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Preface

Enzyme kinetics has undergone very rapid growth and development during the past fifteen years and has been well received by the biochemical community. A cursory glance at the current biochemical literature reveals the increasing popularity of enzyme kinetics; yet, there are very few books available to guide the enzymologist who wishes to conduct kinetic experiments.

This monograph was undertaken to provide the fledgling kineticist with an outline of contemporary initial rate enzyme kinetics. A large portion of the material contained in this book is presented in a second-year, graduate-level course in biochemistry at Iowa State University. I have found that the presentation in this course has enabled students without a strong background in mathematics to undertake initial rate studies at the research bench. The monograph obviously is more comprehensive than any course could be, and should permit similar accomplishment.

As the title implies, the major emphasis of this monograph is on initial rate enzyme kinetics. I considered at length the advisability of including chapters on integrated rate equations and on the theory and application of rapid reaction kinetics, such as rapid-mixing stopped-flow, and temperature-jump kinetics. These, however, are topics that would require a good deal of space to develop if they were to be helpful to the beginner. Some deviation from initial rate kinetics was required when the topics of cooperativity and allostery were broached. A very large fraction of the research in this area of biochemistry has involved static binding measurements, and the current literature clearly reflects this. It was necessary, therefore, to introduce these topics within the framework of the simpler equilibrium binding models before the kinetics of allostery and cooperativity were considered.

It will become quite obvious that a number of topics are omitted that might have been included in Chapter IX. In an area of research such as allostery, which is in a state of flux at this writing, concepts that are not widely accepted or clearly defined are either treated superficially or not included.

Books on kinetics usually cover theory and interpretation of data in the literature, but rarely present the experimental protocol. Chapters III and VI are devoted in large measure to instruction on setting up and carrying out initial rate and isotope-exchange experiments. Although these sections may not provide enlightenment for the advanced student, they may serve to lower

the energy barrier to potential experimentalists who wish to use their theoretical knowledge for practical ends.

Acknowledgement

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Ames, January 1975 HERBERT J. FROMM

Contents

Chapter I

Nomenclature, Definitions, and Evolution of the Kinetic Mechanism

Kinetics is now an integral part of enzymology. This statement would not have been valid fifteen or even ten years ago; however, within the last few years biochemists have begun to appreciate the enormous potential that the kinetic approach offers to the study of enzyme reaction mechanisms. The fact that in many types of kinetic studies one need not have a pure protein, that elaborate equipment is usually not required, and that the experimental protocol is relatively simple, are important factors in the increasing popularity of kinetics. Although it is true that "kinetics cannot prove anything" in terms of enzyme mechanisms, it is also true that, when considered in the context of current physical and organic chemical theory, such cliches become less meaningful.

Steady-state kinetics is and has been used to investigate the mechanism of enzyme action (how enzymes and their substrates interact), the functional groups on the enzyme involved in catalysis, mapping of the active enzyme site, the types of intermediates involved in the catalytic process in certain instances, and kinetic parameters that are essential for an understanding of enzyme regulation and control.

It is unfortunate that, in many instances, initial rate or kinetic studies are not undertaken until a good deal of chemical data have been accumulated in studies of enzyme mechanisms. Although it is essential that both types of information be available when evaluating enzyme catalysis, it is usually more profitable to do the kinetic studies relatively early in the experimental phase of the investigation. This point is well described by the history of the studies of yeast hexokinase during 1956 - 1962.

After Mildred COHN had shown that the hexokinase reaction involves phosphoryl rather than phosphate group transfer from ATP to acceptor glucose (1), ÅGREN and ENGSTRÖM reported finding phosphate associated with the enzyme after reaction with ATP (2). They concluded that the enzyme reaction involves participation of a phosphoryl-enzyme (E-P) covalent intermediate. Their proposal can be described in terms of the following pathway of enzyme and substrate interaction:

$$E + ATP \rightleftharpoons EX \rightleftharpoons E\text{-}P + ADP \qquad (I\text{-}1)$$

$$E\text{-}P + glucose \rightleftharpoons EY \rightleftharpoons E + glucose\text{-}6\text{-}P. \qquad (I\text{-}2)$$

In this series of reactions, E, EX, and EY are taken to be free enzyme, Michaelis complex EX, and Michaelis complex EY, respectively.

It is clear from Eq. (I-2) that, in the presence of hexokinase, but in the absence of nucleotide substrates, there should be a glucose⇄glucose-6-P exchange reaction. A similar partial exchange should be observed for ADP and ATP in the absence of the sugar substrates. NAJJAR and McCOY (3) investigated this problem and were unable to discern the requisite glucose⇄glucose-6-P exchange. On the basis of this observation, they discarded the Ågren-Engström proposal in favor of a mechanism that involves an obligatory glucosyl-enzyme intermediate; i.e.,

$$\text{E-glucose} + \text{ATP} \rightleftharpoons \text{E-glucose-6-P} + \text{ADP} \qquad \text{(I-3)}$$

$$\text{E-glucose-6-P} + \text{glucose} \rightleftharpoons \text{E-glucose} + \text{glucose-6-P}. \qquad \text{(I-4)}$$

In the mechanism of yeast hexokinase action as proposed by NAJJAR and McCOY (3) and described by Eqs. (I-3) and (I-4), glucose displaces glucose-6-P from the enzyme, and the partial glucose⇄glucose-6-P exchange reaction would not occur. On the other hand, KAUFMAN (4) reported in 1955 that yeast hexokinase did not exhibit a demonstrable ADP⇄ATP exchange in the absence of sugar substrates, and this finding was clearly at variance with the proposals involving the obligatory enzyme-phosphoryl and enzyme-glucosyl intermediates.

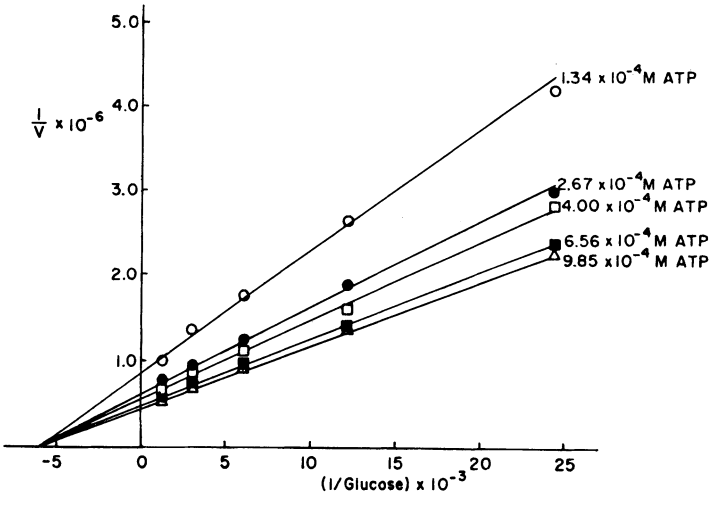


Fig. I-1. Plot of reciprocal of initial reaction velocity (V) versus reciprocal of molar concentration of glucose. The ATP concentrations are shown on the graph. V was determined as a function of glucose concentration, which was varied in the range from 7.88×10^{-4}M to 4.10×10^{-5}M. Velocities are expressed as moles per liter of product formed per minute

These possibilities were reinvestigated chemically by COLOWICK's (5) and BOYER's (6) groups, and no evidence for covalent intermediates was obtained. Both HAMMES and KOCHAVI (7) and FROMM and ZEWE (8) were able independently to show very clearly in 1962 that both glucose and MgATP must reside on hexokinase simultaneously before product formation could occur.

The experimental protocol used to distinguish between mechanisms involving covalent intermediates and those involving concerted type mechanisms is illustrated in Figs. I-1 and I-2. Experimentally, one substrate is held constant at different fixed concentrations, and the other substrate is varied. In order to determine the type of intermediate involved, one need establish merely whether the family of curves intersect or are parallel. In the case of yeast hexokinase, the data required to make this determination were obtained in only a few hours and are described in Figs. I-1 and I-2. It is evident that, had these initial rate experiments been carried out in 1956, a good deal of extraneous information regarding the kinetic mechanism of hexokinase action would not have accumulated in the literature.

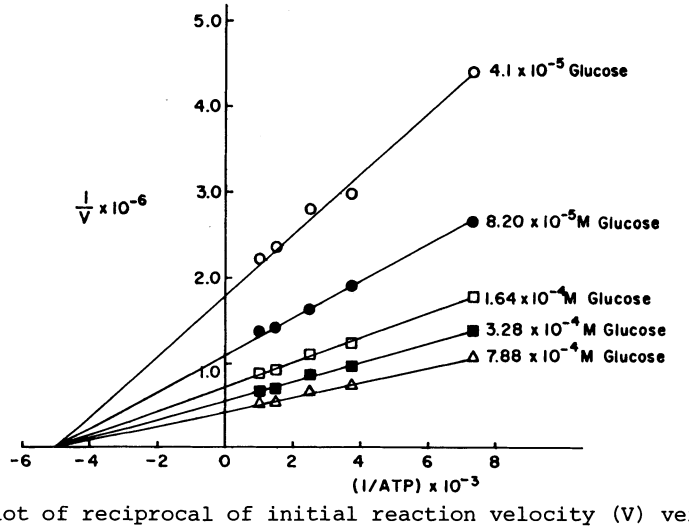


Fig. I-2. Plot of reciprocal of initial reaction velocity (V) versus reciprocal of molar concentration of ATP. The concentrations of glucose are shown on the graph. V was determined as a function of ATP concentration, which was varied in the range from 9.85 × 10^{-4}M to 1.34 × 10^{-4}M. V is expressed as in Fig. I-1

A. Nomenclature

The nomenclature of CLELAND (9) will be used throughout this book to describe enzyme and substrate interactions. Substrates will be indicated by the letters A, B, and C, and products by P, Q, and R. Substrates A, B, and C will add to the enzyme in that order, and products P, Q, and R will dissociate, with P leaving first and R leaving last. In certain kinetic pathways, sub-

strate addition and product release will not occur in any particular order, and these mechanisms are called random mechanisms.

CLELAND (9) refers to those enzyme forms that can break down in a unimolecular step to substrates or products, or those enzyme forms which can isomerize to these forms, as transitory complexes. These complexes are of the binary, ternary, or quarternary type and will be described by the substrate molecules with which they are associated; e.g., EA, EPQ, EAQ, etc. Central complexes are those transitory complexes that can only decompose in a unimolecular step to substrates or products, or transitory complexes that isomerize to such enzyme forms. Central complexes cannot participate in bimolecular reactions. An illustration of transitory complexes can readily be provided by using liver alcohol dehydrogenase as an example:

$$E + NAD \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\text{-}NAD \qquad \text{(I-5)}$$

$$E\text{-}NAD + ethanol \underset{k_4}{\overset{k_3}{\rightleftharpoons}} (E\text{-}NAD\text{-}ethanol) \rightleftharpoons (E\text{-}NADH\text{-}acetaldehyde) \underset{k_6}{\overset{k_5}{\rightleftharpoons}} acetaldehyde + E\text{-}NADH \qquad \text{(I-6)}$$

$$E\text{-}NADH \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E + NADH. \qquad \text{(I-7)}$$

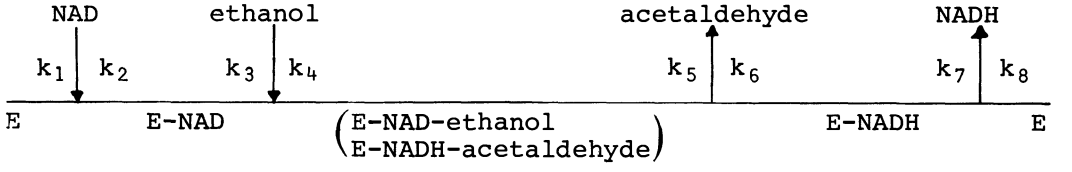
In this kinetic mechanism, all compounds except the free enzyme, E, substrates, and products are transitory complexes. The central complexes, indicated by parentheses, are (E-NAD-ethanol) and (E-NADH-acetaldehyde). Any complex or complexes intermediate between these two also are central complexes. The term "stable complex" is used to describe transitory complexes that are not central complexes.

The terms Uni, Bi, and Ter will be used to describe the number of substrates and products involved in the reaction. These terms will be used in conjunction with types of mechanisms; i.e., "Ordered" and "Random." Thus, a mechanism will be described as an Ordered Bi Ter or Random Uni Bi mechanism. In the former case this will mean that two substrates add to the enzyme in an obligatory fashion; i.e., A first and then B and dissociate from the enzyme in the order P, Q, and R. On the other hand, in the latter pathway, only one substrate (A) will interact with enzyme, and the products will dissociate in random fashion; i.e., P followed by Q or vice versa.

If all substrates must reside at the active site of the enzyme before product can be formed, the mechanism will be referred to as "Sequential"; however, if an enzyme reacts with one substrate to yield a product that dissociates before the next substrate adds, the mechanism will be referred to as "Ping Pong." Thus, the sequence of substrate addition in a Bi Uni Uni Bi Ping Pong mechanism will be A, B, P, C, Q, R.

When stable enzyme forms that are part of the enzyme-substrate pathway isomerize, there is an alteration in the rate equation relative to the mechanism in which isomerization does not occur. These mechanisms are "Iso" mechanisms and give rise to such terms as "Iso Ordered" or "Iso Ping Pong" when a single stable enzyme form isomerizes. In the case of mechanisms in which more than one stable enzyme form isomerizes, terms such as "Di-Iso" and "Tri-Iso" are used.

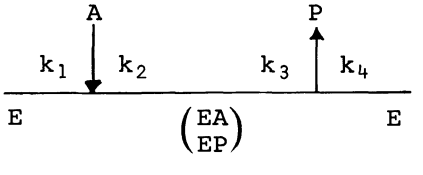
Rate equations and enzyme and substrate pathways of interaction will be presented as suggested by CLELAND (9). With alcohol dehydrogenase, which is described by Eqs. (I-5) to (I-7), the following model is used:



Scheme I-1

The enzyme is presented as a horizontal line, and substrate addition and product release are described by vertical arrows. These arrows may include either rate constants (lower case, "k") or dissociation constants (upper case, "K"). The various enzyme intermediates appear below the horizontal line, with the central complexes in parentheses.

The symbols for maximal velocity, dissociation and Michaelis constants, and apparent equilibrium constant are described by the familiar Uni Uni mechanism of Michaelis and Menten described by Scheme I-2.



Scheme I-2

The rate expression for this mechanism is

$$v = \frac{V_1 V_2 (A - \frac{P}{K_{eq}})}{K_a V_2 + V_2 A + \frac{V_1 P}{K_{eq}}} \qquad \text{(I-8)}$$

where v, V_1, V_2, K_a, and K_{eq} are initial reaction velocity, maximal forward velocity, maximal reverse velocity, Michaelis constant for A, and the apparent equilibrium constant, respectively.

The equilibrium constant may be described in terms of substrate and product ratios, rate constants, or kinetic parameters; e.g.,

$$K_{eq} = \frac{P}{A} = \frac{k_1 k_3}{k_2 k_4} = \frac{V_1 K_p}{V_2 K_a}. \qquad \text{(I-9)}$$

It should be remembered that if a proton is generated in the reaction, K_{eq} will equal the thermodynamic equilibrium constant divided by the hydrogen ion concentration.

Additional points of nomenclature will be required as new concepts are introduced. In addition, rate equations will be presented when applicable either in the text or in the *Appendix*. A comparison of the various nomenclatures now in vogue is shown in Table I-1.

Table I-1. Nomenclature comparison of kinetic parameters for sequential Bi Bi mechanisms

	This Monograph	DALZIEL (10)	ALBERTY (11)
Michaelis constant for A [a]	K_a	Φ_1/Φ_0	K_A
Michaelis constant for B	K_b	Φ_2/Φ_0	K_B
Dissociation constant for EA complex	K_{ia}	Φ_{12}/Φ_2	K_{AB}/K_B
Maximum velocity	V_1	e/Φ_0	V_f
Turnover number	V_1/E_0	$1/\Phi_0$	$V_f/(E)_0$

[a] A adds first for ordered mechanisms.

B. Evolution of Initial Rate Kinetics

The idea that the enzyme and substrate must unite for a finite time before catalysis can occur is the recognized basis of contemporary enzyme kinetics. O'SULLIVAN and TOMPSON (12) were among the first to suggest such interaction, based upon their studies of invertase action on sucrose in 1890. They stated that, "when invertase hydrolyses cane-sugar, combination takes place between the two substances, and the invertase remains in combination with the invert-sugar. The combination breaks up in the presence of molecules of cane-sugar."

O'SULLIVAN and TOMPSON (12) also presented data that suggested that the invertase reaction exhibited first-order kinetics relative to the substrate. They concluded that the system followed the law of mass action. In 1892 BROWN (13) observed that the same reaction, when catalyzed by a yeast preparation, displayed kinetics independent of sucrose concentration; i.e., the system displayed zero-order kinetics relative to sucrose. These results were clearly in conflict with the concept that enzyme catalysis

adheres to the law of mass action. In 1902 BROWN (14) was able to synthesize the concept of enzyme kinetics that today is universally accepted and that accounts for the typical rectangular hyperbolic response observed when initial velocity is plotted versus substrate concentration. He proposed that the enzyme and substrate interact to form a complex that persists for a finite time before decomposing to form product with regeneration of the active enzyme. He explained the first-order kinetics by suggesting that, at low substrate concentration, the enzyme could turn over more substrate than was being presented to it. Zero-order kinetics, according to BROWN, were a result of greater amounts of substrate than the enzyme could convert to product.

HENRI (15), in attempting to summarize the state of enzyme kinetics in 1903, pointed out that enzymes do not alter the equilibrium constant, but do enhance reaction rates in direct proportion to their concentration, and in addition, are present in kinetic studies at much lower levels than are the substrates. Using these points as the basis of his reasoning, HENRI was able to propose a rate expression that has the exact form of the well-known Michaelis-Menten (16) equation. He also integrated this equation and indicated how it could be used to follow the entire time course of an enzyme catalyzed reaction. This procedure provided the basis for the well known integrated rate expressions.

HENRI (15) and MICHAELIS and MENTEN (16), in order to explain enzyme kinetics using a mathematical formulation, assumed that the enzyme and substrate were in equilibrium with the complex of enzyme and substrate. The reaction velocity was assumed to be governed by the decomposition of the enzyme-substrate complex. BRIGGS and HALDANE (17) incorporated the concept of the steady-state into the derivation of the Michaelis-Menten equation in 1925. This point will be elaborated in Chapter II; however, it is useful to point out here that, in both approaches to the derivation of the kinetic equation, the concentration of the binary complex remains essentially constant, but for different reasons.

If we set P in Eq. (I-8) equal to zero, the result is the easily recognized Michaelis-Menten equation,

$$v = \frac{V_1 A}{K_a + A}. \qquad \text{(I-10)}$$

This expression serves to explain the rectangular hyperbola obtained when v is plotted against A and also why the kinetics are first-order with respect to A at low levels of A but zero-order in substrate at high concentrations of A.

K_a is the Michaelis constant for substrate and is defined as the substrate concentration when the initial velocity is equal to one-half the maximal velocity; i.e., $v = 1/2\ V_1$. Figure I-3 illustrates a typical velocity-substrate profile and shows how K_a may be calculated.

If one uses the equilibrium assumption, K_a is the dissociation constant for the breakdown of the EA complex into its component parts. It is not possible to define this constant definitively if steady-state conditions prevail, except to cite the definition already described in terms of substrate concentration, V_1, and v.

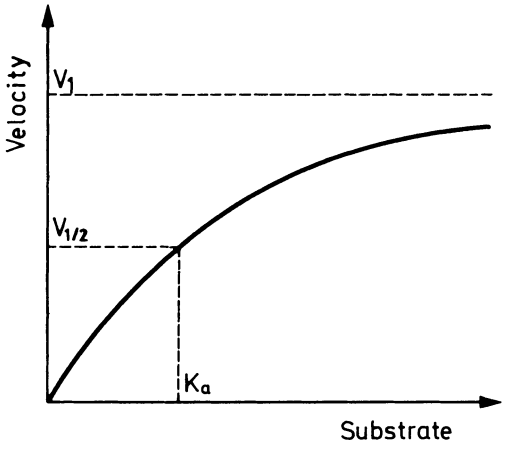


Fig. I-3. Plot of velocity versus substrate concentration. V_1 and K_a represent maximal velocity and and Michaelis constant, respectively

Equation (I-10) seems to adequately explain the observations of BROWN (14) and HENRI (15) with invertase. It should be pointed out that the rate equation for Scheme I-2, as well as the kinetic mechanism, considers the hydrolysis reaction to be a unimolecular reaction when, in fact, the reactants are sucrose and water. The latter substrate does not enter overtly into the velocity expression because its concentration, 55 M, does not change in the reaction. Similar arguments can be made regarding a number of other nonsubstrate factors known to influence the velocity of enzyme catalyzed reactions but that remain constant during a reaction; e.g., temperature, pH, ionic strength, etc.

At low substrate concentration; i.e., where $K_a >> A$, Eq. (I-10) is reduced to:

$$v = \frac{V_1 A}{K_a} \qquad \text{(I-11)}$$

while, at high substrate concentration when $A >> K_a$, Eq. (I-10) is altered to give

$$v = V_1 . \qquad \text{(I-12)}$$

Under conditions that satisfy Eq. (I-11), the reaction is first-order relative to substrate whereas the kinetics appear to approximate zero-order at high substrate concentration (Eq. I-12). These conclusions serve to explain the rather typical findings observed with enzymes as illustrated in Fig. I-3.

The kinetic models proposed by HENRI (15), BROWN (14), and MICHAELIS and MENTEN (16) are not the only schemes that give rise to data of the type shown in Fig. I-3. Consider, for example, LANGMUIR's proposal in 1916 of the adsorption of a gas on a solid support (18). He made the following assumptions: a) Only one molecule can be adsorbed per site, b) there is no site-site interaction; i.e., the presence of a molecule on one site does not affect neighboring sites, and c) the number of sites are fixed.

If we let $\bar{Y}$ represent the fraction of sites bound, then $(1-\bar{Y})$ is the fraction unoccupied. The rate of adsorption by substrate A will equal some constant k_1 multiplied by A times the factor $(1-\bar{Y})$, or

$$\text{adsorption rate} = k_1 A(1-\bar{Y}). \qquad \text{(I-13)}$$

On the other hand, adsorbed molecules will tend to leave the support at a rate equal to the fraction of occupied sites, $\bar{Y}$, multiplied by some rate constant k_2, or

$$\text{desorption rate} = k_2\bar{Y}. \qquad \text{(I-14)}$$

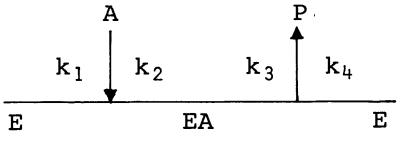
At equilibrium, these two rates will be equal by definition, and

$$k_2\bar{Y} = k_1 A(1-\bar{Y}) \quad \text{or} \qquad \text{(I-15)}$$

$$\bar{Y} = \frac{k_1 A}{k_2 + k_1 A}. \qquad \text{(I-16)}$$

A plot of $\bar{Y}$ versus A will give the type of curve shown in Fig. I-3. What makes this model so interesting are the assumptions, which are identical to those required to describe the initial rate kinetics of an enzyme catalyzed reaction. The fractional saturation of sites, $\bar{Y}$, is analogous to EA/(E + EA) in Scheme I-3. It will be shown in Chapter II that EA/(E + EA) v/E_0, where E_0 is the total enzyme concentration.

The original proposal regarding kinetic mechanisms espoused by HENRI, BROWN, MICHAELIS, and many other early workers is outlined in Scheme I-3.



Scheme I-3

Cursory examination of Scheme I-3 reveals some serious inadequacies in attempting to describe an enzymatically catalyzed chemical reaction by this pathway. Even if one replaces EA with EX, it is clearly simplistic to assume that both substrate and product react in a single step to form the *same* binary complex.

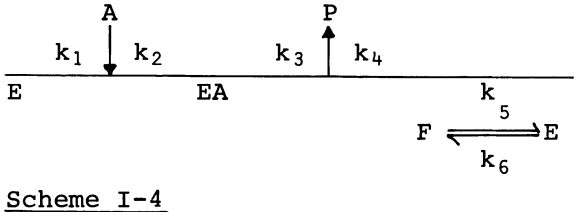
Such an assumption is in obvious disagreement with current transition state theory of chemical reactions. HALDANE (19) has provided us with a more realistic kinetic mechanism to describe substrate and product interactions with enzymes. His proposal is described by the pathway outlined in Scheme I-2.

In Scheme I-2 it is seen that the binary complex of enzyme and substrate is capable of undergoing some sort of transition, defined previously as an isomerization. The rate equation for Scheme I-2 is identical in form to that of Scheme I-3. The composition of the kinetic parameters, K_a and V_1, will not differ for the two mechanisms under consideration, but a choice of mechanism can be made kinetically by using approaches that involve variation of kinetic parameters with pH (20).

If the isomerization reaction is included in the kinetic pathway illustrated by Scheme I-2; i.e., $(EA) \underset{k_6}{\overset{k_5}{\rightleftharpoons}} (EP)$, two additional rate constants, k_5 and k_6, will be incorporated into the rate equation. It is possible when studying this pathway in the forward reaction to obtain values for V_1 and K_a, and, in the reverse reaction, V_2 and K_p. It is clearly not possible from initial rate studies to evaluate the rate constants for the isomerization step as there are only four known quantities, V_1, V_2, K_a, and K_p, and six unknown rate constants. The constants, k_5 and k_6, are incorporated into the four determinable kinetic parameters and cannot be evaluated. These points will be elaborated in Chapter II.

It is often stated that kinetics does not permit one to gain insight into reactions that occur in the central complexes. Although it is true that one cannot gain information of this type directly from initial rate experiments, the point seems to be overstated. It is possible, by using transition state analogs and alternative substrates, for example, in conjunction with initial rate studies, to arrive at definitive conclusions on the chemistry involved in the transition state. Consider as a case in point the sugar specificity of the hexokinase reaction. PURICH et al. (21) were able, from a knowledge of sugar substrate specificity, to come to certain conclusions regarding the probable structure of the hexose in the transition state.

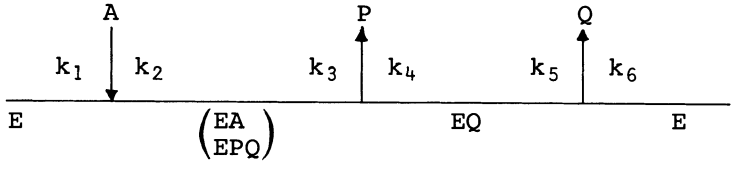
Other types of isomerization may yield rate equations that do differ in form; i.e., give rise to unique terms, when compared with analogous mechanisms in which such transitions do not occur. A relatively simple case in point is the pathway outlined in Scheme I-4.



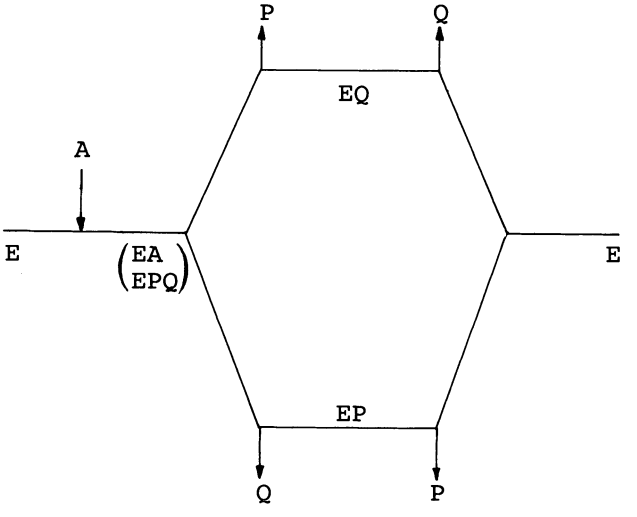
Scheme I-4

The velocity expression for the mechanism illustrated in Scheme I-4 is identical in form to those of Schemes I-2 and I-3 when P is zero; however, in the presence of product the rate equation for Scheme I-4 will contain a unique term, (A)(P), which is not found with the other mechanisms. Similarly, when considering bireactant enzyme mechanisms, if isomerizations occur that do not involve central complexes, it may be possible to detect such transitions from initial rate studies.

It is obvious that very few enzyme systems exhibit Uni Uni mechanisms of the type illustrated in Schemes I-2 and I-3. Two relatively frequently encountered one-substrate pathways are depicted in Schemes I-5 and I-6. These are the Ordered Uni Bi (Scheme I-5) and Random Uni Bi models (Scheme I-6).



Scheme I-5



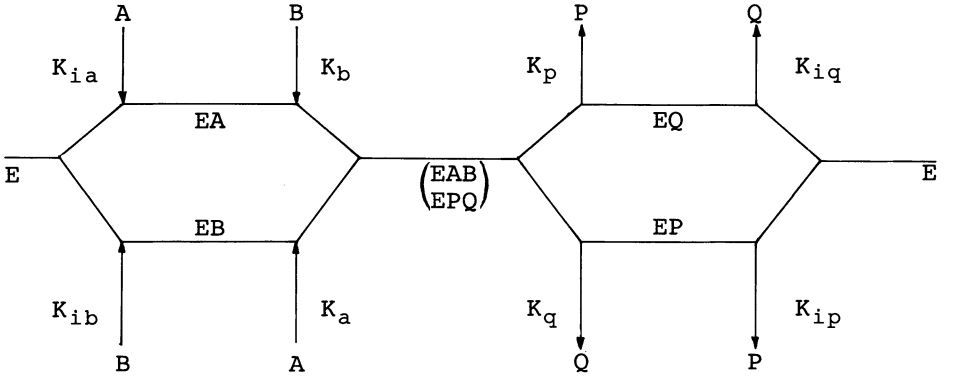
Scheme I-6

The initial rate equation for the Uni Bi mechanisms is identical in form to that of the Uni Uni example when studied from the A substrate side of the reaction[1]. In the presence of product, however, a choice can be made between the Ordered and Random cases. It also is possible to make a distinction between these possibilities from studies of the reverse reaction as will be indicated later.

[1] This statement assumes that the rate limiting step for the Uni Bi reactions is the breakdown of the central complexes.

Two Substrate Systems

Most of the enzymes studied kinetically using the steady-state approach are of the bireactant type, A + B⇌P + Q. In 1930, HALDANE (19) proposed a model for bireactant systems that seems to fulfill the criteria cited earlier for enzyme catalyzed reactions. This mechanism, which is clearly the simplest and most general case for a two substrate system, is the rapid equilibrium Random Bi Bi kinetic model described in Scheme I-7.



Scheme I-7

For this pathway of enzyme and substrate interaction, all steps are assumed to equilibrate rapidly relative to the interconversion, or isomerization, of the central ternary complexes[2]. The forward rate equation for this mechanism has the form

$$\frac{V_1}{v} = 1 + \frac{K_a}{A} + \frac{K_b}{B} + \frac{K_{ia}K_b}{(A)(B)}. \qquad \text{(I-17)}$$

It can be seen from Eq. (I-17) that saturation by either substrate will reduce the rate expression to that of a one-substrate system; e.g., if B → ∞ at any finite concentration of A, the rate equation will reduce to:

$$\frac{V_1}{v} = 1 + \frac{K_a}{A}. \qquad \text{(I-18)}$$

It also is clear from Eq. (I-17) that, if one plots V_1/v versus $1/A$ at *different* fixed levels of B, there will result a family of linear lines that intersect to the left of the ordinate axis.

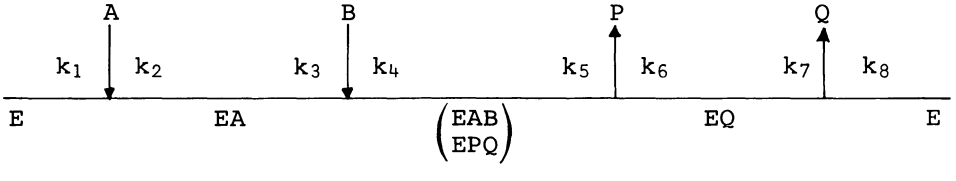
---

[2] K_{ia}, K_{ib}, K_{ip}, and K_{iq} represent dissociation constants for the EA, EB, EP, and EQ complexes, respectively. The following relationships hold from thermodynamic considerations: $K_{ia}K_b = K_{ib}K_a$ and $K_{iq}K_p = K_{ip}K_q$.

In this graph, the intercept term will be $(1 + K_b/B)$, and the slope term $(K_a + K_{ia}K_b/B)$. Thus, as B decreases, both intercepts and slopes will increase. A similar result is to be expected when V_1/v is plotted as a function of the reciprocal of B at various fixed levels of A. Figs. I-1 and I-2 illustrate the types of data to be expected from the use of Eq. (I-17).

Kinetic mechanisms that give rise to curves of this type are referred to as *Sequential* mechanisms - so designated by CLELAND (9). It is of interest to point out that, if the last term in Eq. (I-17) is small relative to other terms in the rate expression, then, at different levels of fixed substrate, the resulting family of curves, when V_1/v is plotted against $1/A$, may appear to give parallel, rather than converging lines; i.e., the $(K_{ia}K_b/B)$ term will not appear in the slope. This point, which has serious implications when considering certain types of kinetic mechanisms, will be discussed in detail in Chapter III.

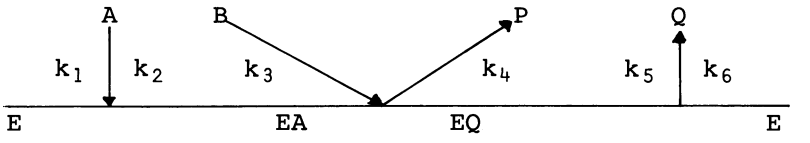
An obvious variation of the mechanism shown in Scheme I-7 involves the case of obligatory order of substrate binding. Mechanistically, this type of pathway requires that one and only one of the two substrates, designated A, can add to the enzyme before the second substrate can be adsorbed to form a productive ternary complex. The simplest explanation of how this sequence of substrate addition occurs is to suggest that the pocket at the active site for B does not exist preceding the presence of A on the enzyme. Presumably, after the formation of a Michaelis type complex between enzyme and A, a conformational change occurs that creates a site on the enzyme for B. This mechanism is summarized in Scheme I-8 and is referred to as the Ordered Bi Bi mechanism. Alcohol dehydrogenase described in Eqs. (I-5) to (I-7) exhibits this type of mechanism.



Scheme I-8

As was pointed out in the previous discussion, it is not possible to differentiate between this model and the case in which one or more central complexes are formed; however, current transition state theory demands that at least two such complexes be formed. SEGAL, KACHMAR, and BOYER (22) proposed this mechanism in 1952, as well as a number of others, for bireactant systems in a paper that has been largely overlooked.

A modification of the Ordered Bi Bi mechanism, the "Theorell-Chance" model (23), does not involve kinetically significant ternary complexes and is shown in Scheme I-9.



Scheme I-9

The difference between the Theorell-Chance and the Ordered Bi Bi mechanism is subtle and has been pointed out by ALBERTY (11). This difference can be appreciated by reference to V_1 and V_2 for these two mechanisms. For the Ordered Bi Bi mechanism (Scheme I-8),

$$V_1 = k_5k_7E_0/(k_5 + k_7) \text{ and } V_2 = k_2k_4E_0/(k_2 + k_4) \qquad (I\text{-}19)$$

where E_0 is the total enzyme concentration.

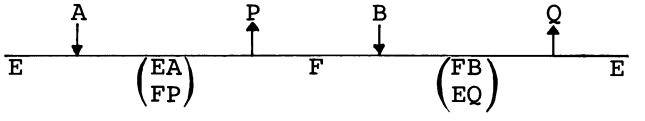
Under conditions where $k_5 >> k_7$ and $k_4 >> k_2$,

$$V_1 = k_7E_0 \quad \text{and} \quad V_2 = k_2E_0 \qquad (I\text{-}20)$$

whereas, originally, the maximal velocities were determined by rate constants involving the breakdown of binary *and* ternary complexes. With these limiting assumptions, V_1 and V_2 are functions of the decomposition of the binary complexes EQ(forward reaction) and EA(reverse reaction) only. In the Theorell-Chance mechanism, the maximal velocity also is governed by those constants involved in the breakdown of the EQ and EA complexes. The binary complex mechanism is really a limiting case of the Ordered Bi Bi mechanism, and it does not imply that ternary complexes of enzyme and substrates do not form.

It is important to note that the kinetic mechanisms described in Schemes I-7 to I-9 all can be described by the same initial rate equation (Eq. I-17); however, it is possible to make a choice from among these possibilities, and indeed many others, from steady-state experiments alone.

If upon substrate addition, the enzyme-substrate A complex undergoes reaction to form product that is released before addition of the second substrate, the mechanism is called Ping Pong Bi Bi. This model, which is depicted in Scheme I-10, was first proposed by G.W. SCHWERT and is contained in a 1953 report by ALBERTY (11).



Scheme I-10

The rate expression for the mechanism of Scheme I-10 is

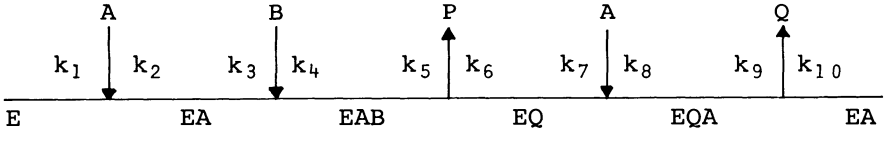
$$\frac{V_1}{v} = 1 + \frac{K_a}{A} + \frac{K_b}{B}. \qquad (I\text{-}21)$$

Rate Eq. (I-21) differs from Eq. (I-17) in that the $K_{ia}K_b/(A)(B)$ term is deleted in the former expression. When V_1/v is graphed as a function of the reciprocal of A at different fixed levels of B, a family of parallel lines is obtained. A similar result is observed when, in the analogous experiment, B is varied at different constant concentrations of A.

In theory, then, it is possible to segregate kinetic mechanisms into Sequential (Eq. I-17) and Ping Pong (Eq. I-21) types. This was very clearly pointed out by ALBERTY (24) in 1956, although he did not use the terms Sequential and Ping Pong.

It has often been implied tacitly that enzymes that exhibit Ping Pong kinetics involve participation of a covalent intermediate; however, such an assumption may not be valid. The cardinal feature of such mechanisms is dissociation of the product of the first substrate before the second substrate binds to the enzyme. The nature of the intermediate cannot be inferred from initial rate experiments alone, and chemical studies are required to establish the nature of the complex of enzyme and substrate or portion thereof. It also is possible, when parallel line data of the type expected for the Ping Pong case are obtained, that the mechanism is actually Sequential. This will occur if the $K_{ia}K_b/(A)(B)$ term of Eq. (I-17) is small relative to other terms in the rate expression. Precisely this type of situation was observed with mammalian brain hexokinase when glucose was a substrate for the enzyme (25); however, Sequential kinetics were obtained with fructose (26). It also is possible to obtain Sequential kinetics even if a covalent intermediate is formed in the reaction between enzyme and substrates - *if the product does not dissociate from the enzyme until the second substrate has been adsorbed.*

Another type of Ping Pong mechanism, which gives converging double reciprocal plots, is illustrated in Scheme I-11 (27).



Scheme I-11

The rate equation for this mechanism is identical in form to Eq. (I-17). There are no known examples of the pathway depicted in Scheme I-11 to date.

One mechanism that apparently does involve a covalent intermediate but that gives rise to initial rate data that seem to be Sequential has been proposed by ARION and NORDLIE (28) for glucose-6 phosphatase-pyrophosphate-glucose phosphotransferase. This kinetic pathway may be described by the outline of Scheme I-10 with one additional step; the breakdown of F to P + E. In Scheme I-10, A, P, F, B, and Q represent pyrophosphate, phosphate, phosphoryl enzyme, glucose, and glucose-6-P. In this

mechanism, the rate constant for the breakdown of the covalent intermediate to free enzyme and orthophosphate is comparable in magnitude to the other rate constants involving product formation.

One feature of the Ping Pong mechanism that should be borne in mind is that the pathway of substrate addition is obligatory and ordered. An adsorption pocket for the second substrate presumably either does not exist on the enzyme until after the first substrate has added to the enzyme, or if it does exist, its conformation is such that there is specificity of substrate binding for E and F in Scheme I-10.

WONG and HANES (29) have proposed a kinetic mechanism for bireactant enzyme cases that they suggest is the simplest general mechanism for such systems. This model (mechanism II) represents a hybrid of the Random Bi Bi and Ping Pong pathways of enzyme and substrate interactions. It is of interest that this mechanism serves as the basis of models proposed as a possible explanation of cooperative substrate interactions for regulatory enzymes.

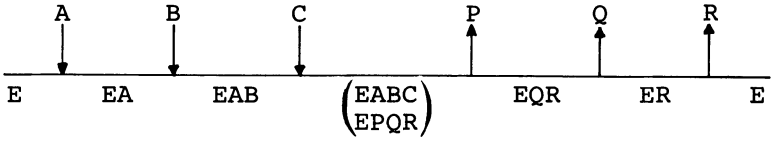
Three Substrate Systems

The kinetic models of three substrate enzyme systems are based upon analogous bireactant cases; however, a historical development of this aspect of kinetics is beyond the scope of this monograph. CLELAND (9) has proposed a number of probable three substrate pathways, and FROMM (30) has extended his treatment to include a few additional cases.

Listed below are the Sequential and Ping Pong models for terreactant enzyme systems other than "Iso" mechanisms.

a) Sequential Mechanisms

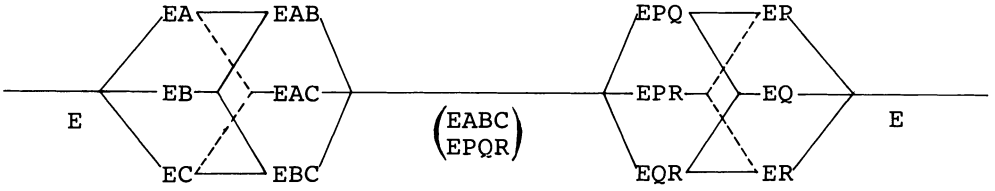
1. Ordered Ter Ter



Scheme I-12

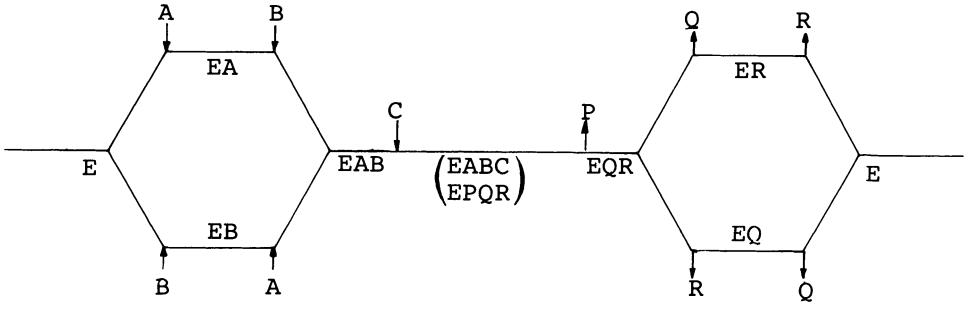
A modification of this pathway is the Ordered Ter Bi mechanism in which R is eliminated and there are ten rate constants.

2. Random Ter Ter (Rapid Equilibrium)



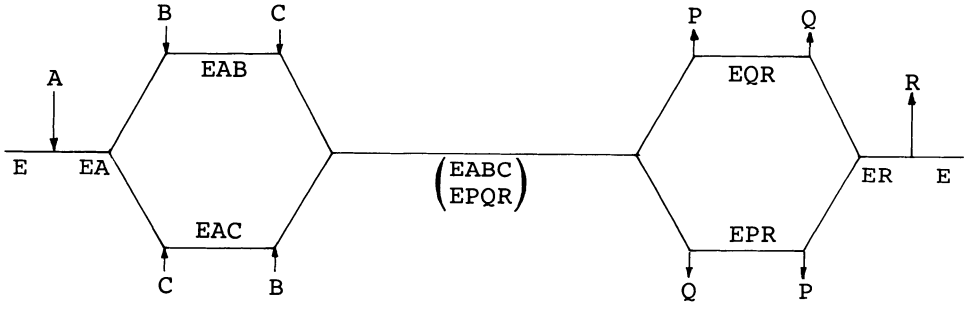
Scheme I-13

3. Random AB (Rapid Equilibrium)



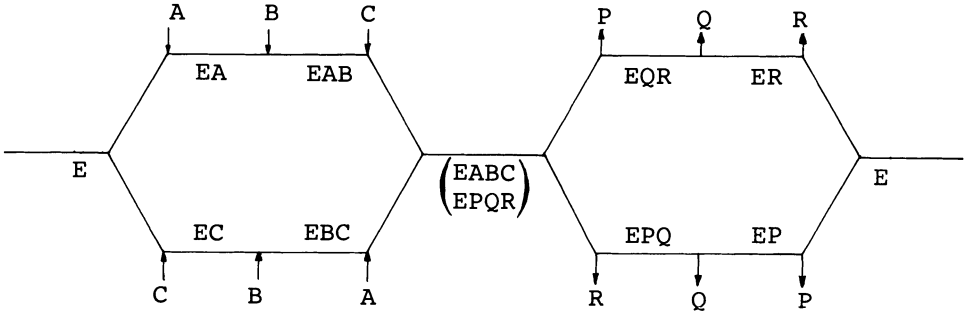
Scheme I-14

4. Random BC (Rapid Equilibrium)



Scheme I-15

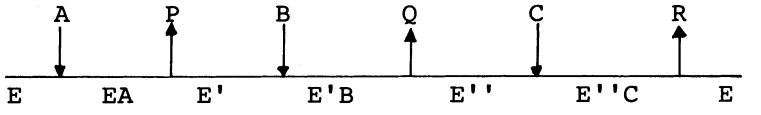
5. Random AC (Rapid Equilibrium)



Scheme I-16

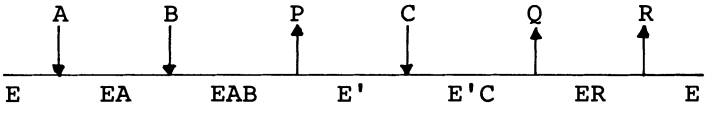
b) Ping Pong Mechanisms

1. Hexa Uni Ping Pong



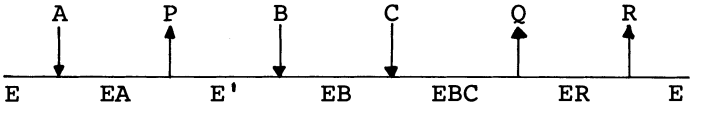
Scheme I-17

2. Ordered Bi Uni Uni Bi Ping Pong



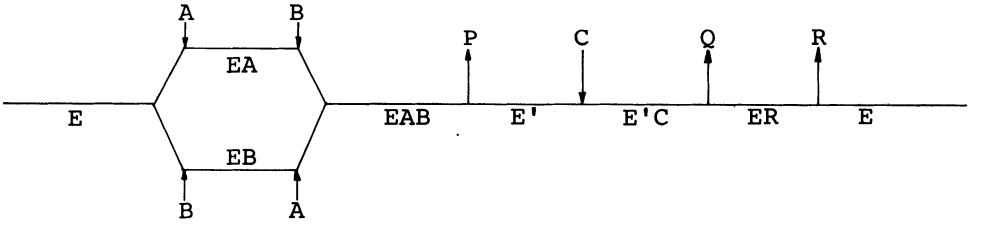
Scheme I-18

3. Ordered Uni Uni Bi Bi Ping Pong



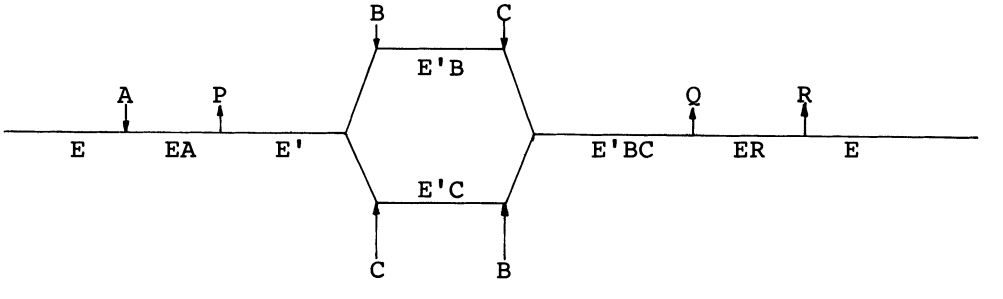
Scheme I-19

4. Random Bi Uni Uni Bi Ping Pong (Rapid Equilibrium)



Scheme I-20

5. Random Uni Uni Bi Bi Ping Pong (Rapid Equilibrium)



Scheme I-21

CLELAND (9) has pointed out that there are really only three different ordered terreactant Ping Pong mechanisms (Schemes I-17, I-18, and I-19). The Uni Uni Bi Bi and Bi Bi Uni Uni Ping Pong mechanisms are equivalent as are the Bi Uni Uni Bi and the Uni Bi Bi Uni Ping Pong mechanisms.

A large number of kinetic models that are variations on the Bi Bi or Ter Ter theme may be invoked. These are not to be con-

Table I-2. Some examples of various enzymes mechanisms

Mechanisms	Enzyme	Reference
I. Bireactant		
A. Ping Pong	Acetate kinase	31
	Adenine phosphoribosyltransferase	32
	Coenzyme A transferase	33
	Glutamic-alanine transaminase	34
	Glucose oxidase	35
	Nucleoside diphosphokinase	36, 37
	Transcarboxylase	38
B. Ordered	Alcohol dehydrogenase	39
	Carbamate kinase	40
	Lactate dehydrogenase (muscle)	41
	Lactate dehydrogenase (liver)	42
	Lactate dehydrogenase (heart)	43
	Purine nucleoside phosphorylase	44
	Ribitol dehydrogenase	45
	5-adenosyl methionine: homocysteine methyltransferase	46
C. Random	Adenylate kinase	47
	Creatine kinase (pH 8)	48
	Galactokinase	49
	Hexokinase (yeast)	8
	Hexokinase (brain)	50
	Phosphorylase b	51
	Pyruvate kinase	52
D. Equilibrium Ordered	Creatine kinase (pH 7)	53
II. Terreactant		
A. Ping Pong	Tyrosine aminotransferase	54
B. Ordered	Malic enzyme	55
	Glyceraldehyde-3-P dehydrogenase	56
C. Random	Adenylosuccinate synthetase	57
	Glutamate dehydrogenase	58
D. Partially Random	Citrate clevage enzyme	59

sidered in this chapter, but include Bi Ter, Ter Bi, Ter Quad, etc., mechanisms. In Schemes I-20 and I-21 it will be assumed that the random binding steps are in rapid equilibrium relative to the other steps in the mechanism.

