)$ (see ref. 3) and uses the graphical plot only to test for linearity, to determine if any point should be discarded, and to visualize the results after the fitted curve is plotted, it makes little difference which plot is used, and the more familiar double reciprocal plot will be used here.

with, and thereby changes the reaction velocity in a manner which can not be eliminated by saturation with the variable substrate.

2. A compound affects the slope of a reciprocal plot when it and the variable substrate either combine with the same form of the enzyme, or are separated in the reaction sequence by a series of reversible steps along which they can interact in such a manner that a change in the concentration of the compound specifically alters the net rate of the step involving the addition of the variable substrate in a manner which can be eliminated by a change in the concentration of the variable substrate. Release of a product at zero concentration (but not at finite concentration), or addition of a substrate at infinite concentration (saturation) are considered irreversible steps for purposes of this analysis.

To use these rules, one simply analyzes for slope and intercept effects separately, and then combines the results to determine the pattern. If the compound whose effects are being analyzed adds to only one enzyme form, the effect is always linear; that is, a replot of slopes or intercepts *versus* concentration for inhibitors, or *versus* reciprocal concentration for substrates and activators, is a straight line. If the compound in question reacts with two (or more) enzyme forms, the effects on slopes or intercepts are determined separately for each point of combination, and the combined effects are given by Rule 3:

3. When combination by a compound at two or more points in a reaction sequence gives by Rules 1 or 2 a multiple effect on slopes or intercepts, the resultant effects will be parabolic (or of higher degree for more than two points of combination) if: (a) the effects produced by all combinations are the same (inhibition or activation), (b) the points of combination are separated in the reaction sequence by reversible steps along which interaction may take place so that an increase in the concentration of the compound specifically causes, as the result of combination at one point, an increase in the steady-state concentration of the enzyme form reacting with this compound at the other (or next) point of combination (and so forth, if there are more than two points of combination). If no reversible sequences connect the points of combination, or if combination at one point does not affect the steady-state concentrations of the enzyme forms combined with at other points, then the resulting effects are linear. When analyzing intercept effects, it should be remembered that the variable substrate is saturating, and its combination with the enzyme is an irreversible step.

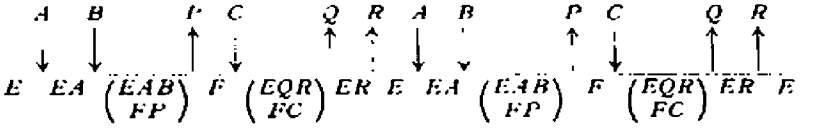
In a mechanism without alternate reaction sequences, when a compound reacts with two different enzyme forms to cause inhibition at one point and activation at another, and both combinations should affect either slope or intercept, then the resulting effects are neither linear nor parabolic. Replots of the slopes or intercepts would be a hyperbola having the vertical axis as one asymptote, and having as the other the expected plot in the absence of the activation if the replot is *versus* the concentration, or the inhibition if the replot is *versus* the reciprocal concentration of the compound under consideration. Such situations result when the compound is a substrate other than the variable one and can act also as a dead end inhibitor.

Initial velocity patterns

Initial velocity patterns are usually obtained by making reciprocal plots for

one substrate (variable substrate) at different fixed concentrations of one of the others (changing fixed substrate), while keeping all other substrates, if there are any, at constant concentration. If the mechanism does not include alternate reaction sequences, and no substrate reacts with more than one enzyme form, then according to Rule 1 the intercepts must always be a linear function of the reciprocal concentration of the changing fixed substrate. Analysis of the slope effects, however, may or may not show a reversible connection between the points of combination of the variable and changing fixed substrates. As a result there are two possible initial velocity patterns: parallel lines when no reversible connection exists, or lines intersecting to the left of the vertical axis when such a connection does exist. Whether the lines cross above, below, or on the horizontal axis depends on the ratio of certain kinetic constants (such as the ratio K_a/K_{ia} in Ordered Bi Bi) rather than on the mechanism. A replot of slopes *versus* the reciprocal concentration of the changing fixed substrate is linear.

As an example of this analysis, consider the Bi Uni Uni Bi Ping Pong mechanism[1]:



(1)

When A is the variable and B the changing fixed substrate, B specifically alters the rate of conversion of E to EA by reacting with EA and converting it into the central complex (EAB). Thus the slope is affected, and the intersecting pattern is observed. When B is the variable and A the changing fixed substrate, a high concentration of A converts E to EA more rapidly and specifically increases the concentration of EA, and thereby the rate of reaction between EA and B. The slope is affected as before. When A is the variable and C the changing fixed substrate, however, the situation is different. Changing the concentration of C does not specifically change the concentration of either E or EA (although these will both change by the same ratio as the result of the general redistribution of the enzyme among its forms as the concentration of C changes), because there is no reversible sequence between the points of combination of A and C. The release of P blocks the sequence on one side, and the release of Q and R blocks the sequence on the other side. The parallel pattern is thus observed.