Table I-2 lists a number of examples for the various kinetic mechanisms described in the text. It should be borne in mind that many kinetic mechanisms have been proposed to date, and this listing represents a very small fraction of the studies available in the literature.

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Chapter II

Derivation of Initial Velocity Rate Equations

The initial rate equation for enzyme catalyzed reactions that describes initial reaction velocity as a function of substrate concentration had its origins in the work of HENRI (1), BROWN (2), MICHAELIS and MENTEN (3) and BRIGGS and HALDANE (4).

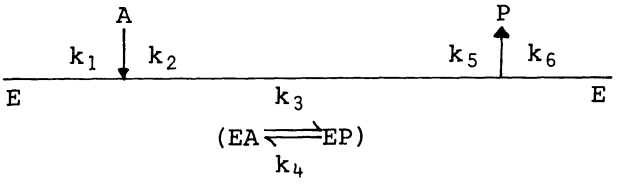
The derivation of initial rate equations involves either steady-state or equilibrium assumptions. In the case of certain kinetic mechanisms, it may be desirable to use a combination of both the steady-state and equilibrium approximations as suggested by CHA (5). When considering derivations, certain implicit assumptions are made regarding velocity, concentration ratio of substrate to enzyme, concentration of product, and attainment of either the steady or equilibrium state.

A. Definitions and Derivations

1. Steady-State

During the steady-state there is no change in concentration of the various enzyme forms involved in the reaction; i.e., the rate of production of a particular form of the enzyme is exactly equal to its rate of conversion to another enzyme intermediate. This approach was first employed by HALDANE in the derivation of the rate equation for a one substrate system (4).

When deriving the rate equation for a kinetic mechanism, one is desirous of obtaining a velocity expression (initial velocity) as a function of substrate concentration. As an illustration of how this type of expression may be derived, consider the following Uni Uni mechanism.



Scheme II-1

In the derivation, the following information is required:

a) conservation of enzyme equation, $E_0 = E + EA + EP$, where E_0 is total enzyme,
b) an expression for velocity, velocity = $dP/dt = -dA/dt = k_5(EP) - k_6P(E) = k_1A(E) - k_2(EA)$,
c) and finally (n-1) nonlinear differential equations for the change of a particular enzyme species with time; i.e., $d(E)/dt$, $d(EA)dt$, and $d(EP)/dt$. The term n refers to the number of enzyme, forms, which in Scheme II-1, is 3.

From the last consideration, only two of the nonlinear differential equations are required for the derivation of the rate equation for the mechanism illustrated in Scheme II-1, if steady-state conditions are assumed. These expressions are:

$$\frac{d(E)}{dt} = -k_1(E)(A) + k_2(EA) + k_5(EP) - k_6P(E) \qquad \text{(II-1)}$$

$$\frac{d(EA)}{dt} = k_1(E)(A) - (k_2 + k_3)(EA) + k_4(EP) \qquad \text{(II-2)}$$

$$\frac{d(EP)}{dt} = k_3(EA) - (k_4 + k_5)(EP) + k_6(E)(P) \qquad \text{(II-3)}$$

If it is assumed that $P \simeq 0$ and the velocity expression involving formation of product (i.e., $v = k_5(EP)$), rather than loss of substrate, $v = k_1(E)(A) - k_2(EA)$ is used, Eqs. (II-2) and (II-3) may be invoked to obtain the equation for velocity as a function of substrate concentration. The expression, $v = k_5(EP)$, is simpler than the equation involving substrate; however, the same final rate equation will be obtained regardless of which term is selcted.

In the steady-state, $d(EA)/dt = d(EP)/dt = 0$ and, if $P \simeq 0$

$$\frac{d(EA)}{dt} = k_1(E)(A) - (k_2 + k_3)(EA) + k_4(EP) = 0, \text{ and} \qquad \text{(II-4)}$$

$$\frac{d(EP)}{dt} = k_3(EA) - (k_4 + k_5)(EP) = 0. \qquad \text{(II-5)}$$

Because $v = k_5(EP)$, all enzyme terms are expressed in terms of EP; i.e.,

$$EA = \frac{(k_4 + k_5)(EP)}{k_3} \quad \text{and} \qquad \text{(II-6)}$$

$$E = \frac{(k_2 + k_3)(EA)}{k_1(A)} - \frac{k_4(EP)}{k_1(A)}. \qquad \text{(II-7)}$$

Negative terms do not appear in initial rate equations, except where all substrates and products are present. The negative term in Eq. (II-7) is eliminated by substituting the expression for EA in Eq. (II-6) into Eq. (II-7). Thus

$$E = \frac{(k_2k_4 + k_2k_5 + k_3k_5)(EP)}{k_1k_3(A)}. \qquad (II-8)$$

Substitution for E and EA is made in the equation, $E_0 = E + EA + EP$, so that

$$E_0 = \frac{(k_2k_4 + k_2k_5 + k_3k_5)(EP)}{k_1k_3(A)} + \frac{(k_4 + k_5)(EP)}{k_3} + EP. \qquad (II-9)$$

Dividing through by EP and collecting terms gives the expression,

$$\frac{E_0}{EP} = \frac{(k_3 + k_4 + k_5)}{k_3} + \frac{(k_2k_4 + k_2k_5 + k_3k_5)}{k_1k_3(A)}. \qquad (II-10)$$

Remembering that $v = k_5(EP)$ or that $EP = v/k_5$ and substituing into Eq. (II-10) gives

$$\frac{E_0}{v} = \frac{(k_3 + k_4 + k_5)}{k_3k_5} + \frac{(k_2k_4 + k_2k_5 + k_3k_5)}{k_1k_3k_5(A)}. \qquad (II-11)$$

This expression may be rearranged to

$$\frac{1}{v} = \frac{1}{V_1}\left(1 + \frac{K_a}{A}\right). \qquad (II-12)$$

It is to be noted that A exists in two forms, A and EA, and, in a sense as EP. By analogy with the enzyme forms, one might consider a conservation of substrate equation. Under most experimental conditions, however, in which initial velocity is monitored, $A >> E_0$, and therefore $A = A_0$.

It is possible to obtain an exact solution to the various differential equations which may be obtained from the mechanism of Scheme I-2, provided certain limiting assumptions are made. These assumptions are that the substrate concentration does not change in the course of the reaction, that product inhibition does not occur, and that the reverse reaction can be ignored. The following first order linear differential equations may therefore be written:

$$d(EA)/dt = k_1(E)(A) - (k_2 + k_3)(EA) \qquad (II-13)$$

$$dP/dt = k_3(EA) \qquad (II-14)$$

By invoking the conservation of enzyme equation, $E_0 = E + EA$, Eq. (II-13) may be rearranged as follows,

$$d(EA)/dt = k_1AE_0 - (k_1A + k_2 + k_3)(EA). \qquad (II-15)$$

An expression for EA is obtained after separating the variables of Eq. (II-15) and integrating.

$$EA = \frac{k_1 A E_0}{(k_1 A + k_2 + k_3)} (1 - e^{-(k_1 A + k_2 + k_3)t}) \qquad \text{(II-16)}$$

The expression for E can be obtained from the conservation of enzyme equation and is

$$E = E_0 \left[1 - \frac{k_1 A}{(k_1 A + k_2 + k_3)} (1 - e^{-(k_1 A + k_2 + k_3)t}) \right]. \qquad \text{(II-17)}$$

It is possible to obtain an equation for P from Eqs. (II-14) and (II-16). When this equation is integrated, Eq. (II-18) is obtained.

$$P = \frac{k_1 k_3 A E_0}{k_1 A + k_2 + k_3} \left[t + \frac{1}{(k_1 A + k_2 + k_3)} (e^{-(k_1 A + k_2 + k_3)t} - 1) \right]. \qquad \text{(II-18)}$$

Equation II-18 provides interesting information on the product-time relationship for an enzyme catalyzed reaction within the context of the mechanism of Scheme I-2 and the limiting assumptions already alluded to. At t = 0, P will also be zero; however, as t increases there will be an exponential increase in the parabolic slope of the product-time progress curve i.e., an induction period usually referred to as the pre-steady state phase of the reaction. At a still later time (where the exponential term in Eq. (II-18) approaches 0), P will be a linear function of t with a slope of $k_1 k_3 A E_0/(k_1 A + k_2 + k_3)$. It is of interest that the slope of this phase of the reaction, the initial velocity or steady-state phase, incorporates certain features of the well known Michaelis-Menten equation.

The question of the validity of the steady-state assumption has been a recurring one for many years. If one considers the simplest Uni Uni mechanism illustrated in Scheme I-2, where the two central complexes are represented by EA collectively, then the following equations pertain:

$$d(EA)/dt = k_1 (E)(A) - (k_2 + k_3)(EA) + k_4 (E)(P) \qquad \text{(II-19)}$$

$$dP/dt = k_3 (EA) - k_4 (E)(P) \qquad \text{(II-20)}$$

The concentrations of EA, P, and A at any time t are described by Eqs. (II-19) and (II-20). Analytic solutions to these equations are not possible; however, both the digital and analog computer can be used to provide exact solutions to these non-linear differential equations. These solutions can be obtained provided that certain parameters, such as the rate constants, and A and E are known. Alternatively, it also is possible through trial and error analysis to match the computer simulations with experimental data when data on ratios of rate constants and substrate and enzyme concentrations are not available. Using these approaches, conclusions on the validity of the steady-state assumption can be arrived at.

CHANCE (6) was among the first to use a computer to determine whether an intermediate of the type suggested in Scheme I-2 is formed in an enzymic reaction. He also attempted to gain some insight into the validity of the steady-state approximation. His simulations suggested that an intermediate enzyme-substrate complex was indeed formed in the peroxidase reaction, but that it was short-lived.

Figures II-1, II-2, and II-3 illustrate the use of the analog computer in obtaining exact solutions to Eqs. (II-19) and (II-20). In these simulations, it was assumed that $k_4 = 0$; thus, the effect of inhibition by product was obviated, and the ultimate solution simplified.

Figure II-1 shows an analog computer plot of the progress curve for the reaction described by Scheme I-2 with $k_4 = 0$ within the time frame 300, which is plotted on the abscissa. In these simulations, the concentrations of both the free enzyme, E, and the enzyme-substrate complex, EA, are enlarged by a factor of 100.

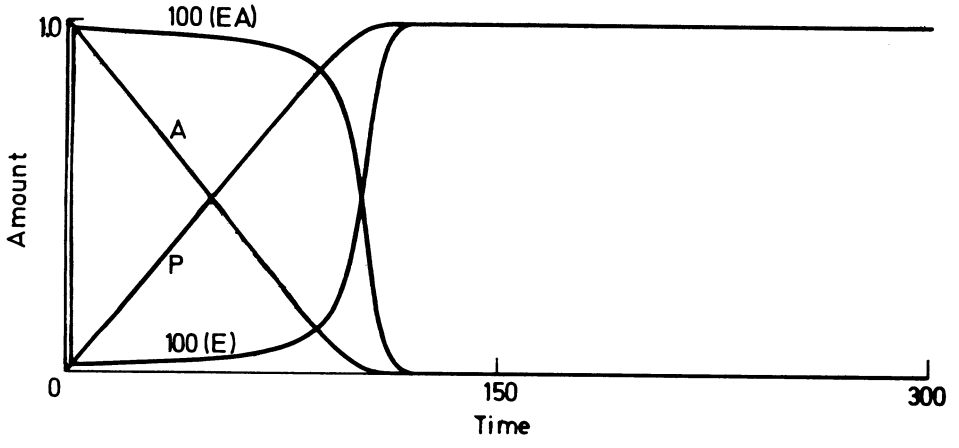


Fig. II-1. Analog computer simulation plot of the concentration of A, P, 100(E) and 100(EA) as a function of time for the reaction: $E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \xrightarrow{k_3} E + P$. The concentrations of E, EA, A, and P vary from 0 to 1. The ratio of A to total enzyme is 100, and $k_1 = 100$, $k_2 = k_3 = 1$. The time interval is from 0 to 300

It can be seen from Figs. II-1, II-2, and II-3 that enzymic reactions can be divided into three different phases. The first (pre-steady-state) is very short and is followed by a somewhat longer phase (steady-state) during which both E and EA remain relatively constant. Finally, these enzyme forms decay during the post-steady-state stage to their pre-steady-state levels.

In Figs. II-1 and II-2 the ratio of substrate to enzyme is 100, and the *numerical* values for k_1, k_2, and k_3 were taken to be 100,

1, and 1, respectively. The results of Fig. II-1 show a rapid transient for A, but none for P. This effect on substrate is caused by formation of the enzyme substrate complex. It also appears that the concentrations of E and EA change slowly with time until approximately 50-60% of the substrate has been utilized. These early effects can be better described by the data of Fig. II-2 in which the scale of Fig. II-1 has been expanded 10-fold. In Fig. II-2, it is quite clear that neither E nor EA

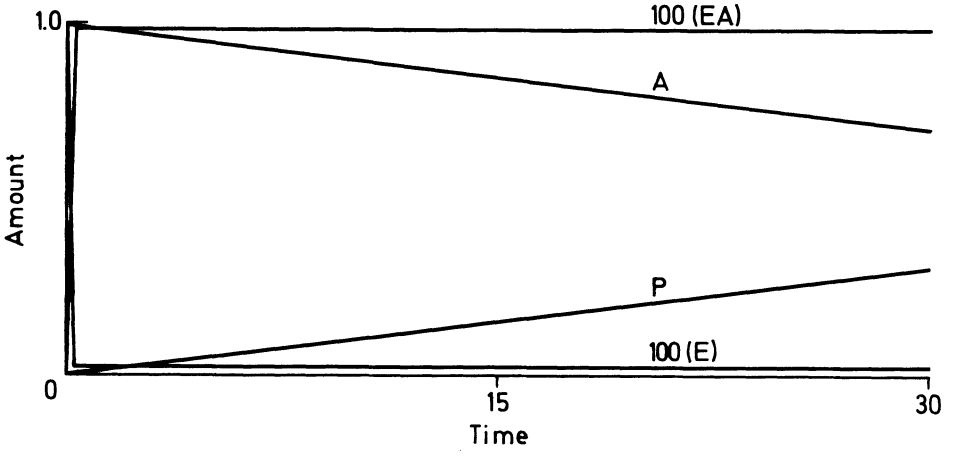


Fig. II-2. Analog computer simulation plot of the concentration of A, P, 100(E) and 100(EA) as a function of time for the reaction:

$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \xrightarrow{k_3} E + P$. The concentrations of E, EA, A, and P vary from 0 to 1. The ratio of A to total enzyme is 100, and $k_1 = 100$, $k_2 = k_3 = 1$. The time interval is from 0 to 30

changes with time, i.e., $d(E)/dt = d(EA)/dt = 0$, and furthermore, P changes linearly with time. When the ratio of substrate to enzyme is increased to 1000, the transient associated with A is barely discernible and there is no evidence for the relatively slow buildup of EA described in Fig. II-2.

It is interesting to compare these results with those obtained when the substrate to enzyme ratio is only 10. This effect can be seen from a comparison of the simulations shown in Figs. II-1 and II-3. It is important to note that the time scale is more rapid, by a factor of ten, in the case of Fig. II-3 relative to the simulations of Fig. II-1.

A number of investigators have addressed themselves to the question of whether the steady-state approximation is a valid assumption when considering the kinetics of enzyme catalyzed reactions (7-11). WONG (10) has found that, when $k_4 = 0$, the steady-state assumption becomes increasingly more valid as the ratio of substrate to enzyme is increased. WONG (10) has also listed a number of experimental conditions that serve to validate the steady-state approximation. Probably the simplest and

most important criterion in this regard is the well-known linear relationship which exists when velocity is graphed as a function of enzyme concentration. If this relationship is not satisfied, initial velocity conditions may not prevail for the enzyme system under study.

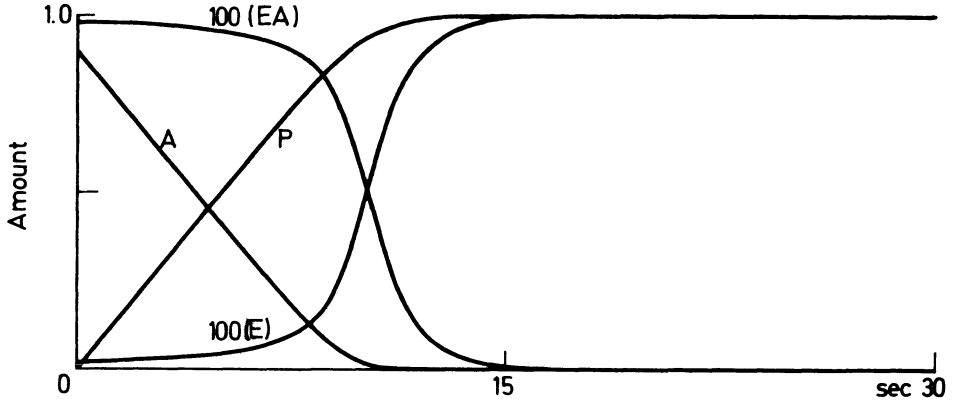


Fig. II-3. Analog computer simulation plot of the concentration of A, P, 100(E) and 100(EA) as a function of time for the reaction: $E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \xrightarrow{k_3} E + P$. The concentrations of E, EA, A, and P vary from 0 to 1. The ratio of A to total enzyme is 10, and $k_1 = 100$, $k_2 = k_3 = 1$. The time interval is from 0 to 30

MORALES and his co-workers (7-9) have for some time been interested in determining conditions under which the steady-state assumption for the enzyme-substrate intermediate holds, and where $k_4 \neq 0$. The points that these workers have found to be of crucial importance are the ratio of substrate to enzyme, which also was considered by WONG (10), and the ratios of the four rate constants to each other.

WALTER (11) has described the error inherent in the steady-state assumption as a function of enzyme and substrate concentration and the four rate constants shown in Scheme I-2. If $k_1 > k_4$, the steady-state assumption will be valid; however, there will be an error associated with this approximation which can be calculated according to Eq. (II-21).

$$\varepsilon_{max} = \left(\frac{4}{27}\right)\left(\frac{E_0}{A_{ss}}\right) \qquad \text{(II-21)}$$

In Eq. (II-21) ε_{max}, E_0, and A_{ss} represent the maximum error theoretically possible using the steady-state assumption, total enzyme, and substrate concentration at the time the error is to be determined, respectively.

Equation II-21 suggests that when $A_{ss} >> E_0$, as is the case for most kinetic experiments under so called steady-state conditions, the steady-state approximation is reasonably valid. These considerations are consistent with the simulations of Figs. II-1, II-2, and II-3 in which $k_4 = 0$.

Under conditions where $k_1 < k_4$, the steady-state approximation is never exact; although, if the ratio of A_{ss} to E_0 is high, a very small error will be introduced into the steady-state assumption unless the ratio of k_4 to k_1 is also very great.

WALTER (11) shows that

$$\varepsilon_{max} = \frac{4}{27} \left(\frac{E_0}{A_{ss}}\right) \left(\frac{k_4}{k_1}\right) \qquad \text{(II-22)}$$

when $k_1 < k_4$. It is clear from Eq. (II-22) that, the greater the ratio $k_4:k_1$, the larger A_{ss} must be relative to E_0 in order to satisfy the steady-state approximation.

These theoretical studies imply that the steady-state assumption is not universally correct. However, in cases where the ratio of substrate to enzyme is relatively great (1000:1), the steady-state assumption would appear to be a valid approximation, provided that the relationship $k_4 >> k_1$ does not hold.

2. Initial Velocity

In considering the derivation of the rate equation for the Uni Uni mechanism shown in Scheme II-1, it was assumed that, during the steady-state phase of the enzyme reaction, $d(EP)/dt \simeq 0$. Velocity (v) can be taken to be $k_1(E)(A)-k_2(EA)$ or $k_5(EP)-k_6(E)(A)$. In the absence of product ($P \simeq 0$), $v = k_5(EP)$, and during the steady-state phase of the reaction, velocity is constant because the concentration of EP does not vary. A similar statement can be made, but for different reasons, if equilibrium is attained.

If one were to make a plot of product formation as a function of time, the graph, Fig. II-4, would result. During the early

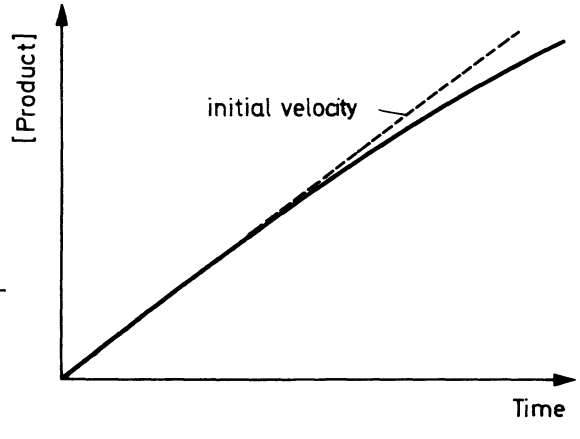


Fig. II-4. Plot of product formed versus time for an enzymatically catalzyed reaction. The linear portion of the curve represents the initial velocity phase of the reaction

part of the reaction, it is clear that there is a linear relationship between product production and time. This is the so-called *initial velocity* portion of the enzyme reaction, and it occurs where $d(EP)/dt = 0$; i.e., where the concentration of EP is constant. It is important to note that experimentally only the initial velocity phase of the reaction is relevant to the steady-state assumption. Obtaining this type of progress curve may be a relatively simple matter if the product (or substrate) is a chromophore, and product formation as a function of time can be monitored continuously in a spectrophotometer. However, it will be necessary to determine product formation at a number of different times after initiation of the reaction if a continuous spectrophotometric assay cannot be used. The specific details of these procedures will be considered in Chapter III.

During the initial velocity phase of the reaction, as illustrated in Fig. II-4, doubling of the reaction time will double the amount of product produced, and this relationship will hold throughout the steady-state phase of the reaction. The dotted line is a tangent to the initial phase of the progress curve, and its slope represents the initial reaction rate.

Other assumptions that one normally makes in deriving initial rate expressions are that $A >> E_0$, and that temperature, pH, and other experimental parameters are constant.

3. The Maximal Velocity (V_1) and Michaelis Constant (K_a)

The form of Eq. (II-11) is very useful for evaluation of certain kinetic parameters. It is convenient to rearrange Eq. (II-11) to obtain V_1 and K_a, such that the term not associated with the variables v and A be numerically equal to one.

$$\frac{k_3k_5(E_0)}{(k_3 + k_4 + k_5)(v)} = 1 + \frac{(k_2k_4 + k_2k_5 + k_3k_5)}{k_1(k_3 + k_4 + k_5)(A)} \qquad \text{(II-23)}$$

$$V_1 = \frac{k_3k_5(E_0)}{(k_3 + k_4 + k_5)} \quad \text{and} \quad K_a = \frac{(k_2k_4 + k_2k_5 + k_3k_5)}{k_1(k_3 + k_4 + k_5)} \qquad \text{(II-24)}$$

This simple manipulation follows from the definitions of V_1 and K_a and is a valid approach regardless of the complexity of the rate equation.

a) V_1 - By definition, $v = V_1$ when $A \to \infty$. The A term will drop out of Eq. (II-23), and the expression for V_1 is obtained.

b) K_a - By definition when $v = 1/2\ V_1$, $A = K_a$. When the rate equation is in the form of Eq. (II-23), K_a will be the term associated with A. Eq. (II-11) may therefore be expressed as

$$\frac{V_1}{v} = 1 + \frac{(k_2k_4 + k_2k_5 + k_3k_5)}{k_1(k_3 + k_4 + k_5)(A)} = 1 + \frac{K_a}{A} \qquad \text{(II-25)}$$

and where $v = 1/2\ V_1$, substituting K_a for A gives the expression for K_a in terms of rate constants.

4. Reverse Reaction Parameters and Rate Constants

It is often necessary to know the rate equation for the reverse reaction; i.e., where A = O and P is now taken to be the substrate. For a symmetrical mechanism of the type shown in Scheme II-1 this is simply done, if the forward reaction rate equation has been derived previously.

A shorthand method involves placing the rate constants into two columns as follows,

1	6
2	5
3	4

The rate constant k_1 in the forward direction corresponds to k_6 in the reverse direction and so forth. Thus for the reverse reaction, Eq. (II-11) is transformed into,

$$\frac{E_0}{v} = \frac{(k_2 + k_3 + k_4)}{k_2k_4} + \frac{(k_2k_4 + k_2k_5 + k_3k_5)}{k_2k_4k_6(P)} \qquad \text{(II-26)}$$

and

$$V_2 = \frac{k_2k_4E_0}{(k_2 + k_3 + k_4)}, \qquad K_p = \frac{(k_2k_4 + k_2k_5 + k_3k_5)}{k_6(k_2 + k_3 + k_4)}. \qquad \text{(II-27)}$$

B. The Equilibrium Assumption

Both the steady-state and equilibrium assumptions are often made when deriving initial velocity equations. How these assumptions differ is illustrated by the example for the Uni Uni mechanism of Scheme II-1. Under steady-state conditions, the concentrations of the various enzyme forms are assumed to remain essentially constant because the rate of conversion of one form to another is equal to its rate of production from yet another enzyme form. In the case of the equilibrium assumption the concentration of enzyme forms also remains constant, but for another reason. Here the flux through one of the steps is slow enough so that the preceding steps equilibrate. If the very slow step is at k_5 (Scheme II-1), the concentration of E is maintained constant because it is in equilibrium with EA and not because of the conversion of EP to E and P. The equilibrium assumption was first used by Michaelis for the derivation of the so-called Michaelis-Menten equation:

$$v = \frac{V_1(A)}{K_{ia} + A} \qquad \text{(II-28)}$$

where K_{ia} is taken to be a dissociation constant.

If the mechanism of Scheme II-1 is treated as if the system were in equilibrium, three distinct equilibria may be recognized,

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA, \qquad K_{ia} = \frac{(E)(A)}{(EA)} = \frac{k_2}{k_1} \qquad \text{(II-29)}$$

$$EA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EP, \qquad K = \frac{(EA)}{(EP)} = \frac{k_4}{k_3} \qquad \text{(II-30)}$$

$$EP \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E + P, \qquad K_{ip} = \frac{(E)(P)}{(EP)} = \frac{k_5}{k_6}. \qquad \text{(II-31)}$$

If we assume $P \simeq 0$, only the expressions $E = k_2(EA)/k_1(A)$ and $(EA) = k_4(EP)/k_3$ are important. In order to derive the initial rate equation, it is necessary to use the following relationships: $v = k_5(EP)$, $E_0 = E + EA + EP$, $EA = k_4(EP)/k_3$ and $E = k_2k_4(EP)/k_1k_3(A)$. The final rate equation is then

$$\frac{E_0}{v} = \frac{(k_3 + k_4)}{k_3k_5} + \frac{k_2k_4}{k_1k_3k_5(A)}. \qquad \text{(II-32)}$$

It is clear from an examination of Eqs. (II-11) and (II-32) that they are of the same form. Actually, it is not possible to distinguish between the steady state and equilibrium approximation for this system. This, however, is not the case when considering certain mechanisms for two and three substrate systems.

It is possible to reduce Eq. (II-11) to Eq. (II-32) by assuming the following inequalities, $k_2 >> k_3$ and $k_4 >> k_5$. When the reverse reaction is considered, however, the assumptions that lead to the reduction involve the inequalitites $k_3 >> k_2$ and $k_5 >> k_4$. It is obvious that such assumptions are contradictory and therefore not valid. Other inequalities that could satisfy the reduction are of the type

$$\frac{(k_3 + k_4)}{k_3k_4} >> \frac{1}{k_3} \quad \text{and} \quad \frac{k_2k_4}{k_1k_3k_5} >> \frac{(k_2 + k_3)}{k_1k_3}. \qquad \text{(II-33)}$$

When the analogous inequality relationships for the back reaction are considered, it becomes obvious that the equilibrium assumption is not valid for the mechanism of Scheme II-1. Similar statements can be made when considering the mechanism for a one substrate system involving a single intermediate; i.e., $E + A \rightleftharpoons EA \rightleftharpoons E + P$. It is clear from this discussion that the equi-

librium assumption, when considered to be a limiting case of the steady-state assumption, will not always be valid, and this approximation must be applied cautiously. It *may* be possible to arrive at the equilibrium form of the Michaelis-Menten equation by reduction of the numerical solution of Scheme II-1 without making conflicting assumptions; however, this has evidently not been attempted.

C. Derivation of Complex Steady-State Rate Equations

The basic procedure for the derivation of steady-state rate equations was presented earlier in this chapter. This method, as well as others (12), depends upon solving a series of nonlinear differential equations. Such solutions are possible by using determinant and matrix methods (13). KING and ALTMAN (14) advanced a schematic approach in 1956 for the derivation of initial rate equations based upon determinants. Their contribution, which has been used extensively by kineticists, has played a most important role in advancing the field of enzyme kinetics. This approach to the derivation of rate equations does, however, become increasingly difficult to use as the number of enzyme forms and the complexity of the kinetic mechanism increase. Many of these problems were circumvented when VOLKENSTEIN and GOLDSTEIN (15) applied graph theory to obtain steady-state equations. The detailed original theoretical basis for their method, which is beyond the scope of this monograph, may be found in the book of MASON and ZIMMERMAN (16). More recently, FROMM (17), has employed a systematic, rather than schematic, modification of the Volkenstein-Goldstein procedure. Its one disadvantage is that it generates "extra" terms in the determinant; however, these can be eliminated conveniently by inspection. The advantage of the systematic over the schematic method for deriving rate expressions is that, in the former case, the determinant terms are generated algebraically, while in the latter procedure, it is necessary to know the number of terms to be generated and then to obtain them by inspection.

Derivation of the rate expression of the mechanism described in Scheme II-2 is presented to illustrate the systematic method.

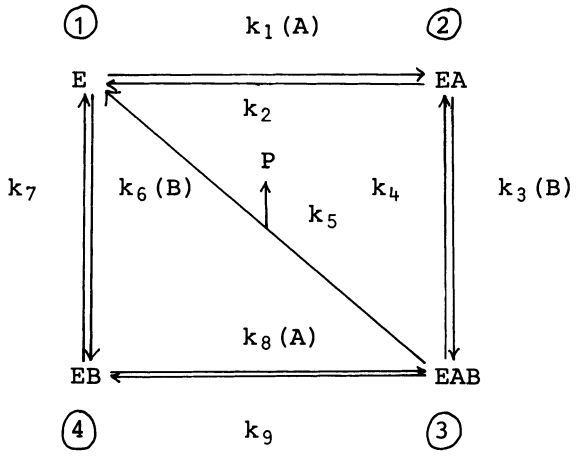
$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \qquad E + B \underset{k_7}{\overset{k_6}{\rightleftharpoons}} EB$$

$$EA + B \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EAB \qquad EB + A \underset{k_9}{\overset{k_8}{\rightleftharpoons}} EAB$$

$$EAB \xrightarrow{k_5} E + \text{products (P)}$$

Scheme II-2

1. The kinetic mechanism is first set up in geometric form as suggested by KING and ALTMAN (14) as shown in Scheme II-3.



Scheme II-3

2. Each enzyme form is numbered as illustrated above; e.g. E is ①, EA is ②, etc.

3. Each circled number above the enzyme form is characterized by one or more arrows that lead *away* from the enzyme form. These are listed in parentheses as a summation of rate constants.

$(1) = k_1(A) + k_6(B)$ $\qquad$ $(3) = k_4 + k_5 + k_9$

$(2) = k_2 + k_3(B)$ $\qquad$ $(4) = k_7 + k_8(A)$

4. To obtain the determinant for an enzyme form, the shortest *one-step* paths to that form from the other enzyme species that contribute directly to it are written down. Thus, for E or 1, we would have $2 \rightarrow 1$, $3 \rightarrow 1$, and $4 \rightarrow 1$. Each path is characterized by a rate constant. For the paths illustrated they would be k_2, k_5, and k_7, respectively.

5. Next to each of these one-step routes is written in parentheses the number in the geometric figure that does *not* appear in the one-step path; e.g., $2 \rightarrow 1(3)(4)$, $3 \rightarrow 1(2)(4)$, and $4 \rightarrow 1(2)(3)$.

6. The determinant for E is then, $E = 2 \rightarrow 1(3)(4) + 3 \rightarrow 1(2)(4) + 4 \rightarrow 1(2)(3)$, and thus $E = k_2(k_4 + k_5 + k_9)(k_7 + k_8A) + k_5(k_2 + k_3B)(k_7 + k_8A) + k_7(k_2 + k_3B)(k_4 + k_5 + k_9)$.

7. The determinant is next expanded, and certain terms are eliminated by inspection. These are a) *redundant terms* - only one particular term is permitted in each determinant, and b) *forbidden terms*. These are of the type $k_1k_2(A)$, $k_3k_4(B)$, $k_6k_7(B)$, and $k_8k_9(A)$. Whenever they appear in a product of rate constants, the whole term is eliminated. Finally, if a closed loop is

generated, it is not included in the determinant. Although mechanisms that produce closed loops are rare, the terms would be of the type $k_5k_6k_8(A)(B)$ for the mechanism above.

8. Expansion of E gives:

a) $$E = k_2k_4k_7 + k_2k_4k_8(A) + k_2k_5k_7 + k_2k_5k_8(A) + k_2k_7k_9 + k_2k_8k_9(A) + k_2k_5k_7 + k_2k_5k_8(A) + k_3k_5k_7(B) + k_3k_5k_8(A)(B) + k_2k_4k_7 + k_2k_5k_7 + k_2k_7k_9 + k_3k_4k_7(B) + k_3k_5k_7(B) + k_3k_7k_9(B). \quad \text{(II-34)}$$

b) The eliminated terms are either redundant or forbidden.

c) $$E = k_2k_4k_7 + k_2k_4k_8(A) + k_2k_5k_7 + k_2k_5k_8(A) + k_2k_7k_9 + k_3k_5k_7(B) + k_3k_5k_8(A)(B) + k_3k_7k_9(B). \quad \text{(II-35)}$$

9. The rate equation for this mechanism, as shown by KING and ALTMAN (14), is,

$$v = \frac{E_0\left[\underline{k}_5(EAB)\right]}{E + EA + EB + EAB} \quad \text{(II-36)}$$

where v, E_0, E, EA, and EAB represent velocity, total enzyme, determinant for E, determinant for EA, determinant for EB, and determinant for EAB, respectively.

10. Determinants for the other enzyme forms are:

$$EA = k_1k_4k_7(A) + k_1k_4k_8(A)^2 + k_1k_5k_7(A) + k_1k_5k_8(A)^2 + k_1k_7k_9(A) + k_4k_6k_8(A)(B) \quad \text{(II-37)}$$

$$EAB = k_1k_3k_7(A)(B) + k_1k_3k_8(A)^2(B) + k_3k_6k_8(A)(B)^2 + k_2k_6k_8(A)(B) \quad \text{(II-38)}$$

$$EB = k_2k_4k_6(B) + k_2k_5k_6(B) + k_2k_6k_9(B) + k_3k_5k_6(B)^2 + k_3k_6k_9(B)^2 + k_1k_3k_9(A)(B) \quad \text{(II-39)}$$

11. The values for the different enzyme forms are finally substituted into Eq. (II-36) to yield the final rate equation.

In the case of the one substrate system involving one intermediate, the mechanism is written as follows:

$$\underset{①}{E} \underset{k_2 + k_3}{\overset{k_1(A) + k_4(P)}{\rightleftharpoons}} \underset{②}{EA} \quad \text{(II-40)}$$

The determinant for E is $2 \rightarrow 1$ and for EA $1 \rightarrow 2$; i.e., $E = (k_2 + k_3)$ and $EA = (k_1A + k_4P)$. These determinants are then substituted into Eq. (II-41) to obtain the final rate expression

$$\frac{v}{E_0} = \frac{k_3(EA) - k_4(E)(P)}{(E) + (EA)}. \qquad \text{(II-41)}$$

D. Derivation of the Rate Equation Using the Rapid Equilibrium Assumption

1. The Random Bi Bi Mechanism

HALDANE was the first to derive a rate equation for the Random Bi Bi mechanism making equilibrium assumptions (18). For the case cited, to illustrate the algebraic procedure for writing rate equations, it can be assumed that all steps of the reaction are in rapid equilibrium relative to product formation from the ternary complex, EAB. Then

$$E + A = EA, \; K_{ia} \qquad EA + B = EAB, \; K_b$$

$$E + B = EB, \; K_{ib} \qquad EB + A = EAB, \; K_a$$

$$EAB \xrightarrow{k_5} E + P$$

Scheme II-4

The velocity expression is $v = k_5(EAB)$ as the last step is rate limiting. Thus all enzyme forms in the conservation of enzyme equation ($E_0 = E + EA + EB + EAB$) must be in terms of EAB.

The various equilibrium expressions are:

$$\frac{(E)(A)}{(EA)} = K_{ia}, \quad \frac{(E)(B)}{(EB)} = K_{ib}, \quad \frac{(EA)(B)}{(EAB)} = K_b, \quad \frac{(EB)(A)}{(EAB)} = K_a \qquad \text{(II-42)}$$

EA and EB are already expressed in terms of EAB,

$$EA = \frac{K_b(EAB)}{B} \quad \text{and} \quad EB = \frac{K_a(EAB)}{A}. \qquad \text{(II-43)}$$

The expression for E can be in terms of either K_{ia} or K_{ib}. (All four equilibrium constants are not independent but are related by the expression, $K_{ia}K_b = K_aK_{ib}$.) Substituting these enzyme forms into the conservations of enzyme equation gives,

$$E_0 = \left[1 + \frac{K_a}{A} + \frac{K_b}{B} + \frac{K_{ia}K_b}{(A)(B)}\right](EAB). \qquad \text{(II-44)}$$

This equation can be rearranged in velocity form as $v = k_5(EAB)$, as follows:

$$\frac{k_5E_0}{k_5(EAB)} = 1 + \frac{K_a}{A} + \frac{K_b}{B} + \frac{K_{ia}K_b}{(A)(B)} = \frac{V_1}{v}, \quad \text{or} \tag{II-45}$$

$$v = \frac{V_1}{1 + \frac{K_a}{A} + \frac{K_b}{B} + \frac{K_{ia}K_b}{(A)(B)}}. \tag{II-46}$$

2. The Ordered Bi Bi Mechanism (Rapid Equilibrium)

If the steps in the kinetic mechanism described by Scheme I-8 are assumed to be rapid relative to the interconversion of the ternary complexes, and if this interconversion can be described by two new rate constants, k_9 and k_{10},

$$EAB \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} EPQ \tag{II-47}$$

then

$$v = k_9(EAB) \tag{II-48}$$

when P = Q = O. The conservation of enzyme equation will now be

$$E_0 = E + EA + EAB \tag{II-49}$$

and the pertinent equilibria:

$$K_{ia} = \frac{(E)(A)}{EA}; \quad K_b = \frac{(EA)(B)}{EAB}. \tag{II-50}$$

Substituting from Eq. (II-50) into Eq. (II-49) and utilizing the velocity expression described by Eq. (II-48) yields the rate equation shown in Eq. (II-51).

$$\frac{V_1}{v} = \frac{k_9E_0}{k_9(EAB)} = 1 + \frac{K_b}{B} + \frac{K_{ia}K_b}{(A)(B)}. \tag{II-51}$$

When considering the rapid Equilibrium Random Bi Bi mechanism (Scheme I-7), the binding of one substrate by the enzyme may or may not lead to the enhancement of binding of the other substrate by the enzyme. If binding of substrate is not affected by the presence of the other substrate on the enzyme, $K_{ia} = K_a$ and $K_{ib} = K_b$. However, if the binding of one substrate by the enzyme does affect binding of the other, $K_{ia} \neq K_a$ and $K_{ib} \neq K_b$.

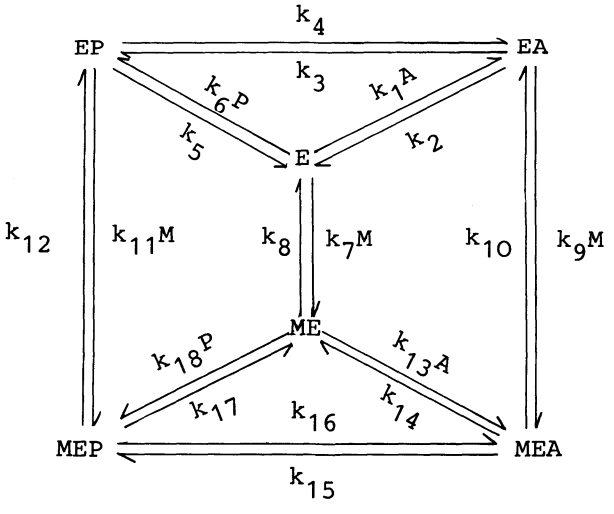
It is important to note that, although it is usually easier to derive rate equations using the equilibrium assumption compared to making the steady-state approximation, these two methods often lead to rate expressions of different form for a single mechanism. Thus, for the steady-state derivation of the Random

Bi Bi mechanism (Scheme II-3), the rate equation contains substrate terms of second degree as indicated by the determinants for the various enzyme forms. On the other hand, the equilibrium assumption gives rise to kinetic equations for this same mechanism that are obviously much different, as shown in Eq. (II-51). A qualitative difference in rate equations will also be obtained with other mechanisms, for example, the Ordered Bi Bi case. Although this mechanism is of the sequential type, Eq. (II-51) differs from Eq. (II-46) in that the K_a/A term is absent in the former equation. It is possible to differentiate between the steady-state and the rapid equilibrium case because of the deleted term in the rate equation (19).

E. Derivation of Initial Rate Equations Using a Combination of Equilibrium and Steady-State Assumptions

CHA (5) has shown that it is sometimes advantageous to use a combination of steady-state and equilibrium assumptions to derive kinetic rate equations. An attempt will be made to illustrate the procedure involved by citing a simple example of the method. The reader is referred to the original article by CHA (5) for further details of this procedure.

Consider the following mechanism in which a single modifier (M) is involved in the catalytic process:



Scheme II-5

The derivation of the initial rate expression is rather laborious even when the method of FROMM (17) is used; however, equilibrium assumptions can be made, which lead to relatively simple operations using CHA's method (5). If one assumes that the steps

EP$\rightleftharpoons$EA and MEP$\rightleftharpoons$MEA are slow relative to all other steps that equilibrate rapidly, then

$$v = k_3(EA) - k_4(EP) + k_{15}(MEA) - k_{16}(MEP). \quad \text{(II-52)}$$

In the derivation, the various equilibria are used to get Eq. (II-52) into an expression that contains only one enzyme species. From the following expression, v can be described in terms of EA.

$$E = k_2(EA)/k_1(A) \quad \text{(II-53)}$$

$$EP = k_2k_6(P)(EA)/k_1k_5(A) \quad \text{(II-54)}$$

$$ME = k_2k_7(M)(EA)/k_1k_8(A) \quad \text{(II-55)}$$

$$MEA = k_9(M)(EA)/k_{10} \quad \text{(II-56)}$$

$$MEP = k_2k_6k_{11}(M)(P)(EA)/k_1k_5k_{12}(A) \quad \text{(II-57)}$$

$$v = \left[\left(k_3 + \frac{k_9k_{15}(M)}{k_{10}}\right) - \left(\frac{k_2k_4k_6(P)}{k_1k_5(A)} + \frac{k_2k_6k_{11}k_{16}(M)(P)}{k_1k_5k_{12}(A)}\right)\right](EA) \quad \text{(II-58)}$$

If both sides of Eq. (II-58) are divided by E_0 and the denominator expressed in terms of EA, remembering that

$$E_0 = E + EA + EP + ME + MEA + MEP \quad \text{(II-59)}$$

the expression for velocity becomes

$$v = \frac{\left[\left(k_3 + \frac{k_9k_{15}(M)}{k_{10}}\right) - \left(\frac{k_2k_4k_6(P)}{k_1k_5(A)} + \frac{k_2k_6k_{11}k_{16}(M)(P)}{k_1k_5k_{12}(A)}\right)\right]E_0}{\left[1 + \frac{k_2}{k_1(A)} + \frac{k_2k_6(P)}{k_1k_5(A)} + \frac{k_2k_7(M)}{k_1k_8(A)} + \frac{k_9(M)}{k_{10}} + \frac{k_2k_6k_{11}(M)(P)}{k_1k_5k_{12}(A)}\right]}. \quad \text{(II-60)}$$

CHA (5) lists a number of examples to illustrate his procedure, and these will not be presented here. It should be noted that different rate equations will result depending upon which segments of the mechanisms are assumed to be in the equilibrium and steady-states. In the derivation of rate equations for Schemes I-20 and I-21, it was assumed that the random substrate binding steps are in rapid equilibrium relative to the other steps which are in the steady-state.

F. Derivation of Steady-State Rate Equations by Using the Digital Computer

A number of programs have been written to aid in the derivation of steady-state rate equations (20). One such method, written in PL/1 language, is presented in the *Appendix*. The use of the computer in this context becomes very important when complex mechanisms are under consideration. It will be shown in Chapter V how the digital computer can be used to generate theoretical plots for one of these complex mechanisms (steady-state Random Bi Bi).

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Chapter III

Experimental Protocol and Plotting of Kinetic Data

A. General Considerations

In order to carry out initial rate studies, it is necessary to be aware of those factors that affect the kinetics of the system under investigation. These include substrate and product concentration, pH, temperature, ionic strength, enzyme stability, activators and inhibitors.

It is very unlikely that the investigator will be totally uninformed about the kinetic properties of an enzyme before carrying out the preliminary experiments that precede the more serious initial rate studies. Information on substrate and product identity is required before the enzyme is actually characterized, and further discussion on these points does not seem to be appropriate. In the course of enzyme purification, an assay that measures either substrate utilization or product accumulation is required. In addition to these data, the kineticist will normally have information on the substrate(s) concentration(s) and pH of his assay available to him before undertaking any experiments on his own.

Initial velocity studies are *normally* carried out with an assay mixture containing buffer and substrate, which is equilibrated or preincubated to a predetermined temperature in an accurately controlled water bath. If the substrate is stable, preincubation can normally be carried out for 10-15 min or until the desired temperature is reached before the enzyme solution, which is ordinarily maintained at 2^{o} - 4^{o} in an ice bath, is added. The ratio of assay to enzyme solution is usually large enough (100:1) to minimize alterations in temperature after enzyme addition. If the substrate is unstable in the assay mixture, it will be necessary to correct for substrate decay with time before meaningful kinetic data can be gathered. It is important then to establish whether the substrate is stable in the absence of enzyme for periods of time that will be used during the initial rate experiments. This can be accomplished by making up the substrate to a concentration that will be used experimentally, adding it to the assay mixture, and then removing samples at different periods of time. This procedure will probably cover a period of many hours, as substrates are normally made up to desired concentrations and then placed in an ice bath before use. Substrate analysis will be either chemical or enzymatic, and the reader is referred to *Methods of Enzymatic Analysis* (1) if the latter procedure is to be used. This protocol will enable the investigator to plot substrate concentration as a function of time, as illus-

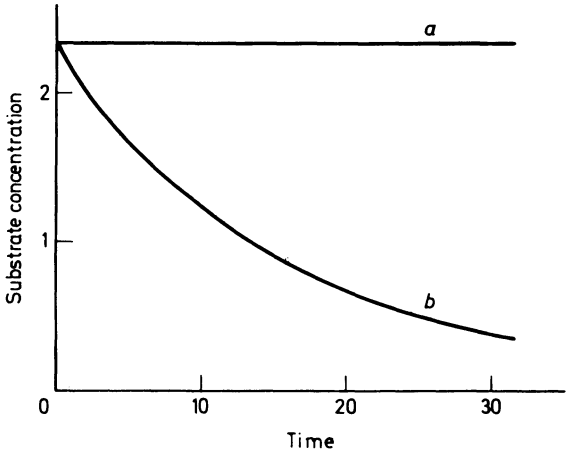

Fig. III-1. Plot of substrate concentration as a function of time. Curve a represents the case in which the substrate is stable with time. Curve b represents a first-order decay process for substrate with time

trated in Fig. III-1. This graph indicates two situations, one in which the substrate is stable and the other in which the substrate decomposes at a rate that is first-order in substrate. If substrate decay is represented by a single first-order process, the following equation may be used to calculate the substrate concentration at any time:

$$A = A_0 e^{-kt}. \qquad \text{(III-1)}$$

A, A_0, k, and t represent substrate concentration at time t, initial substrate concentration, first-order rate constant for decomposition and time t, respectively. The first-order rate constant can be obtained from a plot of the log of substrate concentration *versus* time, as depicted by Fig. III-2, where k = -2.303 × slope. The decomposition of substrate may be

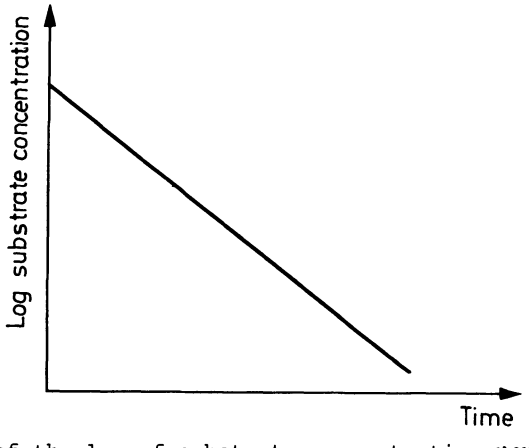

Fig. III-2. Plot of the log of substrate concentration *versus* time for a first-order process. The slope of the line is used to evaluate the rate constant, k, as follows: k = -2.303 × slope

a second or higher-order process, or it may be composed of more than a single first-order decay. The type of decomposition that occurs may be evaluated by using equations available in any physical chemistry text.

Even if one is fully capable of correcting for substrate decomposition, the investigator is still faced with the problem of the decomposition product and its effect on the kinetics of the system. This effect may be evaluated in a number of ways. If for example, the nature of the product is unknown, it may be possible to do a kinetic study with freshly prepared substrate in the presence and absence of the decomposition product. Even if the product proves to be innocuous, it will be desirable to test for conditions, such as pH or temperatures, that preclude destruction of the substrate with time. In this context, TU et al. (2) found that 1,5-gluconolactone, a compound that they wished to use as an inhibitor of the phosphorylase reaction, decomposes rapidly in the presence of glucose-1-P at alkaline pH. They were able to circumvent this problem by lowering the pH to 6.0 and by using enough enzyme so that the assays could be completed in a few minutes. It was also necessary for these investigators to make up the lactone immediately before it was to be used.

It is not unusual for enzyme preparations to undergo slow, progressive inactivation during a kinetic experiment. Inactivation ordinarily results from dilution of the stock enzyme preparation to a concentration of protein required to obtain initial velocities. This may be a result of surface denaturation, or in some instances, adsorption of the enzyme on glass surfaces. This latter effect can be obviated by dilution of the enzyme into plastic test tubes. The investigator should determine enzyme activity of the diluted enzyme preparation at a series of intervals over the time required for the entire kinetic experiments using a standard assay solution. If inactivation with time does occur, a correction to zero time can be made by using the approach outlined for substrate decomposition; however, this is not the procedure of choice. This correction can be made by reference to Fig. III-2 in which it is assumed that loss of activity is a first-order process (of course it may not be, and more involved calculations may be required).

It is clearly more desirable to establish conditions under which inactivation can be precluded. This end may be achieved by making a minimal dilution, usually, but not necessarily, with the buffer of the assay mixture and by using any of the excellent micropipettes currently available commercially to deliver the small volume of enzyme. If this proves impractical and high dilution of the enzyme is required, a stabilizing compound may have to be added to the diluting buffer.

Enzymes are often stabilized by substrate, and inactivation can often be precluded by dilution of the stock enzyme solution into buffer containing one of the substrates of a multisubstrate system. The investigator should be careful to record the exact

amount of substrate added along with enzyme to the assay mixtures so that a proper correction can be made for added substrate.

Bovine serum albumin at a concentration of 1-2 mg/ml of buffer is a very useful enzyme stabilizing compound. Here, too, however, one must exercise caution in the use of albumin to insure that it does not bind enough substrate or enzyme to cause complications. Although it is not possible to exhaust the variety of methods used to prevent inactivation upon dilution, it might be mentioned that high ionic strength solutions (achieved either with salts or buffer), ethylenediamine tetracetate (1-10 mM), sucrose, glycerol, and β-mercaptoethanol have been used routinely for this purpose. It is important to emphasize that the solution used for dilution of the enzyme should be buffered to prevent inactivation.

Some enzymes exhibit anomalous kinetic behavior when the reaction is initiated with enzyme. An example of this type of response is observed with coenzyme A-linked aldehyde dehydrogenase (3) which yields a sigmoidal progress curve of product formation with time, as shown in Fig. III-3. Although these effects are of great interest, it is necessary to obviate them if meaningful initial rate studies are to be carried out. In the case of coenzyme A-linked aldehyde dehydrogenase, it was found that, when the enzyme was first incubated with NAD, β-mercaptoethanol, and either acetaldehyde or coenzyme A and the reaction initiated 15 min later with the substrate that had been omitted, a normal initial velocity response was obtained (curve D, Fig. III-3).

One of the great advantages of the kinetic approach to studying reaction mechanisms is that the investigation does not require large amounts of pure enzyme, although the use of a pure enzyme is highly desirable. It is imperative, however, that the enzyme preparation be devoid of extraneous activities that can attack either the substrates or the products of the reaction under study. In the case of a one substrate system, the possible occurrence of "side reactions" with the enzyme preparation should be investigated. In the case of reactions of higher order, each substrate and reaction product can be incubated with the enzyme under simulated experimental conditions in the absence of the other substrates and product. One must be certain that, if the enzyme prepration does in fact contain enzyme activities that can utilize either the substrates or products of the reaction under investigation, these activities are relatively minor. If the contaminating enzyme activities are serious, further purification of the enzyme under study will be required.

Many enzymes exist as isoenzymes, and the serious kineticist will always attempt to carry out experiments with no more than a single form of the enzyme during any single experimental study. A mixture of isozymes may give rise to non-Michaelis-Menten kinetic patterns (see Chapter IX.2). If the enzyme is not already known to be homogeneous, the experimentalist should analyze the enzyme preparation for isozymes. The most direct procedure used to analyze for isozymes involves disc gel electrophoresis (4) followed by a specific staining procedure directly on the gel. For most enzymes, such procedures are not available and alternative methods can

be used. Procedures such as starch block electrophoresis (5) and ion exchange chromatography on diethylaminoethylcellulose (6) have been used for this purpose. In the starch block method, after separation of the proteins by electrophoresis using starch as the supporting medium, samples from different parts of the starch block are removed, soaked in buffer to elute protein, and then assayed for enzymatic activity. In both the electrophoretic and chromatographic methods, the isozymes will migrate at different rates, and their presence is readily recognized. It is conceivable that these procedures will not detect very subtle differences in protein homology; however, an appreciation of this type of heterology would probably require very careful amino acid sequence analysis or gene mapping, if the isozymes can be separated.

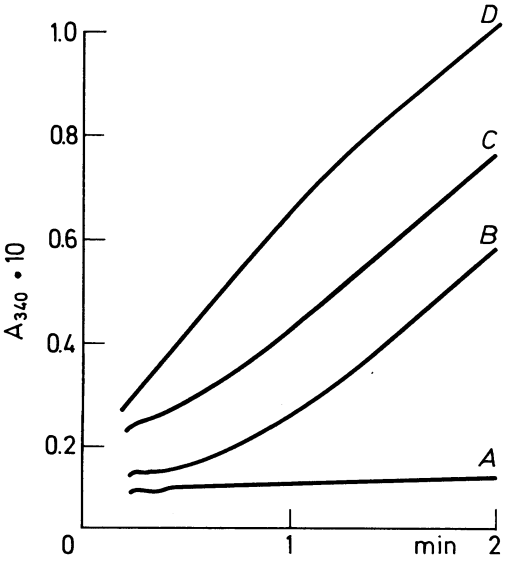

Fig. III-3. Plot of absorbance (A_{340}) as a function of time for aldehyde dehydrogenase. Assay mixtures contained enzyme, 0.4 mM NAD^+, 10 mM β-mercaptoethanol, 8.16 μM CoA, 16 mM acetaldehyde, and 22.5 mM Tris-Cl buffer, pH 8.1. Curve A represents the case in which the enzyme was added to the complete reaction mixture from which β-mercaptoethanol was omitted. In Curve B, the enzyme was added to the complete reaction mixture to initiate the reaction. In Curve C, the enzyme was incubated for 15 min with the complete reaction mixture from which NAD^+ was omitted. The reaction was started with NAD^+. Curve D represents the case in which incubation was carried out for 15 min with enzyme, β-mercaptoethanol, NAD^+ and either CoA or acetaldehyde. The reaction was initiated with the substrate that had been omitted from the reaction mixture

B. Analysis of Radioactive Substrates and Determination of Radiopurity

It is now well established that [^{14}C] compounds undergo decomposition in aqueous solution. All laboratory workers recognize the

need for determining chemical purity of compounds that they use experimentally. On the other hand, once analyzed, "stable" compounds are usually not subjected to reanalyses at regular intervals. A radioactive compound may decompose chemically (as opposed to radioactive decay) even though its nonradioactive counterpart is chemically stable. This effect is probably the result of free radical formation, which occurs from the action of radiation on the solvent. These free radicals in turn attack the $[^{14}C]$ compound. The ultimate result of this type of decomposition is a progressive increase in the apparent specific activity of the radioactive substrate.

SILVERSTEIN and BOYER (7) found, for example, that a solution of $[C^{14}]$ pyruvate decomposed rapidly with time even in the frozen state (-15^{o}). Their assays for pyruvate were both chemical and enzymatic. These workers found that the $[C^{14}]$ pyruvate, after purification by chromatography, could be stabilized by a variety of compounds of which 0.1 M HCl was the most effective.

The studies of SILVERSTEIN and BOYER (7) and others suggest that radioactive substrates be analyzed for purity before they are used. Furthermore, procedures for stabilization of these compounds should be sought if the investigator does not plan to use the purified substrates immediately after they are prepared.

C. pH Effects

Some idea of the proper pH to be employed in usual inital velocity experiments can ordinarily be obtained from the literature on the assay system used during purification. This value may require modification; however, it is of importance to note that the pH need not be the optimum pH for the reaction. Of greater significance in this regard is the effect of pH on the stability of the enzyme, substrates, and auxiliary compounds used in the experimental protocol. Thus, if an enzyme exhibits a pH optimum at 10.5 - a pH where either the enzyme or substrate is unstable, it may be necessary to lower the pH to a point where stability is no longer a factor and yet velocity can still be determined conveniently.

The choice of buffer is an important factor that requires a good deal of consideration. It is important to choose a buffer that does not inhibit the reaction under consideration. Acetate, for example, is known to inhibit the liver alcohol dehydrogenase reaction, and its choice for this system would not be a wise one. The pK value of the buffer must also be reckoned with; it is not uncommon to read a paper in which Tris (tris(hydroxymethyl)aminomethane) buffer is used at pH 7.0 even though the pK is about 8.1. Ideally, one should use a buffer whose pK is equal to the pH required. The concentration of the buffer is also important. Enzymes may be inhibited by high or low ionic strength, and the concentration of buffer can be used to obviate this effect. Often variation of the substrate concentration, particularly in

cases where the Michaelis constants are above millimolar, may cause alterations in ionic strength that require compensation either with salt, an innocuous or spectator ion, or by maintaining the concentration of buffer high enough so that this effect is minimized. Finally, in this regard, it is important to indicate that the use of improper buffers may seriously compromise the results of certain kinetic studies. For example, when studying systems that require a nucleoside triphosphate and a divalent ion for activity, the type, pH, and ionic strength of the buffer become critical. This point will be elaborated upon in Section F.

It is important that the investigator be aware of the pH of the stock reagent and enzyme solutions to be used for the kinetic experiments. Many of these materials have very strong buffering capacities, and the pH could change with changes in their concentration. For example, most solid preparations of NAD^+ and nucleoside triphosphates are strongly acid when dissolved in water. To circumvent this problem, the reagent should be dissolved in the buffer that is to be used for the kinetic experiments and the pH adjusted with the aid of a pH meter.

In kinetic studies in which both the forward and reverse reaction are to be investigated, it is often found that a favorable pH in one direction is a poor pH choice for the other direction. When it is required that the system be investigated in both directions, it will be necessary to choose a compromise pH. Comparisons of kinetic results at two different pH values, one for the forward and another for the reverse reaction, are often meaningless.

D. Substrate Concentration

The range of substrate concentration for initial rate experiments must be established from preliminary experiments before serious kinetic studies can be undertaken. It is important that the investigator choose a level of substrate that causes at least a two-fold velocity dependence when it is varied. It also is important to preclude substrate inhibition, a phenomenon which is usually associated with high levels of substrate, unless that effect is to be studied specifically. It is also essential that meaningful velocity determinations be obtained. It is not uncommon to find a study in the literature in which velocities reported are so low as to be at the outer sensitivity limits of the instrument used to monitor the rates.

If the substrate concentration is varied from 1/2 K_m to 5 times K_m, for a one substrate system that obeys the Michaelis-Menten rate law, the velocity change will be approximately three-fold. Ideally, it is most desirable to vary the substrate concentration over a great concentration range (100-fold); however, this procedure may require extremely sensitive assay methods and also the use of different concentrations of enzyme, a procedure that should be avoided if possible in a single experiment. If the substrate

concentration is varied approximately ten-fold (i.e., from 1/2 the K_m to five times K_m) a single concentration of enzyme can usually be used. This concentration range of substrate will, in most instances, generate data that will permit the investigator to make a choice of mechanism from among the types outlined in Chapter I.

In the case of bireactant enzyme systems, the dissociation constant may be much greater or much less than the K_m. When these two parameters are not too dissimilar, the substrate concentration may be varied as suggested for a one substrate system. When studying the kinetics of a bireactant enzyme system, it will be necessary to vary the substrate concentration in the range of the dominant kinetic parameter in order to observe velocity dependence upon substrate concentration. The general equation for a sequential mechanism is:

$$\frac{V_1}{v} = 1 + \frac{K_a}{A} + \frac{K_b}{B} + \frac{K_{ia}K_b}{(A)(B)}. \qquad \text{(III-2)}$$

If we assume that K_a = 20 μM, K_{ia} = 200 μM, and K_b = 1 mM, when B is held constant at different concentrations in the range 0.5-5 mM, the Michaelis constant term, K_a, will be minimized. Only when B is very low, relative to K_b, will the Michaelis constant term for A become significant. Thus, when B is fixed near its Michaelis constant level, substrate A should be varied in the region of its dissociation constant (K_{ia}). When the reverse relationship pertains (i.e., $[K_a] >> K_{ia}$), A must be varied in the concentration range of its K_a.

Substrate purity, standardization, and handling must be carefully considered before any kinetic investigation. The purity of each substrate, *including the water*, should be scrupulously checked. Substrates can be assayed either chemically or enzymatically. With regard to this last point, the book *Methods of Enzymatic Analysis* (1) is an excellent reference in this context. Specifications regarding reagent grades as stated by the supplier should be accepted cautiously.

Many biological compounds are unstable, and conditions for stabilizing these reagents should be sought. If freezing of reagents in solution is required, the reagents may be thawed in a water bath at room temperature, after which they should be vigorously shaken to insure adequate mixing. Solid reagents which are stored in a dessicator in the freezer should be permitted to reach room temperature before the dessicator and reagent bottles are exposed to air. This procedure precludes condensation of moisture in the reagent bottles.

It is essential to demonstrate that the enzyme preparation does not cause degradation of substrates in unwanted reactions. Thus, for example, if NADH is a substrate in a bireactant enzyme system, the entire assay mixture, including the enzyme, but minus the other substrate should be assayed under expected experimental conditions to evaluate the stability of the NADH. If the sub-

strate is utilized in a side reaction, further purification of the enzyme to eliminate the contaminating activity will be required.

E. Studies of Forward and Reverse Reactions

It may often be necessary to study the initial rate kinetics of systems in both directions. Frequently, enzymes exhibit different pH optima and maximum velocities for the forward and reverse reactions. The use of a compromise pH has already been alluded to; however, the amount of enzyme required for these experiments deserves special consideration. When the velocities in the two directions differ, two different levels of enzyme will usually be required, one for each direction. It will be necessary to refer the velocities to a single enzyme concentration if the findings are to be meaningfully interpreted. By using a standard assay mixture (for one side of the reaction), the effect of dilution may be evaluated by plotting initial velocity against enzyme concentration. The kinetic data for the two reactions may then be adjusted to a single theoretical enzyme concentration. It is important to note that this procedure does not alter the Michaelis constants for the system; it merely adjusts the maximal observed velocities.

In most reports, kinetic data are depicted in the form of double reciprocal plots (8, 9) of 1/velocity *versus* 1/substrate concentration. In order to obtain a relatively even distribution of points along the 1/substrate axis, serial dilutions of the stock solution can be made as follows: to 1 ml of stock substrate are added either 0, 2, 4, 6, or 8 ml of water. This procedure will provide five different concentrations of substrate of 1, 1/3, 1/5, 1/7, and 1/9 the concentration of the stock solution. When these fractions are inverted, the concentration will approximately fulfill the criterion of having equal point distribution along the abscissa. It should be remembered that the lowest substrate level should be approximately 1/2 the Michaelis constant. To anyone with even superficial experience with enzyme assays, it is obvious that those substrate levels that exhibit the lowest velocities are the least accurate and reproducible. This is a consequence of at least two factors, a) any small constant error will be magnified when velocities are themselves relatively small, and b) the reciprocal of say a 10% error at high substrate, where velocity approaches zero-order kinetics relative to substrate, will be small, while such an error will be very great at low substrate concentrations. These problems can be minimized by doing replicate analysis at low concentrations of substrate, by using statistical analysis in which the various velocities are weighted (10), or by varying the enzyme or substrate level (in the direction of the Michaelis constant) to minimize these deficiencies.

Concentrations of substrates and buffers should be manipulated so that solutions can be dispensed with volumetric pipettes if possible. If graduated pipettes are required, "long-tip" Mohr

pipettes in which as much of the entire graduated volume of the pipette should be used, if possible. Thus, if a volume of 0.08 ml is to be dispensed, a 0.1 ml rather than a 0.2 ml pipette should be used.

Assay solution will ordinarily contain 2.0 ml or 3.0 ml before enzyme is added or, if micropipettes are used, 1.0 ml or less. For a bireactant system in which there are three components of 1 ml each, a 13 × 1 cm test tube may be used. The buffer should be added first so that there is no chance that it may be contaminated with substrate. The solution is not to be mixed after adding the first substrate, and the pipette containing the second substrate should be placed at a point much above the point of addition of the first substrate. The assay solution should be mixed either by tapping the tube or with the aid of a vortex mixer *only* after all components of the mixture have been added. The tube is then sealed with parafilm and placed in the water bath.

It might be useful to describe in some detail how to set up a kinetic experiment for a two substrate system in which five points are required to describe each double reciprocal plot and in which there are five such lines. This experiment, involving substrate A (1.0 ml) and substrate B (1.0 ml) will require twenty-five tubes. If standards are needed to evaluate substrate and/or enzyme decomposition, additional assay solutions will be required; however, solving of these problems will be left to the ingenuity of the investigator.

Stock solutions of A and B are diluted to give 10 ml of concentrations marked 1 in Fig. III-4. The following solutions are mixed: (3) 2 ml of solution 1 + 4 ml H_2O; (5) 2 ml of solution 1 + 8 ml of H_2O; (7) 1 ml of solution 1 + 6 ml of H_2O; (9) 1 ml of

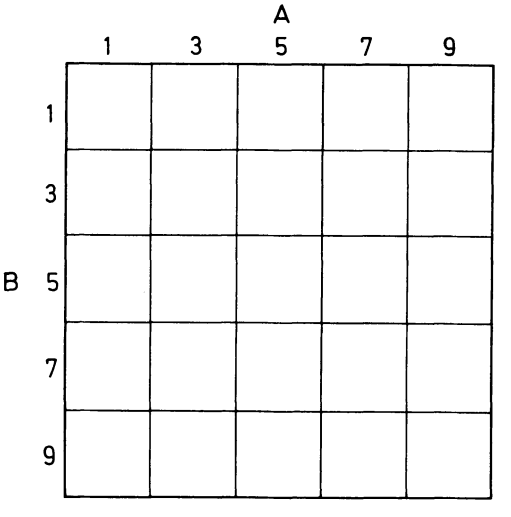

Fig. III-4. Illustration of the arrangement of twenty-five reaction mixture test tubes to which have been added 1.0 ml buffer. Additional details are contained in the text

solution 1 + 8 ml H_2O. After thorough mixing, the substrate solutions are added as indicated in Fig. III-4. To illustrate this point, one would pipette 1.0 ml of solution A-9 into the five tubes in the vertical column of Fig. III-4 (marked 9). Using another 1.0 ml pipette, substrate A-7 would be dispersed into each of the five test tubes in vertical column 7. This procedure is continued until substrate A is added to all twenty-five tubes. One ml of substrate B-9 is then distributed to the tubes in the horizontal column marked B-9, starting with the tube containing the *lowest* concentration of substrate A (A-9). In this manner, substrate is added to all tubes required for the kinetic experiment.

A somewhat different approach is used when kinetic experiments are to be carried out for three substrate systems. Experiments exactly analogous to those described for the two substrate case would require too many assays to be practical. Two different types of protocols are in vogue when doing three substrate kinetics. The advantages and limitations of these two procedures are available in the literature (11). The method to be described requires only three different experiments, each one of which will require either twenty or twenty-five assays. The substrates for a terreactant system are designated A, B, and C as indicated in Chapter I. For these studies, A will be varied over a nine-fold concentration range, as indicated for the two substrate reaction; however, B and C will be fixed at five different levels over a ten-fold concentration range. The concentration of substrates B and C will be 5 times their Michaelis constants at the highest fixed level and decrease to 1/2 the Michaelis constant at the lowest fixed level. The intermediate concentrations may be at any values that give a velocity dependence with changes in concentration. It is important to emphasize that *the concentrations of B and C are to be in a constant ratio*. Let us assume that the Michaelis constants for B and C are 0.1 mM and 1 mM, respectively. Tubes containing the highest fixed concentration of B and C would contain 0.5 mM B and 5 mM C, while at the lowest fixed levels, the tubes would contain 0.05 mM and 0.5 mM, B and C, respectively. Experimentally, one would mix substrates B and C *together* for the highest so-called fixed concentration and then serially dilute this mixture for each fixed level of these substrates. The pipetting sequence and substrate and buffer volumes are similar to those described for the bireactant system.

F. Studies of Nucleotide Dependent Enzymic Reactions

Nucleotide dependent transphosphorylation reactions require the 1:1 complex of divalent metal ion and nucleotide as the true substrate (12). The free nucleotide is generally a potent inhibitor of the reaction, as is the uncomplexed metal ion. FROMM et al. (13) have found that excess Mg^{2+} inhibits the yeast hexokinase reaction in which $MgADP^{1-}$ and glucose-6-P are substrates and brain hexokinase is markedly inhibited by ATP^{4-} with $MgATP^{2-}$ and glucose as substrates (14).

It is important when investigating the kinetics of transphosphorylases to recognize these problems of substrate inhibition and also to design experiments in which these effects are precluded. It is of interest that investigators sometimes assume that, if the Mg^{2+} to ATP^{4-} ratio is high, assuming Mg^{2+} inhibition is not observed, the active substrate species, $MgATP^{2-}$, will not dissociate enough as substrate dilutions are made to change its concentration. Occasionally, this assumption proves to be wrong, and rather bizarre kinetic results are obtained. For example, BACHELARD (15) found that substrate-saturation curves of brain hexokinase for $MgATP^{2-}$ were sigmoidal at subsaturating concentrations of glucose when the Mg^{2+}:ATP ratio was unity. On the other hand, he observed that, when this ratio was five, the system exhibited a normal hyperbolic response. Hill plots (16) of these data indicated that the number of binding sites for $MgATP^{2-}$ varied from 1.05 to 1.8, depending upon the Mg^{2+} concentration, and the investigator proposed an allosteric site for Mg^{2+}. Recalculation of the concentration of $MgATP^{2-}$ in the assay mixtures led to the conclusions that the anomalous kinetics were merely a manifestation of an incorrect assignment of the concentration of $MgATP^{2-}$ and the system really followed Michaelis-Menten kinetics (17).

A number of factors can affect the concentration of the metal-nucleotide complex. These include the concentration of the two species, buffer and salt effects (ionic strength), temperature, and pH. The following protocol describes how one should adjust the total metal ion concentration to minimize these inhibitory effects, assuming Mg^{2+} is the divalent cation.

1. Determine the optimum Mg^{2+} concentration by carrying out rate studies as a function of total Mg^{2+} (Mg_0^{2+}) at the highest and lowest levels of the substrates. The type of result to be expected is depicted in Fig. III-5.

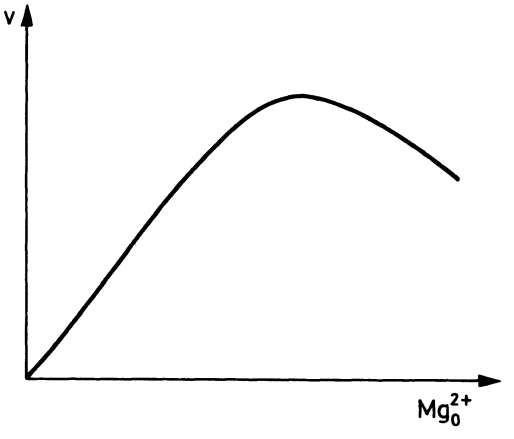

Fig. III-5. Plot of initial velocity (v) *versus* total magnesium ion (Mg_0^{2+}) for a transphosphorylase. The concentration of nucleotide and other substrates are held constant

Such studies should be carried out in buffers for which there is adequate information on the stability constants for those compounds that bind metal ion. GOOD et al. (18) have described a number of buffers that either bind no Mg^{2+} or bind very little Mg^{2+}. One of these buffers is N-2-hydroxyethylpiperazine-N^1-2-ethanesulfonic acid or HEPES. Table III-1 lists the stability constants for Mg with a number of biologically important compounds as determined spectrophotometrically by using the 8-hydroxyquinoline procedure of BURTON (19). It is important to note that conditions of buffer concentration, pH, and temperature are specified.

Table III-1. Stability constants for Mg-anion complexes [a]

Compound	Stability Constant (K) [b]
$MgATP^{2-}$	100,000
$MgADP^{1-}$	10,000
MgAMP	∿ 110
MgP	∿ 100

[a] From the data of RUDOLPH and FROMM (20)

[b] Analyses were in 20 mM HEPES, pH 7.7 and 28°.

These values will vary with buffer; e.g., in 0.1 M triethanolamine bromide, the K for $MgATP^{2-}$ is 70,000 M^{-1} (21), whereas the K is 20,000 M^{-1} and 2,000 M^{-1} in 0.05 M tris-hydroxymethylaminomethane chloride, pH 7.6, for $MgATP^{2-}$ and $MgADP^{1-}$, respectively.

The very dramatic alterations in the stability constants for $MgATP^{2-}$ and $MgADP^{1-}$ observed with changes in ionic strength have been demonstrated by NOAT et al. (22).

2. Calculate the free uncomplexed Mg^{2+} concentration that corresponds to the optimal Mg^{2+} determined as described in Fig. III-5.

From the reaction

$$Mg^{2+} + ATP^{4-} \rightleftharpoons MgATP^{2-}. \qquad \text{(III-3)}$$

The stability constant K is

$$K = \frac{(MgATP^{2-})}{(Mg^{2+})_{Free}(ATP^{4-})_{Free}}. \qquad \text{(III-4)}$$

Note that

$$Mg_0^{2+} = \text{total } Mg^{2+} = Mg_{Free}^{2+} + MgATP^{2-} \qquad \text{(III-5)}$$

$$ATP_0^{4-} = \text{total } ATP^{4-} = ATP_{Free}^{4-} + MgATP^{2-}. \qquad \text{(III-6)}$$

Thus:

$$K = \frac{(MgATP^{2-})}{(Mg_0^{2+} - MgATP^{2-})(ATP_0^{4-} - MgATP^{2-})}. \qquad \text{(III-7)}$$

Let $X = MgATP^{2-}$

$$K = \frac{X}{(Mg_0^{2+} - X)(ATP_0^{4-} - X)}. \qquad \text{(III-8)}$$

The value for K can be obtained from the literature or by experiment (see Table III-1), and Mg_0^{2+} and ATP_0^{4-} can be obtained from data of the type shown in Fig. III-5. It is possible, therefore, to solve the quadratic Eq. (III-8). If the concentration of $MgATP^{2-}$ is known, Mg_{Free}^{2+} can be determined from Eq. (III-5).

3. If the Mg^{2+} that corresponds to the optimal uncomplexed Mg^{2+} is known, then the amount of Mg^{2+} to be added to each reaction mixture may be calculated. For example, what must the Mg_0^{2+} be to maintain Mg_{Free}^{2+} at 1 mM for any concentration of nucleotide (ATP_0^{4-})?

$$K = \frac{(MgATP^{2-})}{(ATP^{4-})_{Free}(Mg^{2+})_{Free}}. \qquad \text{(III-9)}$$

If Mg_{Free}^{2+} is 10^{-3} M, then

$$K\ (10^{-3}M) = \frac{(MgATP^{2-})}{(ATP^{4-})_{Free}} = \frac{(MgATP^{2-})}{(ATP_0^{4-} - MgATP^{2-})}. \qquad \text{(III-10)}$$

If K and ATP_0 are known, the amount of Mg^{2+} associated with $MgATP^{2-}$ can be calculated from Eq. (III-10). Mg_0^{2+} can be calculated from

$$Mg_0^{2+} = Mg_{Free}^{2+} + MgATP^{2-} = 1\ mM + MgATP^{2-}. \qquad \text{(III-11)}$$

The following example may be a useful exercise. Calculate the Mg_0^{2+} required in 0.05 M buffer to maintain 1 mM free Mg^{2+} if ATP_0 is 5 mM. If the stability constant is $2 \times 10^4\ M^{-1}$, Mg_0^{2+} is 5.76 mM.

A more complicated calculation is required when two cation binding species are present in a reaction mixture simultaneously. Let us now calculate the concentration of Mg_0^{2+} required to maintain Mg_{Free}^{2+} at 1 mM when ATP_0^{4-} and ADP_0^{3-} are both 5 mM in 20 mM HEPES buffer, pH 7.7 at 28°.

Total Mg_0^{2+} must now be calculated from the expression

$$Mg_0^{2+} = Mg_{Free}^{2+} + MgATP^{2-} + MgADP^{1-}. \qquad \text{(III-12)}$$

$MgATP^{2-}$ can be calculated from Eq. (III-10) and $MgADP^{1-}$ from the expression

$$K\ (10^{-3}\ M) = \frac{MgADP^{1-}}{(ADP_0^{3-} - MgADP^{1-})}. \qquad \text{(III-13)}$$

The concentration of $MgATP^{2-}$ is 4.95 mM and $MgADP^{1-}$ is 4.55 mM. The concentration of Mg_0^{2+} from Eq. (III-12) is therefore 10.5 mM.

The calculations show that, when the Mg_{Free}^{2+} is maintained at 1 mM, 99% of the ATP is bound, whereas only 91% of the ADP is associated with Mg^{2+}. This figure for $MgADP^{1-}$ may be unacceptable in kinetic studies, and Mg^{2+} may therefore be maintained at a higher free concentration. Thus, at 2 mM Mg_{Free}^{2+}, $MgATP^{2-}$ is 99.5% of ATP_0^{4-} and $MgADP^{1-}$ is 95% of the ADP_0^{3-}.

4. It was mentioned earlier that pH has a pronounced effect on metal binding to nucleotides. This phenomenon is unrelated to the effect of pH on the other facets of the catalytic process (see Chapter VIII). It is now well established that the pK_a for the secondary phosphoryl dissociation in ATP is about 7 (23). The stability constant for $MgHATP^{1-}$ is 31 M^{-1} (23); however, the values varies with experimental conditions. It is clear, that, even at pH 8, 9% of the ATP_0 exists as $HATP^{3-}$, and the effect of this latter compound on the reaction kinetics cannot be predicted with certainty in the absence of detailed experiments. Fortunately, the potential presence of $HATP^{3-}$ at lower pH values (between pH 7 and pH 8) can be obviated by taking advantage of the fact that Mg^{2+} will shift the reaction $HATP^{3-} \rightleftharpoons H^+ + ATP^{4-}$ to the right because of its much greater affinity for the tetranegative anionic form of the nucleotide.

The different adenylate and Mg^{2+} species are:

$$ATP_0 = ATP^{4-} + HATP^{3-} + MgATP^{2-} + MgHATP^{1-} \qquad \text{(III-14)}$$

$$Mg_0^{2+} = Mg_{Free}^{2+} + MgATP^{2-} + MgHATP^{1-}. \qquad \text{(III-15)}$$

If we let K_1, K_2, and K_a represent *dissociation constants* for $MgATP^{2-}$ $MgHATP^{1-}$, and $HATP^{3-}$, then

$$Mg_{Free}^{2+} = Mg_0^{2+} - MgATP^{2-}(1 + K_1(H^+)/K_2K_a) \qquad \text{(III-16)}$$

and thus

$$\frac{ATP_0}{MgATP^{2-}} = 1 + \frac{K_1(H^+)}{K_aK_2} + \frac{K_1(1 + (H^+)/K_a)}{\left[Mg_0^{2+} - MgATP^{2-}(1 + K_1(H^+)/K_aK_2)\right]}. \qquad \text{(III-17)}$$

If $K_1 << K_2$ and $H^+ << K_a$, as will be the case when considering the K's as dissociation constants and pH >> pK_a, Eq. (III-17) will reduce to Eq. (III-9). Equation (III-17) can be used to determine whether a significant fraction of the ATP exists as either $MgHATP^{1-}$ or as $HATP^{3-}$. For example, if K_1, K_2, K_a, pH, ATP_0, and Mg_0^{2+} are taken to be 10^{-5} M, 10^{-2} M, 10^{-7} M, 7.7, 5 mM, and 7 mM, respectively, essentially all the ATP exists as $MgATP^{2-}$

This will not be the case at lower pH and where $(H^+)/K_a$ is much higher than in this example.

G. The Kinetic Assay

The enzymic reaction is usually initiated by adding enzyme to the assay mixture which had been thermally equilibrated to the desired temperature; however, the reaction may be started with substrate when preincubation of the enzyme is required. It is important that the starting component be added very rapidly and the complete assay mixture thoroughly mixed immediately thereafter. The former requirement may be accomplished by adding the enzyme with a constriction micropipette after which the assay mixture is inverted twice to insure proper mixing or with the adder-mixer device of BOYER and SEGAL (24) in which the enzyme is added directly to the spectrophotometer cell and which permits complete mixing within two seconds.

There are two ways in which velocity can be monitored after the reaction has begun. The method of choice is the continuous assay; the stop-time assay, although theoretically the equivalent of the continuous assay, requires many more analyses and manipulations.

1. The Continuous Assay

If a chromophore is either generated or consumed in the course of an enzymic reaction, the assay will usually be amenable to continuous assay analysis. This method requires a good recording spectrophotometer, such as the Beckman DU spectrophotometer equipped with the Gilford optical density converter or any one of the many excellent spectrophotometers sold by Cary Instruments with a 0-0.1 absorbance slide wire. Facile control of the recorder chart speed is a most important factor in the continuous assay procedure. Ideally, one should attempt to obtain a recorder tracing of approximately 45°, and this can be achieved either by varying the enzyme concentration or the recorder chart speed from assay to assay. It is clearly more desirable to work with a single concentration and volume of enzyme and to vary the recorder speed. The linear portion of the velocity progress curve should be long enough (minimally six inches) so that an accurate tangent to the initial velocity phase of the recorder tracing can be made with a straight edge ruler and sharp pencil. All the assays are then expressed in some convenient standard form, such as μ moles product formed per minute.

Although many enzymic reactions do not result in the formation (or loss) of a chromophore, the product of the reaction may be used as the substrate in another reaction, the product of which is a chromophore. Under proper conditions, these two reactions may be *coupled* so that a continuous assay may be used. An example of the coupled assay involves the use of the $NADP^+$-linked

enzyme glucose-6-phosphate dehydrogenase to continuously monitor the hexokinase reaction, as follows:

$$MgATP^{2-} + glucose \rightleftharpoons MgADP^{1-} + glucose\text{-}6\text{-}phosphate^{2-} + H^{+} \qquad \text{(III-18)}$$

$$glucose\text{-}6\text{-}phosphate^{2-} + NADP^{+} \rightleftharpoons \delta\text{-}gluconolactone\text{-}6\text{-}phosphate^{2-} + NADPH + H^{+}. \qquad \text{(III-19)}$$

Although enzymologists have been using coupled reactions for many years, and although chemists have long had an interest in consecutive reactions, it was not until 1969 that McCLURE (25) formalized the concepts involved in coupled enzymic reactions. Consider for example the following sequence of reactions:

$$A \xrightarrow[E_1]{k_1} P \xrightarrow[E_2]{k_2} Q. \qquad \text{(III-20)}$$

Where E_1 is the enzyme being investigated and E_2 is the auxiliary enzyme, let it be assumed that the first reaction is zero-order with respect to A (A does not change in concentration appreciably) and irreversible (P is removed as it is formed). Let it be further assumed that the second reaction is first-order relative to P ($P << K_p$) and irreversible (auxiliary enzymes are usually chosen so that the equilibrium point lies far in the direction of product) and that any substrate for the second reaction besides P is saturating. It is possible, with these assumptions, to calculate the amount of E_2 required to carry out a theoretically correct coupled assay.

For the reaction sequence shown in Eq. (III-20),

$$\frac{dP}{dt} = k_1 - k_2 P. \qquad \text{(III-21)}$$

Integrating between the limits t = 0 and t = t and P = 0 and P = P, Eq. (III-22) is obtained.

$$P = \frac{k_1}{k_2}\left(1 - e^{-k_2 t}\right). \qquad \text{(III-22)}$$

As $t \to \infty$, P approaches the steady state (P_{ss}); i.e.,

$$P_{ss} = \frac{k_1}{k_2}. \qquad \text{(III-23)}$$

Equation (III-22) can be arranged to

$$2.303 \log\left[1 - \frac{k_2 P}{k_1}\right] = -k_2 t. \qquad \text{(III-24)}$$

With these equations in hand, we can calculate the amount of E_2 required to reach the steady state for P (P_{ss}) in a certain period of time.

Substituting Eq. (III-23) into (III-24) gives,

$$2.303 \log \left[1 - \frac{P}{P_{ss}}\right] = -k_2 t. \qquad \text{(III-25)}$$

In the first-order enzymic process in Eq. (III-20), where $K_p >> P_{ss}$,

$$v_2 = \frac{V_2 P_{ss}}{K_p} = k_2 P_{ss}. \qquad \text{(III-26)}$$

Substituting from Eq. (III-26) into Eq. (III-25) provides an equation that allows calculation of the amount of E_2 required to provide a fractional attainment of the steady-state phase of the coupled process at any time, t.

$$V_2 = \frac{-2.303(K_p) \log \left[1 - \frac{P}{P_{ss}}\right]}{t}. \qquad \text{(III-27)}$$

If, for example, one wishes to obtain 99% of the steady state in 5 sec (1/12 min) and K_p is 0.1 mM, V_2 = 5.53 mM/min or 5.53 IU/ml of E_2.

McCLURE (25) points out some of the limitations in the use of Eq. (III-27); i.e., in the use of the coupled assay. First and foremost is the assumption that $P_{ss} << K_p$. This assumption is the basis for Eq. (III-26) and provides that the reaction will be first-order relative to E_2. Figure III-6 illustrates the attainment of the steady-state in 5 sec as outlined in the preceding example for a coupled enzyme system. Note that, when P_{ss} is attained, dQ/dt is constant and initial velocity conditions are achieved.

McCLURE (25) has also considered in detail the conditions required to assay continuously with two auxiliary enzyme systems; i.e., for a case involving measurement of ADP production in the hexokinase reaction.

$$MgADP^{1-} + \text{phosphoenolpyruvate}^{3-} + H^+ \overset{E_2}{\rightleftharpoons} \text{pyruvate}^{1-} + MgATP^{2-} \qquad \text{(III-28)}$$

$$\text{pyruvate}^{1-} + NADH + H^+ \overset{E_3}{\rightleftharpoons} \text{lactate}^{1-} + NAD^+. \qquad \text{(III-29)}$$

The fundamental assumptions are similar to those already considered with a single auxiliary enzyme, and the reader is referred to the article by McCLURE (25) for the theory and details of these coupled enzymic assays.

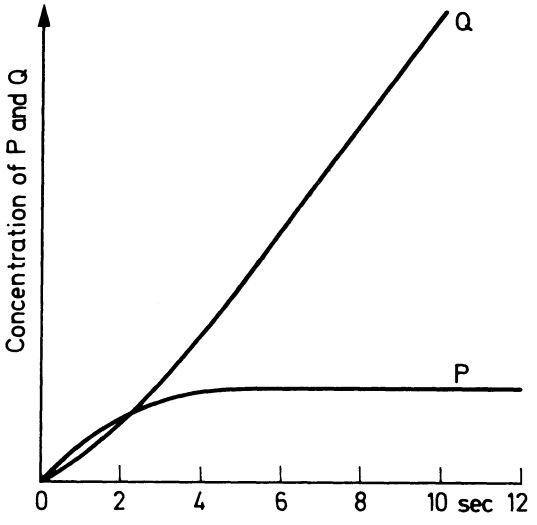

Fig. III-6. Plot of concentration of compounds P and Q (as defined in Eq. (III-20)) as a function of time. It is assumed in this plot that P attains a steady-state concentration in 5 sec. Note that after 5 sec the change of concentration of Q with time is constant

When using the coupled assay, it is useful to prepare a complete reaction mixture minus the enzymes. The auxiliary enzyme can then be added to determine whether it is contaminated either with the enzyme under study or with one of its isozymes. For example, I have found that commercial preparations of yeast glucose-6-P dehydrogenase are occasionally contaminated with yeast hexokinase. These enzyme preparations cannot be used for experiments with other types of hexokinases (e.g., mammalian), and a special protocol is required for enzyme addition, even when the two enzymes are obtained from the same yeast preparation.

It is also essential that the components of the auxiliary system neither inhibit nor activate the enzyme system under study. This point may be checked by assaying the enzyme in the absence and presence of the auxiliary system (without the auxiliary enzyme). Finally, it may be useful to determine the velocity using a stop-time assay (described later) and the continuous assay in parallel experiments at high, low, and intermediate levels of substrates to insure that similar results are being obtained in the two assays. It may be of interest to note that in systems which measure ADP production (Eqs. (III-28) and (III-29)), there will usually be some ADP in the ATP, and compensating amounts of auxiliary substrate and coupling enzymes should be preincubated with the system under study before the enzyme is added.

In many reactions, protons will be either generated or used in the course of the enzymic reactions. These reactions can be monitored by sensitive recording pH meters. Recording fluorometers may be used in place of spectrophotometers if a fluorescing species is found on either side of the chemical reac-

tion; however, special problems involving self-quenching may arise, particularly when loss of fluorescing compound with time is measured (26). This is less of a problem when considering spectrophotometry; however, technical problems may also arise when the loss of absorbing species is monitored in the spectrophotometer. For example, in a dehydrogenase reaction, when NADH is present initially, it will often be necessary to compensate for the high initial absorbance. This may be accomplished with a reagent blank in double beam instruments, but care should be exercised not to use an excessive absorbance, which might lead to stray-light artifacts or a sluggish pen response. The former problem can be recognized by determining whether Beer's Law is followed with the chromophore under study. Single beam recording spectrophotometers, such as the Gilford, are also adversely affected by stray-light artifacts, and the investigator would be well advised to recognize the limitations of these instruments.

With some enzyme systems, it may be found that the initial velocity phase of the reaction is simply too short to measure. This can occur, assuming the substrate concentration is essentially unchanged, if accumulated product is a potent inhibitor of the reaction. One way to circumvent this problem, without the aid of rapid reaction devices, is to remove the product by coupling the reaction to another enzyme system as already suggested. In some cases, it may even be desirable to remove both products; i.e., in the hexokinase reaction the auxiliary enzymes glucose 6-phosphate dehydrogenase and pyruvate kinase may be used along with $NADP^+$ and phosphoenolpyruvate. As described, this series of enzymes will permit stop-time assaying of the hexokinase reaction with the simultaneous removal of ADP and regeneration of ATP. It is also possible to use cells of longer light path with the continuous assay and decrease the enzyme concentration, or alternatively, to make measurements at substrate concentrations below the Michaelis constant.

2. The Stop-Time Assay

In many instances it is neither possible nor desirable to use a continouous spectrophotometric assay to monitor initial velocities. For example, the availability of radioactive substrates permits the investigator to carry out various types of initial velocity experiments in which the enzymic reaction is terminated before assay. In order to determine initial rates under these conditions, a minimum of four determinations is required at different times after initiation of the reaction. An assay blank, or zero time determination, is an absolute necessity with the stop-time protocol. This will permit the product *versus* time progress curve to pass through the origin.

The following description indicates in a general way how the stop-time assay protocol is carried out. If n reaction mixtures are to be used, then (n + 1) volumes of assay solutions are made up in a test tube for each substrate concentration, mixed, and equilibrated to temperature in a water bath. An aliquot of the

solution is then removed for blank analysis, after which the starting reagent - either enzyme or substrate - is added at time zero. Using a stop watch, aliquots of the assay mixture are removed at known increments of time, and the reactions terminated. A variety of procedures may be used to stop the reaction; however, each system will have special requirements, and a lengthy discussion of the various methods that have been used to achieve this end does not seem to be fruitful. Conditions such as boiling in a water bath and addition of reagents such as acids (trichloroacetic and perchloric), bases and compounds such as $AgNO_3$ are most commonly employed to terminate enzyme reactions. It is also possible to add the reaction mixture directly to some paper supporting material, after which either electrophoresis or chromatography may be carried out. It is absolutely essential to establish that the reaction stopping procedure is effective. This point may be ascertained by comparing a reaction mixture lacking one of the components of the enzymic reaction with one in which the missing component is added after conditions are used to terminate the reaction. For example, if the reaction is to be begun with enzyme and stopped with $AgNO_3$, two identical assay mixtures are treated first with stopping reagent, mixed, and the enzyme added to one of them. The solutions are then incubated for a period of time during which a discernible amount of product would be expected to be produced. If none is formed when a comparison of the two reaction mixtures is made, the stopping technique can be used with the system.

It is ordinarily only necessary to establish conditions of initial reaction velocity at the highest, lowest, and intermediate substrate concentrations. Once the amount of enzyme, incubation time, and temperature have been determined with *certainty*, the same number of reaction mixtures that were employed for the continuous spectrophotometry assays may be used. In the case of a twenty-five tube experiment, the reactions may be initiated at one minute intervals and terminated after twenty-five minutes at exactly one minute intervals, provided of course that conditions are used in which initial velocities persist for at least twenty-five minutes.

It is sometimes advantageous to remove a reaction product to prolong the initial velocity phase of the reaction. How this may be accomplished is illustrated in the assay for hexokinase. As indicated earlier, the continuous assay procedure for this enzyme couples the hexokinase reaction to the glucose-6-P dehydrogenase reaction. It is possible to also include pyruvate kinase, KCl, and phosphoenolpyruvate to rephosphorylate ADP formed in the hexokinase reaction. Procedures of this type, if properly carried out, serve to provide highly reliable initial velocity data.

Replicate kinetic analyses are highly desirable; however, they do present serious technical problems when working with unstable enzymes and substrates. Ideally, the kineticist should carry out enough determinations at a single substrate concentration to permit a statistical analysis of the initial velocity data to be made.

H. Plotting Methods

One and Two Substrate Systems. After the initial velocity data have been obtained as outlined in the previous section, they are graphed to evaluate the kinetic mechanism and certain kinetic parameters. Plots of initial velocity as a function of substrate concentration for enzyme catalyzed reactions that adhere to Michaelis-Menten kinetics are hyperbolic. It is extremely difficult to accurately estimate the asymptote to the plateau portion of the velocity *versus* substrate curve, which is actually the maximal velocity. In order to circumvent this problem, use has been made of reciprocal plots, which serve to transform the Michaelis-Menten Equation into linear form.

The most commonly used form of the Michaelis-Menten Equation is the double reciprocal or Lineweaver-Burk plot (8, 9).

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1}\left(\frac{1}{A}\right). \qquad \text{(III-30)}$$

Another transformation of this equation, as proposed by HANES (27), involves multiplication of Eq. (III-30) by A to yield Eq. (III-31).

$$\frac{A}{v} = \frac{K_a}{V_1} + \frac{A}{V_1}. \qquad \text{(III-31)}$$

The Michaelis-Menten Equation may be rearranged yet another way, as shown by WOOLF (8) and HOFSTEE (28) in Eq. (III-32).

$$v = V_1 - \frac{K_a v}{A}. \qquad \text{(III-32)}$$

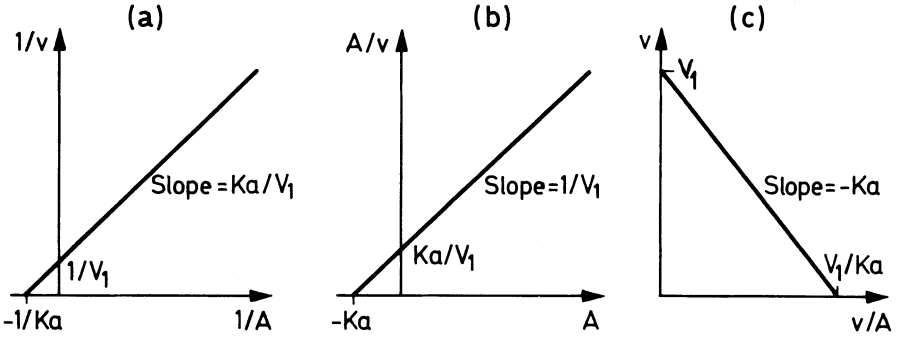

Fig. III-7. Plots of 1/v *versus* 1/A (a), A/v *versus* A (b), and v *versus* v/A (c) for the simple Michaelis-Menten Equation. The graphs illustrate the three different linear plotting methods and how the kinetic parameters are obtained

The plots described by Eqs. (III-30) to (III-32) are shown in Fig. III-7.

In order to characterize the intersection of the curves on the abscissa, evaluation of Eq. (III-31) where A/v = 0 will be illustrated.

$$\frac{A}{v} = 0 = \frac{K_a}{V_1} + \frac{A}{V_1} \qquad \text{(III-33)}$$

$$A = - K_a$$

Although the double reciprocal or Lineweaver-Burk plot has been the overwhelming choice of enzymologists and enzyme kineticists for graphing initial rate data, its use has come into serious question. WILKINSON (10) and DOWD and RIGGS (29) have pointed out some shortcomings inherent in this transformation relative to the equations described by Eqs. (III-31) and (III-32). For example, Wilkinson's analysis of linear rate Eqs. (III-30) and (III-31) indicates that the double reciprocal plot exhibits a greater variation in weighting than does the graph of A/v *versus* A. In these studies, WILKINSON assumed that the variance of velocity is reasonably constant and found that 1/v exhibits a greater variance in accuracy than A/v over the range 1/3 to 3 times the Michaelis constant concentration of A. DOWD and RIGGS (29) came to similar conclusions regarding the relative merits of the three linear transformations of the Michaelis-Menten Equation, and they suggest that the Lineweaver-Burk plot should be abandoned. Although this writer accepts many of the arguments regarding the relative inadequacies of the double reciprocal method, it seems neither appropriate nor realistic to express kinetic data in terms of Eqs. (III-31) and (III-32) in this monograph because of the almost exclusive use of the Lineweaver-Burk method at present. The Hanes plot itself presents certain limitations. For example, consider the case in which the substrate is varied from 1/2 to 5 times K_a in a relatively even distribution of concentrations. In the A/v *versus* A plot, most of the data points will be above the K_a in a region in which there is little velocity dependence if equal spacing on the abscissa is made. In the case of the Lineweaver-Burk and Hofstee plots, most of the experimental points lie in a region in which velocity is highly dependent upon substrate concentration. If the suggestion of WILKINSON (10) is accepted and substrate is varied in the range 1/3 to 3 times the Michaelis constant, it may be experimentally difficult to measure the lowest velocities accurately. These are technical problems rather than theoretical ones, but they do require consideration when the various plotting procedures are evaluated.