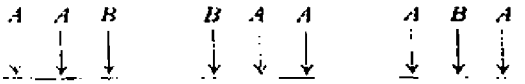
If the concentration of P is not zero, but has a finite value, then a reversible connection is established between C and A. An increase in the concentration of C lowers the steady-state concentration of F; this lowers the rate of reaction between F and P and the steady-state concentration of $(EAB\text{-}FP)$. As the concentration of this central complex falls, so does the rate of its decomposition to form EA, and the concentration of EA. A rise in the concentration of C thus specifically lowers the steady-state concentration of EA, and raises the net rate of combination of E with A. A decrease in slope results.

If B were saturating in the system, however, the reversible connection between A and C would again be broken. The steady-state level of EA would be zero already, and changing the concentration of C would have no effect. The parallel pattern would be observed, even though P were present. If B were the variable and C the

changing fixed substrate (or *vice versa*), the presence of P would always establish a reversible link and the intersecting pattern, regardless of the concentration of A.

This type of analysis also tells whether any substrates can be changed together with their concentrations at constant ratio. Any substrates not reversibly connected can be varied together as variable substrate without giving a non-linear reciprocal plot or as changing fixed substrate without giving non-linear replots of slopes and intercepts. Thus in Bi Uni Uni Bi, A and C or B and C can be varied together, but not A and B. If A and B are varied together as variable substrate, the reciprocal plots ($1/v$ *versus* $1/A$ or $1/B$) are parabolas. If A and B are varied together as changing fixed substrate, the replots of slopes and intercepts (*versus* $1/A$ or $1/B$) are likewise parabolas.

This theory will also predict what happens when a substrate is added two or more times during the reaction sequence. When such a substrate is the variable substrate it gives linear reciprocal plots if the points of addition of the substrate are not reversibly connected, but parabolas if the two points are reversibly connected (or higher degree functions if there are more than two points of addition reversibly connected). If such a substrate is the changing fixed substrate, replots of slopes and intercepts will be linear if the two points of addition are not reversibly connected, and parabolic if they are. It must be remembered, however, that the intercepts represent the points at which the variable substrate is saturating, and thus a parabolic intercept replot is observed only when there is a reversible sequence between the points of addition of the changing fixed substrate which does not include the addition of the variable substrate. For instance in the following partial reaction sequences, with B as variable substrate, replots of slopes *versus* $1/A$ are always parabolic, but replots of intercepts will be parabolic only for the first two mechanisms:



If A is the variable substrate, reciprocal plots ($1/v$ *versus* $1/A$) will always be parabolas except in the third case, where the lines would be straight if B were saturating, and parabolic if it were not.

Product inhibition patterns

Products that react with only one enzyme form always give linear inhibitions, which may be competitive (slopes only vary), uncompetitive (intercepts only vary), or non-competitive (both vary). In non-competitive cases, the lines cross to the left of the vertical axis, and as with the similar initial velocity pattern, the intersection point may be above, below, or on the horizontal axis, depending on the ratio of certain kinetic constants. If two or more molecules of a product are generated by a reaction so that the product normally combines with more than one enzyme form, the total effect is the sum of the various slope and intercept effects predicted separately. In accordance with Rule 3, if slope or intercept effects result from two points of combination, the effect is parabolic if the two points are reversibly connected, and linear if they are not.

A product inhibitor is capable of interacting along a reversible series of steps with variable substrates both upstream and downstream of it in the reaction se-

quence, so the application of Rules 1, 2 and 3 is straightforward. For example, consider the following analysis of product inhibition in the Bi Uni Uni Bi Ping Pong mechanism (Mechanism 1):

P as inhibitor, *A* varies: *P* reacts with *F*, and *A* with *E* in the sequence, so the intercept will vary with *P*. *P* and *A* can interact along the reversible sequence *E–EA–(EAB)-F*, so the slope varies also, and the inhibition is non-competitive. Saturation with *B* has no effect on the intercept term, but interupts the sequences between *A* and *P* so that the slope term no longer varies with *P* and the inhibition uncompetitive. Saturation with *C* eliminates inhibition by *P*, since *P* reacts with *F*, and when *C* is very large the steady-state concentration of *F* remaining in the system is nearly zero.

P as inhibitor, *B* varies: *P* and *B* react with different enzyme forms, so the intercept varies. The sequence *EA–(EAB)–F* is reversible, so the slope varies also, giving non-competitive inhibition. Saturation with *A* has no effect, but saturation with *C* eliminates inhibition by *P*, as before.