An important procedure in plotting kinetic data is the use of data weighting as suggested by WILKINSON (10). The need for a procedure of this type can be gotten by reference to Fig. III-8. In this figure, velocity *versus* substrate concentration is plotted on one set of axes and the reciprocals of these functions on the other. It is assumed for the hypothetical data of

Fig. III-8 that the standard deviations (σ_i) all equal 0.01 and the σ values are shown on the graph. In the hyperbolic curve, the σ_i values are all of similar magnitude, whereas in the double reciprocal plot, the standard deviations increase as the velocities decrease. This effect can be compensated for by statistically modifying the standard deviation as suggested by WILKINSON (10).

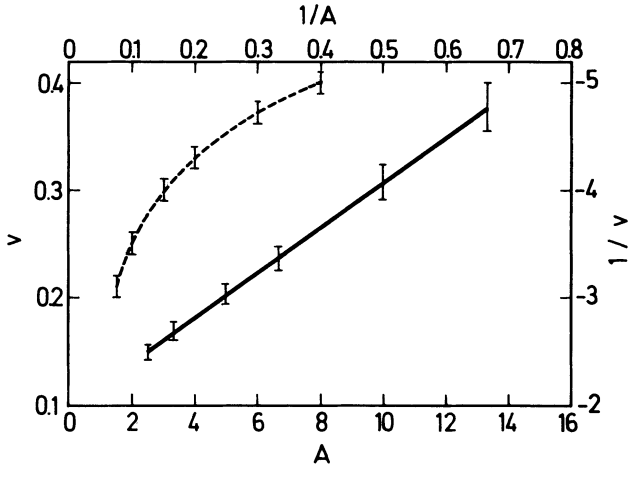

Fig. III-8. Plot of initial velocity (v) *versus* substrate concentration (A) for hypothetical data which conform to the equation $v = V_1A/(K_a + A)$ (dashed line). Also shown in the figure is a plot of reciprocal initial velocity (1/v) *versus* reciprocal of substrate concentration (1/A) for the same hypothetical data used to describe the hyperbola. The linear transformation is the equation $\frac{1}{v} = \frac{1}{V_1}\left(1 + \frac{K_a}{A}\right)$ and is described by the solid line. The standard deviation σ_1 is taken to be constant and equal to 0.01

Since the weight$_x \sim \frac{1}{\sigma_x^2}$ (30), then for $x = 1/v$, $\sigma_x = -(1/v^2)\sigma_v$ and thus $\sigma_x 2 = (1/v^4)\sigma_v 2$. The weighting factor then involves taking the initial velocity to the fourth power,

$$\text{weight } 1/v = \frac{\frac{v_i^4}{\sigma_{v_i}^2}}{\frac{1}{N}\sum_i \frac{v_i^4}{\sigma_{v_i}^4}} . \qquad \text{(III-34)}$$

When straight lines are plotted to a linear transformation of the Michaelis-Menten Equation, either by eye or by a least-squares fit, each point is given equal weighting. When a weighted least-squares fit is done as suggested by WILKINSON (10) by computer, there would appear to be very little advantage of using one linear transformation in preference to either of the others. CLELAND (31) was the first to use weighting factors to give a "best fit" to various kinetic equations of kinetic models by using the digital computer. CLELAND's program is currently available and can be found in a review article by him on this subject (31). Experimental data, usually done in duplicate, but where each velocity is treated separately, are fitted to specific equations by using a Fortran program. In the presentation of the data, the lines are drawn through the experimental points by computer calculated fits to certain rate equations. As a first approximation, lines are drawn through the data points by hand to eliminate rate equations that obviously do not conform to particular data.

Cleland's computer program gives standard errors for slopes and intercepts of data fitted by a particular equation, and if a question arises as to whether a slope or intercept change occurs, the t test of significance may be used to evaluate the results.

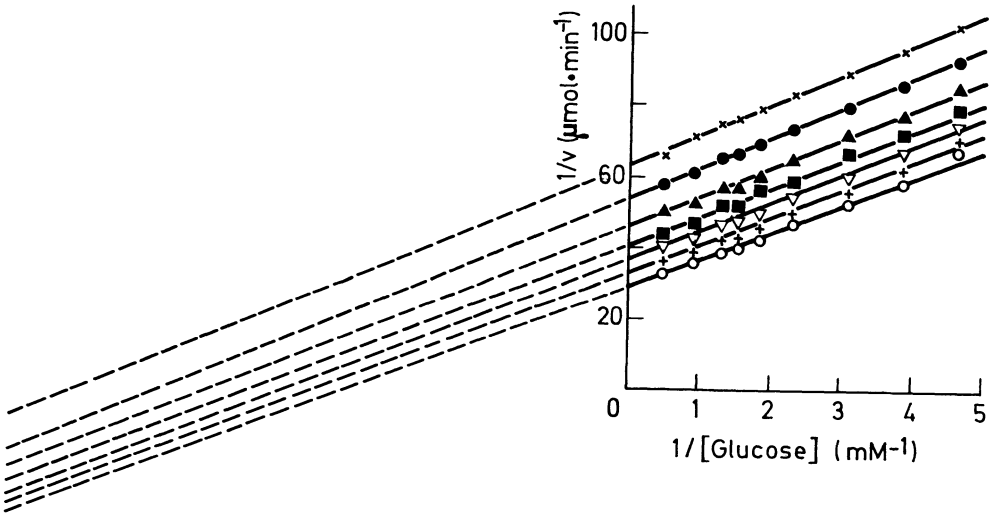

Fig. III-9. Plot of reciprocal of initial reaction velocity (v) *versus* reciprocal concentration of glucose at different concentrations of the inhibitor 9-(β-D-glucopyranosyl)-adenine-6'-triphosphate. The enzyme was yeast hexokinase and ATP was held constant at 0.38 mM. The solid lines were taken from the data of HOHNADEL and COOPER (32). The broken lines were added to the original figure to indicate the subtle convergence of the family of "parallel" lines

There are at the present time highly sophisticated "model testing" rather than "model fitting" programs available. Consider for example the two very real problems outlined in the data of Figs. III-9 and III-10 that the kineticist often encounters. In Fig. III-9 are presented data on yeast hexokinase which were obtained by HOHNADEL and COOPER (32). The determination of whether these curves are indeed parallel or are in fact convergent is cruical to an understanding of the mechanism of yeast hexokinase (see Chapter IV). Figure III-10 represents curves for noncompetitive inhibition, which intersect closely enough to the axis of ordinates so that one may ask whether the inhibition pattern is in fact competitive. It is not really possible to answer these

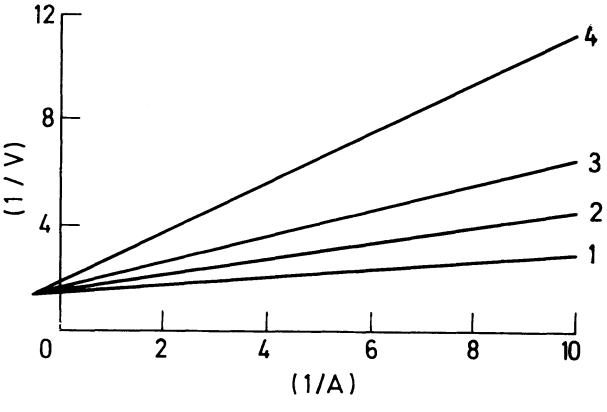

Fig. III-10. Plot of reciprocal of initial reaction velocity (v) *versus* reciprocal of the concentration of substrate (A) in the presence (lines 2, 3, and 4) and absence (line 1) of a noncompetitive inhibitor

questions with a model-fitting computer program; rather, a model-testing approach must be used. The difference between competitive, noncompetitive, and uncompetitive inhibition is associated with the inhibition terms in the basic rate equation. The most general kinetic expression in this context is the equation for noncompetitive inhibition,

$$\frac{1}{v} = \frac{1}{V_1}\left(1 + \frac{I}{K_{ii}}\right) + \frac{K_a}{V_1}\left(1 + \frac{I}{K_i}\right)\left(\frac{1}{A}\right) \qquad \text{(III-35)}$$

where I is the concentration of inhibitor and K_i and K_{ii} represent dissociation constants for complexes EI and EAI, respectively, which occur in the presence of a noncompetitive inhibitor (Chapter IV).

The more terms present in any rate equation, as independent variables, the better the fit will be. Thus, Eq. (III-35) will give a better fit to the data of Figs. III-9 and III-10 than will an equation of uncompetitive inhibition in the case of Fig. III-9,

where $K_i >> I$, or of competitive inhibition in the case of Fig. III-10, where $K_{ii} >> I$. The central question is, however, whether these extra terms in the rate equation are truly essential, in a statistical sense, in describing the kinetic data. A number of different statistical tests are available which provide answers to these questions. They include the F test (30) and the C_p-statistic (33). In the former test, the appropriateness of including an extra term in the fitting function is made. The F test is also useful in testing the entire fit of the data of a particular model. The C_p statistic is based on the idea that the selection of a good subset of independent variables in a multiple linear regression should be based on the total squared error. The C_p statistic is an estimate of the total squared error.

Procedures for computer plotting of kinetic data that permit model (kinetic equation) testing and fitting are presented in the *Appendix*. The computer program described is in OMNITAB II language and allows the nonprogrammer to use the high-speed digital computer easily and accurately.

Another plotting procedure which shows great promise is the method proposed by EISENTHAL and CORNISH-BOWDEN (34). The impressive advantages of this graphical approach are that calculations are not required for evaluation of kinetic parameters, and, perhaps more importantly, it is not necessary to weight the velocities. It is also insensitive to velocities that are classified as outliers (i.e., aberrant observations).

The plotting method is based on the linear transformation of the Michaelis-Menten expression shown in Eq. (III-36),

$$\frac{V_1}{v} - \frac{K_a}{A} = 1. \qquad \text{(III-36)}$$

It is possible to plot Eq. (III-36) in $V_1 K_a$ space as a straight line with intercepts v and A on the y and x axes, respectively. This is illustrated in Fig. III-11. The ordinate axis is V_1 and the abscissa K_a. Each kinetic determination is recorded in terms of -A on the K_a axis and v on the V_1 axis. These points are then connected and a straight line extended into the first quadrant. Additional data points are collected and the intersection point of the lines is used to evaluate K_a directly (Fig. III-11).

EISENTHAL and CORNISH-BOWDEN have indicated that real experimental data which contain a degree of error will not intersect at a common point (34). This effect is displayed in Fig. III-12. The best value for K_a is the *median* of the vertical broken lines in Fig. III-12. If there are an even number of determinations, the value for K_a will be the average of the two middle values. The intersection of different lines at a common point is treated by weighting the point by the following relationship:

$$\text{number of intersections} = \frac{n}{2}(n - 1) \qquad \text{(III-37)}$$

where n = number of lines.

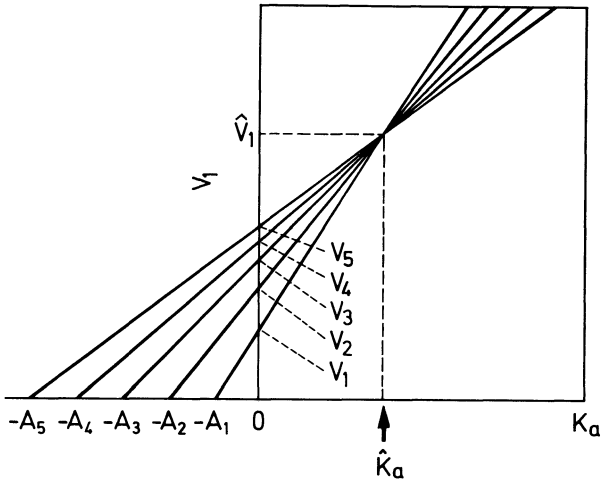

Fig. III-11. Plot of V_1 *versus* K_a as an example of the direct plot method (34). Each line represents one observation of A and v, and has intercepts -A and v on the K_a and V_1 axes, respectively. The point of intersection of the lines gives the coordinates of the best fit values, $\hat{K}_a$ and $\hat{V}_1$

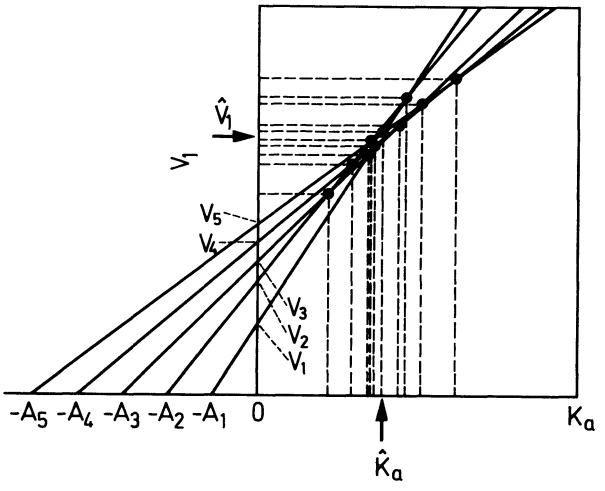

Fig. III-12. The unique intersection point of Fig. III-11 degenerates into ten points [i.e., 1/2 n (n - 1), with n = 5] when the lines are subject to error. Each intersection provides an estimate of K_a and an estimate of V_1. The best estimates, $\hat{K}_a$ and $\hat{V}_1$, are taken as the medians of the two sets of estimates

The degree of accuracy of the kinetic data can be appraised by the deviation of the various lines in the plot from a common point of intersection. This direct plotting method can also be used to evaluate various types of reversible inhibition and to choose between Sequential and Ping Pong mechanisms. The authors have presented a statistical basis for the direct plotting procedure (35).

I. Graphical Procedures

A number of graphical procedures, which can be done at the desk, have been employed for the evaluation of kinetic parameters for two (36-38) and three (11, 39) substrate systems. It is also possible to come to a definitive conclusion on mechanisms for terreactant systems by using this approach (20). The procedures also permit the investigator to segregate kinetic mechanisms by inspection into two classes, either Ping Pong or Sequential.

The graphical procedure of DALZIEL (36) is particularly useful for illustrating replotting methods and how kinetic parameters may be obtained from kinetic data. Consider the typical rate equation for a bireactant system as illustrated in Eq. (III-38).

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)} + \frac{K_b}{V_1(B)} + \frac{K_{ia}K_b}{V_1(A)(B)}. \qquad \text{(III-38)}$$

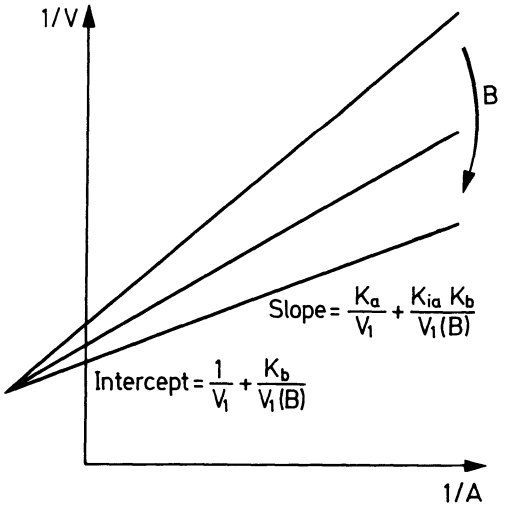

Fig. III-13. Plot of reciprocal of initial reaction velocity (v) *versus* the reciprocal of the concentration of substrate (A) in the presence of different fixed concentrations of substrate B. The values for intercepts and slopes from Eq. (III-38) are shown on the figure

Reciprocal of initial velocity data may be plotted against the reciprocal of substrate A at different fixed concentrations of substrate B, and the slopes and intercepts evaluated as shown in Fig. III-13. These slopes and intercepts are replotted as a function of 1/B in a so-called secondary plot illustrated in Figs. III-14a and III-14b. This method permits evaluation of the parameters V_1, K_a, K_b, and K_{ia}. It is possible from the same initial velocity data to make a primary plot of 1/v *versus* 1/B

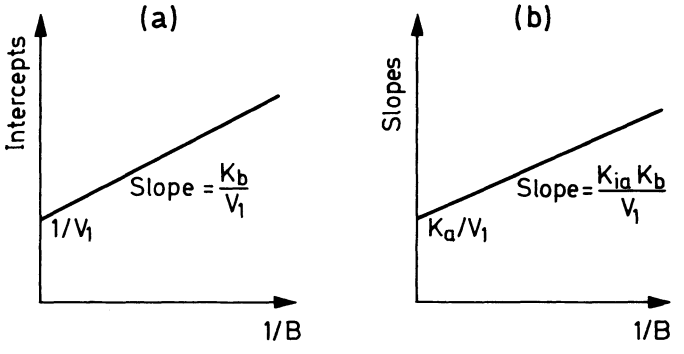

Fig. III-14. (a) A secondary plot of intercept *versus* the reciprocal of the concentration of B from the data of Fig. III-13. (b) A secondary plot of slope *versus* the reciprocal of the concentration of B from the data of Fig. III-13

and secondary plots of intercepts and slopes against 1/A. An evaluation of the four kinetic parameters can then be made, and a comparison can then be obtained between the two plotting procedures. In theory, the two plotting methods should give identical values for the kinetic parameters, and the investigator will get some insight into the validity of the original kinetic data by comparing the values for the four kinetic parameters.

It is possible, with a knowledge of the kinetic parameters, for many uni- and bireactant systems to evaluate the individual rate constants. In the case of the simple Michaelis-Menten Equation for a Uni Uni mechanism, one can evaluate four kinetic parameters from studies of the forward and reverse reaction at a single pH; i.e., V_1, V_2, K_a, and K_b. With these four knowns only four rate constants can be determined. In the case of bireactant systems, the eight kinetic paramters that can be obtained by initial rate experiments in both directions at a single pH will permit evaluation of only eight rate constants. In some cases, it is possible to determine the rate constants from experiments in a single direction only. For example, for the Ordered Bi Bi mechanism involving only binary complexes (see Scheme I-9), the rate equation contains the usual four kinetic parameters and four rate constants. It will also be shown in Chapter V that, when initial rates are carried out in the presence of one product, it is sometimes possible to determine the dissociation constant for that product.

GARCES and CLELAND (40) have suggested that, under certain circumstances, it is advantageous to vary the concentrations of the substrates A and B in a constant ratio. This is a particularly useful procedure when the primary plots of Fig. III-13 are suggestive of a Ping Pong mechanism; i.e., parallel lines are in evidence. In certain cases the convergence of the double reciprocal plots may be so subtle that the curves seem to be parallel. In the case of the Ping Pong mechanism, the rate equation does not contain the (A)(B) term, and plots of 1/v *versus* the reciprocal of either substrate at fixed concentrations of the other substrate yield parallel lines. This equation is:

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)} + \frac{K_b}{V_1(B)}. \qquad \text{(III-39)}$$

When A and B are varied in a constant ratio, A = a(B) where a is some constant. Substituting this relationship into Eq. (III-39) gives the rate equation in terms of only one substrate,

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)} + \frac{aK_b}{V_1(A)}. \qquad \text{(III-40)}$$

When 1/v is graphed as a function of substrate A, with A and B varied in a constant ratio, the resulting curve will be linear. In the case of a Sequential mechanism, however, the rate equation will contain substrate squared terms, and the curve will be parabolic-up with a minimum in the second quadrant to the left of the 1/v axis. For example, when A = a(B) is substituted for B into Eq. (III-38), the rate expression is,

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)} + \frac{aK_b}{V_1(A)} + \frac{aK_{ia}K_b}{V_1(A)^2}. \qquad \text{(III-41)}$$

If $\frac{K_{ia}K_b}{V_1}$ is extremely small relative to other terms in the rate expression, Eq. (III-41) will resemble Eq. (III-40), and little will be gained by using the outlined procedure. It would seem, however, that, if the factor (a) is relatively large (i.e., A >> B), the last term in Eq. (III-41) would become discernible, if indeed it exists. Using this approach, it will be necessary to do two experiments, one where A >> B and the other where B >> A in an attempt to discern curvature. It is also important in these experiments to be certain that neither A nor B is held at a level that may cause substrate inhibition. Substrate inhibition for a Ping Pong mechanism could give rise to nonlinear, hyperbolic-up curves, when the substrates are varied in a constant ratio; however, the minima of these lines in a double reciprocal plot will be obvious in the first quadrant and the inhibited lines will approach the 1/v axis as the asymptote. As already stated, in the case of a Sequential mechanism when both substrates are varied, nonlinear, parabolic-up curves will be observed that will exhibit a minimum to the left of the vertical

axis. Figs. III-15a and III-15b represent hyperbolic-up and parabolic-up functions, respectively.

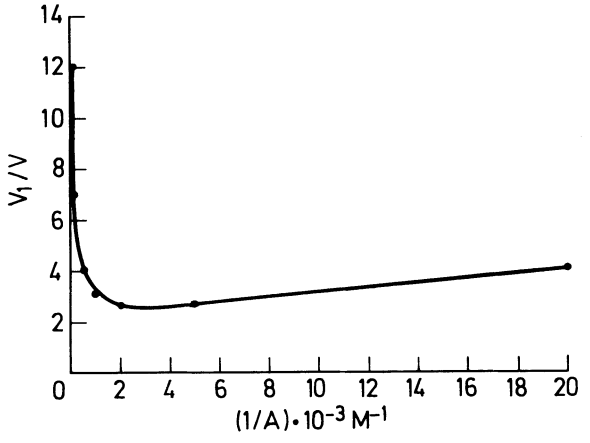

Fig. III-15a

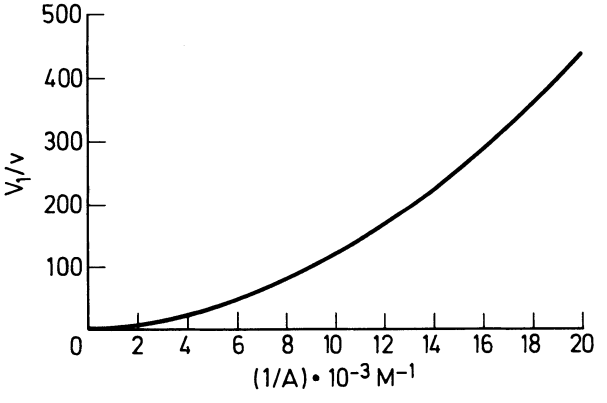

Fig. III-15b

Fig. III-15. (a) Theoretical plot of V_1/v *versus* 1/A for the equation: $\frac{V_1}{v} = 1 + \frac{A}{K_i} + \frac{K_a}{A} + \frac{K_b}{B}$. The ratio K_b/B was taken as 1 and K_i and K_a are 10^{-3} M and 10^{-4} M, respectively. The substrate was varied in the concentration range 5×10^{-5} M to 10×10^{-3} M. The curve is an example of a hyperbolic-up function. The coordinates of the minimum are: $\left[1/(K_aK_i)^{1/2}\right]$, $(1/V_1)\left[1 + (K_b/B) + 2(K_a/K_i)^{1/2}\right]$ for a 1/v *versus* 1/A plot. (b) Theoretical plot of V_1/v *versus* 1/A for the equation: $\frac{V_1}{v} = 1 + \frac{K_a}{A} + \frac{aK_b}{A} + \frac{aK_{ia}K_b}{(A)^2}$. The constants K_a, K_b, and K_{ia} were assumed to equal 10^{-3} M and a was taken to be 1. The substrate was varied in the concentration range 5×10^{-5} M to 10×10^{-3} M. The curve is an example of a parabolic-up function. The coordinates of the minimum are: $\left[-(K_a + aK_b)/2aK_{ia}K_b\right]$, $(1/V_1)\left[1 - (K_a + aK_b)^2/4aK_{ia}K_b\right]$ for a 1/v *versus* 1/A plot

Another procedure which may be used to make a choice between Ping Pong and Sequential mechanisms, involves the use of alternative substrates. When considering the hexokinase reaction, for example, glucose and fructose would be referred to as alternative substrates. By plotting 1/v *versus* 1/ATP, i.e., (1/A), the slopes of the lines could be determined with subsaturating fixed levels of glucose and fructose separately. If the mechanism is Ping Pong, the slopes of both plots will be the same, whereas they will be different with Sequential mechanisms. The rationale for this difference is as follows: In the Ping Pong mechanism (Scheme I-10), the slope of the double reciprocal plot = $(k_2 + k_3)/k_1k_3E_0$, regardless of the nature of the second substrate B, which would be either glucose or fructose. This relationship would not be valid for Sequential mechanisms, and the slope would vary with the nature of the substrate B. Similar effects are to be expected when B is the varied substrate and alternative substrates for A are used.

J. The Point of Convergence of Sequential Double Reciprocal Plots as a Criterion of Kinetic Mechanism

LUECK et al. (41) have shown how information on the kinetic mechanism may be obtained from a knowledge of where double reciprocal plots converge relative to the abscissa.

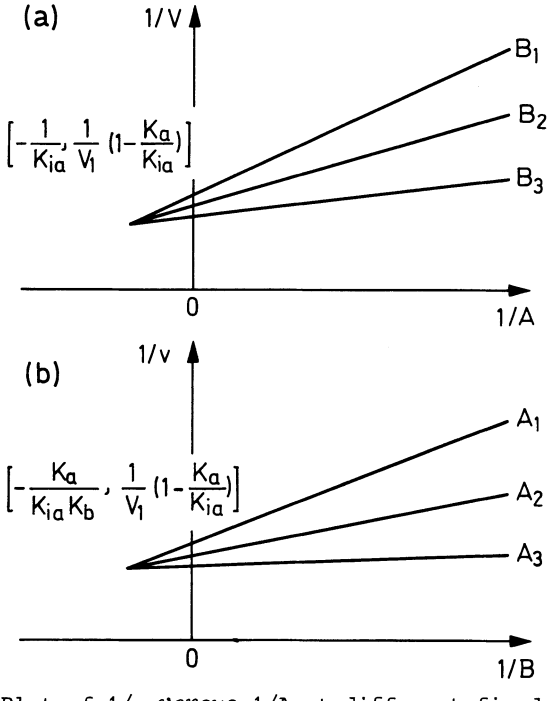

Fig. III-16. (a) Plot of 1/v *versus* 1/A at different fixed levels of substrate B. The coordinates of the point of intersection of the family of straight lines are indicated on the graph. (b) Plot of 1/v *versus* 1/B at various fixed levels of substrate A. The coordinates of the point of intersection of the family of straight lines are indicated on the graph

The coordinates of the intersection point of the curves of Figs. III-16a and III-16b indicate that the 1/v coordinates are the same in both the 1/A and 1/B plots. This is one criterion that must be satisfied by all mechanisms of the Bi Bi Sequential type. The same constraint applies in the reverse direction when comparing 1/v *versus* 1/P or 1/Q plots. It can be shown, however, that a relationship exists between the intersection points in both the forward and reverse direction and the kinetic mechanism.

When considering the 1/v coordinate, it can be shown that intersection may be on ($K_a = K_{ia}$) above ($K_a << K_{ia}$) or below ($K_a >> K_{ia}$ the abscissa. The significance of these intersection points is manyfold.

When intersection of the curves occurs on the X-axis, the Michaelis constant (K_a) equals the dissociation constant (K_{ia}). When intersection is above the abscissa, the dissociation constant is greater than the Michaelis constant. The curves in Figs. III-16a and III-16b will intersect below the 1/substrate axis when the dissociation constant is less than the Michaelis constant.

LUECK et al. (41) have shown that it is possible to make a choice of mechanism from among the three sequential type bireactant pathways shown in Schemes I-7, I-8, and I-9, along with the "ISO" mechanisms associated with the latter two cases, from evaluation of the points of intersection of double reciprocal plots in both directions.

Exactly how this may be accomplished is illustrated by a few examples. In the case of the Scheme I-9 mechanism, $1/v_1 = \frac{1}{E_0}(1/k_5 - 1/k_2)$ and $1/v_2 = \frac{1}{E_0}(1/k_2 - 1/k_5)$ where v_1 and v_2 represent initial velocity coordinates in the forward and reverse direction, respectively. If the lines converge on the abscissa in the forward direction, they must also converge on the abscissa in the reverse direction. This is true because, if $1/v_1 = 0$, $1/k_5 = 1/k_2$ and in the opposite direction $1/v_2$ must equal zero. Furthermore, for this mechanism $V_1 = (k_5E_0) = V_2 = (k_2E_0)$. If, on the other hand, intersection occurs above the axis in one direction (i.e., $1/k_5 > 1/k_2$), then it must occur below the axis in the reverse direction.

No constraints are placed upon the rapid equilibrium Random Bi Bi pathway because no relationship other than the equilibrium constant ties together the forward and reverse reactions.

In order to illustrate a "forbidden" relationship between the forward and reverse reactions, consider the Ordered Bi Bi (Scheme I-8) case where intersection occurs on the abscissa in one direction, designated forward, where $1/v_1 = \frac{1}{E_0}(1/k_5 + 1/k_7 - 1/k_2) = 0$. Thus, $1/k_2 = 1/k_5 + 1/k_7$. In the reverse reaction can $1/v_2$ also equal zero? Here $1/v_2 = \frac{1}{E_0}(1/k_2 + 1/k_4 - 1/k_7) = 0$. Substituting for $1/k_2$, $1/k_5 + 1/k_7 + 1/k_4 - 1/k_7 = 0$, and therefore $1/k_5 = -1/k_4$, a condition that is kinetically impossible.

This last identity reveals that in the case of the Ordered Bi Bi mechanism, the family of intersecting lines of Fig. III-16 cannot converge on the abscissa in both directions. Using this approach, LUECK et al. (41) were able to conclude from the data of DeLa-FUENTE and SOLS (42) that the kinetic mechansim of yeast hexokinase could not be Ordered Bi Bi. Table III-2 illustrates how one may choose between the various two substrate systems described in the table from inspection of primary plots in both directions.

Table III-2. Types of intersections of double reciprocal plots to be expected for sequential bireactant mechanisms

Mechanisms	(References)	Intersection of lines relative to abscissa				
		Forward direction		Reverse direction		
				Above	On	Below
Theorell-Chance	(I-9)	Above	+	F[a]	F	+
and		On	+	F	+[b]	F
Iso Theorell-Chance		Below	+	+	F	F
Random Bi Bi	(I-7)	Above	+	+	+	+
		On	+	+	+	+
		Below	+	+	+	+
Ordered Bi Bi	(I-8)	Above	+	+	+	+
and		On	+	+	F	F
Iso Ordered Bi Bi		Below	+	+	F	F

[a] F means that the type of intersection is forbidden.
+ indicates that the type of intersection is permissable.
[b] Maximal velocity in the forward direction, V_1, must equal that of the reverse direction, V_2.

K. Protocol and Data Plotting for Three Substrate Systems

As in the case of bireactant systems, three substrate enzymic mechanisms may be divided into two classes - Ping Pong and Sequential. Experimentally, as indicated earlier, it is necessary to vary one substrate while holding the other two substrates in a fixed ratio at different levels, in the range of their Michaelis constants. In the case of terreactant systems, there will be three such experiments. Plots of data for Sequential mechanisms will yield converging line data, while for Ping Pong mechanisms, one or more of the plots will give parallel lines. When the primary plot data are regraphed to give secondary plots, it is not only possible to evaluate certain kinetic parameters as we saw in the case of bireactant systems, but for certain

systems it is also possible to appraise the kinetic mechanism (20). The mechanics of the graphical manipulations may be best illustrated by reference to the rapid equilibrium Random Ter Ter rate expression which is described as Eq. (III-42)

$$\frac{V_1}{v} = 1+\frac{K_a}{A}+\frac{K_b}{B}+\frac{K_c}{C}+\frac{K_bK_{ca}}{(A)(B)}+\frac{K_cK_{ba}}{(A)(C)}+\frac{K_cK_{ab}}{(B)(C)}+\frac{K_{ia}K_cK_{ab}}{(A)(B)(C)} \qquad \text{(III-42)}$$

where $V_1 = k_1(E_0)$ and K_{ab}, K_{cb}, K_{ba}, K_{ca}, K_{ac}, and K_{bc} represent dissociation constants for the ternary complex dissociations: EAB = EA + B, K_{ab}; ECB = EC + B, K_{cb}; EAB = EB + A, K_{ba}; EAC = EC + A, K_{ca}; EAC = EA + C, K_{ac}; and EBC = EB + C, K_{bc}, respectively, for the rapid equilibrium Random Ter Ter mechanism of Scheme I-13[3].

If in one of the initial rate experiments substrate A is varied and substrates B and C are maintained in a constant ratio at different levels in the range of their Michaelis constants, B = a(C). Substituting this equality into Eq. (III-42) yields the relationship shown in Eq. (III-43).

$$\frac{V_1}{v} = 1+\frac{K_a}{A}+\frac{K_b}{(a)(C)}+\frac{K_c}{C}+\frac{K_bK_{ca}}{(a)(A)(C)}+\frac{K_cK_{ba}}{(A)(C)}+\frac{K_cK_{ab}}{(a)(C)^2}+\frac{K_{ia}K_cK_{ab}}{(a)(A)(C)^2} \qquad \text{(III-43)}$$

The primary plot for the experiment in which substrate A is varied is shown in Fig. III-17. The replots of the intercepts and slopes of Fig. III-17 *versus* 1/C will be parabolic-up; however, the minima of the curves will be to the left of the vertical axis and the extrapolated portion of the curve to infinite C (i.e., 1/C) can be made readily to evaluate $\frac{1}{V_1}$ and K_a. Values of K_b and K_c are obtained from primary and secondary graphs involving the substrates B and C. With a knowledge of these four parameters, it is possible to evaluate the other three by simple substitution into the double reciprocal plot equations. For example, if V_1, K_b and K_c are known, it is possible to calculate K_{ab} from the intercept of Fig. III-17. The other kinetic parameters can be determined by using the appropriate thermodynamic identities.

Another procedure for determining the various kinetic parameters involves saturating the enzyme with one of the substrates and then studying the initial velocites, as in the case of bireactant systems. The major limitation of this approach involves the possibility of substrate inhibition. Let us assume that A is the varied substrate and that velocities are determined at

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[3] Equation (III-42) can be expressed in a number of different forms depending upon certain thermodynamic relationships. These include the following: $K_{ab}K_c = K_{ac}K_b$; $K_{cb}K_a = K_{ca}K_b$; and $K_{bc}K_a = K_{ba}K_c$. Other identities can also be obtained. For the dissociation constants of the ternary complex, the first letter of the subscript is the substrate in the binary complex, whereas the second subscript letter represents free substrate.

different fixed concentrations of substrate B. From Eq. (III-42), the intercept of the primary plot will be

$$\frac{1}{V_1}\left(1 + \frac{K_b}{B} + \frac{K_c}{C} + \frac{K_c K_{ab}}{(B)(C)}\right) \qquad \text{(III-44)}$$

and the slope,

$$\frac{1}{V_1}\left(K_a + \frac{K_b K_{ca}}{B} + \frac{K_c K_{ba}}{C} + \frac{K_{ia} K_c K_{ab}}{(B)(C)}\right). \qquad \text{(III-45)}$$

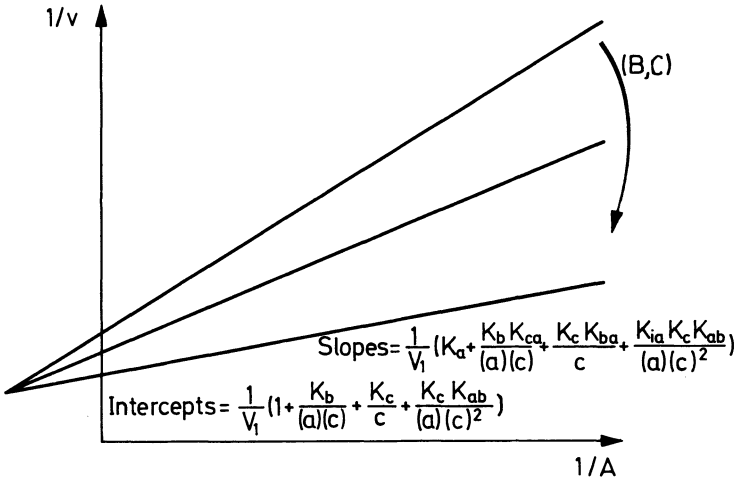

Fig. III-17. Plot of 1/v *versus* the concentration of 1/A. The other substrates (B and C) were held at three different fixed concentrations in a constant ratio. The equations for slopes and intercepts are shown on the figure

If substrate C is at a saturating concentration, then the replot of intercepts *versus* 1/B will yield a linear curve with an intercept of $\frac{1}{V_1}$ and a slope of $\frac{K_b}{V_1}$. Similarly a replot of the slope as a function of 1/B at saturating C will give an intercept of $\frac{K_a}{V_1}$ and a slope of $\frac{K_b K_{ca}}{V_1}$. A similar experiment can then be carried out under conditions in which C is *not* saturating to evaluate K_c, K_{ab}, K_{ba}, and K_{ia} from a knowledge of V_1, K_a, K_b, and K_{ca}. It is very unlikely that all substrates will cause inhibition; however, if this situation does in fact pertain, it will be necessary to carry out experiments as already outlined where one substrate is varied and the other two held in a constant ratio at different fixed concentrations, and in a concentration range where substrate inhibition is not in evidence.

The experimental protocol described above in which one substrate is varied and the other two held in a constant ratio at different concentrations is useful for making a choice of mechanism from a number of possibilities suggested in Chapter I (20). When slope and intercept replots of kinetic data of the type displayed in Fig. III-17 are made, it is possible to exclude a number of terreactant kinetic mechanisms from consideration. Exactly how this can be done is illustrated in Table III-3.

Table III-3. Graphical method for differentiating between various three-substrate enzymic mechanisms

Mechanism[a]	(Reference)	Substrate A		Substrate B		Substrate C	
		Slope replot[b]	Intercept replot[b]	Slope replot	Intercept replot	Slope replot	Intercept replot
Ordered Ter Ter	(I-12)	N[c]	N	N	L[d]	N	N
Random Ter Ter	(I-13)	N	N	N	N	N	N
Partially Random (AB Random)	(I-14)	NO[e]	N	NO	N	N	C[f]
Partially Random (BC Random)	(I-15)	NO	N	N	L	N	L
Partially Random (AC Random)	(I-16)	N	N	NO	L	N	N
Hexa Uni Uni Ping Pong	(I-17)	C	L	C	L	C	L
Ordered Bi Uni Uni Bi Ping Pong	(I-18)	L	L	L	L	C	N
Ordered Uni Uni Bi Bi Ping Pong	(I-19)	C	N	L	L	L	L
Random Bi Uni Uni Bi Ping Pong	(I-20)	L	L	L	L	C	N
Random Uni Uni Bi Bi Ping Pong	(I-21)	C	N	L	L	L	L

[a] The rate equations can be found in Chapter III, *Appendix* I, and Ref. (20).
[b] Slope and intercept values are determined from primary plots (Fig. III-17) and are replotted against the different fixed substrate concentrations.
[c] N refers to parabolic replots with non-zero intercepts on the vertical axis.
[d] L refers to a linear replot.
[e] NO refers to parabolic replots which intersect the origin.
[f] C refers to replots which give a constant slope or intercept.

It can be seen from Table III-3 that many of the terreactant mechanisms have unique intercept and slope replot patterns. This approach to the study of terreactant systems has recently been applied to the enzyme adenylosuccinate synthetase from bacteria (20) and yeast (43). It was found that when any of the substrates was varied in the concentration range of its Michaelis constant at different fixed levels of the other two substrates (which were also in the region of their Michaelis constants) which were held in a constant ratio, the Lineweaver-Burk plots were converging. These findings eliminated Ping Pong mechanisms. It was not possible to exclude the Ordered Ter Ter, or the fully or partially Random Ter Ter mechanisms from these data directly. However, when slope and intercept replots were made from these results, it was possible to eliminate additional terreactant mechanisms. Exactly how this analysis was carried out is described by using certain of the results of RUDOLPH and FROMM (20). Figure III-18 describes a primary double reciprocal plot in

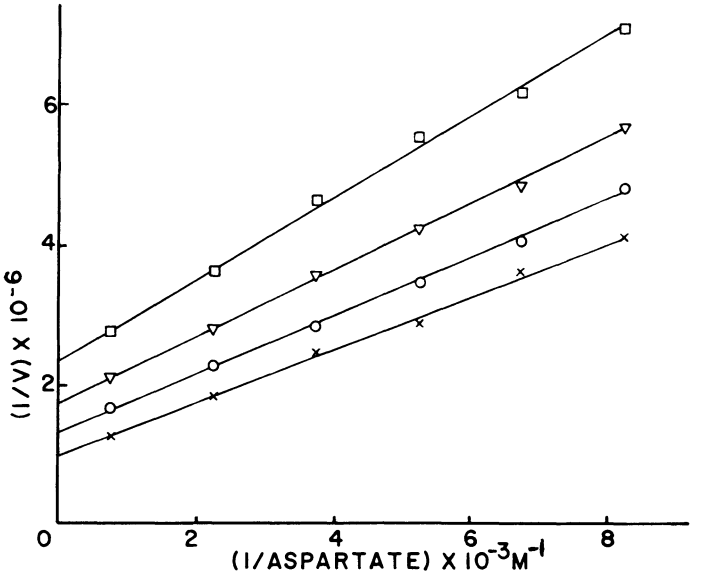

Fig. III-18. Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the molar concentration of aspartate. The respective concentrations of GTP and IMP were: □, 0.027 and 0.0375 mM; ▽, 0.037 and 0.050 mM; ○, 0.054 and 0.075 mM; and X, 0.109 and 0.150 mM. The aspartate concentration was varied from 0.12 to 1.31 mM

which aspartate is the varied substrate. In Figs. III-19 and III-20 intercept and slope replots, respectively, are shown as a function of 1/IMP by using the primary plot data of Fig. III-18. The secondary plots could have been plotted against 1/GTP because, in these experiments, GTP and IMP were held in a constant ratio. The findings of the secondary graphs indicate that the data are parabolic concave-up and do not intersect the origin. These results, along with data obtained from the other two

primary plots, may then be referred to the patterns described in in Table III-3.

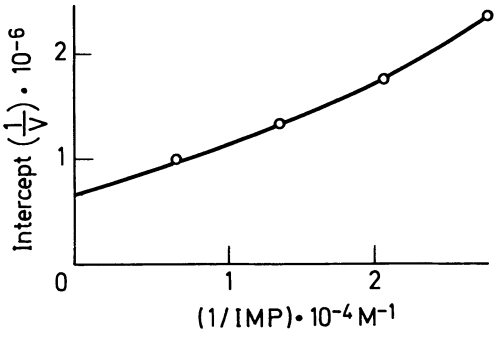

Fig. III-19. Secondary plot of intercepts *versus* the reciprocal of the molar concentration of IMP. The intercepts were obtained from the extrapolated curves where 1/aspartate = 0 in Fig. III-18

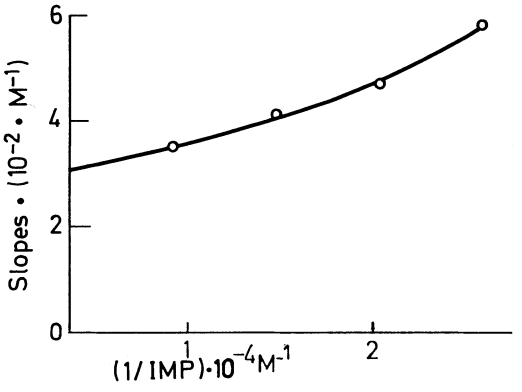

Fig. III-20. Secondary plot of the slopes *versus* the reciprocal of the molar concentration of IMP. The slopes were obtained from the curves exhibited in Fig. III-18

It is important to point out that, whereas in theory this analysis alone may permit one to arrive at a definitive conclusion regarding terreactant mechanisms as evidenced by the unique patterns illustrated in Table III-3, in practice this approach should be coupled with procedures outlined in Chapters IV, V, and VI. The reason for this is the fact that the replots in certain cases give rise to parabolas which are sometimes difficult to distinguish from linear curves. This problem can be obviated to some extent by varying the concentration of the fixed substrates over a wide range. The method is particularly useful in cases where the replots may or may not give rise to curves that intersect the origin.

L. Graphical Methods for Differentiating between Steady-State and Equilibrium Ordered Bi Bi Mechanisms

The initial rate equation for the Ordered Bi Bi mechanism (Scheme I-8) making steady-state assumptions is described by Eq. (III-38). SEGAL et al. (44) have pointed out that the analogous rate expression in which equilibrium assumptions are made lacks the K_a/A term; i.e.,

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_b}{V_1(B)} + \frac{K_{ia}K_b}{V_1(A)(B)} \quad . \qquad \text{(III-46)}$$

When double reciprocal plots are made for Eq. (III-46) the 1/v *versus* 1/B plot of different fixed concentrations of A will give rise to a family of straight lines that intersect *on* the 1/v axis. The analogous 1/A plot will show intersection to the left of the axis of ordinates, and its slope will be $K_{ia}K_b/V_1(B)$. A secondary plot of the slopes will therefore intersect the origin. Thus there will be fundamental differences between the primary and secondary plots for the steady-state and rapid equilibrium Ordered Bi Bi mechanisms.

SCHIMERLIK and CLELAND (45) have found that, at pH 7.0, creatine kinase, which exhibits a rapid equilibrium Random Bi Bi mechanism at pH 8.0, is rapid equilibrium Ordered Bi Bi from the creatine side of the reaction. The kinetic mechanism remains Random Bi Bi from the creatine phosphate side of the reaction at pH 7.0. These findings indicate that mechanisms may change with pH, and they also serve to validate the analogous partially random terreactant mechanisms (11) that have come into question by DALZIEL (46) on theoretical ground.

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Chapter IV

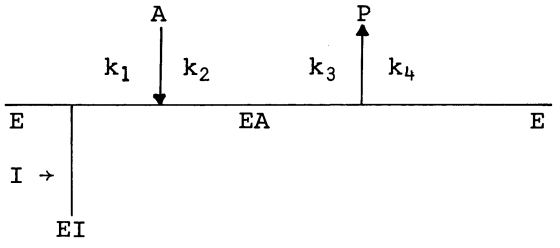
Use of Competitive Substrate Analogs and Alternative Substrates for Studying Kinetic Mechanisms

Very many factors are capable of causing enzyme inhibition; i.e., they are capable of slowing down the rates of enzymically catalyzed reactions. These factors include denaturation and interaction of the enzyme with so-called irreversible and reversible inhibitors. This monograph will be restricted to the latter class of enzyme inhibitors, those whose action can be reversed by either dilution or dialysis. The reader is directed to the book by BAKER (1) for a discussion of irreversible inhibitors. The purpose of this chapter is to show how inhibitors may be used to characterize kinetic mechanisms and also to provide information on the nature of enzyme and substrate interaction. Inhibitors may combine with one or more enzyme forms. If these enzyme-inhibitor complexes cannot undergo further reaction, they are called "dead end" inhibitors. On the other hand, if the complexes of enzyme and inhibitor can react with substrate to form product at a reduced rate, they are called "partial" inhibitors. This nomenclature was originally suggested by CLELAND (2).

A. Competitive Inhibition

MICHAELIS and MENTEN (3) and Van SLYKE and ZACHARIAS (4) were among the first to describe dead end competitive inhibition. By definition, a competitive inhibitor competes with the substrate for the same active site on the enzyme. Furthermore, there is usually, although not necessarily, a structural similarity between the two compounds. Finally, the inhibition is completely reversed when the enzyme is saturated with substrate, provided that the inhibitor is not also at a saturating level. It is of interest to note that the inhibitor may span a smaller or greater portion of the active site adsorption pocket relative to that occupied by the substrate. Examples in the literature indicate that competitive substrate inhibitors may be bound either more or less strongly than the substrate; however, the majority of inhibitors fall into the latter class.

The derivation of the rate expression for competitive inhibition in a unireactant system, based upon the stated assumptions, is as follows:



Scheme IV-1

$$v = k_3(EA); \quad E_0 = E + EA + EI; \quad \frac{(E)(I)}{EI} = K_{is}; \tag{IV-1}$$

Thus

$$E_0 = E + EA + \frac{(E)(I)}{K_{is}} = E\left(1 + \frac{I}{K_{is}}\right) + EA \tag{IV-2}$$

$$v = \frac{V_1(A)}{K_a\left(1 + \frac{I}{K_{is}}\right) + A} \tag{IV-3}$$

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1}\left(1 + \frac{I}{K_{is}}\right)\left(\frac{1}{A}\right). \tag{IV-4}$$

In the derivation of Eq. (IV-3), it is assumed that an equilibrium exists between enzyme (E) and inhibitor (I). An identical relationship exists if steady-state conditions are assumed; however, caution must be exercised in extending these limiting cases to other types of inhibition, e.g., for noncompetitive inhibition steady-state and equilibrium assumptions result in very different rate expressions.

It can be seen from the derivation of Eq. (IV-3) that the free enzyme component E of the conservation of enzyme equation is multiplied by the factor $(1 + I/K_{is})$. When dealing with more complex rate equations, it is necessary to multiply the determinant of that enzyme form that reacts with inhibitor by the factor, $(1 + I/K_{is})$.

In Fig. IV-1 is shown a graph of reciprocal velocity as a function of reciprocal of substrate concentration at different concentrations of inhibitor I as described by Eq. (IV-4). The plot illustrates how the inhibition constant K_i may be calculated.

It is also possible to graph the data of Fig. IV-1 as a function of inhibitor concentration; i.e., 1/v against I. Rearrangement of the equation for competitive inhibition gives Eq. (IV-5).

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)} + \left[\frac{K_a}{V_1 K_{is}(A)}\right](I). \tag{IV-5}$$

This equation is in linear form when 1/v is plotted as a function of I, and the inhibition is referred to as linear-competitive.

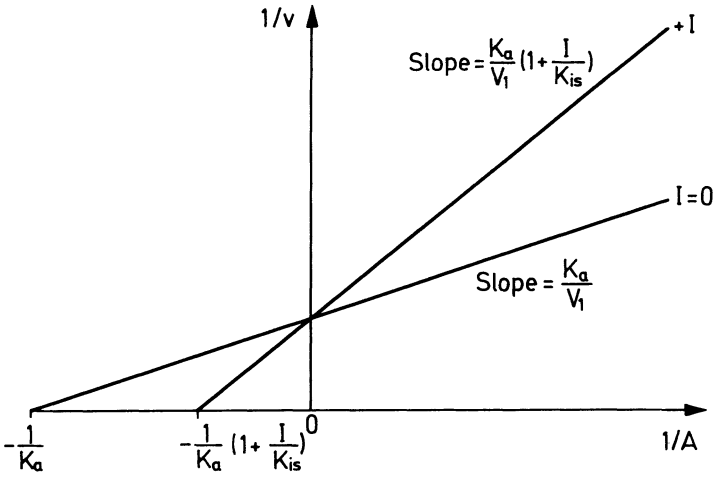


Fig. IV-1. Double reciprocal plot of 1/v *versus* 1/A in the presence and absence of a competitive inhibitor

Actually, it is possible to visualize inhibition kinetics of the competitive type even when the inhibitor and substrate do not compete for the same topological site, provided binding is mutually exclusive. It is important to note that the kinetic mechanism in Scheme IV-1 does not specify that A and I compete for the same site. Rather, it shows that these two entities cannot bind the enzyme simultaneously. It is this very type of argument that weakens the idea that when substrate protects against enzyme inactivation, it necessarily follows that the inhibitor, in this case of the irreversible type, binds at the active site. Inactivation can occur, for example, provided that, when the inhibitor binds, the substrate does not have access to the active site. Alternatively, when substrate binds the enzyme, the altered conformation of the enzyme does not permit inhibitor binding.

In the subsequent discussion of competitive inhibition, it will be assumed that both substrate and inhibitor compete for the same enzyme locus. This assumption must be tempered with its inherent limitations.

A cursory consideration of the foregoing discussion suggests that competitive inhibition is readily discernible from kinetic studies; however, this may not always be true. Consider the case proposed by DALZIEL (5) in which a competitive inhibitor is a contaminant in the substrate preparation. DALZIEL found that the kinetic parameters for liver alcohol dehydrogenase were altered when commercial preparations of NAD^+ were purified by ion exchange chromatography compared with unpurified preparations of the coenzyme. He identified the contaminant as ADP-ribose and intensively investigated the kinetic effect of a competitive inhibitor that varies with the substrate in a constant ratio. Variation of the

substrate concentration will cause a similar variation in the concentration of inhibitor.

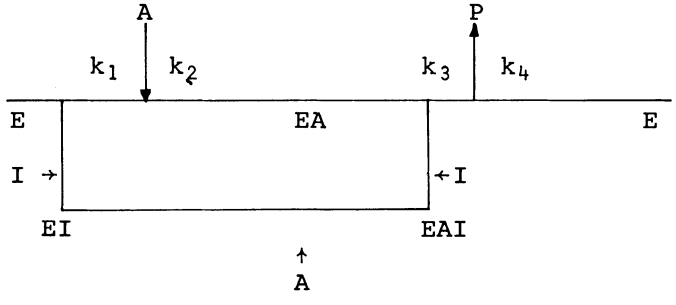
It follows that, because inhibitor and substrate are in a constant ratio, I = (X)(A), where (X) is taken to be some fraction. If (I) is substituted into Eq. (IV-5), Eq. (IV-6) is obtained after rearrangement.

$$\frac{1}{v} = \frac{1}{V_1}\left[1 + \frac{K_a(X)}{K_{is}}\right] + \left(\frac{K_a}{V_1}\right)\left(\frac{1}{A}\right). \qquad \text{(IV-6)}$$

When 1/v is plotted against 1/A, a straight line will be obtained, and it will not be possible to tell whether the substrate contains the inhibitor; i.e., whether the term $K_a(X)/K_{is}$ is present. This type of competitive inhibition alters the maximal velocity but not the Michaelis constant in the case of unireactant systems. This point emphasizes the importance of using pure substrates in kinetic experiments. An expression identical in form to Eq. (IV-4) is obtained if the inhibitor reacts with the substrate to form a substrate-inhibitor complex which does not react with the enzyme.

B. Partial Competitive Inhibition

One of the cardinal features of competitive inhibition is the substrate's ability to reverse the affect of a finite amount of inhibitor when the substrate concentration is saturating. A similar reversal can be achieved when the mechanism of inhibition is completely different from simple linear competitive inhibition. This class of inhibition is termed partial competitive and is discussed in some detail by DIXON and WEBB (6). In this type of inhibition, the inhibitor does not bind at the active catalytic site, but rather at another, or secondary, site. If it is assumed that a ternary complex of enzyme, substrate, and inhibitor can form and, further, that this complex can break down to products at the same rate as the productive binary (EA) complex, partial competitive inhibition results. These assumptions may be formalized as follows:



Scheme IV-2

If it is further assumed that the breakdowns of the EA and EAI complexes occur at the same rates, i.e., $v = k_3(EA + EAI)$, and that the formation of complexes EA, EI, and EAI in Scheme IV-2 involves rapid equilibria, then Eq. (IV-7) is obtained.

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_{ia}\left[1 + \frac{(I)}{K_i}\right]}{V_1\left[1 + \frac{(I)}{K_{ii}}\right]} \cdot \left(\frac{1}{A}\right) \quad . \qquad \text{(IV-7)}$$

The following relationships hold for Eq. (IV-7): $v = k_3[(EA) + (EAI)]$; $K_{ia} = (E)(A)/(EA)$; $K_i = (E)(I)/(EI)$; $K_{ii} = (EA)(I)/(EAI)$; $K_{iii} = (EI)(A)/(EAI)$. Note that the affinity of I differs for E and EA.

A Lineweaver-Burk plot of 1/v against 1/A for this case at different levels of (I) cannot be differentiated from the linear-competitive inhibition mechanism. However, a replot of the slopes does not give a linear curve; it is instead hyperbolic-up or -down, depending upon the relationship between K_i and K_{ii}. Figure IV-2 illustrates the case in which the replot of slopes as a function of inhibitor concentration is hyperbolic-up. In this simulation $K_i > K_{ii}$; i.e., the affinity of the enzyme for the inhibitor is less than the affinity when substrate is bound

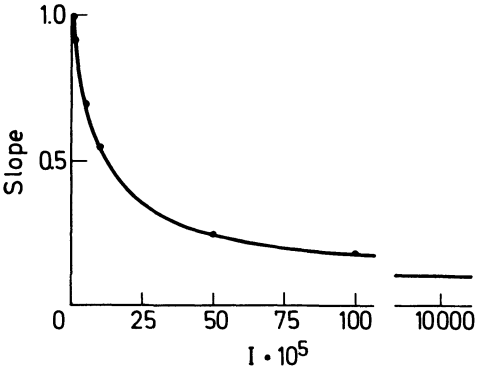


Fig. IV-2. Slope *versus* inhibitor replot of Eq. (IV-7). The slope $\left[\frac{K_{ia}(1 + I/K_i)}{V_1(1 + I/K_{ii})}\right]$ was evaluated by assuming $K_{ia} = 10^{-3}$M, $V_1 = 10^{-3}$M/min, $K_i = 10^{-3}$M, and $K_{ii} = 10^{-4}$M. The concentrations of the inhibitor I are shown on the abscissa. The horizontal asymptote has a value of 0.1

to the enzyme. When $K_i < K_{ii}$ the slope of the hyperbola will increase as the concentration of inhibitor is raised. It is of interest to note that for simple partial competitive inhibition of the type illustrated by Eq. (IV-7), if binding of the inhibitor is not influenced by the association of enzyme and substrate; i.e., $K_i = K_{ii}$, the effect of the inhibitor is not man-

ifested. Similarly, if inhibitor binding does not affect substrate binding ($K_{ia} = K_{iii}$), partial competitive inhibition will not affect the kinetics of the system.

If $(1 + I/K_{ii}) > (1 + I/K_i)$, competitive activation will occur; i.e., there will be a decrease in the slope of the double reciprocal plot of 1/v *versus* 1/A as the concentration of I increases. This effect will cause a decrease in the apparent dissociation constant with no effect on the maximal velocity V_1. Activation will occur provided that I has a greater affinity for EA than for E. If the reverse is true, partial competitive inhibition will occur.

If steady-state rather than equilibrium assumptions are made in deriving the rate expression which pertains to Scheme IV-2, the resulting equation is extremely complex and contains second degree terms in both substrate and inhibitor. Another interesting facet of the mechanism of Scheme IV-2 is the case in which the rate constants for the breakdown of the EA and EAI complexes are different. If velocity is now expressed as $v = k(EA) + k'(EAI)$, where $k' = k(X)$, then the equation for Scheme IV-2 may be written as

$$\frac{k(E_0)}{v} = \frac{\left[1 + \frac{(I)}{K_{ii}}\right]}{\left[1 + \frac{(X)(I)}{K_{ii}}\right]} + \frac{K_{ia}\left[1 + \frac{(I)}{K_i}\right]}{\left[1 + \frac{(X)(I)}{K_{ii}}\right]}\left(\frac{1}{A}\right). \qquad \text{(IV-8)}$$

It is clear that the family of curves that results when 1/v is plotted against 1/A in the presence of (I) will not converge on the vertical axis, nor will the intercept and slope replots as a function of (I) be linear. If $k' >> k$, activation will occur, and the apparent K_{ia} will decrease when (I) is present as predicted by Eq. (IV-8). When $k' << k$, inhibition will result, and there will be an increase in the apparent dissociation constant.

C. Noncompetitive Inhibition

MICHAELIS and his coworkers (7, 8) recognized that reversible inhibition could occur in which the inhibitory effect could not be reversed when the enzyme is saturated with substrate. This type of inhibition is referred to as noncompetitive, and the fundamental assumption made when considering this phenomenon is that the inhibitor binds at a site other than the substrate binding site, with the result that the enzyme-inhibitor complex does not break down to form product when the substrate is associated with the enzyme. Noncompetitive inhibition is readily rationalized with the assumption that the inhibitor, when present on the enzyme, distorts the conformation of either the substrate or the enzyme, or both, so as to preclude catalysis.

In Scheme IV-2 is shown the mechanism of noncompetitive inhibition.

If it is assumed that all steps of the reaction are in rapid equilibrium relative to the conversion of EA to product, then $K_{is} = (E)(I)/(EI)$, $K_{ii} = (EA)(I)/(EAI)$, and $K_{iii} = (EI)(A)/(EAI)$.

Equation (IV-9) represents the rate expression for noncompetitive inhibition.

$$\frac{1}{v} = \frac{1}{V_1}\left[1 + \frac{(I)}{K_{ii}}\right] + \frac{K_{ia}}{V_1}\left[1 + \frac{(I)}{K_{is}}\right]\left(\frac{1}{A}\right) \qquad \text{(IV-9)}$$

The inhibition described by Eq. (IV-9) is linear noncompetitive, and in a double reciprocal plot in which 1/A is varied at different fixed concentrations of the inhibitor, I, there will be an increase in intercept, $\left(\frac{1}{V_1}\right)\left(1 + \frac{I}{K_{ii}}\right)$, and slope, $\left(\frac{K_{ia}}{V_1}\right)\left(1 + \frac{I}{K_{is}}\right)$. Simple noncompetitive inhibition of the type illustrated by Eq. (IV-9) is linear because replots of either slopes or intercepts against different concentrations of inhibitor give straight lines.

The four dissociation constants, K_{ia}, K_{is}, K_{ii}, and K_{iii}, are not independent but rather are related by the following equation:

$$K_{is} \cdot K_{iii} = K_{ia} \cdot K_{ii}. \qquad \text{(IV-10)}$$

The same rate expression will result then whether noncompetitive inhibition is described as shown in Eq. (IV-9) or whether a substitution is made to include K_{iii}.

If steady-state, rather than equilibrium, conditions prevail for the mechanism depicted in Scheme IV-2, the resultant rate equation will be highly complex and will contain terms which are second degree for both the substrate and inhibitor. What is rather interesting is that so many cases of classical noncompetitive inhibition (i.e., rapid equilibrium) are on record. Obviously, either the nonlinearity that one might expect from the steady-state treatment of Scheme IV-2 is so small as to be undetectable, or the equilibrium assumption is indeed appraoched.

When one graphs kinetic data that are consistent with Eq. (IV-9) the curves may converge above, below, or on the *abscissa* as shown in Fig. IV-3. The (x,y) coordinates of the intersection point for noncompetitive inhibition are $\left[(-K_{is}/K_{ia}K_{ii}), (1/V_1)(1 - K_{is}/K_{ii})\right]$. If $K_{is} = K_{ii}$ (that is, the binding of the inhibitor to the enzyme is not affected by the presence or absence of the substrate on the enzyme), the curves will intersect on the 1/A axis. On the other hand, intersection may occur above ($K_{is} < K_{ii}$) or below ($K_{is} > K_{ii}$) the *abscissa*. In these latter two cases, the inhibition will be referred to as mixed *inhibition*.

Another graphing procedure that has been used extensively to depict competitive and noncompetitive inhibition is the Dixon

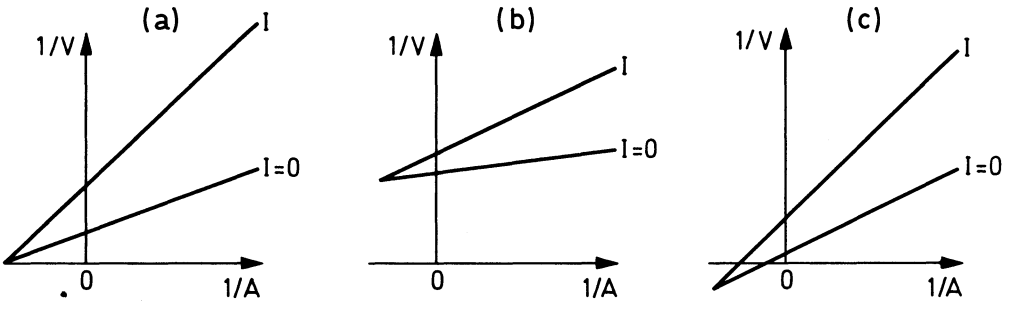


Fig. IV-3. Double reciprocal plots of noncompetitive inhibition. (a) $K_{is} = K_{ii}$ (b) $K_{is} < K_{ii}$, (c) $K_{is} > K_{ii}$

plot (9). The experimental protocol that permits the use of these graphs requires that 1/v be determined as a function of inhibitor at different fixed levels of substrate. For a competitive inhibitor, the coordinates of intersection of the curves are $(-K_{is}, 1/V_1)$ (see Scheme IV-1 and Eq. (IV-3)); i.e., the curves intersect above the *abscissa*. In the case of a noncompetitive inhibitor, the coordinates of the point of intersection of the lines of the Dixon plot for Eq. (IV-9) are $\left[-K_{is}, 1/V_1 (1 - K_{is}/K_{ii})\right]$. It is obvious that the curves may intersect above, below, or on the abscissa. If $K_{is} < K_{ii}$, it will not be possible to make a choice between this case and that for competitive inhibition. It is for this reason that it is advisable to use the Lineweaver-Burk plot in preference to the Dixon plot for studying competitive and noncompetitive enzyme inhibition. Further discussion on this point can be found elsewhere (10).

When considering the subject of competitive inhibition, it was shown how the presence of a competitive inhibitor in the substrate preparation will effect the Michaelis-Menten equation. By analogy with that discussion, consider the case where a noncompetitive inhibitor is present in the substrate preparation; i.e., the inhibitor and substrate will vary in a constant ratio. If I = (X)(A) where X is taken to represent a fraction, and the factor (X)(A) substituted for (I) in Eq. (IV-9), the result is Eq. (IV-11).

$$\frac{1}{v} = \frac{1}{V_1}\left[1 + \frac{(X)K_{ia}}{K_{is}} + \frac{(X)(A)}{K_{ii}}\right] + \frac{K_{ia}}{V_1} \cdot \left(\frac{1}{A}\right) \qquad \text{(IV-11)}$$

Figure IV-4 illustrates the type of graph to be expected when 1/v is plotted against 1/A. This curve is hyperbolic-up and predicts that inhibition will be complete at infinite substrate concentration. What is interesting about this effect is that it may in fact be responsible for the phenomenon usually interpreted as substrate inhibition - a concept that will be discussed at length in Chapter V.

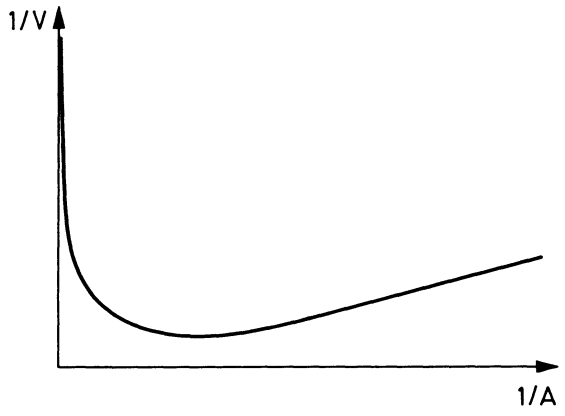
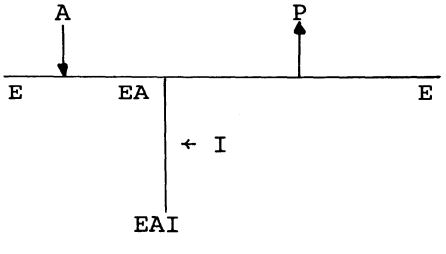


Fig. IV-4. Plot of the reciprocal of initial velocity as a function of the reciprocal of substrate for the mechanism described by Eq. (IV-11)

D. Uncompetitive Inhibition

Another type of reversible inhibition, but one which is very rarely, if ever, encountered in unireactant systems, is uncompetitive inhibition. In the case of a one substrate system, if the inhibitor combines with the enzyme-substrate complex exclusively, the result is uncompetitive inhibition. The effect of the inhibitor is to decrease the maximal velocity without affecting the true dissociation constant. Uncompetitive inhibition is illustrated by the reactions of Scheme IV-3.



Scheme IV-3

The rate equation for uncompetitive inhibition is depicted in Eq. (IV-12), where $K_{ii} = (EA)(I)/(EAI)$.

$$\frac{1}{v} = \frac{1}{V_1}\left[1 + \frac{I}{K_{ii}}\right] + \frac{K_{ia}}{V_1} \cdot \left(\frac{1}{A}\right) \qquad \text{(IV-12)}$$

The form of the rate expression is the same regardless of whether steady-state or equilibrium assumptions are made. The very distinctive plot for uncompetitive inhibition is shown in Fig. IV-5.

A replot of the intercepts $(\frac{1}{V_1})(1 + \frac{I}{K_{ii}})$ for Eq. (IV-12) shows that the inhibition is linear.

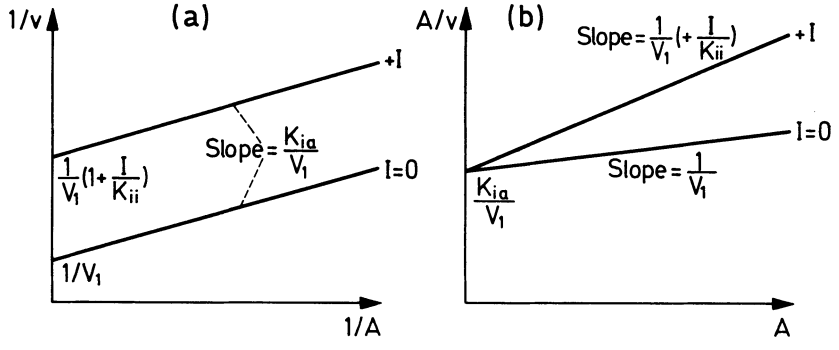


Fig. IV-5. (a) Double reciprocal plot of uncompetitive inhibition. (b) A/v *versus* A plot for uncompetitive inhibition

The intersection point of the family of curves on the abscissa is $-(1 + I/K_{ii})/K_{ia}$. When I = 0, the true dissociation constant will be obtained; however, in the presence of uncompetitive inhibitor, the apparent K_{ia} will change as the concentration of I is altered.

Equation (IV-12) may also be cast in a form that permits more precise evaluation of uncompetitive inhibition. Parallel line data are at best difficult to distinguish from noncompetitive inhibition in which slope changes are slight. It is suggested that, when suspected parallel line data are obtained, they be plotted as indicated in Fig. IV-5b to determine whether the lines do indeed intersect on the A/v axis. This specific problem and analogous questions as to whether inhibition is really competitive, noncompetitive or uncompetitive can be resolved statistically by using model testing procedures such as the F test or the Cp statistic (see Chapter III). The computer programs presented in the *Appendix* that bear on this point allow the kineticist to make a choice from among these three different models.

E. Nonlinear Enzyme Inhibition

Nonlinear enzyme inhibition may be obtained from replots of primary double reciprocal plots under a number of circumstances. These include multiple dead end inhibition, substrate and product inhibition, partial inhibition, and allostery. The following discussion will be restricted to the multiple dead end type.

When an inhibitor adds to different enzyme forms (e.g., Scheme IV-2), inhibition is linear; however, when there is multiple

inhibitor binding to a single enzyme form or to enzyme forms which are connected, replots of either slopes or intercepts against inhibitor may be nonlinear. If the following equilibria are added to the simple Uni Uni mechanism of Scheme I-3,

$$E + I = EI,\ K_i;\ EI + I = EI_2,\ K_{ii} \qquad \text{(IV-13)}$$

the rate equation obtained is,

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_{ia}}{V_1}\left[1 + \frac{I}{K_i} + \frac{I^2}{K_iK_{ii}}\right]\left(\frac{1}{A}\right). \qquad \text{(IV-14)}$$

Equation IV-14 describes parabolic-competitive inhibition. The slope of this equation is

$$\text{slope} = \frac{K_{ia}}{V_1}\left[1 + \frac{I}{K_i} + \frac{I^2}{K_iK_{ii}}\right] \qquad \text{(IV-15)}$$

and a plot of slope as a function of I will give rise to a parabola. It is also possible to obtain parabolic-uncompetitive inhibition. In this case only the intercept will be affected by inhibitor. In the event that parabolic noncompetitive inhibition is encountered, either the slope or the intercept, or both, may contain inhibitor terms of greater than first degree.

Nonlinear noncompetitive inhibition may affect slopes and intercepts; in which case it will be called S-parabolic I-parabolic noncompetitive inhibition. If the replots of intercept against I are linear, whereas the slope replot is parabolic, the inhibition is called S-parabolic I-linear noncompetitive inhibition (2).

Parabolic inhibition will also result from interactions of the type:

$$E + I = EI,\ K_i;\ EI + A = EIA,\ K_{ii};\ EIA + I = EI_2A,\ K_{iii}. \qquad \text{(IV-16)}$$

Slope and intercept replots *versus* inhibitor may be of a more complicated nature. CLELAND (2) has referred to some of these as 2/1, 3/2, etc., functions. Equation (IV-17) illustrates an example of a S-2/1 function, in which a second order polynomial is divided by a first order polynomial.

$$\text{slope} = \frac{K_{ia}\,(1 + aI + bI^2)}{V_1(1 + cI)} \qquad \text{(IV-17)}$$

It would appear that it would be difficult to distinguish a plot of slope *versus* inhibitor concentration for Eq. (IV-17) from linear replots.

F. The Use of Substrate Analogs for Studying Kinetic Mechanisms

1. Bireactant Enzymic Systems

Although competitive inhibitors have been used extensively for many years, their value as tools for making a choice of kinetic mechanism from among a number of possible alternatives was not realized until 1962 when FROMM and ZEWE (11) suggested that competitive inhibitors of substrates could be used to differentiate between random and ordered mechanisms. Furthermore, in the latter case, a determination of the substrate binding order could be made from such experiments. This protocol is quite likely the simplest approach for making a choice of mechanism between Ordered and Random Bi Bi possibilities. In addition, it has the advantage of permitting the kineticist to come to definitive conclusions from studies of reactions in a single direction only. Its obvious limitation involves the requirement that a dead end competitive inhibitor be available for each substrate.

Let us consider first the case of the random mechanism to determine how the dead end competitive inhibitor affects the kinetics of the system. The rapid-equilibrium random pathway of enzyme and substrate interaction (Random Bi Bi) is illustrated in Scheme I-7. In the case of a competitive inhibitor for substrate A, the inhibitor, I, would participate at every step in the kinetic mechanism in which the substrate normally reacts. Thus the following interactions of enzyme with inhibitor I might be expected:

$$E + I = EI,\ K_i;\ EB + I = EIB,\ K_{ii};\ EI + B = EIB,\ K_{iii} \qquad \text{(IV-18)}$$

When the expressions EI and EIB are added to the conservation of enzyme term, and the rate equation derived for the effect of competitive inhibitor of substrate A, the following initial rate relationship is obtained.

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)}\left[1 + \frac{I}{K_{ii}}\right] + \frac{K_b}{V_1(B)} + \frac{K_{ia}K_b}{V_1(A)(B)}\left[1 + \frac{I}{K_i}\right] \qquad \text{(IV-19)}$$

When double reciprocal plots of 1/v as a function of 1/A are made at different fixed concentrations of inhibitor only the slope term of the rate expression is altered; i.e.,

$$\text{slope} = \frac{K_a}{V_1}\left[1 + \frac{I}{K_{ii}}\right] + \frac{K_{ia}K_b}{V_1(B)}\left[1 + \frac{I}{K_i}\right]. \qquad \text{(IV-20)}$$

On the other hand, when B is the variable substrate, double reciprocal plots at different fixed levels of inhibitor will exhibit increases in both slopes and intercepts.

$$\text{Intercept} = \frac{1}{V_1}\left[1 + \frac{K_a}{A}\left(1 + \frac{I}{K_{ii}}\right)\right] \qquad \text{(IV-21)}$$

$$\text{Slope} = \frac{1}{V_1}\left[K_b + \frac{K_{ia}K_b}{A}\left(1 + \frac{I}{K_i}\right)\right] . \qquad \text{(IV-22)}$$

Equation (IV-19) predicts then that a dead end competitive inhibitor for substrate A of the Random Bi Bi mechanism is a noncompetitive inhibitor of substrate B.

If now a dead end competitive inhibitor for substrate B is used, the following interactions are to be expected:

$$E + I = EI,\ K_i;\ EA + I = EAI,\ K_{ii};\ EI + A = EAI,\ K_{iii} \qquad \text{(IV-23)}$$

The rate equation for the effect of a dead end competitive inhibitor of substrate B is described by Eq. (IV-24).

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)} + \frac{K_b}{V_1(B)}\left[1 + \frac{I}{K_{ii}}\right] + \frac{K_{ia}K_b}{V_1(A)(B)}\left[1 + \frac{I}{K_i}\right] . \qquad \text{(IV-24)}$$

It is clear from Eq. (IV-24) that a dead end competitive inhibitor of substrate B will show noncompetitive inhibition relative to substrate A. In summary then, for the Random Bi Bi mechanism, a competitive inhibitor for either substrate will act as a noncompetitive inhibitor for the other substrate. These observations are consistent with the symmetry inherent in the random mechanism. Similar inhibition patterns are to be expected for the rapid equilibrium Random Bi Uni mechanism (Scheme I-6).

Experimentally, it is very important that the fixed, or nonvaried, substrate be held at a subsaturating level, preferably in the region of its Michaelis constant. If, for example, when considering Eq. (IV-19), the concentration of substrate A is held very high when B is the variable substrate, it is possible that the intercept increases to be expected in the presence of inhibitor may not be discernible, and the inhibition may appear to be competitive with respect to either substrate. It is important to note that, when replots of slopes and intercepts are made as a function of inhibitor concentration for the type of inhibition illustrated, the replots will be linear.

Very few Random Bi Bi mechanisms are truly rapid equilibrium random in both directions; however, this condition will be approximated in the "slow direction". When steady-state conditions prevail (i.e., when the interconversion of the ternary complexes is not slow relative to other steps of the kinetic mechanism) it may be supposed that the initial rate plots in double reciprocal form would not be linear. This is to be expected because of the second degree substrate terms generated under steady-state conditions; however, SCHWERT (12) has suggested that the deviation from linearity might be too subtle to discern. A similar point was also made by WRATTEN and CLELAND (13), and RUDOLPH and

FROMM (14) concluded from computer simulations of the steady-state Random Bi Bi mechanism proposed for yeast hexokinase that the kinetics approximate the limiting equilibrium assumption. These workers also found that the competitive inhibition patterns proposed for the rapid equilibrium case would be indistinguishable from the situation in which steady-state conditions prevail.

In the case of the Ordered Bi Bi mechanism, competitive dead end inhibitors of the first substrate to add to the enzyme give inhibition patterns relative to the other substrate that are distinctively different from the pattern obtained when a competitive dead end inhibitor of the second substrate is employed. It is this very point that permits the kineticist to make a choice between Random and Ordered Bi Bi mechanisms (11).

In the case of the Ordered Bi Bi mechanism, substrate A adds only to free enzyme (Scheme I-8). The competitive dead end inhibitor by analogy should add only to this enzyme form. In addition, it is assumed that the conformation of the enzyme has been distorted enough by the inhibitor so as to preclude addition of substrate B to the enzyme-inhibitor complex.

The competitive dead end inhibitor for substrate A may react as follows with the enzyme:

$$E + I = EI, \quad K_i = (E)(I)/(EI) \tag{IV-25}$$

If the conservation of enzyme equation for the Ordered Bi Bi mechanism is modified to account for the additional complex EI, the initial rate expression is

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)}\left[1 + \frac{I}{K_i}\right] + \frac{K_b}{V_1(B)} + \frac{K_{ia}K_b}{V_1(A)(B)}\left[1 + \frac{I}{K_i}\right]. \tag{IV-26}$$

Equation (IV-26) predicts that the competitive inhibitor for substrate A, the first substrate to add in the ordered mechanism, will be noncompetitive relative to substrate B. On the other hand, for this mechanism, a dead end competitive inhibitor for substrate B would be expected not to react with free enzyme, but rather with the EA binary complex. This interaction may be described by the following relationships:

$$EA + I = EAI, \quad K_i = (EA)(I)/(EAI) \tag{IV-27}$$

The kinetic expression obtained when this effect is included in the Ordered Bi Bi mechanism is

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)} + \frac{K_b}{V_1(B)}\left[1 + \frac{I}{K_i}\right] + \frac{K_{ia}K_b}{V_1(A)(B)}. \tag{IV-28}$$

It is quite clear that a dead end competitive inhibitor for the second substrate will yield *uncompetitive* inhibition relative to substrate A. This unique inhibition pattern allows a distinction to be made between ordered and random bireactant kinetic mechanisms and permits determination of the substrate binding order in the former case. These points are summarized in Table IV-1.

Table IV-1. Use of competitive inhibitors for determining bireactant kinetic mechanisms

Mechanism	Competitive inhibitor for substrate	1/A plot	1/B plot
Random Bi Bi	A	C[a]	N[b]
and			
Random Bi Uni	B	N	C
Ordered Bi Bi	A	C	N[c]
and			
Ordered Bi Uni	B	U[d]	C
Ping Pong Bi Bi	A	C	U
	B	U	C

[a] Refers to a Lineweaver-Burk plot that shows competitive inhibition.
[b] Refers to a Lineweaver-Burk plot that shows noncompetitive inhibition.
[c] In the ordered mechanism convergence may be on, above or below the abscissa; however, the point of intersection with the inhibitor must have the same ordinate as a family of curves in which the other substrate is substituted for the inhibitor.
[d] Refers to a Lineweaver-Burk plot that shows uncompetitive inhibition

When considering competitive substrate inhibitors, the possibility is automatically excluded that the inhibitor may bind to an enzyme-product complex. In the case of the rapid equilibrium Random Bi Bi mechanism, a competitive inhibitor for substrate B could in theory bind the EQ complex; however, this complex occurs after the rate limiting step and is not part of the kinetic equation. Similarly, although this binary complex is kinetically important in the Ordered Bi Bi case, if an EQI complex did form, inhibition would be noncompetitive rather than competitive relative to substrate B. Under these conditions, the approach would not be viable technique, and another inhibitor should be sought.

The use of dead end competitive inhibitors for choosing between the Random and Ordered Bi Bi mechanisms has been employed with many enzyme systems. The basic protocol involves segregation of

mechanisms into either the Ping Pong or Sequential class from initial rate experiments. After the Sequential nature of the system has been established, the dead end competitive inhibitors may be used to establish whether the kinetic mechanism is Random or Ordered.

Two examples can be used to illustrate this point. FROMM and ZEWE (11) reported that yeast hexokinase is sequential when they demonstrated that double reciprocal plots of 1/v *versus* $1/MgATP^{2-}$ at different fixed concentrations of glucose converged to the left of the axis of ordinates. From the same data, they observed that, when 1/v was plotted as a function of 1/glucose at different fixed concentrations of $MgATP^{2-}$, the resulting family of curves also converged to the left of the 1/v axis. In addition both sets of primary plots intersected on the abscissa. These investigators also demonstrated that AMP, a competitive dead end inhibitor for $MgATP^{2-}$, was a noncompetitive inhibitor with respect to glucose. From these experiments, it was concluded that the kinetic mechanism for yeast hexokinase was either Random Bi Bi or Ordered Bi Bi with $MgATP^{2-}$ as the initial substrate to add to the enzyme. If glucose were to add to hexokinase before $MgATP^{2-}$ in an Ordered Bi Bi mechanism, AMP inhibition would have been uncompetitive with respect to glucose.

The same investigators employed oxalate, a dead end competitive inhibitor of L-lactate, to help establish the kinetic mechanism of the muscle lactate dehydrogenase reaction (15). They observed that oxalate was uncompetitive with respect to NAD^+ and concluded from these findings and other studies that the kinetic mechanism was Iso-Ordered Bi Bi with the nucleotide substrates adding to the enzyme first.

It should be pointed out that dead end competitive inhibitors cannot be used to differentiate between normal and Iso mechanisms. Nor can they be used to make a choice as to whether ternary complexes are kinetically important in Ordered mechanisms; i.e., they cannot be used to differentiate between the pathways of Schemes I-8 and I-9.

In studies in which dead end competitive inhibitors are employed, it is often useful to evaluate the various inhibition constants. This can be done in a number of ways, and a few of the methods that may be used will be illustrated.

It is possible to evaluate either K_i or K_{ii} in Eqs. (IV-19) and (IV-24) from secondary plots of slopes and intercepts versus inhibitor concentration. It can be seen from Eq. (IV-22) that a plot of slope versus I will give a replot in which the slope of the secondary plot is

$$\text{Slope} = \frac{K_{ia}K_b}{V_1K_i(A)}. \qquad \text{(IV-29)}$$

K_i may also be evaluated by determining the intersection point of the secondary plot on the abscissa; i.e., where slope = 0.

In this case

$$I = - K_i \left(1 + \frac{(A)}{K_{ia}}\right). \qquad \text{(IV-30)}$$

The advantage of using Eq. (IV-30) rather than Eq. (IV-29) is that it is not necessary to evaluate V_1. Presumably, data for A and K_{ia} will be in hand.

The value for K_{ii} can be determined with a knowledge of A and K_a by evaluating the point of intersection on the abscissa of data from Eq. (IV-21). In this case where the intercept = 0 in the replot,

$$I = - K_{ii} \left(1 + \frac{A}{K_a}\right). \qquad \text{(IV-31)}$$

Methods similar to those described for the Random Bi Bi mechanism can be used to determine the dissociation constants in the case of the Ordered Bi Bi mechanism. For example, K_i can be evaluated from either a slope or intercept *versus* inhibitor replot using Eq. (IV-26) in which B is the variable substrate. It is of interest to note that the inhibition constant *must* be the same for this mechanism regardless of whether the determination is made from the slope or intercept. This may or may not be true for the Random pathway depending upon whether $K_i = K_{ii}$. It will be possible to determine K_i in Eq. (IV-28) readily using the methods already described.

It has already been shown that Dixon plots (9) may not be used to unambiguously differentiate between competitive and noncompetitive inhibition. In the case of competitive inhibition in bireactant systems, it is not possible to evaluate the inhibition constants unless the mechanism dependent rate equation is known. The inhibition constant, K_{is} is equal to -I for a unireactant system, and is obtained from the x coordinate of the Dixon plot (9). Similar manipulation can usually not be made with bireactant systems. The competitive inhibitor illustrated in Eqs. (IV-19) and (IV-24) gives an x coordinate in the Dixon plot of $-\left[K_a + (K_{ia}K_b/B)\right] / \left[(K_a/K_{ii}) + (K_{ia}K_b/B \cdot K_i)\right]$. Only when $K_i = K_2$ will $I = -K_i$ (Eq. IV-26). Evaluation of the inhibition constant using the Dixon plot is not possible directly even when only one term in the rate expression is affected. In Eq. (IV-28), the x coordinate equals $-K_i \left[1 + (K_{ia}/A)\right]$.

The competitive substrate inhibitors cited above have been referred to as "dead end" inhibitors (2). The question arises as to what happens if the inhibitors are not of the dead end type, i.e., if the enzyme-inhibitor complexes of the ordered mechanisms act in a manner similar to those analogous complexes in the random mechanism. This possibility was considered by HANSON and FROMM in 1965 (16). If in the Ordered mechanisms, the EI complex permitted substrate B to add, the additional reaction would be

$$EI + B = EIB, \; K_{ii} = (EI)(B)/(EIB) \qquad \text{(IV-32)}$$

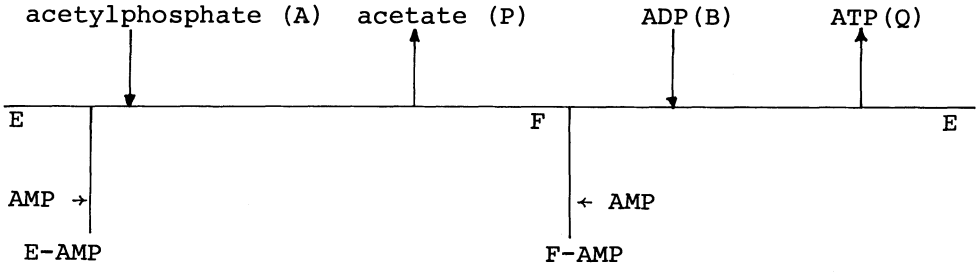
and Eq. (IV-26) would be modified as shown in Eq. (IV-33)

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)}\left[1 + \frac{I}{K_i} + \frac{(I)(B)}{K_i K_{ii}} + \frac{K_{ia}K_b(I)}{K_a K_i K_{ii}}\right] + \frac{K_b}{V_1(B)} + \frac{K_{ia}K_b}{V_1(A)(B)}\left[1 + \frac{I}{K_i}\right] \qquad \text{(IV-33)}$$

Inhibition relative to substrate A would of course be competitive; however, a 1/v *versus* 1/B plot would give concave-up hyperbolic inhibition. This effect is obviously readily distinguishable from the case in which a dead end binary complex is formed.

Although competitive inhibitors cannot be used to differentiate between different types of Ordered Bi Bi mechanisms (i.e., ternary complex, Theorell-Chance and the appropriate "Iso" types), they may be employed as support for the Bi Bi Ping Pong mechanism. It has already been pointed out how the Ping Pong and Sequential bireactant mechanisms may be segregated from initial rate data alone. In Table IV-1 are shown the types of patterns to be expected when double reciprocal plots are made of kinetic data in the presence and absence of competitive inhibitors. The symmetry inherent in the Ping Pong mechanism gives rise to the unique inhibition patterns illustrated in Table IV-1 for this mechanism.

Finding a competitive inhibitor for an enzyme system that exhibits a Ping Pong pathway is usually a more formidable task than in the case of a Sequential mechanism. A specific example of this problem is illustrated by the acetate kinase reaction (17), whose mechanism is illustrated in Scheme IV-4.



Scheme IV-4

When AMP inhibition was analyzed, it was found to be a noncompetitive inhibitor of ADP and acetylphosphate, indicating that it was competing with the substrates for two different forms of the enzyme (free enzyme and the phosphoryl-enzyme intermediate). These effects are illustrated in Scheme IV-4 and described by Eq. (IV-34)

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)}\left[1 + \frac{I}{K_i}\right] + \frac{K_b}{V_1(B)}\left[1 + \frac{I}{K_{ii}}\right] \qquad \text{(IV-34)}$$

where $E + I = EI$, $K_i = (E)(I)/(EI)$; $F + I = FI$, $K_{ii} = (F)(I)/(FI)$ (IV-35)

2. Terreactant Systems

The kinetic mechanisms for enzymes that utilize three substrates may be divided into two types, Sequential and Ping Pong. In 1967 it was shown how competitive inhibitors may be used to make a choice from among a number of alternatives (18). Listed below are the various terreactant mechanisms, assumptions, and rate equations that have been derived based upon these assumptions. Table IV-2 lists the patterns to be expected for the various kinetic pathways based upon the rate equations. Reference should be made to Chapter I for additional details and definitions. The basic assumption made is that the competitive inhibition is of the dead end type.

Table IV-2. Competitive inhibition patterns for various three-substrate mechanisms [a]

Mechanism [b]	Competitive inhibitor for substrate	1/A plot	1/B plot	1/C plot
I-12	A	C [c]	N [d,e]	N [d,e]
	B	U [f]	C	N [g]
	C	U	U	C
I-13	A	C	N	N
	B	N	C	N
	C	N	N	C
I-14	A	C	N	C [h]
	B	N	C	C [i]
	C	U	U	C
I-15	A	C	N	N
	B	U	C	N
	C	U	N	C
I-16	A	C	N	N
	B	N	C	N
	C	N	N	C
I-17	A	C	U	U
	B	U	C	U
	C	U	U	C
I-18	A	C	N [j]	U
	B	U	C	U
	C	U	U	C

Table IV-2 (continued)

Mechanism	Competitive inhibitor for substrate	1/A plot	1/B plot	1/C plot
I-19	A	C	U	U
	B	U	C	N [k]
	C	U	U	C
I-20	A	C	N	U
	B	N	C	U
	C	U	U	C
I-21	A	C	U	U
	B	U	C	N
	C	U	N	C

[a] The various interactions of the competitive inhibitor are given in text along with the inhibited rate equation.
[b] The numbers refer to the mechanism listed in Chapter I.
[c] C refers to a Lineweaver-Burk plot that shows competitive inhibition.
[d] N refers to a Lineweaver-Burk plot that shows noncompetitive inhibition.
[e] If EI reacts with B to form EIB, the plots would be nonlinear.
[f] U refers to a Lineweaver-Burk plot that shows uncompetitive inhibition.
[g] If EAI reacts with C to form EAIC, the plot would be nonlinear.
[h] If EIB reacts with C to form EIBC, the plot would be noncompetitive.
[i] If EIA reacts with C to form EIAC, the plot would be noncompetitive.
[j] If EI reacts with B to form EIB, the plot would be nonlinear.
[k] If E'I reacts with C to form E'IC, the plot would be nonlinear.

a) Ordered Ter Ter (Mechanism I-12)

α) Competitive inhibitor for A.

$$E + I = EI,\ K_i \tag{IV-36}$$

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)}\left[1 + \frac{I}{K_i}\right] + \frac{K_b}{V_1(B)} + \frac{K_c}{V_1(C)} + \frac{K_{ia}K_b}{V_1(A)(B)}\left[1 + \frac{I}{K_i}\right] + \frac{K_{ib}K_c}{V_1(B)(C)} + \frac{K_{ia}K_{ib}K_c}{V_1(A)(B)(C)}\left[1 + \frac{I}{K_i}\right]. \tag{IV-37}$$

β) Competitive inhibitor for B.

$$EA + I = EAI,\ K_i \tag{IV-38}$$

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)} + \frac{K_b}{V_1(B)}\left[1 + \frac{I}{K_i}\right] + \frac{K_c}{V_1(C)} + \frac{K_{ia}K_b}{V_1(A)(B)} + \frac{K_{ib}K_c}{V_1(B)(C)}\left[1 + \frac{I}{K_i}\right] + \frac{K_{ia}K_{ib}K_c}{V_1(A)(B)(C)}. \tag{IV-39}$$

γ) Competitive inhibitor for C.

$$EAB + I = EABI,\ K_i \tag{IV-40}$$

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)} + \frac{K_b}{V_1(B)} + \frac{K_c}{V_1(C)}\left[1 + \frac{I}{K_i}\right] + \frac{K_{ia}K_b}{V_1(A)(B)} + \frac{K_{ib}K_c}{V_1(B)(C)} + \frac{K_{ia}K_{ib}K_c}{V_1(A)(B)(C)}. \tag{IV-41}$$

b) Random Ter Ter (Rapid Equilibrium) (Mechanism I-13)

α) Competitive inhibitor for A.

$$E + I = EI,\ K_i \qquad EI + C = EIC,\ K_{iv} \qquad EIB + C = EIBC,\ K_{vii} \tag{IV-42}$$

$$EI + B = EIB,\ K_{ii} \qquad EIC + B = EIBC,\ K_v \qquad EC + I = EIC,\ K_{viii} \tag{IV-43}$$

$$EIB + C = EIBC,\ K_{iii} \qquad EB + I = EIB,\ K_{vi} \qquad EBC + I = EIBC,\ K_{ix} \tag{IV-44}$$

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)}\left[1 + \frac{I}{K_{ix}}\right] + \frac{K_b}{V_1(B)} + \frac{K_c}{V_1(C)} + \frac{K_bK_{ca}}{V_1(A)(B)}\left[1 + \frac{I}{K_{viii}}\right] + \frac{K_cK_{ba}}{V_1(A)(C)}\left[1 + \frac{I}{K_{vi}}\right] + \frac{K_{ab}K_c}{V_1(B)(C)} + \frac{K_{ia}K_cK_{ab}}{V_1(A)(B)(C)}\left[1 + \frac{I}{K_i}\right]. \tag{IV-45}$$

β) Because of the symmetry of the Random Ter Ter mechanism, a competitive inhibitor for substrate B will give the same inhibition patterns relative to substrates A and C that the competitive inhibitor of A causes with respect to substrates B and C.

c) Random AB (Rapid Equilibrium) (Mechanism I-14)

α) Competitive inhibitor for A.

$$E + I = EI,\ K_i; \qquad EB + I = EIB,\ K_{iii} \tag{IV-46}$$

$$EI + B = EIB,\ K_{ii}; \quad EIB + C = EIBC,\ K_{iv} \tag{IV-47}$$

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_aK_c(I)}{V_1K_{iii}K_{iv}(A)} + \frac{K_c}{V_1(C)} + \frac{K_aK_c}{V_1(A)(C)}\left[1 + \frac{I}{K_{iii}}\right]$$

$$+ \frac{K_bK_c}{V_1(B)(C)} + \frac{K_{ia}K_bK_c}{V_1(A)(B)(C)}\left[1 + \frac{I}{K_i}\right]. \tag{IV-48}$$

β) Competitive inhibitor for B.

$$E + I = EI,\ K_i; \qquad EA + I = EAI,\ K_{iii} \tag{IV-49}$$

$$EI + A = EAI,\ K_{ii}; \quad EAI + C = EAIC,\ K_{iv} \tag{IV-50}$$

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_bK_c(I)}{V_1K_{iii}K_{iv}(B)} + \frac{K_c}{V_1(C)} + \frac{K_aK_c}{V_1(A)(C)} +$$

$$\frac{K_bK_c}{V_1(B)(C)}\left[1 + \frac{I}{K_{iii}}\right] + \frac{K_{ia}K_bK_c}{V_1(A)(B)(C)}\left[1 + \frac{I}{K_i}\right]. \tag{IV-51}$$

γ) Competitive inhibitor for C.

$$EAB + I = EABI,\ K_i \tag{IV-52}$$

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_c}{V_1(C)}\left[1 + \frac{I}{K_i}\right] + \frac{K_aK_c}{V_1(A)(C)} + \frac{K_bK_c}{V_1(B)(C)} +$$

$$\frac{K_{ia}K_bK_c}{V_1(A)(B)(C)}. \tag{IV-53}$$

d) Random BC (Rapid Equilibrium) (Mechanism I-15)

α) Competitive inhibitor for A.

$$E + I = EI,\ K_i; \quad EI + B = EIB,\ K_{ii}; \quad EI + C = EIC,\ K_{iii} \tag{IV-54}$$

$$EIB + C = EIBC,\ K_{iv}; \quad EIC + B = EIBC,\ K_v \tag{IV-55}$$

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_{ia}K_cK_{ab}(I)}{V_1K_iK_{ii}K_{iv}(A)} + \frac{K_b}{V_1(B)} + \frac{K_c}{V_1(C)} + \frac{K_{ia}K_cK_{ab}(I)}{V_1K_iK_{iii}(A)(B)} +$$

$$\frac{K_{ia}K_cK_{ab}(I)}{V_1K_iK_{ii}(A)(C)} + \frac{K_cK_{ab}}{V_1(B)(C)} + \frac{K_{ia}K_cK_{ab}}{V_1(A)(B)(C)}\left[1 + \frac{I}{K_i}\right]. \tag{IV-56}$$

β) Competitive inhibitor for B.

$$EA + I = EAI,\ K_i;\quad EAI + C = EAIC,\ K_{ii};\quad EAC + I = EAIC,\ K_{iii} \tag{IV-57}$$

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_cK_{ab}}{V_1(B)}\left[\frac{K_b}{K_cK_{ab}} + \frac{I}{K_iK_{ii}}\right] + \frac{K_c}{V_1(C)} + \frac{K_cK_{ab}}{V_1(B)(C)}\left[1 + \frac{I}{K_i}\right] + \frac{K_{ia}K_cK_{ab}}{V_1(A)(B)(C)}. \tag{IV-58}$$

γ) Competitive inhibitor for C.

Because substrates B and C add randomly to the enzyme, the inhibition pattern relative to substrates A and B with a competitive inhibitor of C will be similar to that described for a competitive inhibitor of substrate B.

e) Random AC (Rapid Equilibrium) (Mechanism I-16)

α) Competitive inhibitor for A.

$$E + I = EI,\ K_i;\qquad EI + B = EIB,\ K_{ii} \tag{IV-59}$$

$$EBC + I = EIBC,\ K_{iii};\quad EIB + C = EIBC,\ K_{iv} \tag{IV-60}$$

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)}\left[1 + \frac{I}{K_{iii}}\right] + \frac{K_c}{V_1(C)} + \frac{K_aK_{cb}}{V_1(A)(B)} + \frac{K_{ia}K_cK_{ab}(I)}{V_1K_iK_{ii}(A)(C)} + \frac{K_cK_{ab}}{V_1(B)(C)} + \frac{K_{ia}K_cK_{ab}}{V_1(A)(B)(C)}\left[1 + \frac{I}{K_i}\right]. \tag{IV-61}$$

β) Competitive inhibitor for B.

$$EA + I = EAI,\ K_i;\qquad EC + I = EIC,\ K_{iii} \tag{IV-62}$$

$$EAI + C = EAIC,\ K_{ii};\qquad EIC + A = EAIC,\ K_{iv} \tag{IV-63}$$

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)} + \frac{K_cK_{ab}(I)}{V_1K_iK_{ii}(B)} + \frac{K_c}{V_1(C)} + \frac{K_aK_{cb}}{V_1(A)(B)}\left[1 + \frac{I}{K_{iii}}\right] + \frac{K_cK_{ab}}{V_1(B)(C)}\left[1 + \frac{I}{K_i}\right] + \frac{K_{ia}K_cK_{ab}}{V_1(A)(B)(C)}. \tag{IV-64}$$

γ) Competitive inhibitor for C.

$$E + I = EI,\ K_i;\qquad EBI + A = EABI,\ K_{iii} \tag{IV-65}$$

$$EI + B = EBI,\ K_{ii};\quad EAB + I = EABI,\ K_{iv} \tag{IV-66}$$

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)} + \frac{K_c}{V_1(C)}\left[1 + \frac{I}{K_{iv}}\right] + \frac{K_aK_{cb}}{V_1(A)(B)} +$$

$$\frac{K_{ia}K_cK_{ab}(I)}{V_1K_iK_{ii}(A)(C)} + \frac{K_cK_{ab}}{V_1(B)(C)} + \frac{K_{ia}K_cK_{ab}}{V_1(A)(B)(C)}\left[1 + \frac{I}{K_i}\right]. \qquad \text{(IV-67)}$$

It is of interest to note that the competitive inhibition patterns for this mechanism are indistinguishable from the Random Ter Ter (Rapid Equilibrium) case.

f) Hexa Uni Ping Pong (Mechanism I-17)

α) Competitive inhibitor for A.

$$E + I = EI,\ K_i \qquad \text{(IV-68)}$$

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)}\left[1 + \frac{I}{K_i}\right] + \frac{K_b}{V_1(B)} + \frac{K_c}{V_1(C)}. \qquad \text{(IV-69)}$$

β) Competitive inhibitors for B or C.