P as inhibitor, *C* varies: *P* and *C* react with the same enzyme form only, so the slope but not the intercept varies, giving competitive inhibition. Saturation with *A* or *B* has no effect.

Q as inhibitor, *A* varies: *Q* and *A* react with different enzyme forms, so the intercept varies. The sequence *ER E* is interrupted by liberation of *R* and the sequence *E–EA–(EAB)–F–(EQR)–ER* by liberation of *P*, so the slope does not vary and the inhibition is uncompetitive. Saturation with *B* or *C* has no effect since neither reacts with *ER*.

Q as inhibitor, *B* varies: *Q* and *B* react with different enzyme forms, so the intercept varies. The sequences between them are blocked by release of *P* and *R* as before, so the inhibition is uncompetitive. Saturation with *A* or *C* has no effect.

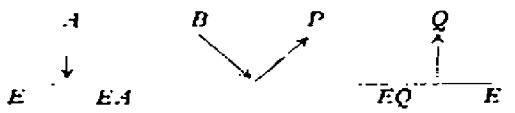
Q as inhibitor, *C* varies: *Q* and *C* react with different enzyme forms, so the intercept varies. The sequence *F (ERQ)-ER* is reversible so the slope varies also, giving non-competitive inhibition. Saturation with *A* or *B* has no effect.

R as inhibitor, *A* varies: *A* and *R* both react only with *E*, so the slope and not the intercept varies, giving competitive inhibition.

R as inhibitor, *B* varies: *R* and *B* react with different enzyme forms, so the intercept varies. The sequence *E–EA* is reversible, so the slope varies also and the inhibition is non-competitive. Saturation with *A* eliminates inhibition by *R*, since the concentration of *E* is reduced to zero. Saturation with *C* has no effect.

R as inhibitor, *C* varies: *R* and *C* react with different enzyme forms, so the intercept varies. The sequences between *R* and *C* are rendered irreversible by release of *P* and *Q*, so the slope does not vary and the inhibition is uncompetitive. Saturation with *A* eliminates inhibition by *R*. Saturation with *B* has no effect.

This type of analysis will also successfully predict inhibition patterns in mechanisms such as the Theorell–Chance mechanism (Mechanism 2) where product inhibition by *P* against *B* as variable substrate is competitive.



(2)

Saturation with B overcomes this inhibition completely by converting all of the EA into EQ and lowering the steady-state concentration of EA essentially to zero. There is no inhibition by P under these conditions, since the inhibitory effect of P is to raise the steady-state level of EA. The intercepts of reciprocal plots are thus not affected and the inhibition is competitive. These rules also predict the competitive product inhibition by either product in the Rapid Equilibrium Random Bi Bi mechanism[1], where saturation with the variable substrate lowers the equilibrium concentrations of the enzyme forms reacting with the inhibitor to zero and thus eliminates the inhibition.

Dead end inhibition patterns

Compounds which are not substrates or products but which react with one or more enzyme forms to give complexes which can not participate in the reaction give dead end inhibition. Because dead end inhibitors can not back up reaction sequences, as can products, they influence the slopes of reciprocal plots only when the point of combination of the variable substrate follows the point of addition of inhibitor in the reaction sequence and is reversibly connected with it. Dead end inhibitors often add to more than one enzyme form. According to Rule 3, however, the inhibitions are always linear because adding the inhibitor at one point never increases the concentration of any of the other normal enzyme forms. Only if the inhibitor combines with an EI complex to form EI_2 is a parabolic inhibition observed. The following analysis of dead end inhibition for the Bi Uni Uni Bi Ping Pong mechanism (Mechanism 1) will serve to illustrate the method

I reacts with E, A varies: I and A react only with the same enzyme form, so the inhibition is competitive.

I reacts with E, B varies: I and B react with different enzyme forms, so the intercept varies. The sequence EI–E–EA is reversible, so the slope varies, giving non-competitive inhibition. Inhibition by I is eliminated by saturation with A, but saturation with C has no effect.

I reacts with E, C varies: No reversible sequence connects E and F, so the inhibition is uncompetitive. Saturation with A eliminates inhibition by I, as before.

I reacts with EA, A varies: EAI and E are not part of a reversible sequence, so the inhibition is uncompetitive. Saturation with B eliminates inhibition by I.

I reacts with EA, B varies: The inhibition is competitive, since both B and I react only with EA.

I reacts with EA, C varies: The release of P interrupts the reversible sequence between EAI and F, so the inhibition is uncompetitive. Saturation with B eliminates the inhibition.

I reacts with *(EAB)*, *(EQR)*, or *ER:* No substrates react with these enzyme forms and reversible sequences are blocked by release of products, so the inhibition is always uncompetitive.