A competitive inhibitor for substrate B will affect the $K_b/V_1(B)$ term of the rate expression, and a competitive inhibitor for substrate C will alter the $K_c/V_1(C)$ term. In each instance, the rate equation will be modified by the factor $(1 + I/K_i)$.

g) Ordered Bi Uni Uni Bi Ping Pong (Mechanism I-18)

α) Competitive inhibitor for A.

$$E + I = EI,\ K_i \qquad \text{(IV-70)}$$

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)}\left[1 + \frac{I}{K_i}\right] + \frac{K_b}{V_1(B)} + \frac{K_c}{V_1(C)} + \frac{K_{ia}K_b}{V_1(A)(B)}\left[1 + \frac{I}{K_i}\right]. \qquad \text{(IV-71)}$$

β) Competitive inhibitor for B.

$$EA + I = EAI,\ K_i \qquad \text{(IV-72)}$$

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)} + \frac{K_b}{V_1(B)}\left[1 + \frac{I}{K_i}\right] + \frac{K_c}{V_1(C)} + \frac{K_{ia}K_b}{V_1(A)(B)}. \qquad \text{(IV-73)}$$

γ) Competitive inhibitor for C.

$$E' + I = E'I,\ K_i \qquad \text{(IV-74)}$$

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)} + \frac{K_b}{V_1(B)} + \frac{K_c}{V_1(C)}\left[1 + \frac{I}{K_i}\right] + \frac{K_{ia}K_b}{V_1(A)(B)}. \qquad \text{(IV-75)}$$

h) Ordered Uni Uni Bi Bi Ping Pong (Mechanism I-19)

α) Competitive inhibitor for A.

$$E + I = EI,\ K_i \tag{IV-76}$$

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)}\left[1 + \frac{I}{K_i}\right] + \frac{K_b}{V_1(B)} + \frac{K_c}{V_1(C)} + \frac{K_{ib}K_c}{V_1(B)(C)}. \tag{IV-77}$$

β) Competitive inhibitor for B.

$$E' + I = E'I,\ K_i \tag{IV-78}$$

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)} + \frac{K_b}{V_1(B)}\left[1 + \frac{I}{K_i}\right] + \frac{K_c}{V_1(C)} + \frac{K_{ib}K_c}{V_1(B)(C)}\left[1 + \frac{I}{K_i}\right]. \tag{IV-79}$$

γ) Competitive inhibitor for C.

$$EB + I = EBI,\ K_i \tag{IV-80}$$

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)} + \frac{K_b}{V_1(B)} + \frac{K_c}{V_1(C)}\left[1 + \frac{I}{K_i}\right] + \frac{K_{ib}K_c}{V_1(B)(C)}. \tag{IV-81}$$

i) Random Bi Uni Uni Bi Ping Pong (Mechanism I-20)

α) Competitive inhibitor for A.

$$E + I = EI,\ K_i;\ EI + B = EIB,\ K_{ii};\ EB + I = EIB,\ K_{iii} \tag{IV-82}$$

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)}\left[1 + \frac{I}{K_{iii}}\right] + \frac{K_b}{V_1(B)} + \frac{K_c}{V_1(C)} + \frac{K_{ia}K_b}{V_1(A)(B)}\left[1 + \frac{I}{K_i}\right]. \tag{IV-83}$$

β) Competitive inhibitor for B.

$$E + I = EI,\ K_i;\ EA + I = EAI,\ K_{ii};\ EI + A = EAI,\ K_{iii} \tag{IV-84}$$

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)} + \frac{K_b}{V_1(B)}\left[1 + \frac{I}{K_{ii}}\right] + \frac{K_c}{V_1(C)} + \frac{K_{ia}K_b}{V_1(A)(B)}\left[1 + \frac{I}{K_i}\right]. \tag{IV-85}$$

γ) Competitive inhibitor for C.

$$E' + I = E'I,\ K_i \tag{IV-86}$$

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)} + \frac{K_b}{V_1(B)} + \frac{K_c}{V_1(C)}\left[1 + \frac{I}{K_i}\right] + \frac{K_{ia}K_b}{V_1(A)(B)}. \tag{IV-87}$$

j) Random Uni Uni Bi Bi Ping Pong (Mechanism I-21)

α) Competitive inhibitor for A.

$$E + I = EI,\ K_i \qquad \text{(IV-88)}$$

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)}\left[1 + \frac{I}{K_i}\right] + \frac{K_b}{V_1(B)} + \frac{K_c}{V_1(C)} + \frac{K_{ib}K_c}{V_1(B)(C)}. \qquad \text{(IV-89)}$$

β) Competitive inhibitor for B.

$$E' + I = E'I,\ K_i;\ E'C + I = E'IC,\ K_{ii};\ E'I + C = E'IC,\ K_{iii} \qquad \text{(IV-90)}$$

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)} + \frac{K_b}{V_1(B)}\left[1 + \frac{I}{K_{ii}}\right] + \frac{K_c}{V_1(C)} + \frac{K_{ib}K_c}{V_1(B)(C)}\left[1 + \frac{I}{K_i}\right]. \qquad \text{(IV-91)}$$

γ) Competitive inhibitor for C.

$$E' + I = E'I,\ K_i;\ E'B + I = E'BI,\ K_{ii};\ E'I + B = E'BI,\ K_{iii} \qquad \text{(IV-92)}$$

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)} + \frac{K_b}{V_1(B)} + \frac{K_c}{V_1(C)}\left[1 + \frac{I}{K_{ii}}\right] + \frac{K_{ib}K_c}{V_1(B)(C)}\left[1 + \frac{I}{K_i}\right]. \qquad \text{(IV-93)}$$

In the derivation of the rate equations for the effects of competitive inhibitors, it was tacitly assumed that only dead end complexes are formed when the inhibitor reacts with an enzyme form. By analogy with two substrate enzyme interactions, there seems to be no reason *a priori* to make this assumption. The more general cases are treated elsewhere (18). In Table IV-2 are presented the types of graphical patterns to be expected from double reciprocal plots for the ten different terreactant mechanisms.

3. Kinetic Studies of Adenylosuccinate Synthetase Using Dead End Inhibitors (19)

The kinetic mechanism of adenylosuccinate synthetase action was found to be Sequential from initial rate experiments (19). RUDOLPH and FROMM (19) used dead end inhibitors in an attempt to establish the type of kinetic mechanism for this Ter Ter system within the Sequential class. The substrates and products of the reaction are:

$$\text{IMP} + \text{GTP} + \text{L-aspartate} \overset{Mg^{2+}}{\rightleftharpoons} \text{adenylosuccinate} + \text{GDP} + P_i \qquad \text{(IV-94)}$$

Succinate was found to be a competitive inhibitor for aspartate, whereas 6-mercaptopurine riboside-5'-phosphate and β, γ-5'-quanylyl methylene diphosphonate were determined to be competitive dead end inhibitors for IMP and GTP, respectively. Experimentally, two substrates were held constant at subsaturating levels, but at concentrations above their experimentally determined Michaelis constants. The third substrate was varied

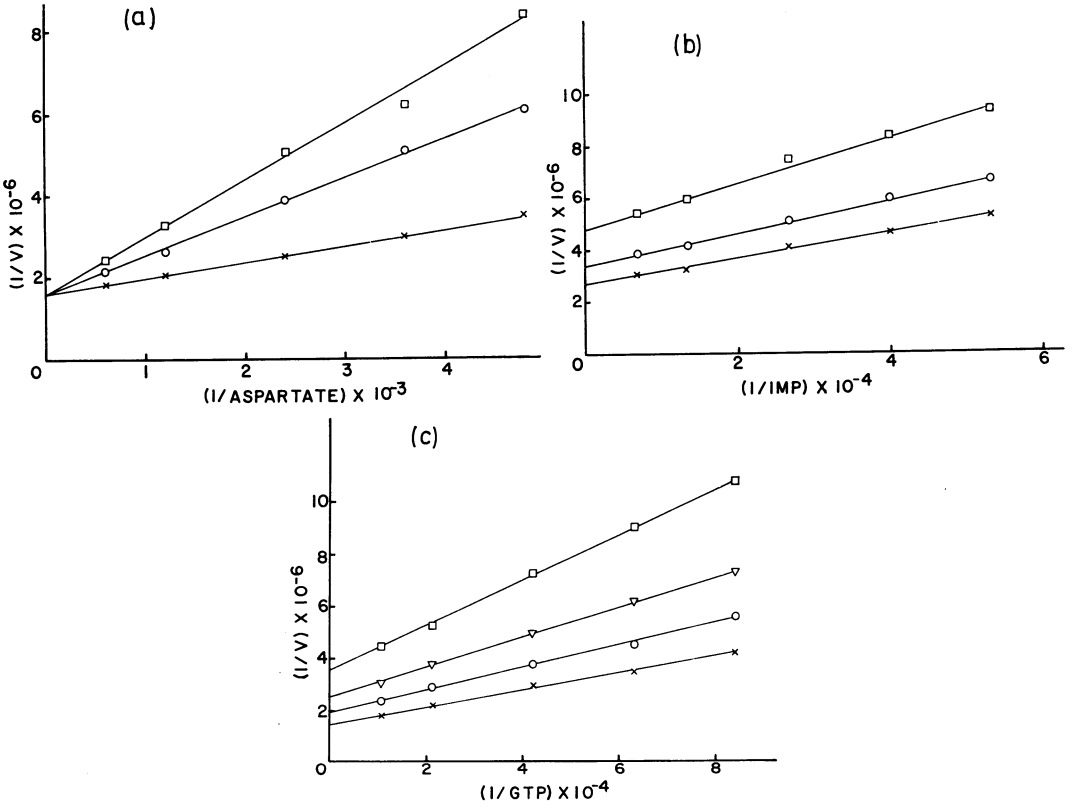


Fig. IV-6 (a). Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the molar concentration of aspartate in the presence and absence of succinate. GTP and IMP concentrations were held constant at 0.033 and 0.15 mM, respectively, and aspartate was varied from 0.208 to 1.67 mM. Succinate concentrations were 0 (X), 10 (O), and 20 mM (□). Other experimental details are described in reference (19).
(b) Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the molar concentration of IMP in the presence and absence of succinate. GTP and aspartate concentrations were held constant at 0.033 and 0.375 mM, respectively, and IMP was varied from 0.019 to 0.15 mM. Succinate concentrations were 0 (X), 10 (O), and 20 mM (□). Other experimental details are described in reference (19).
(c) Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the molar concentration of GTP in the presence and absence of succinate. IMP and aspartate concentrations were held constant at 0.15 and 1.0 mM, respectively, and GTP was varied from 0.012 to 0.095 mM. Succinate concentrations were 0 (X), 12.5 (O), 25 (▽), and 50 mM (□). Other experimental details are described in reference (19)

from about its Michaelis constant concentration to about eight times its Michaelis constant. These experiments were carried out in the presence and absence of the dead end inhibitors. Figures IV-6(a), 6(b), and 6(c) illustrate the inhibition plots obtained with the dead end inhibitor succinate. These results indicate that succinate, a competitive inhibitor for aspartate, is a noncompetitive inhibitor for GTP and IMP. The other competitive dead end inhibitors were also found to exhibit noncompetitive inhibition relative to the other two substrates. Analysis of these findings, with the aid of Table IV-2, indicates that two mechanisms, the Random Ter Ter (Mechanism I-13) and the Partially Random AC (Mechanism I-16), are the only terreactant mechanisms consistent with the inhibition data. It was possible to exclude the partially random mechanism for adenylosuccinate synthetase by replotting the initial rate data according to the protocol outlined in Table III-3.

G. Cleland's Rules for Dead End Inhibition

The inhibition patterns described in this chapter have been codified into a set of rules by CLELAND (20). These rules, along with illustrative examples, are described in this section.

In the derivation of the equation for the mechanism of Scheme I-3, the determinants for E and EA are $(k_2 + k_3)$ and $k_1(A)$, respectively, when P = 0 (Chapter II). When substitutions are made for E and EA in the velocity expression

$$v = \frac{k_3(EA)E_0}{E_0} \qquad \text{(IV-95)}$$

the resulting rate expression is of the form

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1}\left(\frac{1}{A}\right). \qquad \text{(IV-96)}$$

The intercept term, V_1, originated from the EA determinant, whereas the slope term, K_a/V_1, comes from the determinant for E. This simple example can be carried one step further for a bireactant system.

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)} + \frac{K_b}{V_1(B)} + \frac{K_{ia}K_b}{V_1(A)(B)}. \qquad \text{(IV-97)}$$

If the assumed mechanism is Ordered Bi Bi, $1/V_1$, $K_a/V_1(A)$, $K_b/V_1(B)$, and $K_{ia}K_b/V_1(A)(B)$, have their origins in the EQ, E, EA, and E determinants, respectively. In the case of the Random Bi Bi mechanism, the enzyme terms will be EAB, EB, EA, and E, respectively.

With this information in hand, the logic of Cleland's rules becomes evident when applied to reversible enzyme inhibition.

Rule 1. An intercept effect on the axis of ordinates in a double reciprocal plot occurs when a reversible inhibitor combines with a form of the enzyme that the variable substrate does not bind. The initial velocity cannot be restored by saturation with the variable substrate, and the effect may or may not be eliminated by saturation with the fixed substrate in bi- or terreactant systems. Saturation with a substrate will serve to eliminate the enzyme species that reacts with the substrate from the rate equation by decreasing its steady-state concentration to zero.

In the case of the unireactant system illustrated by Eq. (IV-96), if a reversible inhibitor combines with EA to form EAI, the intercept will be affected and inhibition will not be reversed at saturating levels of substrate A. Similarly, for the Ordered Bi Bi mechanism described by Eq. (IV-97), saturating the system with substrate A will not affect the intercept term $\left[1/V_1 + K_b/V_1(B)\right]$. If the reversible inhibitor combines with EQ, raising the level of the fixed substrate B will not affect the intercept; however, if the reversible inhibitor combines with the EA complex, saturating with B will eliminate the intercept effect. It follows then that, if the intercept effect is caused by combination of inhibitor and the EQ complex, the intercept effect cannot be eliminated by saturation with either substrate. It is only when the enzyme-inhibitor complex is reversibly connected to one of the enzyme forms associated with substrate that saturation with substrate will be effective in reversing the intercept effect.

These interactions lead to linear inhibition, i.e., if intercepts are plotted *versus* inhibitor concentration, a straight line will result. It is only when the inhibitor adds more than once to the same enzyme form that nonlinear inhibition results. If, for example, EQI_2 formed from the sequence of reactions, $EQ + I = EQI$, $EQI + I = EQI_2$, the replot of intercept *versus* I would be parabolic concave-up.

Rule 2. A *slope* effect in a double reciprocal plot occurs when the inhibitor either combines with the same enzyme form that the variable substrate would normally combine with or, alternatively, reacts with an enzyme form that alters the concentration of an enzyme species with which the variable substrate reacts. In this latter case, there must be a reversible connection between the two different enzyme species.

The first of these points can be described by referral to Eq. (IV-97). If the inhibitor reacts with the same enzyme form that the variable substrate combines with (e.g., E in the case of a 1/A plot), there will be a slope effect. This same pathway can be used to illustrate another point. Substrate A will alter the concentration of complex EA, and if B is the variable substrate, different levels of A would be expected to cause a slope effect relative to B. Similarly, as the concentration of B is changed, if A is the variable substrate, there will be a slope effect. This is because the concentration of E depends upon the concentration of EA, which in turn depends upon the level of substrate B.

Changes in the concentration of product P will cause slope changes when A is the variable substrate in a double reciprocal plot. The reason for this is that all steps between the addition of A and the release of P are reversibly related. On the other hand, if

P is finite and B is saturating ($B \to \infty$), P will not alter the slope in a 1/v *versus* 1/A plot. Under these conditions, the step between EA + B$\rightleftharpoons$EAB will be irreversible, and no connection will exists between E and EQ.

Rule 3. If a compound combines with more than one enzyme form, Rules 1 and 2 must be applied to evaluate the multiple effects. The effect of the compound on the intercept and slope will be multiplied if the different enzyme forms are reversibly connected; i.e., if the compound causes an increase in the steady-state concentration of a particular enzyme form and it also combines with another enzyme form that is reversibly connected to the enzyme species whose concentration was raised.

Consider as an example of this effect the Ordered Bi Bi mechanism of Scheme I-8. When the product P is present along with substrates A and B, the concentration of the central complex will increase as will the concentration of the binary complex EA. The product P, if it can also react with EA, will have in effect reacted at two points in a reversible reaction sequence, and the inhibition will be parabolic with respect to P (on slopes when B is varied and on intercepts when A is varied).

Dead end inhibitors can act as parabolic inhibitors (see Rule 2); however, they are not capable of increasing the steady-state concentrations of enzyme species that participate in catalysis.

Additional information on the application of these rules can be obtained either by reference to CLELAND's original article on this subject (20), or to PLOWMAN's book on enzyme kinetics (21).

H. The Stereochemical Nature of Enzyme and Substrate Interaction

Insight into the relative importance of substituent groups on the substrate and its stereochemical nature in the enzyme-substrate complex may be gained from kinetic studies of enzyme inhibitors and alternative substrates. Although it is clearly beyond the scope of this monograph to detail the types of experiments required to address this problem completely, the fundamental approach must be noted. One of the most elegant articles on this subject is the review by MEISTER on glutamine synthetase (22). MEISTER and his coworkers studied the kinetics of a large number of analogs of glutamine that function either as substrates or as inhibitors of the synthetase reaction. With this information and space-filling models of substrates and substrate analogs, it was possible to ascribe a role to each functional group in the glutamine molecule. Although it may readily be conceded that this approach to the study of enzymology is more of a problem for the organic chemist than for the kineticist, it does indi-

cate that these two disciplines may be exploited for mutual advantage.

PURICH et al. (23) used kinetic data on dead end inhibitors and alternative substrates to obtain information on the active conformation of the sugar substrates for the hexokinase reaction. The Michaelis constants for glucose, mannose, and fructose were reported to be 84μM, 109μM, and 628μM, respectively (24). Galactose is not a substrate for hexokinase and exhibits very weak binding (25). On the other hand, 2-deoxyglucose is reported to have a Michaelis constant and maximal velocity similar to glucose (25). 2-N-acetylglucosamine, a dead end competitive inhibitor of glucose, binds approximately 100-times more weakly than does glucose (26), whereas 2-aminoglucose is almost as active as glucose in the hexokinase reaction (25).

Figure IV-7 illustrates a model for substrate conformation that incorporates the known aspects of substrate and inhibitor specificity in the hexokinase system. The figure shows the structural similarities between C1 β-D-glucopyranose and the corresponding conformer of β-D-fructofuranose. It will be noted that the hydroxyl

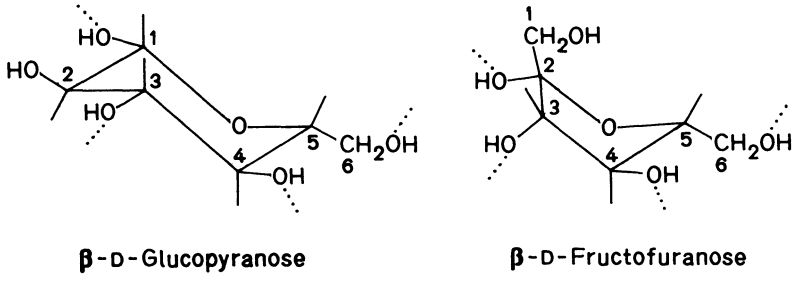


Fig. IV-7. Proposed model of the interaction of the C1 conformers of β-D-glucopyranose and β-D-fructofuranose with the enzyme hexokinase. The dashed lines represent points of enzyme and substrate interaction

substituents on carbon atoms 1, 3, 4, and 6 of glucose are oriented in approximately the same way as the hydroxyl groups located on carbon atoms 2, 3, 4, and 6 of fructose. If it is assumed that these positions are the specificity-imparting groups on the hexose (as indicated by the dotted lines), one can readily understand the relative unimportance of the hydroxyl substituent at carbon atom 2 of glucose, mannose, and 2-deoxyglucose. The relative importance of the hydroxyl substituent at carbon 1 of glucose can be appreciated by the fact that 1,5-anhydro-D-gulcitol and 1,5-anhydro-D-mannitol are bound less tightly than their respective hexoses (25). The fact that these anhydro sugar alcohols can be very slowly phosphorylated, however, indicates that the presence of a hydroxyl group in this position is not required absolutely. On the other hand, the 4

position of glucose seems essential in that galactose is bound very poorly and is not phosphorylated. It would be of interest to know whether 4-deoxyglucose can be bound and phosphorylated; this information would permit one to determine whether the inactivity of galactose is due to the axial orientation of the hydroxyl group or to the lack of a hydroxyl group in the equitorial orientation for binding. Finally, the inability of hexokinase to act on N-acetylglucosamine can be attributed to interaction of the bulky N-acetyl substituent with the groups on the enzyme responsible for recognizing the hydroxyl group at carbon atom 1 or 3 of glucose.

By definition, competitive inhibitors and substrates exhibit mutually exclusive binding. This is, however, not absolutely true; i.e., if the substrate and inhibitor can bind simultaneously in close proximity, the inhibition will obviously not be competitive. On the other hand, one could visualize two competitive inhibitors of a substrate that exhibit mutually exclusive binding or, alternatively, nonexclusive binding. A rationale for kinetic studies with multiple inhibitors has been provided by YONETANI and THEORELL (27) from their investigations of alcohol dehydrogenase. Experiments with multiple inhibitors may provide information on the stereochemistry of the active site relative to the binding of the substrate molecule. They may also suggest whether one portion of the substrate facilitates binding of another part of the substrate molecule to the enzyme. If two competitive inhibitors are present with enzyme and substrate simultaneously, the following interactions can reasonably occur:

$$E + A \rightleftharpoons EA \rightleftharpoons E + P$$

$$E + I_1 \rightleftharpoons EI_1, \; K_i$$

$$E + I_2 \rightleftharpoons EI_2, \; K_{ii}$$

$$EI_1 + I_2 \rightleftharpoons EI_1I_2, \; K_{iii}$$

$$EI_2 + I_1 \rightleftharpoons EI_1I_2, \; K_{iv}$$

Scheme IV-5

The rate expression that accounts for the effect of two competitive inhibitors is,

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)}\left[1 + \frac{I_1}{K_i} + \frac{I_2}{K_{ii}} + \frac{(I_1)(I_2)}{K_iK_{iii}}\right]. \qquad \text{(IV-98)}$$

If the following substitution is made, $K_{iii} = \alpha K_{ii}$, Eq. (IV-98) is transformed into Eq. (IV-99), as $K_iK_{iii} = K_{ii}K_{iv}$.

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)}\left[1 + \frac{I_1}{K_i} + \frac{I_2}{K_{ii}} + \frac{(I_1)(I_2)}{(\alpha)K_iK_{ii}}\right]. \qquad \text{(IV-99)}$$

where α, the interaction coefficient, is equal to K_{iv}/K_i. If $K_{iv} > K_i$ ($\alpha > 1$), the presence of inhibitor I_2 on the enzyme inhibits binding of inhibitor I_1. If $K_{iv} < K_i$ ($\alpha < 1$), the in-

hibitor I_2, when present on the enzyme, facilitates binding of the other inhibitor, I_1. When $K_i = K_{iv}$ ($\alpha = 1$), the presence of the inhibitor I_2 on the enzyme does not affect binding of the other inhibitor, I_1.

If binding of the two inhibitors is mutually exclusive, the complex EI_1I_2 is not formed, and the term containing K_{iii} in Eq. (IV-98) is deleted. Under these conditions, plots of $1/v$ *versus* I_1 at different fixed levels of I_2 or of $1/v$ against I_2 at different constant levels of I_1 will give parallel lines. However, if the lines converge, inhibitor binding is not exclusive. If a graph of $1/v$ is made as a function of I_1 at different levels of I_2, the intercept will equal $1/V_1\left[1 + K_a/A + K_a(I_2)/K_{ii}(A)\right]$. A replot of the intercept as a function of I_2 will give a straight line with a slope of $K_a/K_{ii}V_1(A)$, and if K_a, V_1, and A are known, K_{ii} may be evaluated. In similar fashion, one may calculate K_i and α from plots of slopes *versus* I_2 and a knowledge of K_{ii}. The significance of the interaction coefficient α has already been detailed.

I. Kinetics of Enzyme Specificity

The question is often asked whether one or two different enzymes are involved in catalysis involving different substrates. DIXON and WEBB (6) have shown how alternative substrates that act as competitive inhibitors may be used to provide an answer to this problem. If a single enzyme is involved, the reactions involving alternative substrates will be:

$$E + A_1 \rightleftharpoons EA_1 \rightleftharpoons E + P_1$$
$$E + A_2 \rightleftharpoons EA_2 \rightleftharpoons E + P_2$$

Scheme IV-6

Under these conditions, A_1 and A_2 will act like competitive inhibitors relative to each other, and when E, A_1 and A_2 are present simultaneously,

$$v_{total} = v_a + v_b \text{ where } v_a = \frac{V_{1a}}{1 + \frac{K_{a1}}{A_1}\left[1 + \frac{A_2}{K_{a2}}\right]} \text{ and} \qquad \text{(IV-100)}$$

$$v_b = \frac{V_{1b}}{1 + \frac{K_{a2}}{A_2}\left[1 + \frac{A_1}{K_{a1}}\right]} . \qquad \text{(IV-101)}$$

The total velocity will be less than the case in which two different enzymes act on the two substrates. Presumably, one could

make a choice between the case of a single nonspecific enzyme and two specific catalysts. There are, however, a number of limitations to this approach. The most serious involves the case in which there are indeed two different enzymes, but where the two substrates act as competitive inhibitors of each other. A more remote possibility is the case where a single protein molecule with two different active sites is involved. In any event, the results of such experiments must be interpreted with caution.

J. The Kinetics of Transition State (Multisubstrate and Geometric) Analogs

WOLFENDEN (28) and LIENHARD (29) have recently outlined how transition state and multisubstrate (sometimes referred to as geometric) analogs may be used to provide information on the chemical events that occur during enzymatic catalysis. If it were possible to design an inactive compound that resembles the transition state, this analog would be expected to bind very tightly to the enzyme. In theory, a good deal of binding energy when enzyme and substrate interact is utilized to alter the enzyme's conformation so that proper geometric orientation for catalysis is provided between enzyme and substrate. Therefore, some of this binding energy is conserved because the geometric analog more closely resembles the transition state than does the substrate.

These suggestions may be formalized by considering the following two reactions

$$E + A \rightleftharpoons EA', \quad K_1 = 10^{-7}M \tag{IV-102}$$

$$EA' \rightleftharpoons EA, \quad K_2 = 10^{4} \tag{IV-103}$$

for the overall reaction

$$E + A = EA \quad K_{ia} = 10^{-3}M. \tag{IV-104}$$

Reaction IV-102 represents the thermodynamically favorable process of enzyme-substrate binding. Reaction IV-103 may be taken to be the enzyme-induced distortion of both the substrate and enzyme leading to the transition state.

WOLFENDEN (28) has considered a number of examples of transition state analogs in unireactant enzyme systems. One example concerns the enzyme proline racemase, which is inhibited 50% at a concentration of the so-called transition state analog, pyrrole-2-carboxylic acid, which is 160-fold lower than that of the substrate. From this observation, it is assumed that the substrate, L-proline, assumes a planar structure in the reaction sequence similar to the transition state analog.

From the perspective of kinetics, this situation is not very clear cut in the case of multisubstrate systems. The geometric analog should bind free enzyme, and in theory for a two substrate system, the analog and substrate should not be able to bind to the enzyme simultaneously. This situation is difficult to check experimentally because it is not easy to determine whether, for example, 50% of the enzyme has substrate bound and the other 50% of the enzyme is associated with both substrate and analog and analog alone.

It becomes fairly clear when considering the effect of multisubstrate or geometrical analogs on the kinetics of bireactant enzyme systems that only in the case of the rapid equilibrium Random Bi Bi mechanism may one obtain unequivocal results, and then only under certain circumstances. Consider, for example, the interaction of the analog and enzyme in an Ordered Bi Bi mechanism. The inhibitor will bind enzyme and will not permit addition of the second substrate. Thus the analog will act like any other competitive inhibitor of substrate A for this mechanism (see Table IV-1); i.e., it will be a noncompetitive inhibitor of substrate B. It certainly does not follow that, if the inhibition constant is lower than the dissociation constant for enzyme and substrate, the inhibitor is a transition state analog. There are many examples in the literature where competitive inhibitors bind more strongly to enzymes than substrates and yet are clearly not transition state analogs. This discussion serves to indicate then that the inhibition patterns provided by geometric analogs are identical to those to be expected for dead end competitive inhibitors of the first substrate of the Ordered Bi Bi mechanism.

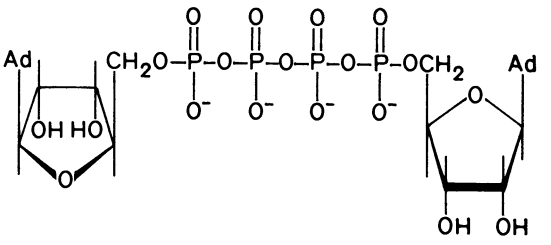
In the case of the Random Bi Bi mechanism, multisubstrate analogs may indeed give unique inhibition patterns (30), and this observation has been used to provide support for the Random Bi Bi mechanism for muscle adenylate kinase (31).

The multisubstrate analog used to test this theory with adenylate kinase was P^1, P^4-di(adenosine-5^1) tetraphosphate (AP_4A)(30). Figure IV-8 illustrates the structure of the analog along with the most probable structure of the substrates AMP and ATP in the transition state. Recently it was shown that AP_5A binds even more strongly to the enzyme than AP_4A (32).

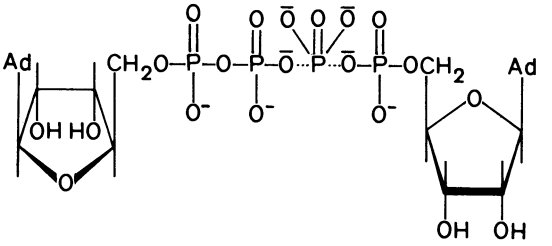
When considering the Random Bi Bi mechanism, the geometric analog should bind exclusively to free enzyme. This binding should effectively preclude binding of substrates A and B, and thus only the E term of the rate equation will be affected by the analog I. The rate expression is, therefore,

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)} + \frac{K_b}{V_1(B)} + \frac{K_{ia}K_b}{V_1(A)(B)}\left[1 + \frac{I}{K_i}\right] \qquad \text{(IV-105)}$$

where K_i is the dissociation constant of the enzyme-multisubstrate inhibitor complex.



AP_4A



Adenylate kinase transition state

Fig. IV-8. Structure of the multisubstrate analog in the adenylate kinase reaction, AP_4A, and of the postulated transition state ATP-AMP intermediate

It is obvious from Eq. (IV-105) that the multisubstrate analog will function as a competitive inhibitor for both substrates. This effect is unique to the Random mechanism and suggests that the inhibitor bridges both substrate binding pockets.

Figure IV-9 depicts the kinetic data obtained with AP_4A in the adenylte kinase reaction. It is clear that the multisubstrate analog functions as a competitive inhibitor with respect to both AMP and ATP.

If the inhibitor binds only at one substrate site in either the Random or the Ordered Bi Bi cases, or if for the latter mechanism the compound does resemble the transition state and substrate B does not add, inhibition patterns will be competitive and non-competitive relative to the two substrates. Thus it will not be possible to differentiate between these two mechanisms based upon these inhibition patterns, nor will it be possible to determine whether the inhibitor is really a transition state analog in the Ordered mechanism. In the case of the Random pathway, the enzyme-inhibitor complex will permit binding of one substrate and the enzyme-substrate complex will allow analog to bind. In summary then, only the unique inhibition pattern illustrated by Eq. (IV-105) allows one to use multisubstrate analogs to unambiguously differentiate between kinetic mechanisms.

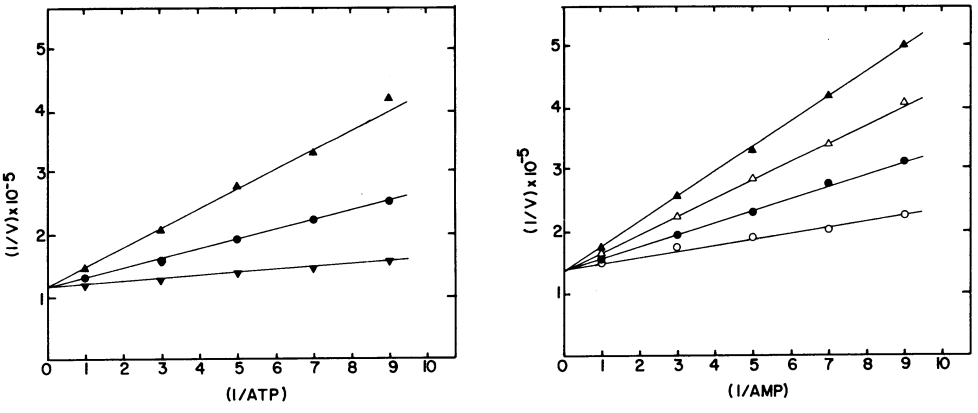


Fig. IV-9 (left). Plot of the reciprocal of the initial reaction velocity (v) *versus* the reciprocal of the millimolar concentration of ATP in the absence and presence of AP_4A. The concentration of AMP was maintained at 0.2 mM, and the ATP was varied in the concentration range of 0.11 to 1.0 mM. The concentrations of AP_4A were none (▼) 0.09 mM (●), and 0.18 mM (▲). The velocity, expressed as the molar concentration of ADP formed in the reaction mixture over a 1-min period after the addition of enzyme at 28°C, in a Cary Model 15 recording spectrophotometer (0 - 0.1 slide wire) was determined in 1.0 ml reaction mixtures by using rabbit muscle adenylate kinase. Each sample contained, in addition to the above components, 50 mM Tris-HCl (pH 8.0), 10 mM cysteine, 75 mM KCl, 0.1 mM NADH, 1.0 mM P-enolpyruvate, excess lactate dehydrogenase (EC 1.1.1.27) and pyruvate kinase (EC 2.7.6.40), and 1.0 mM free uncomplexed Mg^{2+}. The amount of total Mg^{2+} (as $MgSO_4$) added to each mixture was computed as described elsewhere (33), and under these conditions less than 5% of the total ATP was uncomplexed. The ATP, AMP, and AP_4A concentrations were assayed spectrophotometrically; a value of $30.8 \cdot 10^3$ was assumed for the molar absorbance of AP_4A. (right) Plot of the reciprocal of the initial reaction velocity (v) *versus* the reciprocal of the millimolar concentration of AMP in the absence and presence of AP_4A. The concentration of ATP was maintained at 0.15 mM, and the AMP was varied in the concentration range from 0.11 to 1.0 mM. The AP_4A concentrations were none (○), 0.05 mM (●), 0.1 mM (△), and 0.15 mM (▲). The other experimental conditions were as described in the legend to Fig. IV-9 (left)

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Chapter V

Product, Substrate, and Alternative Substrate Inhibition

The study of initial rate kinetics in the presence of product has proven to be a boon to the field of enzyme kinetics. Quite probably, no other single experimental protocol has received greater attention since its inception than product inhibition kinetics. Unfortunately, certain complicating factors, such as abortive ternary complex formation and substrate inhibition, have tended to limit the usefulness of this technique. Nevertheless, kinetic investigations in the presence of product remain a formidable tool available to the kineticist interested in making a choice of mechanism from among the usual possibilities. Furthermore, a good deal of insight may be obtained from such experiments regarding the binding of substrates and products at the active site and, in certain cases, at allosteric sites.

Although investigators studied steady-state kinetics of enzymes in the presence of product for a variety of reasons before 1958, ALBERTY (1), in his classical paper of that year, showed how this technique could be extended for studying kinetic mechanisms. He demonstrated theoretically how, by studying bireactant systems in the presence of product, a choice of mechanism could be made from among the Theorell-Chance, Ordered Bi Bi, and Random Bi Bi mechanisms. He also showed that this protocol permits one to unambiguously choose between the substrate that adds initially in the ordered mechanisms and substrate B. Until that time, it was necessary for the experimentalist to rely upon nonkinetic procedures, such as static binding studies, in order to guess the identity of substrate A and substrate B. Another procedure used extensively for this purpose involved an earlier suggestion by ALBERTY (2) in which the apparent equilibrium constant was correlated with kinetically determined parameters such as maximal velocities and Michaelis constants. This latter technique requires that the enzyme system be reversible and that precise data be obtained for both the equilibrium constant and kinetic parameters.

A. Product Inhibition Experiments

1. Experimental Protocol

ALBERTY (1) suggested that, when initial rate experiments are carried out for bireactant systems in the presence of a *single* product, the kinetic patterns will differ for mechanisms I-7,

I-8, and I-9. Experimentally, the protocol involves holding one substrate constant at subsaturating levels and varying the other substrate in the region of its Michaelis constant at different fixed concentrations of one of the products. This procedure is then repeated so that the fixed substrate in the first experiment is varied and the other substrate is fixed at subsaturating levels. The same procedure is then extended to the other product. It is worth reemphasizing that, in all experiments, the reaction mixtures contain all substrates but only a single product.

In certain experiments, it may be useful to hold the fixed substrate at saturating concentrations rather than at its approximate Michaelis constant concentration. In many instances this procedure is precluded by substrate inhibition; however, if inhibition does not occur, saturation by fixed substrate is a useful diagnostic tool in studies of product inhibition (3).

2. One Substrate Systems

The value of using product inhibition kinetics to make a choice of mechanism can readily be demonstrated with one substrate systems. The rate equation for the simple Michaelis-Menten model (Scheme I-3) in the presence of product is,

$$v = \frac{V_1 V_2 (A - \frac{P}{K_{eq}})}{V_2 K_a + V_2 A + \frac{V_1 P}{K_{eq}}}. \qquad \text{(V-1)}$$

The analogous rate expression for the Iso Uni Uni mechanism (Scheme I-4) is identical to Eq. (V-1) except that the term V_2AP/K_{iip} is added to the denominator. V_1, V_2, K_a, K_p, and K_{iip} are taken to be maximal velocity for the forward reaction, maximal velocity for the reverse reaction, Michaelis constant for the forward reaction, Michaelis constant for the reverse reaction, and an inhibition constant equal to $(k_3 + k_5)/k_4$, respectively.

The extra term associated with the Iso mechanism relative to the pathway that conforms to Eq. (V-1) permits a distinction to be made between these two possibilities. DARVEY (4) has indicated that it may not be possible to choose between these two mechansims from double reciprocal plots of kinetic experiments in the presence and absence of product. Simulation studies with these two equations shows, as indicated in Figs. V-1a and V-1b, that product inhibition is not reversed by substrate with the Iso mechanism. In these simulations, K_{eq} was assumed to be unity; however, if the equilibrium was displaced in the direction of product formation, the curves illustrated in Figs. V-1a and V-1b would tend to be more linear. From these simulations, it seems that a choice can readily be made between the possibilities considered here.

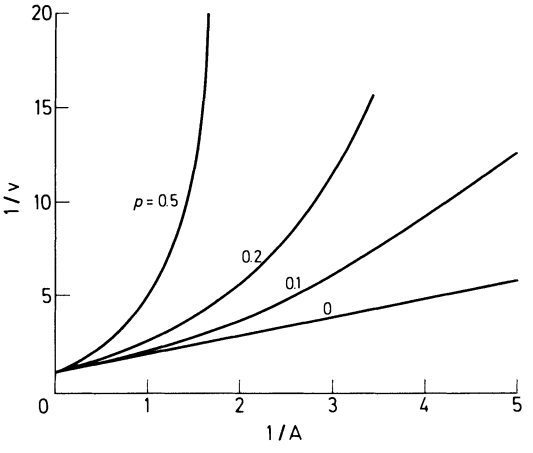


Fig. V-1 (a). Simulation plot of 1/v *versus* 1/A for the mechanism of Eq. (V-1) in the absence and presence of product (P). The parameters V_1, V_2, K_a, and K_p were taken to be unity. The values for product are shown on the graph

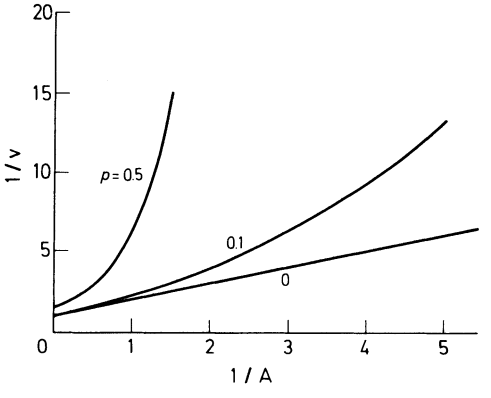


Fig. V-1 (b). Simulation plot of 1/v *versus* 1/A for the mechanism of Scheme I-4. The rate equation is described by Eq. (V-1) except that the term V_2AP/K_{iip} is added to the denominator. The kinetic parameters were assigned values of unity as in Fig. V-1 (a). K_{iip} was assumed to be 1

The Ordered Uni Bi (Mechanism I-5) mechanism may be used as an example to illustrate exactly how the product inhibition equations are derived. The *Appendix* lists the total rate expression for this mechanism, and in the derivation of the product inhibition equations, either P or Q is set equal to zero.

Equation (V-2) describes the case in which P is present and Q is omitted from the reaction mixtures.

$$\frac{1}{v} = \frac{1}{V_1}\left[1 + \frac{P}{K_{ip}}\right] + \frac{1}{V_1}\left[K_a + \frac{V_1 K_q (P)}{V_2 K_{eq}}\right]\left(\frac{1}{A}\right). \qquad \text{(V-2)}$$

When Q is present in the kinetic experiment and P = O, the rate expression is,

$$\frac{1}{v} = \frac{1}{V_1} + \frac{1}{V_1}\left[K_a + \frac{V_1 K_p(Q)}{V_2 K_{eq}}\right]\left(\frac{1}{A}\right). \qquad \text{(V-3)}$$

If one is certain that the mechanism being dealt with is Ordered Uni Bi, product inhibition experiments will serve to identify which product is P and which is Q. It can be seen from Eq. (V-2), when a double reciprocal plot of 1/v *versus* 1/A is made, in the presence and absence of P, the product will affect both the slope and the intercept. It will appear then to be a noncompetitive inhibitor of the substrate. On the other hand, product Q will only alter the slope when 1/v is graphed *versus* 1/A in the presence and absence of Q; i.e., inhibition will be competitive.

Another type of Uni Bi mechanism involves random binding by products. This mechanism is illustrated by the pathway shown in Scheme I-6. If the rate limiting step is assumed to be breakdown of the central complexes, with all other steps in rapid equilibrium, the rate equation in the absence of product will be the usual Michaelis-Menten equation with K_{ia} substituted for K_a. When either product is present, it will bind free enzyme, e.g.,

$$E + P = EP,\ K_{ip} \qquad \text{(V-4)}$$

and the initial rate equation in the presence of P will be

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_{ia}}{V_1(A)}\left[1 + \frac{P}{K_{ip}}\right]. \qquad \text{(V-5)}$$

From the symmetry of mechanism I-6 when Q is substituted for P, P and K_{ip} in Eq. (V-5) will be replaced by Q and K_{iq}, respectively.

The product inhibition patterns for the rapid equilibrium Random Uni Bi mechanism are both competitive with respect to substrate. On the other hand, the Ordered Uni Bi mechanism gives product inhibition patterns in which Q and P are competitive and noncompetitive inhibitors, respectively, with respect to the substrate. It can be seen then that this kinetic approach allows one to differentiate between these two different mechanisms.

If, in the case of the Random Uni Bi mechanism, steady-state rather than equilibrium assumptions are made, a rather complex rate expression is obtained, which is described by Eq. (V-6):

$$\frac{1}{v} = \frac{1}{V_1}\left[1 + a(P)\right] + \frac{(b + c(P))}{V_1 d(A)}\left[1 + \frac{P}{K_p}\right]. \qquad \text{(V-6)}$$

The coefficients a through d in Eq. (V-6) represent combinations of rate constants. From the symmetry of the Random Uni Bi mechanism, the rate equation in the presence of product Q will be similar to that of Eq. (V-6) except that the coefficients will

be different and P and K_p will be replaced by Q and K_q, respectively. Inspection of the various product inhibition equations reveals that one can readily differentiate between the ordered and random mechanisms, and, furthermore, in the case of the ordered mechanism, the sequence of substrate addition to the enzyme may be ascertained.

It can be seen from Eq. (V-2) that product P will act noncompetitively relative to the substrate, whereas product Q (Eq. (V-3)) is a competitive inhibitor of A. Replots of slopes and intercepts reveal that the products are linear inhibitors. In the case of the steady-state Random Uni Bi mechanism, the products will be noncompetitive inhibitors. The intercept replots will be linear, but slope replots will be parabolic concave up.

3. Two Substrate Systems

ALBERTY (1) derived rate equations for the mechanism of Schemes I-7, I-8, and I-9, which illustrate the utility of initial rate studies with product present. These kinetic expressions are listed as follows

a) Rapid Equilibrium Random Bi Bi (Mechanism I-7)

α) *A + B + Q*

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)} + \frac{K_b}{V_1(B)} + \frac{K_{ia}K_b}{V_1(A)(B)}\left[1 + \frac{Q}{K_{iq}}\right] \qquad \text{(V-7)}$$

β) *A + B + P*

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)} + \frac{K_b}{V_1(B)} + \frac{K_{ia}K_b}{V_1(A)(B)}\left[1 + \frac{P}{K_{ip}}\right] \qquad \text{(V-8)}$$

It can be seen from Eq. (V-7) that, when either substrate A or B is varied and the other substrate held constant at subsaturating concentrations, product Q will seem to be a competitive inhibitor of the varied substrate. When the fixed substrate is saturating, at subsaturating levels of product, product inhibition will not be manifested. It can be appreciated from symmetry considerations that both products act as competitive inhibitors in the Random Bi Bi mechanism. Finally, slope replots give rise to linear competitive inhibition for this mechanism.

It will be shown below (Sec. V-4) that for this mechanism, one product and one substrate are expected to bind simultaneously to the enzyme.

b) Ordered Bi Bi (Mechanism I-8)

α) *A + B + Q*

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)}\left[1 + \frac{Q}{K_{iq}}\right] + \frac{K_b}{V_1(B)} + \frac{K_{ia}K_b}{V_1(A)(B)}\left[1 + \frac{Q}{K_{iq}}\right] \qquad \text{(V-9)}$$

β) A + B + P

$$\frac{1}{v} = \frac{1}{V_1}\left[1 + \frac{P}{K_{ip}}\right] + \frac{K_a}{V_1(A)} + \frac{K_b}{V_1(B)}\left[1 + \frac{K_q(P)}{K_{iq}K_p}\right] + \frac{K_{ia}K_b}{V_1(A)(B)}\left[1 + \frac{K_q(P)}{K_{iq}K_p}\right] \qquad \text{(V-10)}$$

Note that K_{ip} is defined as $(k_5 + k_7)/k_6$.

Equation (V-9) indicates that the product inhibitor Q will act as a competitive inhibitor of A, and as a noncompetitive inhibitor of B. Both inhibitions are of the linear type when slope and intercept replots are made. On the other hand, in the presence of product P, inhibition with respect to both substrates will be linear noncompetitive.

When the enzyme is saturated by substrate A, inhibition by Q will not be observed. When the enzyme is saturated by substrate A, P will give linear noncompetitive inhibition with respect to B; however, saturation by substrate B will lead to uncompetitive inhibition with respect to substrate A. This description of product inhibition effects leads to the conclusion that a differentiation can be made between the Random and Ordered Bi Bi mechanisms and furthermore, in the latter case, substrates A and B and products P and Q can be defined by the inhibition patterns.

c) Theorell-Chance Mechanism (Scheme I-9)

α) A + B + Q

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)}\left[1 + \frac{Q}{K_{iq}}\right] + \frac{K_b}{V_1(B)} + \frac{K_{ia}K_b}{V_1(A)(B)}\left[1 + \frac{Q}{K_{iq}}\right] \qquad \text{(V-11)}$$

β) A + B + P

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)} + \frac{K_b}{V_1(B)}\left[1 + \frac{P}{K_{ip}}\right] + \frac{K_{ia}K_b}{V_1(A)(B)}\left[1 + \frac{P}{K_{ip}}\right] \qquad \text{(V-12)}$$

Note that K_{ip} is defined as k_5/k_4.

Equation (V-11) shows that product Q is a competitive inhibitor for A and a noncompetitive inhibitor with respect to substrate B. The product P as indicated by Eq. (V-12) acts as a competitive inhibitor of B, but it is noncompetitive with respect to substrate A. Saturation experiments by A will nullify inhibition by Q, whereas if B is the saturating substrate inhibition by P will not be observed. If B is saturating in the presence of Q, the product will display competitive inhibition with respect

to A. Similarly, if the system is saturated by A, in the presence of P, inhibition will be competitive with respect to B.

Table V-1 illustrates the product inhibition patterns to be expected for the mechanisms illustrated by Eq. (I-7), (I-8), and (I-9). It can be seen by reference to the table that a choice between these three mechanisms can be made by using the protocol of product inhibition.

Table V-1. Product inhibition patterns for bireactant systems [a,b]

Mechanism	Product	Varied Substrate	
		A	B
Theorell-Chance	P	NC[c](NC)[d]	C[e](C)
and			
iso Theorell-Chance	Q	C(C)	NC(NC)
Ordered Bi Bi	P	NC[c](NC)	NC[e](NC)
and			
iso Ordered Bi Bi	Q	C(C)	NC(NC)
Random Bi Bi	P	NC(C)	C(C)
	Q	C(C)	NC(C)
Ping Pong Bi Bi	P	NC[e](NC)	NL[f](C)
	Q	C(C)	NL[f](NC)

[a] In this analysis those terms that result in substrate inhibition due to abortive ternary complex formation are not considered; e.g., the B/V_1K_{Ib} term in Eq. (V-21)
[b] The abbreviations are C (Competitive), NC (Noncompetitive), and NL (Nonlinear).
[c] Intercept replots against inhibitor are parabolic concave up.
[d] Inhibition patterns in parenthesis indicate no abortive ternary complex formation.
[e] Slope replots against inhibitor are parabolic concave up.
[f] Hyperbolic concave up. It is assumed that abortive binary complexes form.

4. Abortive Ternary Complex Formation

FROMM and NELSON (5, 6) undertook product inhibition experiments in 1961 with the enzyme ribitol dehydrogenase from *Aerobacter aerogenes*. It became clear almost at the outset of these studies that the product inhibition findings obtained in the laboratory could not be explained on the theoretical grounds proposed a few years earlier (1). It was soon recognized that the kinetic

studies were complicated by the formation of inactive complexes, called abortive ternary complexes, and modification of the theory of product and substrate inhibition was required.

Abortive complexes are dead end complexes and are of the type enzyme-substrate-product within the context of this discussion. It should be emphasized that the product in the complex is the product of the substrate that may not bind to the free enzyme. Many of these complexes have been characterized using optical techniques. One of the best examples of an abortive ternary complex is enzyme-NAD^+-pyruvate, which occurs in the muscle lactate dehydrogenase reaction (7, 8). In Fig. V-2 is shown a difference spectrum tracing for the enzyme-NAD^+-pyruvate abortive (7). The kinetics of formation of this particular complex and its implications in the mechanism of enzyme action and regulation constitute an interesting subject of enzyme research (9).

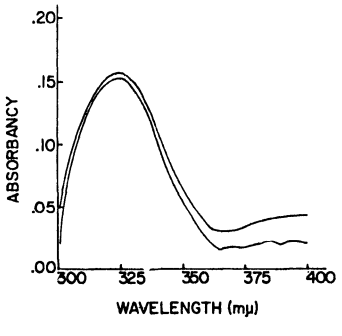


Fig. V-2

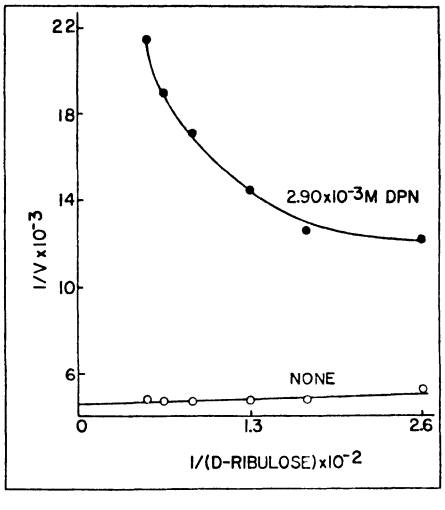


Fig. V-3

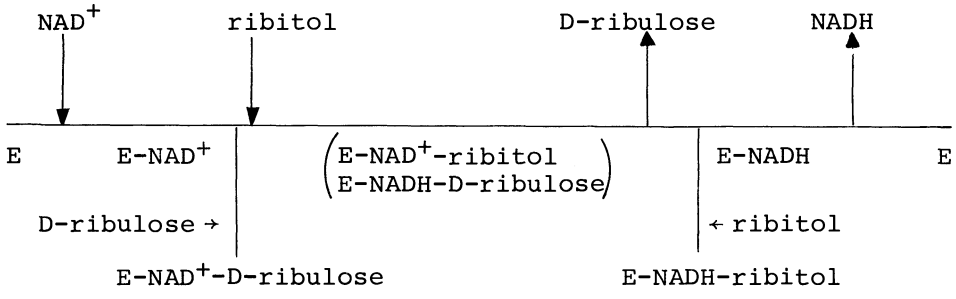
Fig. V-2. Spectrum of an abortive ternary complex. Lactic dehydrogenase, 17.2 mg; NAD, 0.715 mM; Tris-chloride buffer (pH 7.6) 95.3 mM; sodium pyruvate, 11.9 mM. Final vol. 2.1 ml. The upper curve was obtained by reading enzyme and pyruvate (reference) against enzyme, NAD, and pyruvate minus water (reference) against NAD. The lower curve was obtained by reading enzyme and NAD (reference) against enzyme, NAD, and pyruvate minus water (reference) against pyruvate. A curve essentially superimposable on the lower curve was obtained by reading enzyme (reference) against enzyme, NAD, and pyruvate minus water (reference) against NAD and pyruvate. The figure is from Reference (7)

Fig. V-3. Plot of reciprocal of initial reaction velocity (v) *versus* reciprocal of molar concentration of D-ribulose. NADH was held constant at 2.73 x 10^{-4} M, and D-ribulose varied in the range of 3.85 x 10^{-3} M to 1.92 x 10^{-2} M. The concentration of NAD^+ is shown on the graph. From the data of FROMM and NELSON (6)

Exactly how abortive ternary complex formation may be recognized and how it may influence product inhibition studies is illustrated in Fig. V-3 with the ribitol dehydrogenase system. The ribitol dehydrogenase reaction is,

$$\text{ribitol} + \text{NAD}^+ \rightleftharpoons \text{D-ribulose} + \text{NADH} + \text{H}^+ \qquad \text{(V-13)}$$

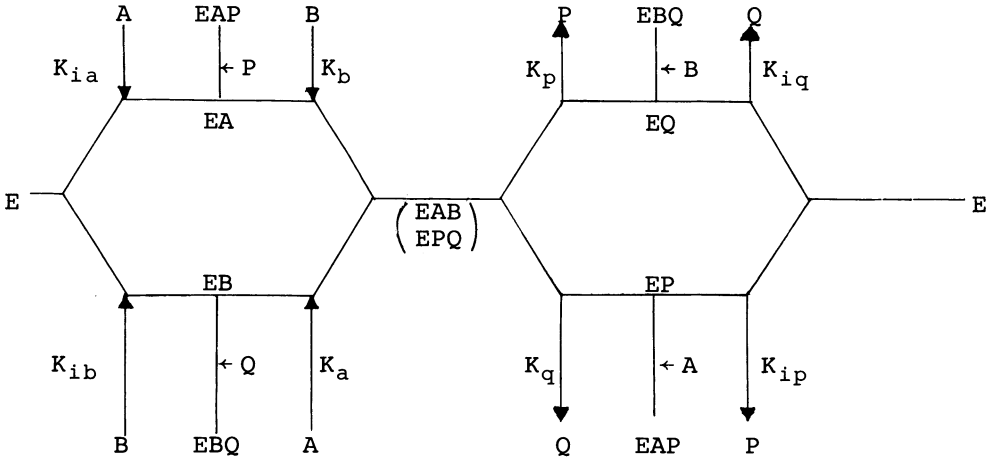
It is quite clear that the hyperbolic concave up results of Fig. V-3 cannot be accounted for by the product inhibition kinetic expressions of Eqs. (V-7) to (V-12). In order to explain these and other observations the following scheme was proposed (5, 6),



Scheme V-1

It will be shown that, when the rate equations for the Ordered Bi Bi mechanism are modified to take into account the abortives described in Scheme V-1, the product inhibition data of Fig. V-3 may be explained. We now know that formation of certain abortive complexes may be kinetically important, whereas others may not form readily. For example, the dissociation constant of the E-NAD$^+$-D-ribulose complex is about 0.5 mM, whereas the dissociation constant for the E-NADH-ribitol complex is 0.43 M (6). These differences in dissociation constants for the different types of abortive complexes that form with a particular enzyme system are common enough in the literature to be expected. This information provides some insight into the enzyme's specificity for certain substrate structures; however, it also serves to complicate product inhibition analysis of kinetic mechanisms. Listed below are the assumptions and rate equations for a number of bireactant mechanisms along with the appropriate rate equations. Table V-1 describes the expected results of these studies.

a) Rapid Equilibrium Random Bi Bi (Mechanism I-7)



Scheme V-2

α) A + B + P

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)} + \frac{K_b}{V_1(B)}\left[1 + \frac{P}{K_{Ip}}\right] + \frac{K_{ia}K_b}{V_1(A)(B)}\left[1 + \frac{P}{K_{ip}}\right] \qquad (V\text{-}14)$$

It can be seen from Scheme V-2 that the product P can add to the free enzyme and it may also add to the EA complex to form the abortive ternary complex EAP. Alternatively, in the random mechanism, A may add to the EP complex. Abortive ternary complex formation may be characterized as follows,

$$EA + P = EAP,\ K_{Ip}; \quad EP + A = EAP,\ K_{Ia}. \qquad (V\text{-}15)$$

Equation (V-14) shows how the abortive EAP complex affects the initial reaction velocity. In the derivation of the rate expression, the two extra complexes, EP and EAP, are included.

β) A + B + Q

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)}\left[1 + \frac{Q}{K_{Iq}}\right] + \frac{K_b}{V_1(B)} + \frac{K_{ia}K_b}{V_1(A)(B)}\left[1 + \frac{Q}{K_{iq}}\right] \qquad (V\text{-}16)$$

By analogy with product P, Q may react as follows,

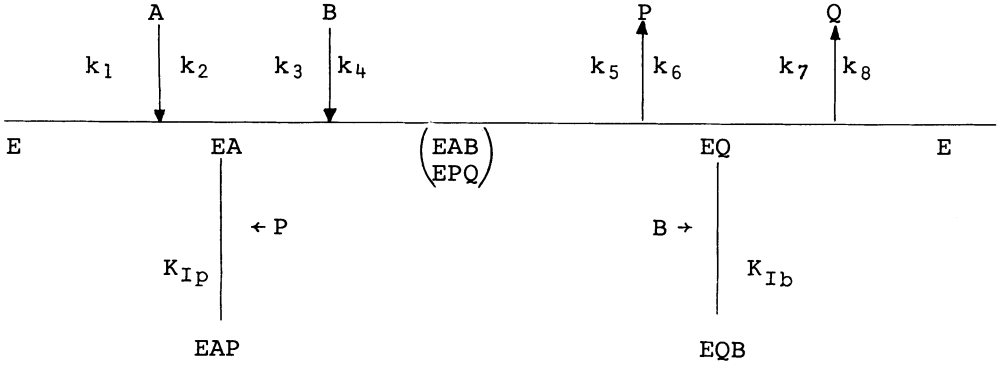
$$E + Q = EQ,\ K_{iq}; \quad EQ + B = EBQ,\ K_{Ib}; \quad EB + Q = EBQ,\ K_{Iq} \qquad (V\text{-}17)$$

It is important to note that there are basic differences in the types of rate equations obtained when abortive complex formation

occurs. When abortives do not form, the product inhibition patterns are competitive with respect to either substrate (Eqs. (V-7) and (V-8)) at subsaturating concentrations of the fixed substrate. Saturating with the nonvaried substrate may be a useful technique in the case of the Random Bi Bi mechanism; however, it may lead to serious complications with ordered mechanisms.

It should be noted that abortives are to be expected in random mechanisms; e.g., between the enzyme, substrate, and the geometrically smaller product.

b) Ordered Bi Bi (Mechanism I-8)



Scheme V-3

α) A + B + P

$$\frac{1}{v} = \frac{1}{V_1}\left[1 + \frac{P}{K_{ip}}\right] + \frac{K_a}{V_1(A)} + \frac{K_b}{V_1(B)}\left[1 + \frac{K_q(P)}{K_{iq}K_p}\right]\left[1 + \frac{P}{K_{Ip}}\right] + \frac{K_{ia}K_b}{V_1(A)(B)}\left[1 + \frac{K_q(P)}{K_{iq}K_p}\right]. \qquad \text{(V-18)}$$

Note that $K_{ip} = (k_5 + k_7)/k_6$.

In Eq. (V-18) it was assumed that the abortive EQB does not form. It may be included in the equation by simply adding the term $B/V_1(K_{IB})$ which is defined below. Equation (V-18) predicts that P will act as a noncompetitive inhibitor of substrates A and B. Closer inspection reveals that the kinetic expression is second order in P. In a plot of 1/v *versus* 1/A, the intercept terms, when graphed as a function of P, will be parabolic concave up. When P is present as a product inhibitor at several different concentrations in a 1/v *versus* 1/B plot, there will be a P^2 term in the slope. A secondary plot of slopes *versus* P will also be parabolic concave up.

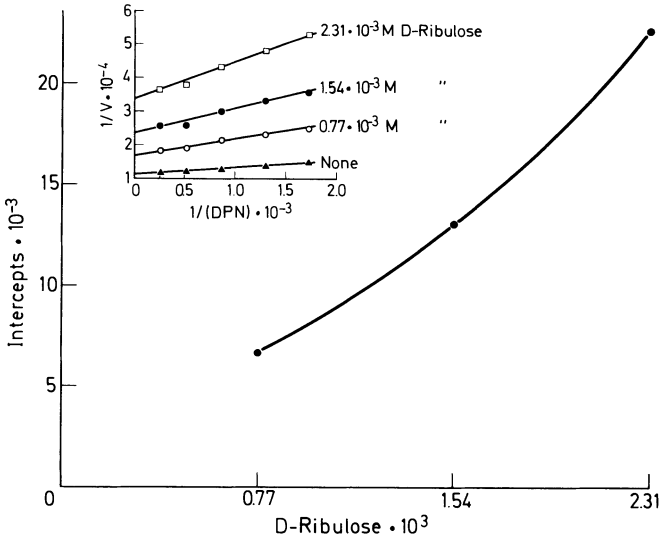


Fig. V-4. Plot of reciprocal of initial reaction velocity (v) *versus* reciprocal of molar concentration of NAD and a replot of the intercepts as a function of D-ribulose concentration. The concentrations of D-ribulose are shown on the graph. Ribitol concentration was held constant at 9.6×10^{-3} M, and NAD varied in the range of 5.76×10^{-4} M to 3.85×10^{-3} M. The data are from Reference (6)

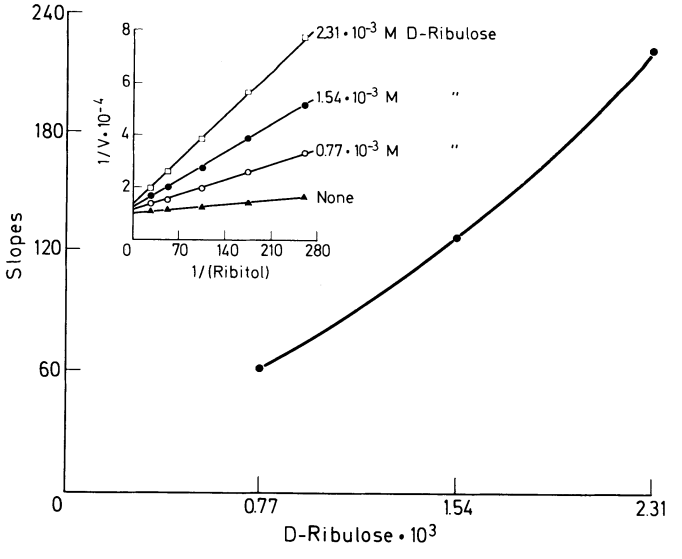


Fig. V-5. Plot of reciprocal of initial reaction velocity (v) *versus* reciprocal of molar concentration of ribitol and a replot of the slopes as a function of D-ribulose concentration. The concentrations of D-ribulose are shown on the graph. NAD^+ concentration was held constant at 2.88×10^{-3} M, and ribitol varied in the range of 3.85×10^{-3} M to 3.85×10^{-2} M. The data are from Reference (6)

Figure V-4 illustrates product inhibition by D-ribulose in the ribitol dehydrogenase reaction when NAD^+ is the varied substrate. Note that the intercepts in the primary plot do not appear to increase in a linear fashion. The nonlinear effect of D-ribulose on the intercepts is also seen in the figure. Thus the inhibition is I-parabolic S-linear.

Figure V-5 depicts the effect of D-ribulose when ribitol is the varied substrate and NAD^+ is held constant. It can be seen that the intercepts appear to increase linearly with product. Figure V-5 also shows that, when the slopes of the primary plot are graphed against D-ribulose, the replot is parabolic concave up. In this case the inhibition is I-linear S-parabolic.

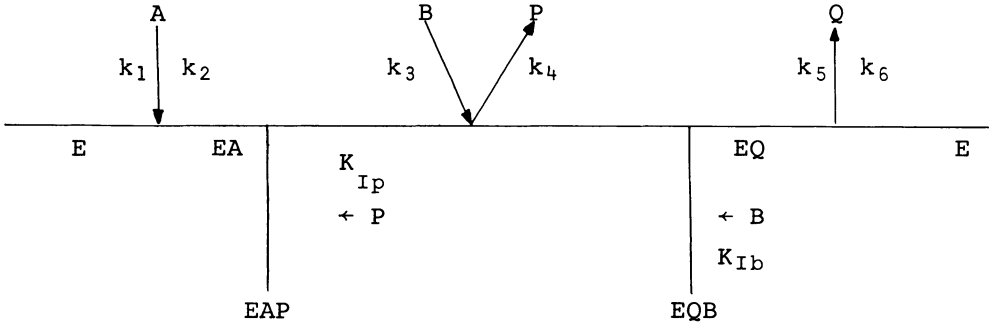
β) A + B + Q

$$\frac{1}{v} = \frac{1}{V_1} + \frac{B}{V_1(K_{IB})} + \frac{K_a}{V_1(A)}\left[1 + \frac{Q}{K_{iq}}\left[1 + \frac{B}{K_{Ib}} + \frac{K_{ia}K_b}{K_aK_{Ib}}\right]\right] + \frac{K_b}{V_1(B)} + \frac{K_{ia}K_b}{V_1(A)(B)}\left[1 + \frac{Q}{K_{iq}}\right]. \qquad \text{(V-19)}$$

In this derivation, $K_{IB} = K_{Ib}(k_5 + k_7)/k_5$.

Equation (V-19) predicts that the product Q will be a competitive inhibitor with respect to substrate A. Inhibition either in the presence or absence of Q will appear to be hyperbolic concave up relative to B. In the absence of Q, the nonlinear effect observed in a 1/v *versus* 1/B plot is a result of the term $B/V_1(K_{IB})$ in Eq. (V-19). Whether this type of substrate inhibition is manifested experimentally depends upon the concentration of B and also upon the value of K_{IB}. Figure V-3 indicates that, in the absence of product NAD^+, there is no discernible inhibition by substrate B; i.e., the factor $B/V_1(K_{IB})$ is relatively small compared with other terms in the rate equation. On the other hand, the term $K_a(Q)(B)/V_1K_{iq}K_{Ib}(A)$ is clearly a dominant function in Eq. (V-19) for the ribitol dehydrogenase system. It should be noted that in Fig. V-3, $NAD^+(Q)$ was 27 times its dissociation constant (K_{iq}). When abortive ternary complexes exhibit relatively high dissociation constants, the inhibitory effect demonstrated in Fig. V-3 does not occur. It should be pointed out that attempting to raise the substrate concentrations to saturating levels may serve to bring out the possible existence of abortive complexes. Unfortunately, the procedure may give rise to nonspecific complexes of the EB type, which one would not suspect as a likely candidate in ordered mechanisms.

c) Theorell-Chance Mechanism (I-9)



Scheme V-4

α) A + B + P

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)} + \frac{K_b}{V_1(B)}\left[1 + \frac{P}{K_{ip}}\right]\left[1 + \frac{P}{K_{Ip}}\right] + \frac{K_{ia}K_b}{V_1(A)(B)}\left[1 + \frac{P}{K_{ip}}\right] \qquad \text{(V-20)}$$

Note that K_{ip} is defined as k_5/k_4.

In the derivation of Eq. (V-20), it is assumed that the abortive EQB does not form; however, the equation can readily be altered to include this abortive.

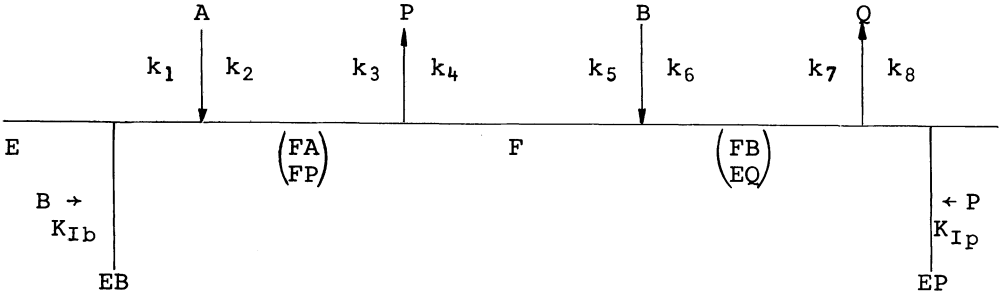
The only real difference between Eq. (V-20) and Eq. (V-18), the analogous expression for the Ordered Bi Bi mechanism, is the absence of the $P/V_1 \cdot K_{ip}$ term in Eq. (V-20). These two mechanisms are readily distinguished by the fact that competitive product inhibition by P will never occur in the mechanism of Scheme V-3, whereas P will seem to be a nonlinear competitive inhibitor of substrate B in the Theorell-Chance mechanism.

β) A + B + Q

$$\frac{1}{v} = \frac{1}{V_1}\left[1 + \frac{B}{K_{Ib}}\right] + \frac{K_a}{V_1(A)}\left[1 + \frac{Q}{K_{iq}}\left[1 + \frac{B}{K_{Ib}} + \frac{K_{ia}K_b}{K_aK_{Ib}}\right]\right] + \frac{K_b}{V_1(B)} + \frac{K_{ia}K_b}{V_1(A)(B)}\left[1 + \frac{Q}{K_{iq}}\right] \qquad \text{(V-21)}$$

Equation (V-21) is essentially identical to Eq. (V-19) for the Ordered Bi Bi mechanism. Thus it is not possible to make a choice between the Theorell-Chance and Ordered Bi Bi mechanisms on the basis of product inhibition studies with product Q.

d) Ping Pong Bi Bi



Scheme V-5

α) A + B + P

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)}\left[1 + \frac{B}{K_{Ib}} + \frac{P}{K_{Ip}}\right] + \frac{K_b}{V_1(B)}\left[1 + \frac{P}{K_{ip}}\right] + \frac{K_{ia}K_b(P)}{V_1K_{ip}(A)(B)}\left[1 + \frac{B}{K_{Ib}} + \frac{P}{K_{Ip}}\right]. \qquad \text{(V-22)}$$

Note that for this mechanism $K_{ia} = k_2/k_1$ and $K_{ip} = k_3/k_4$.

If the primary plot of 1/v *versus* 1/B is not hyperbolic concave up, the B/K_{Ib} term can be deleted from Eq. (V-22). If we assume that the EB binary complex is not kinetically important, the 1/v *versus* 1/A plots in the presence of product P will appear to be noncompetitive. Replots of slopes and intercepts *versus* P will be S-parabolic, I-linear. In the case of 1/v *versus* 1/B plots when P is present, the inhibition will also be noncompetitive. The replots against P will be S-parabolic, I-linear.

In the event that abortive binary complexes EB and EP do not form, P will be a linear noncompetitive inhibitor of substrate A and a linear competitive inhibitor of B.

β) A + B + Q

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)}\left[1 + \frac{B}{K_{Ib}} + \frac{Q}{K_{iq}}\right] + \frac{K_b}{V_1(B)} + \frac{K_{ib}K_a(Q)}{V_1K_{iq}(A)(B)}. \qquad \text{(V-23)}$$

Note that $K_{ib} = k_6/k_5$ and $K_{iq} = k_7/k_8$.

If the abortive binary complex forms in the Ping Pong Bi Bi system, the primary double reciprocal plot when B is the variable substrate will be hyperbolic concave up. If this abortive does not form, inhibition by Q will be linear competitive with respect to A and linear noncompetitive with respect to substrate B.

Table V-1 illustrates the product inhibition patterns to be expected for some Bi Bi mechanisms when abortive ternary complexes are formed. When evaluating the product inhibition patterns expected from the rate equations, it was assumed that the concentrations of substrates were held at a level so as to preclude substrate inhibition. For example, Eq. (V-19) predicts that, in the *absence* of product, substrate B will cause hyperbolic concave up substrate inhibition. It is possible to carry out experiments conveniently in the region where substrate inhibition is minimal and yet where it is possible to discern inhibition caused by abortive ternary complex formation.

The possibility that abortive ternary complexes may form, limits the utility of product inhibition kinetics. For example, it is not possible to make a choice between the Theorell-Chance mechanism in the absence of abortive ternary complex formation and the Random Bi Bi case with production of these complexes. It is difficult to predict in advance the likelihood that abortives will form, and a definitive conclusion on this point may only be reached in the laboratory; however, kinetic and spectral investigations suggest that most enzyme systems do form abortive ternary complexes.

In the derivation of product inhibition rate equations, it is tacitly assumed that the product fits neatly into one of the substrate-product pockets. This may indeed be a reasonable approximation with many enzymes, e.g., pyridine linked anerobic dehydrogenases. However, for an enzyme such as yeast hexokinase, glucose 6-phosphate may be expected to bind at both the glucose and ATP sites. Under these conditions the product inhibition pattern may be noncompetitive relative to either substrate. If substrate inhibition does not occur, the type of binding by the product may be investigated by saturating the enzyme with one substrate and determining the kinetics of product inhibition relative to the other substrate. WETTERMARK et al. (10) found, for example, that when hexokinase was saturated with glucose, inhibition by glucose 6-phosphate was competitive relative to ATP. It was clear from these and other product inhibition experiments (11) that binding by glucose 6-phosphate was primarily at the ATP site. From studies of this kind, it was concluded that the phosphorylated product binds primarily to the γ-phosphoryl group portion of the ATP site, or at the prosphoribosyl part of the ATP site of yeast hexokinase.

Table V-2 illustrates the product inhibition patterns to be expected for a number of terreactant enzyme system. To obtain the patterns listed in Table V-2 it was assumed that abortive complex formation does not occur. The usefulness of product inhibition experiments is seriously compromised if such complexes form.

For experiments of terreactant systems two substrates are held at approximately their Michaelis constant levels and the other substrate varied. Experiments are carried out with and without product. The product inhibition patterns in most cases shown in Table V-2 seem unique, and there is no need to carry out ex-

periments in which one of the substrates is held constant at a "saturating" concentration. This procedure often results in substrate inhibition through abortive complex formation, and its use should be attended with caution.

Table V-2. Product inhibition patterns for terreactant systems

Mechanism	Product	Varied substrate		
		A	B	C
Ordered Ter Ter (I-12)	P	NC[a]	NC	NC
	Q	U	U	U
	R	Comp	NC	NC
Random Ter Ter (I-13)	P	Comp	Comp	Comp
	Q	Comp	Comp	Comp
	R	Comp	Comp	Comp
Partially Random AB (I-14)	P	-	-	-
(C last on)	Q	Comp	Comp	Comp
	R	Comp	Comp	Comp
Partially Random BC (I-15)	P	-	-	-
(A first on)	Q	-	-	-
	R	Comp	Comp	Comp
Partially Random AC (I-16)	P	Comp	Comp	Comp
(B second on)	Q	-	-	-
	R	Comp	Comp	Comp
Hexa Uni Ping Pong (I-17)	P	NC	Comp	U
	Q	U	NC	Comp
	R	Comp	U	NC
Ordered Bi Uni Uni Bi Ping Pong (I-18)	P	NC	NC	Comp
	Q	U	U	NC
	R	Comp	NC	U
Ordered Uni Uni Bi Bi Ping Pong (I-19)	P	NC	Comp	NC
	Q	U	NC	NC
	R	Comp	U	U
Random Bi Uni Uni Bi Ping Pong (I-20)	P	NC	NC	Comp
	Q	U	U	Comp
	R	Comp	Comp	U
Random Uni Uni Bi Bi Ping Pong (I-21)	P	NC	Comp	Comp
	Q	U	NC	NC
	R	Comp	U	U

[a] The abbreviations are: NC (noncompetitive), U (uncompetitive), and Comp (competitive).

5. Calculation of Rate Constants from Product Inhibition Experiments

A knowledge of the values of the rate constants associated with an enzymic mechanism may give the kineticist insight into the catalytic mechanism as well as into the nature of the rate limiting steps in the reaction sequence. One advantage of doing product inhibition experiments is that the investigator may calculate the individual rate constants from experiments in a single direction only provided he has information on the K_{eq}. To illustrate this point, consider Eqs. (V-18) and (V-19) without the abortive complexes EAP and EQB. In the absence of product, the parameters V_1, K_a, K_b, and K_{ia} may be evaluated as described in Chapter III. In the presence of product Q, plots of 1/v against 1/B at different levels of Q permit one to make both slope and intercept replots. The intercept on the 1/v axis for the noncompetitive product inhibition plot of Eq. (V-19) is

$$\text{Intercept} = \frac{1}{V_1}\left[1 + \frac{K_a}{A}\left(1 + \frac{Q}{K_{iq}}\right)\right]. \qquad \text{(V-24)}$$

K_{iq} which is k_7/k_8 can be evaluated from Eq. (V-24) from a knowledge of V_1 and K_a. This value can also be compared with the number determined from the slope replot,

$$\text{Slope} = \frac{1}{V_1}\left[K_b + \frac{K_{ia}K_b}{A}\left[1 + \frac{Q}{K_{iq}}\right]\right]. \qquad \text{(V-25)}$$

The rate constant k_1 can be determined from the equation,

$$k_1 = \frac{V_1}{K_a E_o}. \qquad \text{(V-26)}$$

If the equivalent weight of the enzyme is not known, all the rate constants will be in terms of enzyme concentration, E_0. It is important in this regard to adjust all kinetic experiments to a constant enzyme concentration (see discussion in Chapter III-E).

The rate constant k_2 can now be calculated from the identity

$$K_{ia} = \frac{k_2}{k_1}. \qquad \text{(V-27)}$$

It can be seen from Eq. (V-18) that, when 1/v is graphed as a function of 1/A in the presence of P, the slope term is

$$\text{Slope} = \frac{1}{V_1}\left[K_a + \frac{K_{ia}K_b}{(B)}\left[1 + \frac{K_q(P)}{K_{iq}K_p}\right]\right]. \qquad \text{(V-28)}$$

If a secondary plot is made of slope *versus* P, the resulting slope term is

$$\text{Slope} = \frac{K_{ia}K_bK_q}{V_1K_{iq}K_p(B)} = \frac{k_2k_4k_6}{k_1k_3k_5k_7(B)E_0}. \qquad \text{(V-29)}$$

If both B and K_{eq} are known, it is possible to calculate k_8 from Eq. (V-29) and the expression,

$$K_{eq} = \frac{k_1k_3k_5k_7}{k_2k_4k_6k_8}. \qquad \text{(V-30)}$$

The rate constant k_7 can then be calculated from the identity

$$K_{iq} = \frac{k_7}{k_8}. \qquad \text{(V-31)}$$

A knowledge of V_1 and k_7 allows determination of k_5 from the equation

$$V_1 = \frac{k_5k_7E_0}{(k_5 + k_7)}. \qquad \text{(V-32)}$$

It can readily be seen from Eq. (V-18) that double reciprocal plots of 1/v *versus* 1/B at different concentrations of P permit evaluation of K_{ip}. With a knowledge of k_5 and k_7, k_6 can be determined from the relationship,

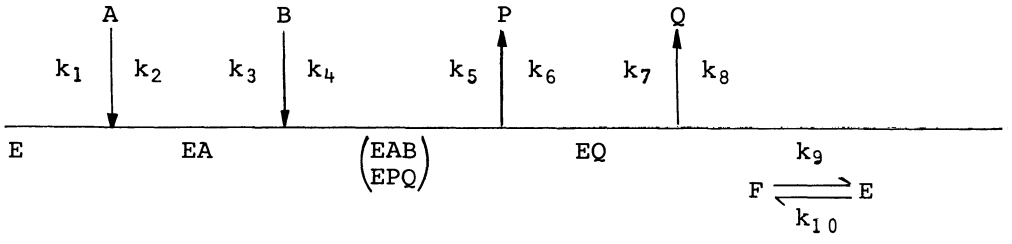
$$K_{ip} = \frac{(k_5 + k_7)}{k_6}. \qquad \text{(V-33)}$$

The remaining two rate constants, k_3 and k_4, may be determined from a knowledge of the known rate constants, K_{eq}, and K_b. These rate constants may also be evaluated by similar, but alternative manipulations.

6. Noncompetitive Product Effects

Cursory scrutiny of the various rate equations for bireactant and terreactant systems in the presence of product indicates that, in all cases, except where substrate inhibition occurs, one of the products will act as a competitive inhibitor of one of the substrates at subsaturating concentrations of the nonvaried substrate. There are at least three types of mechanisms in which this effect does not occur. One involves mechanisms in which a stable enzyme form isomerizes so that substrate A, which goes on the enzyme first, and product Q, which leaves the enzyme last in ordered mechanisms, combine with different enzyme forms.

Consider for example the Iso mechanism depicted in Scheme V-6.



Scheme V-6

In the presence of the product Q, the rate equation for this mechanism is

$$\frac{1}{v} = \frac{1}{V_1}\left[1 + \frac{Q}{K_{Iq}}\right] + \frac{K_a}{V_1(A)}\left[1 + K_E\left[1 + \frac{Q}{K_{iq}}\right]\right] + \frac{K_b}{V_1(B)} + \frac{K_{ia}K_b}{V_1(A)(B)}\left[1 + K_E\left[1 + \frac{Q}{K_{iq}}\right]\right] . \qquad \text{(V-34)}$$

In Eq. (V-34), $K_{Iq} = k_7k_9/k_8$ and $K_E = k_{10}/k_9$. The striking feature of this kinetic equation is that the Lineweaver-Burk plots will always show a linear intercept increase as the concentration of Q is increased. No analogous assumptions regarding isomerizations are actually required to establish the fact that inhibition by product P does not permit competitive product inhibition. Saturating concentrations of fixed substrate will not eliminate the intercept increases observed when considering Eq. (V-34).

It is possible that under certain conditions the rapid equilibrium Random Bi Bi mechanism may also respond to substrate and product inhibition in a manner that may preclude competitive product effects. Consider for example that, in Scheme V-2, EA can combine with Q to form EAQ as follows:

$$EA + Q = EAQ;\ K_{Iq}'. \qquad \text{(V-35)}$$

Equation (V-16) may then be modified to give Eq. (V-36).

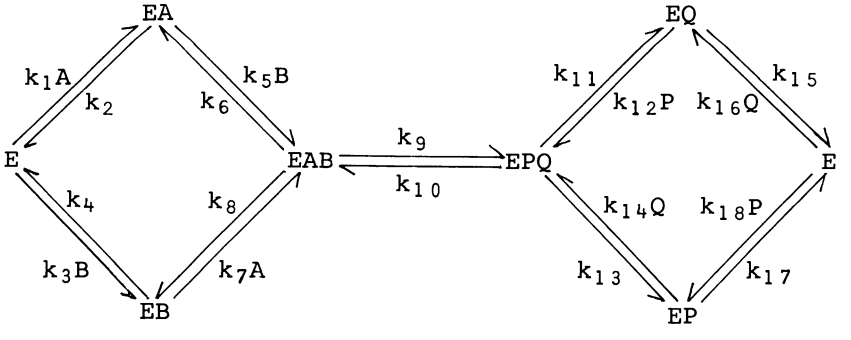
$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)}\left[1 + \frac{Q}{K_{Iq}}\right] + \frac{K_b}{V_1(B)}\left[1 + \frac{Q}{K_{Iq}'}\right] + \frac{K_{ia}K_b}{V_1(A)(B)}\left[1 + \frac{Q}{K_{iq}}\right] \qquad \text{(V-36)}$$

By analogy, Eq. (V-14) could be altered to include an EBP abortive, and the $K_a/V_1(A)$ term would be modified by a factor such as $(1 + P/K_{Ip}')$ where K_{Ip}' would be the dissociation constant for the EBP abortive.

If the hexokinase reaction is assumed to be rapid equilibrium Random Bi Bi, the following abortives would prevent one or both of the products from acting as a competitive product inhibitor: enzyme-glucose-glucose-6-P, enzyme-ATP-glucose-6-P, enzyme-glucose-ADP, and enzyme-ATP-ADP. It is rather doubtful that all four abortives could form in product inhibition experiments; however, the possibility does exist and should be considered in the absence of competitive product inhibition. In these particular examples, glucose-6-P is a product of both substrates and could resonably occupy either substrate site, thus allowing either substrate to bind the enzyme simultaneously with glucose-6-P. A complex of enzyme, glucose, and ADP is also quite reasonable as an abortive for hexokinase; however, the last abortive is somewhat difficult to rationalize. Here too, it is possible that the ribosyl moiety of ADP could bind at the glucose site while ATP occupies its normal locus on the enzyme.

The mechanism outlined in Scheme I-7 assumes that all steps in the reaction equilibriate rapidly relative to the interconversion of the ternary complexes. It is reasonable to assume that this limiting assumption will not always prevail. Under these circumstances, the rate equation will assume a more complex character, i.e., Eq. (V-37). At first glance it would seem from the second degree terms in the rate equation that double reciprocal plots of 1/v *versus* 1/substrate should appear nonlinear. In 1954 SCHWERT (12) made some calculations that led him to conclude that the expected nonlinearity for this mechanism could be so subtle as to be undetectable. DALZIEL (13), CLELAND and WRATTEN (14), and RUDOLPH and FROMM (15) have presented methods for reduction of the steady-state rate expression to that obtained if the limiting equilibrium assumption is made.

The steady-state Random Bi Bi mechanism is illustrated in Scheme V-7 and has the form:



Scheme V-7

$$v = \frac{K_1AB + K_2AB^2 + K_3A^2B}{K_4 + K_5A + K_6B + K_7AB + K_8A^2 + K_9B^2 + K_{10}A^2B + K_{11}AB^2} \qquad \text{(V-37)}$$

It is possible to reduce Eq. (V-37) to the expression obtained if the rapid equilibrium assumption is made provided $k_2 > k_5(B)$ and $k_4 > k_7(A)$. It would appear that these inequality relationships are dependent upon the concentrations of substrates A and B; however, the substrate levels cannot alter the relationship between the rate constants, except where A and B equal infinity. Realistically, for enzyme-catalyzed reactions, the upper limit for substrate concentration is in the range of 1 M. Thus, if the inequality holds it will do so over the entire experimental substrate concentration range.

The characteristics of the steady-state Random Bi Bi mechanism were tested using the IBM 360/65 computer by RUDOLPH and FROMM (16). They programmed Eq. (V-37), which contains eight numerator and seventy-six denominator terms, in FORTRAN/IV. Kinetic data provided by DELAFUENTE and SOLS (17) for yeast hexokinase were used, and a value of 10 was assumed for the ratio of $k_9:k_{10}$. This value was chosen because it is close to that obtained from the apparent equilibrium constant (18). To calculate a relative velocity, the unimolecular rate constants were given values relative to k_9, and the bimolecular rate constants were defined by the assumed dissociation constants. With the rate constants given in Fig. V-6, the theoretical reciprocal plot depicted

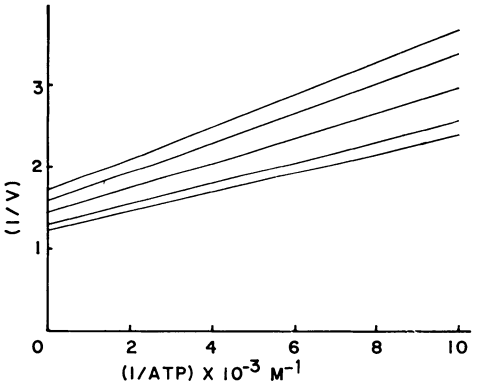


Fig. V-6. A double reciprocal plot of the calculated relative initial velocity of yeast hexokinase *versus* different substrate concentrations assuming a steady-state random mechanism. ATP and glucose concentrations were varied from 0.1 to 1.0 mM. The assumed rate constants were: k_1, 2,500,000 M^{-1} sec^{-1}; k_2, 500 sec^{-1}; k_3, 1,250,000 M^{-1} sec^{-1}; k_4, 100 sec^{-1}; k_5, 12,500 M^{-1} sec^{-1}; k_6, 1 sec^{-1}; k_7, 25,000 M^{-1} sec^{-1}; k_8, 5 sec^{-1}; k_9, 1 sec^{-1}; k_{10}, 0.1 sec^{-1}; k_{11}, 1 sec^{-1}; k_{12}, 125 M^{-1} sec^{-1}; k_{13}, 5 sec^{-1}; k_{14}, 2,500 M^{-1} sec^{-1}; k_{15}, 500 sec^{-1}; k_{16}, 250,000 M^{-1} sec^{-1}; k_{17}, 100 sec^{-1}; k_{18}, 12,500 M^{-1} sec^{-1}. Velocity (v) is the relative number calculated by the computer

in Fig. V-6 is obtained. It was found that, as the outer rate constants (k_2, k_4, k_{15}, k_{17}) were made larger than k_9, the lines became more linear. In this case it was assumed that the dissociation constants for a particular substrate were not affected by the presence of the other substrate. This

is inferred by the fact that the experimental reciprocal plots intersect at or near $1/v = 0$(17, 19, 20). As will be shown, this is not a necessary assumption for the steady-state mechanism, and it was found that, when the dissociation constants were varied independently of each other, the calculated plots also simulated the experimental situation. Linear plots are also obtained if either k_9 or k_{11} and k_{17} are made much lower than the other rate constants. It seems that, even when the assumptions made to reduce the complicated rate equation to Eq. (I-17) are not satisfied, but the values are about equal, the reciprocal plots will remain linear.

It is known that both glucose-6-P and ADP are noncompetitive product inhibitors for either substrate with yeast hexokinase. In order to determine whether such effects are consistent with the steady-state mechanism, the steady-state equation describing product inhibition of hexokinase was derived. The formation of an abortive complex EBQ in the presence of product Q was assumed. The postulated interactions are

$$EB + Q \underset{k_{20}}{\overset{k_{19}}{\rightleftharpoons}} EBQ \qquad \text{(V-38)}$$

$$EQ + B \underset{k_{22}}{\overset{k_{21}}{\rightleftharpoons}} EBQ. \qquad \text{(V-39)}$$

The total rate equation has 45 numerator and 672 denominator terms, which include many squared and cubed concentration terms. By assuming that $k_2 > k_5(B)$, $k_4 > k_7(A)$, $k_{15} > k_{21}(B)$, $k_{17} > k_{14}(Q)$, and $k_4 > k_{19}(Q)$, the rate equation may be reduced to the following form:

$$v = \frac{K_1AB}{K_2 + K_3Q + K_4A + K_5B + K_6AB + K_7BQ + K_8ABQ}. \qquad \text{(V-40)}$$

Once again the K's represent various combinations of rate constants.

The total rate equation was programmed as described for the initial rate simulation, and representative plots are depicted in Figs. V-7 and V-8. It can be seen that the product is a noncompetitive inhibitor of both substrates, and this is true whether Q is ADP or glucose-6-phosphate. Only when the unimolecular rate constants for the abortive complex formation are much smaller than the outer rate constants would the inhibition approach competitive. Various combinations of dissociation constants and rate constants were tested and found to generally give similar results to those shown.

The simulations also predict that the effects of competitive inhibitors are the same as suggested by the rapid equilibrium assumption. That is, a competitive inhibitor of one substrate will be noncompetitive relative to the other substrate.

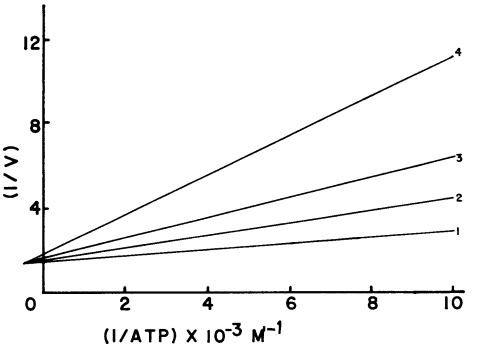


Fig. V-7

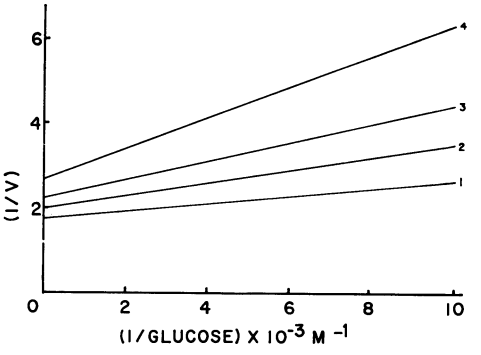


Fig. V-8

Fig. V-7. A double reciprocal plot of the calculated relative initial velocity of yeast hexokinase *versus* the reciprocal of ATP concentration at different assumed levels of ADP. Glucose was assumed constant at 0.2 mM and ATP was varied from 0.1 to 1.0 mM. ADP concentrations were 0 (1), 2.5 (2), 5 (3), and 10 mM (4). The rate constants were the same as in Fig. V-6 with the addition of k_{19}, 100,000 M^{-1} sec^{-1}; k_{20}, 500 sec^{-1}; k_{21}, 20,000 M^{-1} sec^{-1}; k_{22}, 100 sec^{-1}

Fig. V-8. A double reciprocal plot of the calculated relative initial velocity of yeast hexokinase *versus* the reciprocal of glucose concentration at different assumed levels of ADP. ATP was assumed constant at 0.2 mM and glucose was varied from 0.1 to 1.0 mM. ADP concentrations were 0 (1), 2.5 (2), 5 (3), and 10 mM (4). The assumed rate constants were the same as for Fig. V-7

B. Substrate Inhibition

The phenomenon of substrate inhibition is very well documented and is ordinarily attributed to abortive or dead end complex formation between the substrate and one or more enzyme forms. A relatively large fraction of those enzyme systems which have been studied exhibit substrate inhibition, but usually in reverse of the usual reaction direction *in vivo*. Substrate inhibition occurs at elevated substrate concentration; however, it is sometimes observed in the region of the Michaelis constant. In most kinetic studies, the region of substrate concentration that gives rise to substrate inhibition is avoided by the investigator, and extrapolations are made through this concentration range as if alterations in the kinetic patterns did not occur. CLELAND (21) has recently suggested that substrate inhibition may give meaningful information on kinetic mechanisms, and this section attempts to demonstrate how this may be achieved.

1. A Simple Model for Substrate Inhibition

In 1930 HALDANE presented a kinetic model to explain substrate inhibition at high concentrations of substrate (22). The pathway of substrate and enzyme interaction is as follows:

$$E + A \rightleftharpoons EA \overset{k_3}{\rightleftharpoons} E + P$$

$$EA + A \rightleftharpoons EA_2 \overset{k_6}{\rightleftharpoons} E + 2P$$

Scheme V-8

The rate equation for substrate inhibition, making equilibrium assumptions, is

$$v = \frac{V_1}{1 + \frac{K_{ia}}{A} + \frac{A}{K_i}} \qquad \text{(V-41)}$$

where K_i is taken to represent the dissociation constant for the inactive EA_2 complex. Equation (V-41) describes a hyperbola when plotted in double reciprocal form, and this is illustrated in Fig. V-9.

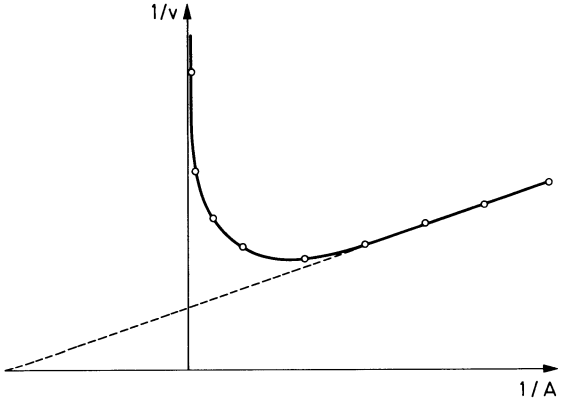


Fig. V-9. Double reciprocal plot of 1/v *versus* 1/A of Eq. (V-41). The experimental points describe substrate inhibition whereas the linear curve and its extrapolated portion are used to calculate the kinetic parameters K_{ia} and V_1

In this treatment, it is assumed that the EA_2 complex is totally inactive; however, a different rate expression (Eq. (V-42)) is obtained if substrate inhibition is not total (partial substrate inhibition).

$$v = \frac{V_1 \left[1 + \frac{V_1'(A)}{V_1 K_i}\right]}{1 + \frac{K_{ia}}{A} + \frac{A}{K_i}} \qquad \text{(V-42)}$$

In this equation V_1 and V_1' represent k_3E_0 and k_6E_0, respectively. Equation (V-42) differs from the expression for substrate inhibition considered previously in that the velocity will not approach zero as A approaches inifinity, and thus inhibition will not be complete. The plot analogous to that of Fig. V-9 will not have the 1/v axis as an asymptote but instead will intersect the ordinate axis. In this treatment it is assumed that $k_3 > k_6$ and that $K_i = (EA)(A)/(EA_2)$.

2. Two Substrate Systems

DALZIEL (23) attempted to explain substrate inhibition in terms of dead end binary complex formation in the case of the mechanisms of Schemes I-8 and I-9. For example, one need only add the reaction,

$$E + B = EB, \; K_{Ib} \qquad \text{(V-43)}$$

to the Theorell-Chance mechanism (Scheme I-9) to obtain the expression,

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)}\left[1 + \frac{K_{ia}K_b}{K_aK_{Ib}} + \frac{B}{K_{Ib}}\right] + \frac{K_b}{V_1(B)} + \frac{K_{ia}K_b}{V_1(A)(B)}. \qquad \text{(V-44)}$$

DALZIEL (23) refers to this type of inhibition as competitive substrate inhibition; i.e., substrates A and B compete for the free enzyme. When double reciprocal plots of 1/v *versus* 1/A are made, the slopes and intercepts will change as the concentration of B is varied. At low B, $B/K_{Ib} < 1$. As B increases, both slopes

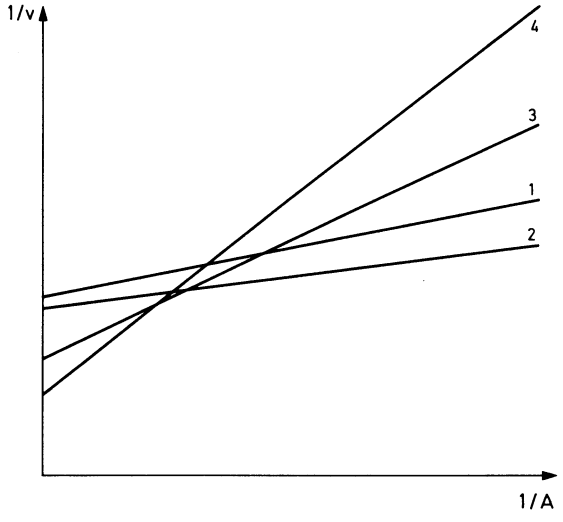


Fig. V-10. Double reciprocal plot of 1/v *versus* 1/A for competitive substrate inhibition. The numbers on the graph indicate increasing concentrations of substrate B

and intercepts will decrease; however, when the factor $B/K_{Ib} > 1$, the slopes will increase, whereas the intercepts will continue to decrease. This effect is illustrated in Fig. V-10. Plots of 1/v *versus* 1/B at different fixed levels of A will tend to be hyperbolic concave up. The nonlinear effect observed in the 1/v *versus* 1/B plot will tend to be minimized as the concentration of A increases.