I reacts with F, A varies: There is no reversible sequence connecting FI and E so the inhibition is uncompetitive. Saturation with C lowers the concentration of F to zero and eliminates the inhibition.

I reacts with F, B varies: The situation is the same, and the inhibition is uncompetitive, and is eliminated by saturation with C.

I reacts with *F*, *C* varies: The inhibition is competitive, since *C* and *I* both react only with *F*.

Note the large number of uncompetitive inhibitions predicted by the above analysis, and the fact that these inhibitions result from combination with enzyme forms other than central complexes. This author feels that dead end combination with central complexes is extremely unlikely, and that all uncompetitive inhibitions observed experimentally can be explained by combination with non-central transitory complexes or stable enzyme forms. The belief that uncompetitive inhibition results from combination with a central complex is one of the very unfortunate results of trying to extend a theory designed for unireactant mechanisms to multireactant ones. This author is aware of no uncompetitive inhibitions observed for true Uni Uni reactions.

Mixed dead end and product inhibition

If a product also reacts in dead end fashion with an enzyme form other than the one it normally reacts with, it gives mixed product and dead end inhibition. The inhibitory effects are predicted separately by Rules 1 and 2, and then the net effects, if multiple ones are predicted, are determined by Rule 3. In order for parabolic effects to be observed, however, the dead end combination must precede the normal combination point with reversible steps in between. Combination at the normal point in the sequence then backs up the reaction and results in an increase in the concentration of the enzyme form that combines to form the dead end complex.

As an example of this type of analysis, consider the following multiple inhibitions by *P* in the Bi Uni Uni Bi mechanism (Mechanism 1) when *B* is the variable substrate. If *P* combines only with *F*, we have linear non-competitive inhibition, as found above. If *P* also reacts with *E*, there will be additional effects on both slope and intercept, since *P* and *B* still combine with different enzyme forms, and there is a reversible sequence between *E* and *EA*. The two points of addition of *P* are reversibly connected, but with the addition of *B* occurring in between. The slope is thus a parabolic, but the intercept a linear function of inhibitor, and we have S-parabolic I-linear non-competitive inhibition. Saturation with *A* eliminates the dead end inhibition by *P* and leaves only the linear non-competitive product inhibition. Saturation with *C* eliminates the product inhibition, but leaves the dead end inhibition, which is also linear non-competitive.

If *P* reacts with *EA* as well as with *F*, an additional effect results only on the slope, since *B* and *P* are both combining with *EA*. The two points of addition of *P* are reversibly connected, so the slope is a parabolic function of inhibitor concentration, and we have again S-parabolic I-linear non-competitive inhibition. Saturation with *A* now has no effect, while saturation with *C* eliminates the product inhibition and leaves only linear competitive inhibition due to the dead end combination with *EA*.

If *P* reacts with the central *(EAB–FP)* complex as well as with *F*, only an additional effect on the intercept is predicted. Since the two points of addition of *P* are reversibly connected, the variation is parabolic and we have S-linear I-parabolic non-competitive inhibition. Saturation with *A* has no effect, but saturation with *C* eliminates the product inhibition, leaving linear uncompetitive inhibition.

When P reacts in dead end fashion with F, (EQR), or ER in addition to the expected combination with F, additional effects on only the intercept are predicted. Since the two points of addition of P are not reversibly connected, or are the same, the intercept will still be a linear function of inhibitor concentration. Saturation with A has no effect, while saturation with C eliminates all inhibition when both combinations are with F, and leaves linear uncompetitive inhibition when the dead end combination of P is with (EQR) or ER.

If we look at multiple interactions by P in the same mechanism with C as variable substrate, one other interesting point is brought out. Combination of a dead end inhibitor with E, EA, or (EAB) would normally not give an effect on the slope, since the release of P is an irreversible step. Dead end inhibition by P does give an effect on the slope here, however, since the presence of P in the system keeps the reaction sequence reversible. Thus combination of P with any of these forms gives parabolic slope variation, although saturation with B reduces this to linear if the combination is with E or EA, and saturation with A does likewise if combination is with E.

DISCUSSION

The value of the methods outlined in this paper is too obvious to need further elaboration here. They have been used routinely in this laboratory for exploring possible mechanisms, and they have proven of special value to students trying to learn something useful about kinetics without getting lost in equations and algebra. While only non-random mechanisms without alternate reaction sequences (excepting Rapid Equilibrium Random mechanisms) have been treated in this paper, preliminary analysis indicates that the general approach can probably be extended to cover alternate product and similar useful types of experiments. This will require a more sophisticated development of the theory of slope and intercept variation than the simple analysis given here, however, and will be left for the future.

ACKNOWLEDGEMENT

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