It is possible to obtain information on how enzymes, substrates, and products interact to form both productive and abortive complexes from kinetic experiments. It is also possible to gain insight into the type of kinetic mechanism from studies of substrate inhibition (21). Cited below are a few examples of substrate inhibition for Sequential and Ping Pong Bi Bi mechanisms and the type of substrate inhibition to be expected for the various mechanisms.

a) Ordered Bi Bi Mechanism

FROMM and NELSON in their studies of product inhibition (5, 6) obtained data that resembled substrate inhibition and that they explained on the basis of abortive ternary complex formation. In the case of the Ordered Bi Bi mechanism, for example, it is possible to get the abortive complex EQB as follows.

$$EQ + B = EQB, \; K_{Ib} \qquad \text{(V-45)}$$

The rate equation, which includes formation of this inhibitory complex, is

$$\frac{1}{v} = \frac{1}{V_1}\left[1 + \frac{B}{K_{Ib}}\right] + \frac{K_a}{V_1(A)} + \frac{K_b}{V_1(B)} + \frac{K_{ia}K_b}{V_1(A)(B)} . \qquad \text{(V-46)}$$

CLELAND (21) refers to this type of inhibition as uncompetitive. Equation (V-46) indicates that plots of 1/v against 1/B will be hyperbolic concave up. When 1/v is graphed against 1/A at different levels of B, the lines will be linear and the family of curves will exhibit decreasing slopes; however, the intercepts will first decrease with B but will increase as the B/V_1K_{Ib} term becomes significant relative to the other intercept terms. Intercept replots *versus* 1/B will be predictably hyperbolic concave up. Figure V-11 illustrates this type of inhibition.

Another type of abortive complex, but one which is much less likely than that described by Eq. (V-45), is

$$EQ + A = EQA, \; K_{Ia}. \qquad \text{(V-47)}$$

When considering this type of substrate inhibition, substrate A may bind either at the B pocket of the enzyme or at some other site that causes inhibition. If association of EQ and A does occur at the B site, obviously the reaction EQ + B = EQB must be weak relative to the interaction of EQ and A.

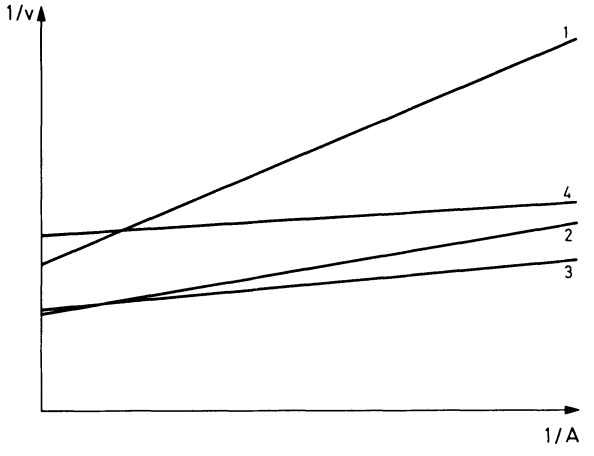


Fig. V-11. Double reciprocal plot of 1/v *versus* 1/A for uncompetitive substrate inhibition in an Ordered Bi Bi mechanism. The numbers on the graph indicate increasing concentrations of substrate B

The kinetic equation for the ordered mechanism in which the EQA complex can occur is

$$\frac{1}{v} = \frac{1}{V_1}\left[1 + \frac{A}{K_{Ia}}\right] + \frac{K_a}{V_1(A)} + \frac{K_b}{V_1(B)} + \frac{K_{ia}K_b}{V_1(A)(B)}. \qquad \text{(V-48)}$$

Lineweaver-Burk plots will be hyperbolic concave up when 1/v is graphed as a function of 1/A. When 1/v is plotted against 1/B at different concentrations of A, a family of linear lines with decreasing slopes and intercepts will be generated; however, as the K_{Ia} term becomes significant the intercepts on the 1/v axis will increase. A replot of the these intercepts *versus* A will yield a concave up hyperbola. K_{Ia} may be evaluated from a series of experiments at "high" A (i.e., 10-times K_a or higher) in order to eliminate the K_a/A and $K_{ia}K_b/(A)(B)$ terms of Eq. (V-48). Plots of 1/v *versus* 1/B will then give a family of parallel lines, and a replot of the intercepts against A will permit calculation of K_{Ia}.

Substrate inhibition by the first substrate to add to the enzyme in the Ordered Bi Bi pathway may occur in an alternative manner, as follows

$$EA + A = EA_2,\ K_{Ia}. \qquad \text{(V-49)}$$

The rate expression for this type of inhibition is

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)} + \frac{K_b}{V_1(B)}\left[1 + \frac{A}{K_{Ia}}\right] + \frac{K_{ia}K_b}{V_1(A)(B)}. \qquad \text{(V-50)}$$

Equation (V-50) predicts that inhibition relative to substrate A in a double reciprocal plot will be hyperbolic concave up. If A is held at increasing fixed concentrations and B varied, the slopes will first decrease and then increase as the inhibitory term becomes dominant; however, the intercepts on the ordinate will decrease. Slope replots of the 1/v *versus* 1/B primary plots as a function of 1/A will be observed to be hyperbolic. These considerations demonstrate that one may readily make a choice between the two mechanisms of substrate inhibition by substrate A.

The two types of substrate inhibition ascribed to substrate B of the Ordered Bi Bi mechanism (Eqs. (V-43) and (V-45)) can be differentiated as suggested by the plots in Figs. V-10 and V-11.

b) Random Bi Bi Mechanism (Rapid Equilibrium)

In theory at least, the Random Bi Bi mechanism should not show substrate inhibition as described for ordered mechanisms. It is not possible to form a kinetically important EQB abortive complex in the mechanism described by Scheme I-7 because the EQ complex is formed after the rate limiting step, which involves isomerization of the central complexes. This would, of course, not be true in the steady-state treatment of this mechanism (see Chapter V-A 6).

One possible explanation of substrate inhibition, if it does occur in the rapid equilibrium Random Bi Bi pathway, is the addition of a second molecule of one of the substrates to a productive binary or ternary complex. This may occur if substrate A, for example, can bind at its own site and at the pocket normally occupied by substrate B, or alternatively, at a topologically remote site. The net affect of these interactions must be the production of an inactive or dead end complex; i.e.,

$$EA + A = EA_2,\ K_{Ia}. \qquad \text{(V-51)}$$

The rate equation for the random mechanism when the EA_2 complex is included is described by Eq. (V-50). The discussion of the kinetic results to be expected for this Ordered Bi Bi rate equation are also applicable to the random mechanism.

If substrate A can induce substrate inhibition in the random mechanism by adding to the EA complex but not at the site for B, it would seem that it could also bind the EAB central complex. If one assumes, that when two molecules of the same substrate reside on a single enzyme molecule, and the resulting complex is inactive, a rate equation can be written to explain substrate inhibition.

$$EA + A = EA_2,\ K_{Ia} \qquad \text{(V-52)}$$

$$EAB + A = EA_2B,\ K_{Ia}', \qquad \text{(V-53)}$$

$$EA_2 + B = EA_2B,\ K_{Ib} \qquad \text{(V-54)}$$

$$\frac{1}{v} = \frac{1}{V_1}\left[1 + \frac{A}{K_{Ia'}}\right] + \frac{K_a}{V_1(A)} + \frac{K_b}{V_1(B)}\left[1 + \frac{A}{K_{Ia}}\right] + \frac{K_{ia}K_b}{V_1(A)(B)}. \quad \text{(V-55)}$$

Equation (V-55) predicts that A will cause hyperbolic concave up inhibition when B is fixed and A is the variable substrate in a double reciprocal plot. When A is held constant at different fixed concentrations and 1/v plotted against 1/B, a complex type of inhibition will result. The intercepts and slopes will first decrease as the level of A is increased; however, this effect will be reversed as the K_{Ia} and $K_{Ia'}$ terms become kinetically important.

c) Ping Pong Bi Bi Mechanism

One of the basic assumptions associated with the Ping Pong mechanism is the concept of a single substrate site. Presumably site B does not exist as such until the enzyme has been modified by reaction of A; however, it is also true that substrate A and Q are capable of competing for free enzyme. According to the mechanism, B is incorporated into Q, and it might be expected that B may also bind to free enzyme at the Q site. This argument is the basis of substrate inhibition for the Ping Pong Bi Bi mechanism.

1. At elevated levels of B, the following interaction may be visualized:

$$E + B = EB,\ K_{Ib} \quad \text{(V-56)}$$

Incorporation of this reaction into the rate expression for the Ping Pong mechanism gives an equation for competitive substrate inhibition (21).

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)}\left[1 + \frac{B}{K_{Ib}}\right] + \frac{K_b}{V_1(B)} \quad \text{(V-57)}$$

Graphs of 1/v against 1/B will be hyperbolic concave up. On the other hand, when A is the varied substrate and B held at different constant levels, the intercepts will decrease as B is increased and the slopes will increase. Fig. V-12 illustrates inhibition by substrate B in this context.

2. If substrate A can combine with the same enzyme form as substrate B, another type of substrate inhibition results. The kinetics of this inhibition are illustrated in Eqs. (V-58) and (V-59).

$$F + A = FA,\ K_{Ia} \quad \text{(V-58)}$$

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)} + \frac{K_b}{V_1(B)}\left[1 + \frac{A}{K_{Ia}}\right] \quad \text{(V-59)}$$

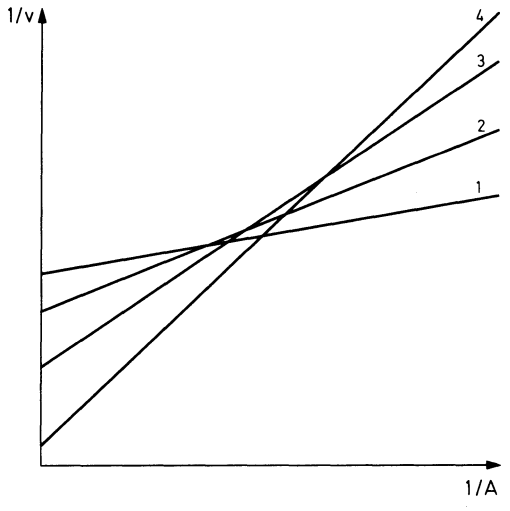


Fig. V-12. Double reciprocal plot of 1/v *versus* 1/A for competitive substrate inhibition in a Ping Pong mechanism. The numbers on the graph indicate increasing concentrations of substrate B

Equation (V-59) is very similar in form to Eq. (V-57), and the discussion relative to Eq. (V-57) is applicable here, except that the substrates are reversed.

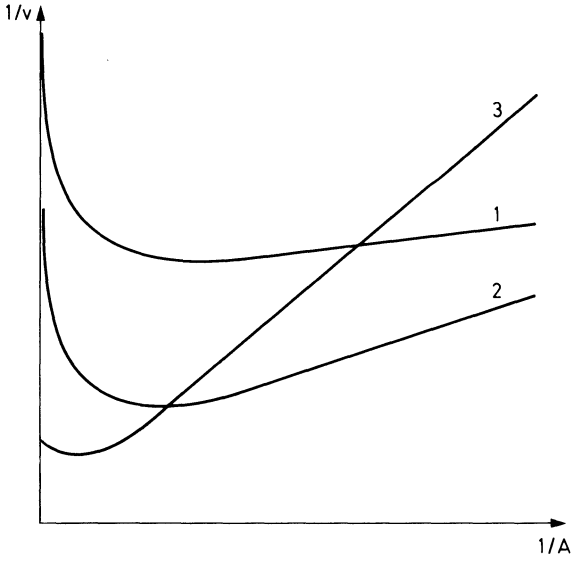


Fig. V-13. Plot of 1/v *versus* 1/A for double substrate inhibition in a Ping Pong mechanism. The numbers on the graph indicate increasing concentration of substrate B

3. With certain enzyme systems, both substrates can form dead end complexes with the enzyme. This effect has been referred to as a double substrate inhibition (21) and represents a combination of Eqs. (V-56) and (V-58) to yield Eq. (V-60).

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)}\left[1 + \frac{B}{K_{Ib}}\right] + \frac{K_b}{V_1(B)}\left[1 + \frac{A}{K_{Ia}}\right] \qquad \text{(V-60)}$$

The type of double reciprocal plot expected for this mechanism is described by Fig. V-13. The analogous 1/v *versus* 1/B plot at different fixed levels of A would be similar to the graph of Fig. V-13.

C. Alternative Substrate Inhibition

It has been long recognized (24) that, when a substrate and an alternative substrate compete for the same enzyme locus, the result is inhibition. The rate decrease is related to the velocities that would be expected if each substrate is assayed in the absence of the other. Two types of experimental protocols may be devised when one studies alternative substrate inhibition: a) the summation of velocities is measured, and b) product formation from only one substrate is determined.

Alternative substrate effects represent yet another procedure for making a choice of mechanism from among the usual possibilities for bi and terreactant mechanisms. The basic theory of the method is as follows. Consider the Uni Uni mechanism with substrate A and alternative substrate A'.

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E + P$$

$$E + A' \underset{k_2'}{\overset{k_1'}{\rightleftharpoons}} EA' \underset{k_4'}{\overset{k_3'}{\rightleftharpoons}} E + P'$$

Scheme V-9

In these discussions alternative substrates, products, and rate constants will be primed relative to the substrates, products, and rate constants. When carrying out experiments of this type, it is possible to measure dP/dt alone and dP/dt + dP'/dt. These two procedures lead to different rate equations.

1. Alternative Substrates Acting as Inhibitors Only

a) $v = dP/dt$

To obtain the rate equation for this case, first write the conservation of enzyme equation. For Scheme V-9 this expression is

$$E_0 = E + EA + EA' \tag{V-61}$$

Next all enzyme forms are gotten in terms of the enzyme species that gives rise to the desired product; i.e., either P or P'. Thus,

$$E_0 = (EA)\left[1 + \left[K_{ia}/A\right](1 + A'/K_{ia'})\right] \tag{V-62}$$

and

$$v = \frac{V_1}{1 + \frac{K_{ia}}{A}\left[1 + \frac{A'}{K_{ia'}}\right]}. \tag{V-63}$$

It is clear from Eq. (V-63) that the alternative substrate A' acts as nothing more than a competitive inhibitor of the substrate. It is important to note that, in these experiments, if the rate with A' is much greater than the rate with A in a bireactant or terreactant system, there may be depletion of the other substrate(s). This possibility would give rise to incorrect values for the other substrate concentrations and should be born in mind when contemplating studies of this type.

b) $$v = dP/dt + dP'/dt = k_3(EA) + k_3'(EA') \tag{V-64}$$

$$v = k_3(EA) + k_3'K_{ia}(A')(EA)/K_{ia'}(A) \tag{V-65}$$

By using the conservation of enzyme expression in Eq. (V-62), the final rate equation is,

$$v = \frac{V_1K_{ia'}(A) + V_1'K_{ia}(A')}{K_{ia}K_{ia'} + K_{ia'}(A) + K_{ia}(A')}. \tag{V-66}$$

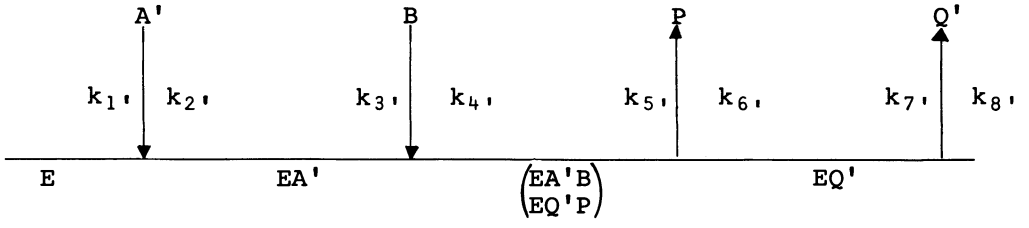
DIXON and WEBB (24) have shown that Eq. (V-66) may be used to answer the question of whether one enzyme is catalyzing the reaction involving A and A' or whether two separate enzymes are responsible. Equation (V-66) predicts that the velocity of the reaction will be intermediate between the velocities observed when A and A' react alone. On the other hand, if the reaction is catalyzed by two different enzymes, the velocity, in theory, of the mixture will be the summation of the individual velocities. Unfortunately, if there are two separate enzymes, it is possible that the nonreactant substrate will cause inhibition of one enzyme; thus, one cannot make the desired choice unambiguously.

2. Bireactant Systems

a) The Ordered Bi Bi Mechanism

Using Scheme I-8 as a model, if in addition to substrates A and B the alternative substrate A' is included in the reaction mixture and initial velocity (dQ/dt) measured, a rate equation is

obtained that differs from that of the Random and Ping Pong Bi Bi mechanisms. If dP/dt is determined, the rate expression will differ from that in which dQ/dt is used to determine velocity.



Scheme V-10

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)}\left[1 + \frac{A'}{K_{ia}'K_b' + K_a'(B)}\left[K_b' + B\right]\right] + \frac{K_b}{V_1(B)} + \frac{K_{ia}K_b}{V_1(A)(B)}\left[1 + \frac{A'}{K_{ia}'K_b' + K_a'(B)}\left[K_b' + B\right]\right] \quad \text{(V-67)}$$

When B' rather than A' is the alternative substrate, the following series of reactions is added to Scheme I-8

$$EA + B' \underset{k_4'}{\overset{k_3'}{\rightleftharpoons}} \begin{pmatrix} EAB' \\ EP'Q \end{pmatrix} \underset{k_6'}{\overset{k_5'}{\rightleftharpoons}} EQ + P' \quad \text{(V-68)}$$

The modified rate equation in the presence of B' is

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)} + \frac{K_b}{V_1(B)}\left[1 + \frac{k_3'(B')}{(k_4' + k_5')}\left[1 + \frac{k_5'}{k_7}\right]\right] + \frac{K_{ia}K_b}{V_1(A)(B)}\left[1 + \frac{k_3'k_5'(B')}{k_2(k_4' + k_5')}\right]. \quad \text{(V-69)}$$

In Eqs. (V-67) and (V-69) velocity is dQ/dt and dP/dt, respectively. Eq. (V-67) indicates that the alternative substrate A' will act competitively with respect to substrate A. On the other hand, when double reciprocal plots of 1/v *versus* 1/B are made in the presence of A', the primary plots will be concave up.

When the alternative substrate B' is used, inhibition will be competitive and noncompetitive with respect to substrates B and A, respectively. Thus, for this mechanism, a choice can be made between substrates A and B from experiments with alternative substrates.

Figure V-14 and Fig. V-15 demonstrate kinetic data for liver alcohol dehydrogenase in which NAD^+ was the substrate and thionicotinamide-NAD^+ the alternative substrate. The analog acts as a competitive inhibitor of NAD^+ (Fig. V-14) and as a nonlinear inhibitor of ethanol (Fig. V-15). These findings are consistent

with the known mechanism and sequence of substrate addition to the enzyme (25).

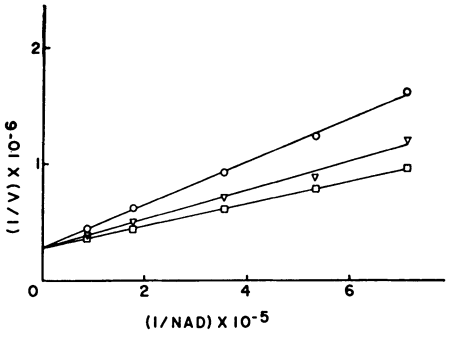


Fig. V-14

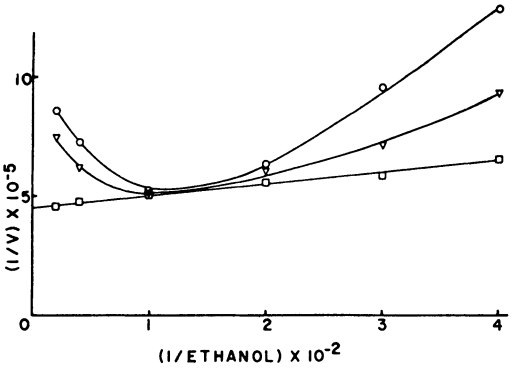


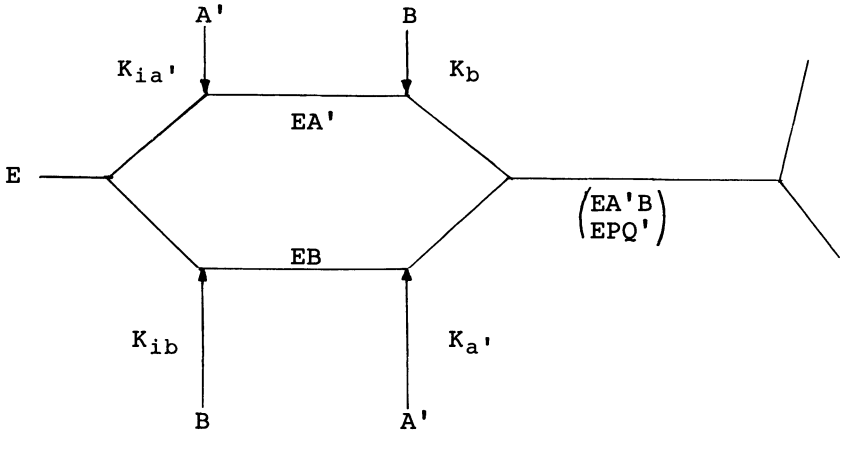
Fig. V-15

Fig. V-14. Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the molar concentration of NAD in the presence and absence of thionicotinamide-NAD. Ethanol was held constant at 3.0 mM and NAD was varied from 1.40 to 11.2 × 10^{-5} M. Thionicotinamide-NAD concentrations were 0 (□), 6.46 (▽), and 12.9 × 10^{-5}M (○)

Fig. V-15. Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the molar concentration of ethanol in the presence and absence of thionicotinamide-NAD. NAD was held constant at 2.48 × 10^{-5} M and ethanol was varied from 2.5 to 50 mM. Thionicotinamide-NAD concentrations were 0 (□), 6.46 (▽), and 12.9 × 10^{-5} M (○)

b) The Rapid Equilibrium Random Bi Bi Mechanism

When an alternative substrate for A, A', is used along with substrates A and B in Scheme I-7, the following additional interactions will occur:



Scheme V-11

The initial velocity equation for the Random Bi Bi mechanism with A, B, and A' present simultaneously is

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)}\left[1 + \frac{A'}{K_{a'}}\right] + \frac{K_b}{V_1(B)} + \frac{K_{ia}K_b}{V_1(A)(B)}\left[1 + \frac{A'}{K_{ia'}}\right]. \qquad \text{(V-70)}$$

It is clear from Eq. (V-70) that A', a competitive inhibitor of A, will exhibit noncompetitive inhibition relative to substrate B. It is possible to obtain an expression in which B' is used as an inhibitor for B. In this case Eq. (V-70) will be modified so that the K_b/B term is multiplied by the factor $(1 + B'/K_{b'})$ and the $K_{ia}K_b/(A)(B)$ term altered by the expression $(1 + B'/K_{ib'})$. Thus in the case of the Random Bi Bi mechanism, the alternative substrate will be a competitive inhibitor for the substrate and a noncompetitive inhibitor for the other substrate pair member. The rate equations for the ordered and random mechanisms permit a choice to be made provided that, in the ordered mechanism, the rate constants do not eliminate the hyperbolic effect described by Eq. (V-67).

It is interesting to note that the rate equations for the Ping Pong Bi Bi mechanism are similar in form to those of the random case, and thus one may not discriminate between these two possibilities by studies of alternative substrates (26).

When considering Eq. (V-67), it might be expected that, although A' is a competitive inhibitor of A, plots of 1/v against 1/B in the presence of A and A' would be hyperbolic concave up as shown in Fig. V-15. RICARD et al. (27) have shown that this effect might not be kinetically discernible. It is therefore suggested that one determine the kinetic parameters described by Eq. (V-67) from experiments of A and A' alone with B. These values may then be substituted into Eq. (V-67) to determine whether nonlinearity will be discernible. If it is, the alternative substrate approach may be used to make a choice from among the various indicated mechanisms. If a linear relationship is obtained, this method may not be used advantageously for this purpose.

3. Terreactant Systems

The rate equations for terreactant systems using alternative substrates have been derived and may be found elsewhere (28). Table V-3 illustrates the types of plots to be expected using this kinetic procedure. The profiles listed in Table V-3 assume that nonlinearity will show up as predicted from the rate equations. This approach is seriously compromised because one does not know in advance whether nonlinearity will be experimentally discernible. The test for this effect will be as indicated for bireactant systems.

When the sum of the velocities of the substrate and alternative substrate are measured, it is theoretically possible to choose from among the Theorell-Chance, Ordered Bi Bi, and rapid equi-

Table V-3. Inhibition patterns for alternative substrate inhibition for various three-substrate mechanisms

Mechanism	Alternative substrate for substrate	1/A plot	1/B plot	1/C plot
Ordered Ter Ter (I-12)	A	C[a]	NL[b]	NL
	B	N[c]	C	NL
	C	N	N	C
Random Ter Ter (Rapid Equilibrium) (I-13)	A	C	N	N
	B	N	C	N
	C	N	N	C
Random AB (Rapid Equilibrium) (I-14)	A	C	N	N
	B	N	C	N
	C	U[d]	U	C
Random BC (Rapid Equilibrium) (I-15)	A	C	N	N
	B	U	C	N
	C	U	N	C
Random AC (Rapid Equilibrium) (I-16)	A	C	N	N
	B	N	C	N
	C	N	N	C
Hexa Uni Ping Pong (I-17)	A	C	N	N
	B	N	C	N
	C	N	N	C
Ordered Bi Uni Uni Bi Ping Pong (I-18)	A	C	NL	N
	B	N	C	N
	C	N	N	C
Ordered Uni Uni Bi Bi Ping Pong (I-19)	A	C	N	N
	B	N	C	NL
	C	N	N	C
Random Bi Uni Uni Bi Ping Pong (I-20)	A	C	N	N
	B	N	C	N
	C	N	N	C
Random Uni Uni Bi Bi Ping Pong (I-21)	A	C	N	N
	B	N	C	N
	C	N	N	C

[a] C refers to a Lineweaver-Burk plot, which shows competitive inhibition.
[b] NL refers to a Lineweaver-Burk plot, which is concave upward in the presence of the alternative substrate.
[c] N refers to a Lineweaver-Burk plot, which shows noncompetitive inhibiton.
[d] U refers to a Lineweaver-Burk plot, which shows uncompetitive inhibtion.

librium Random Bi Bi mechanisms (29). The theory, rate expressions, and experimental protocol for this procedure are available in the literature (29, 30); however, they will not be considered in detail here. This procedure suffers from the same limitations inherent in the method involving alternative substrates in which product derived from the substrate alone is measured.

D. Alternative Product Inhibition

Investigators have occassionally studied alternative product inhibition in an attempt to obtain information on the kinetic mechanism. FROMM and ZEWE (19), for example, used mannose-6-P as an alternative product along with substrates ATP and glucose in the hexokinase reaction. They found that mannose-6-P is a noncompetitive inhibitor of both substrates when glucose-6-P production is monitored. In their proposed mechanism for yeast hexokinase (rapid equilibrium Random Bi Bi), the alternative product could react with the free enzyme, and the two binary complexes of enzyme and substrate. The initial rate equation would be modified so that all three substrate terms (A, B, and AB) would be affected by the alternative product. These experiments not only showed that there was not a simple competition between mannose-6-P and the substrates for the free enzyme, but also indicated that product may bind to the enzyme even when a substrate is already bound.

WRATTEN and CLELAND (31) have made a detailed study of liver alcohol dehydrogenase using alternative products. The kinetic mechanism for this enzyme was established by these investigators to be Ordered Bi Bi (25). By using the alternative product kinetic approach, they were able to exclude the Theorell-Chance mechanism as a viable possibility. In addition, WRATTEN and CLELAND were able to demonstrate the formation of abortive ternary complexes in the alcohol dehydrogenase reaction.

E. Multisite Ping Pong Mechanisms

The classical Ping Pong mechanism illustrated in Scheme I-10 exhibits distinctive product inhibition patterns as indicated by Eqs. (V-22) and (V-23) and as shown in Table V-1. NORTHROP (32) has investigated the kinetics of transcarboxylase from *Propionibacterium shermanii* and found the kinetic mechanism to be Ping Pong as judged by initial rate experiments in the absence of product (32) and by the presence of the requisite partial exchange reactions (33); however, anomalous product inhibition patterns were observed (32).

Partial exchange and chemical studies indicate the following sequence of reactions for transcarboxylase:

$$\text{Methylmalonyl-CoA} + \text{E-biotin} \rightleftharpoons \text{propionyl-CoA} + \text{E-biotin-CO}_2 \qquad \text{(V-71)}$$

$$\text{E-biotin-CO}_2 + \text{pyruvate} \rightleftharpoons \text{E-biotin} + \text{oxalacetate} \qquad \text{(V-72)}$$

Product inhibition studies revealed that propionyl-CoA is a competitive inhibitor of methylmalonyl-CoA and vice versa. The two ketoacids were also found to be mutually competitive.

NORTHROP (32) has provided a plausible explanation of the partial exchange reactions as well as the initial rate product inhibition patterns. Figure V-16 summarizes NORTHROP's model for

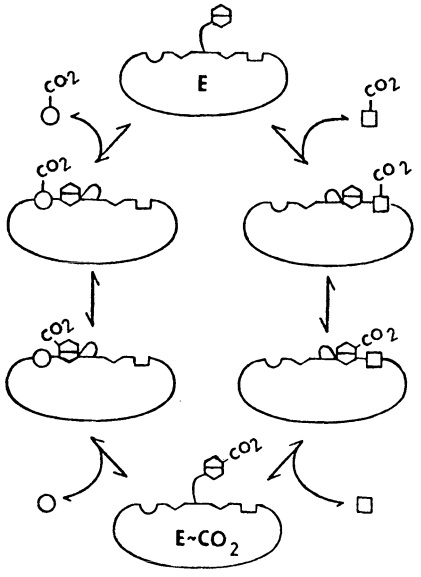


Fig. V-16. Pictorial model of the transcarboxylase reaction (32). Free circle, pyruvate; carboxylated circle, oxalacetate; free square, propionyl-CoA; carboxylated square, methylmalonyl-CoA; hexagonal structure, biotin; carboxylated hexagonal structure, carboxyl-biotin; E, one of possibly six reactive enzyme centers of transcarboxylase. The forward reaction is read in a clockwise direction. Not shown on the diagram are numerous nonproductive complexes between enzyme and substrates

transcarboxylase. Transcarboxylase in the schematic presented is a multisite-enzyme. It can be seen that methylmalonyl CoA ($\square$-CO_2) and propionyl-CoA ($\square$) are capable of binding at the same site. The diagram also shows that pyruvate (O) and oxalacetate (^{O}-CO_2) may also occupy the same site, but the ketoacid and CoA thioester sites are different. An important feature of the multisite mechanism is the ability of biotin (or carboxyl biotin) to bridge the gap between the two sites; i.e., to move between the two sites.

CLELAND (34) has recently extended NORTHROP's multisite treatment of transcarboxylase to the more complex pyruvate dehydrogenase system.

F. Enzymes with Identical Substrate-Product Pairs

A number of enzymes produce a product that is virtually indistinguishable from the substrate used in the reaction. Examples

of enzymes of this type are those that require a primer as a substrate and where the elongated product cannot be distinguished from the substrate.

CHAO et al. (35) studied the kinetics of maltodextrin phosphorylase from *E. coli* and recognized that when orthophosphate is used as a product inhibitor along with the substrates glycogen and glucose-1-P, all of the substrates for the forward and reverse reaction are present. These authors derived initial rate expressions for a number of bireactant mechanisms in which it was assumed that the product glycogen was always present with the substrates, one of which was also glycogen. They established the kinetic mechanism to be rapid-equilibrium Random Bi Bi using dead end inhibitor substrate analogs (Chapter IV) and experiments involving isotope exchange (Chapter VI).

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Chapter VI

Isotope Exchange

One of the single most useful procedures for studying the kinetic mechanism of enzyme action involves the technique of isotope exchange, a procedure pioneered by P.D. BOYER (1). These studies can provide supporting evidence for initial velocity experiments and may also be used for probing enzyme mechanisms involving measurement of rates of loss, or exchange, of all possible atoms or functional groups of substrates, determination of various possible primary and secondary isotope effects, and definition of modifier effects on the catalytic power of regulatory enzymes. The procedure has also been conveniently used for studies of kinetic mechanisms at concentrations of enzyme that might prevail in the cell. The one obvious limitation of the method involves enzyme systems that are kinetically irreversible; however, even here it may still be possible to study product-substrate exchange during the forward reaction.

Isotope exchange kinetic experiments have been used to make a choice of mechanism from among various possibilities and also to evaluate the flux rates through mechanisms involving branched (random) pathways. To illustrate these points, first consider the Ordered Bi Bi Mechanism (Scheme I-8) and second the Random Bi Bi Mechanism (Scheme I-7). Let it be assumed that the Ordered Bi Bi Mechanism is at chemical equilibrium, a tracer amount of substrate A is added (at a concentration that will not perturb the equilibrium) and its rate of conversion, or exchange, with product Q is monitored. If the concentration of the B-P pair is increased in an equilibrium ratio so as not to disturb the equilibrium of the system, the exchange rate would be expected to increase; however, a concentration range will be approached where enzyme (free enzyme) will not be available for reaction with A and Q. At infinite B and P, all the enzyme will exist as EAB and EPQ and the A$\longleftrightarrow$Q will drop to zero. On the other hand, if one measures the B$\longleftrightarrow$P exchange for this mechanism and if A and Q are increased in an equilibrium ratio, the enzyme will be forced into the binary complexes EA and EQ. These forms of the enzyme can react with substrate and product to form the productive ternary complexes, and the B$\longleftrightarrow$P exchange will not be inhibited at elevated levels of A and Q.

Other types of exchanges may be visualized for the Ordered Bi Bi mechanism (i.e., B$\longleftrightarrow$Q and A$\longleftrightarrow$P); however, all four exchanges are not to be expected. For example with lactate dehydrogenase, the exchangable pairs are: lactate-pyruvate, NAD-NADH, and lactate-NADH, but not NAD-pyruvate. At any rate, if the B$\longleftrightarrow$Q exchange could be measured for this mechanism, it too would be inhibited and finally decrease to zero as the levels of A and P

are elevated because of the decrease in the concentration of free enzyme available to react with Q. The exchange patterns to be expected for the Ordered Bi Bi mechanism are illustrated in Figs. VI-1 and VI-2.

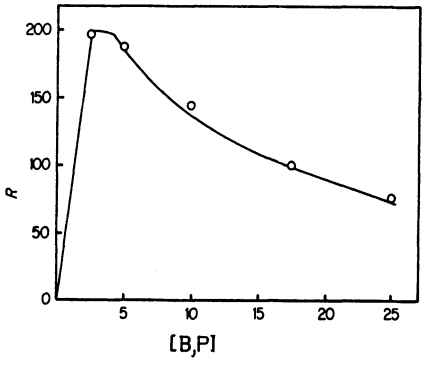


Fig. VI-1

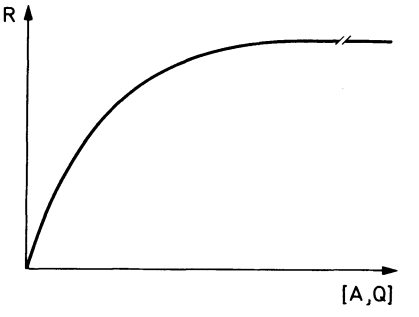


Fig. VI-2

Fig. VI-1. Rate of isotope exchange, R, at equilibrium for the A⇌Q exchange of an ordered mechanism as a function of the concentration of B and P which are maintained in an equilibrium ratio. A similar type of graph is to be expected when the B⇌Q exchange is plotted as a function of [A/P] concentration. The A⇌P exchange rate will exhibit the same type of pattern when B and Q are increased in an equilibrium ratio

Fig. VI-2. Rate of the B⇌P isotope exchange, R, for an ordered mechanism as the concentrations of A and Q are raised in an equilibrium ratio

How the isotope exchange rate at equilibrium of a substrate-product pair is affected in branched mechanisms is best depicted by using the Random Bi Bi mechanism as an example. Here if one determines the A⇌Q exchange as B and P are raised, the exchange rate reaches a maximal velocity, levels off, and is not depressed at elevated concentrations of the B-P pair. The exchanges of A⇌Q are not reduced as in the case of the Ordered Bi Bi mechanism, because there are alternative reaction pathways that the labeled substrate can follow in its conversion to product. When B and P approach infinity, the enzyme will exist as EB and EP; however, both A and Q can add to these enzyme forms to form the productive ternary complexes that permit the conversion of A to Q and *vice versa*. By analogy, the B⇌P exchange is not inhibited as the concentration of the A-Q substrate-product pair approaches saturation. Both exchange patterns for the random mechanism resemble the results of Fig. VI-2.

When considering the mechanism illustrated in Scheme I-7, the Random Bi Bi interaction pathway, it is assumed that all steps in the sequence equilibrate rapidly relative to the central ternary complexes. Because this isomerization step is rate limiting, it follows that all substrate⇌product exchange rates should be

the same. This has been found to be true in some Random Bi Bi cases (creatine kinase)(2), but not in others (yeast hexokinase)(3).

For the latter enzyme system, it was observed that the glucose⇌glucose-6-phosphate exchange exceeds the ADP⇌ATP exchange by approximately 50%. These results suggest that the interconversion of the ternary complexes is not rate limiting and that, although the kinetic mechanism is Random Bi Bi, the equilibrium assumption is not correct. Isotope exchange thus permits one to gain insight into the relative flux rates of the alternative pathways and also to obtain information on the relative magnitudes of certain portions of the kinetic mechanisms. These ends can be achieved to some extent by studies of the A⇌Q, B⇌P exchanges and the A⇌P or B⇌Q exchanges where applicable.

A. Abortive Complex Formation

Abortive complex formation serves to complicate studies of product inhibition kinetics, and the same is true in the case of isotope exchange at equilibrium. It has been suggested that the formation of such complexes makes it difficult to differentiate between ordered and random mechanism from studies of isotope exchange (4); however, RUDOLPH and FROMM (5) have shown that the possible formation of such complexes does not limit the usefulness of isotope exchange studies.

In a qualitative sense one can differentiate between ordered and random mechanisms, because in the former case the outer substrate-product pair exchange can be *completely* inhibited as the inner substrate-product pair becomes saturating. No analogous inhibition is observed with the branched mechanism. When abortive ternary complexes are formed for a system that exhibits a random kinetic mechanism, isotope exchange rates at equilibrium may or may not be depressed depending upon which substrate-product pair concentration becomes saturating. This is illustrated by the rate equations for isotope exchange at equilibrium shown in Table VI-1. Let us first consider the case of the rapid equilibrium Random Bi Bi mechanism in which the central complexes are rate limiting. If one measures the A⇌Q exchange and abortives EBQ and EAP form and if the B-P substrate-product pair is raised in an equilibrium ratio, the measured exchange will plateau at elevated B and P and inhibition will not be discernible. The rates will, of course, be less than if the two abortives did not exist; however, this effect will not be qualitatively observable. On the other hand, if the B-Q substrate-product pair is raised in a constant ratio, one will observe a depression of the A⇌Q exchange because of abortive ternary complex formation. In this latter illustration, the $K_a(Q)/K_{Iq}(A)$ term will approach infinity and the exchange rate will go to zero.

It will be shown that, for the Random and Ordered Bi Bi mechanisms, if one wishes to obviate the complicating effects of abortive complex formation, one should raise the B-P pair when

Table VI-1. Isotope exchange rate equations for bireactant ordered and random mechanisms at chemical equilibrium with abortive complexes

Mechanism	Exchange	Rate Equation
Random (Rapid Equilibrium)	any	$R = \dfrac{V_1}{1 + \dfrac{K_a}{A}\left[1 + \dfrac{Q}{K_{Iq}}\right] + \dfrac{K_b}{B}\left[1 + \dfrac{P}{K_{Ip}}\right] + \dfrac{K_{ia}K_b}{(A)(B)}\left[1 + \dfrac{P}{K_{ip}} + \dfrac{Q}{K_{iq}} + \dfrac{(P)(Q)}{K_{ip}K_q}\right]}$
Ordered Bi Bi[a]	A⇌Q	$R = \dfrac{V_1(A)(B)}{\left[K_{ia}K_b + K_aB + \dfrac{K_{ia}K_bK_q(P)}{K_{iq}K_p}\right]\left[1 + \dfrac{A}{K_{ia}}\left[1 + \dfrac{P}{K_{Ip}} + \dfrac{B}{K_{eb}}\right] + \dfrac{Q}{K_{iq}}\left[1 + \dfrac{B}{K_{Ib}}\right]\right.}$
	B⇌Q	$R = \dfrac{V_1(B)}{\left[K_b + \dfrac{K_bK_q(P)}{K_{iq}K_p}\right]\left[1 + \dfrac{K_{ia}}{A}\left[1 + \dfrac{Q}{K_{iq}} + \dfrac{(B)(Q)}{K_{iq}K_{Ib}}\right] + \dfrac{P}{K_{Ip}} + \dfrac{B}{K_{eb}}\right.}$
	A⇌P	$R = \dfrac{V_1(A)(B)}{\left[K_{ia}K_b + K_a(B)\right]\left[1 + \dfrac{A}{K_{ia}}\left[1 + \dfrac{P}{K_{Ip}} + \dfrac{B}{K_{eb}}\right] + \dfrac{Q}{K_{iq}}\left[1 + \dfrac{B}{K_{Ib}}\right]\right]}$
	B⇌P	$R = \dfrac{V_1(B)}{K_b\left[1 + \dfrac{K_{ia}}{A}\left[1 + \dfrac{Q}{K_{iq}} + \dfrac{(B)(Q)}{K_{iq}K_{Ib}}\right] + \dfrac{P}{K_{Ip}} + \dfrac{B}{K_{eb}}\right.}$

[a] $K_{eb} = k_4/k_3$

studying the A⟷Q exchange and *vice versa*. If one is interested in investigating abortive complex formation, then the alternative protocol may be used; i.e., measurement of the A⟷Q exchange while raising the B-Q substrate product pair or *vice versa*. The disadvantage of carrying out the procedure that permits abortive complex formation to manifest itself kinetically is that depression of exchanges is a characteristic of ordered and not random mechanisms. Thus one cannot differentiate between these two cases when abortive complex formation is allowed to influence the kinetic patterns for isotope exchange. Raising all substrates and products in an equilibrium ratio will result in exchange patterns of the type to be expected when abortives do not form; however, this procedure causes changes in specific activity that must be compensated for either by increasing the level of radioactive tracer or by using a very narrow concentration range so as to preclude the problem of isotope dilution.

Table VI-1 also illustrates the rate equations for the Ordered Bi Bi mechanism involving a single productive ternary complex and abortive complexes EQB and EAP. It is clear that for the A⟷Q exchange, if the B-P pair is raised, the exchange will be inhibited. This will be true whether abortives form or not. This is also clearly the situation for the A⟷P exchange when B and Q are increased. On the other hand, when A and Q are increased and the B⟷P exchange measured, there will not be a depression of the exchange, regardless of whether the abortives form. It follows from this discussion that abortive ternary complex formation is not a deterrent to isotope exchange studies at equilibrium. Their presence may also be discernible if desired from proper experiments as outlined here.

B. Derivation of Rate Equations

Most of the enzyme systems studied by isotope exchange techniques have been at chemical equilibrium. CLELAND and his coworkers (2, 6) have carried out experiments in which the systems are displaced from equilibrium; however, the use of the two approaches require different assumptions in the derivation of the various rate equations for isotope exchange.

Rules for the derivation of rate equations for systems at equilibrium and for systems not at equilibrium are illustrated below.

1. The Equilibrium Case: Ping Pong Bi Bi

The A⟷P portion of the Ping Pong Bi Bi system in the presence of enzyme will be at equilibrium as soon as enzyme is added, i.e., there can be no net change in the concentrations of A and P. In these experiments, A, P and enzyme are added, the system permitted to equilibriate, and a tracer amount of substrate added. Aliquots of the reaction mixture are removed at different times

after addition of the tracer, the reaction terminated, and the amount of tracer in the product determined.

For the Ping Pong Bi Bi mechanism:

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} F + P. \qquad \text{(VI-1)}$$

The velocity of the exchange reaction is: $v^* = k_3(EA^*)$ where the * indicates a labeled species. The k_4 step is not included in the velocity expression because P is not labeled initially, and the amount of P* returning to EA* in the initial velocity phase of the reaction (conditions under which the isotope is distributed in the nonradioactive pool) is essentially zero.

Next an expression is obtained for the enzyme form (E), which reacts with the labeled substrate (A*) in terms of the enzyme form in the velocity expression (EA*) from steady-state consideration. Thus,

$$d(EA^*)/dt = k_1(E)(A^*) - (k_2 + k_3)(EA^*) = 0 \qquad \text{(VI-2)}$$

and

$$EA^* = \frac{k_1(A^*)(E)}{(k_2 + k_3)}. \qquad \text{(VI-3)}$$

Substituting for EA* in the velocity expression gives

$$v^* = \frac{k_1 k_3(A^*)(E)}{(k_2 + k_3)} \qquad \text{(VI-4)}$$

and dividing both sides of this equation by E_0, total enzyme, leads to the expression:

$$\frac{v^*}{E_0} = \frac{k_3(A^*)(E)}{(k_2 + k_3)(E_0)}. \qquad \text{(VI-5)}$$

If the reaction is *not* to be carried out at equilibrium, the value for the determinant for E, obtained by the KING-ALTMAN (7) or modified graph theory method of FROMM (8), is substituted into Eq. (VI-5). E_0 will be the sum of the determinants for E, EA, and F if the exchange is carried out in the absence of B and Q.

For systems at chemical equilibrium, the conservation of enzyme expression ($E_0 = E + EA + F$) is obtained as a function of the enzyme term in the numerator of Eq. (VI-5). The various equilibria for the enzyme forms for the first partial reaction of the Ping Pong Bi Bi mechanism are:

$$EA = \frac{k_1(A)(E)}{k_2} \qquad \text{and} \qquad F = \frac{k_1k_3(A)(E)}{k_2k_4(P)} \tag{VI-6}$$

and

$$E_0 = (E)\left[1 + \frac{k_1A}{k_2} + \frac{k_1k_3A}{k_2k_4P}\right] \tag{VI-7}$$

and finally

$$\frac{v^*}{E_0} = \frac{k_1k_3(A^*)}{(k_2 + k_3)\left[1 + \frac{k_1(A)}{k_2} + \frac{k_1k_3(A)}{k_2k_4(P)}\right]}. \tag{VI-8}$$

Equation (VI-8) represents the expression for isotope exchange at equilibrium for the A⟷P exchange. This exchange could also be measured in the presence of substrate B and product Q; however, the conservation of enzyme term would have to be expanded to include an additional enzyme form under these conditions. It should be remembered that the half-reactions in Ping Pong mechanisms are at chemical equilibrium in the absence of the other substrate-product pair.

It can be seen from Eq. (VI-8) that the concentration of the labeled substrate A appears in the numerator of the equation, whereas the total concentration of A appears in the denominator. If A is described in terms of specific activity (counts/time/unit of concentration), it is not necessary to make a distinction between A in the numerator and denominator of Eq. (VI-8). Under these conditions v^*, the steady-state exchange velocity, is replaced by R, the A⟷P exchange rate (9). The significance of R will be described in detail later in this chapter.

Although Eq. (VI-8) was derived by assuming that the radioactive substrate is A, an identical expression is obtained when labeled P is used and its rate of conversion to A determined. This relationship is true for all exchanges between substrate and product pairs for systems at equilibrium.

2. The Steady-State Case: Ordered Bi Bi (Theorell-Chance)

To further illustrate the method of deriving rate expressions for isotope exchange, the B⟷Q exchange for the Theorell-Chance mechanism is presented as an example. This type of exchange could occur between the 2-hydrogen of lactate and NADH in the lactate dehydrogenase reaction.

The B⟷Q exchange velocity is

$$v^* = k_5(EQ^*). \tag{VI-9}$$

It is now necessary to obtain an equation for EQ* in terms of EA, the enzyme form that reacts with B*; i.e.,

$$d(EQ^*)/dt = k_3B^*(EA) - k_4P(EQ^*) - k_5(EQ^*) = 0 \qquad \text{(VI-10)}$$

and

$$EQ^* = \frac{k_3(B^*)(EA)}{(k_4P + k_5)}. \qquad \text{(VI-11)}$$

Substituting for EQ* in Eq. (VI-9) and multiplying both sides of the equation by E_0 yields

$$\frac{v^*}{E_0} = \frac{k_3k_5(B^*)(EA)}{(k_4P + k_5)E_0}. \qquad \text{(VI-12)}$$

If the system is assumed to be at chemical equilibrium, the expression for the B$\longleftrightarrow$Q exchange is

$$\frac{R}{E_0} = \frac{k_1k_3k_4k_5(A)(B)(P)}{(k_4P + k_5)(k_1k_4AP + k_2k_4P + k_1k_3AB)}. \qquad \text{(VI-13)}$$

On the other hand, if the system is displaced from equilibrium it will be necessary to substitute the determinant for EA in the numerator and those for E, EA, and EQ obtained by the method of FROMM (8) into the denominator of Eq. (VI-12) to obtain the rate expression for the isotope exchange rate between B and Q. This equation is described by Eq. (VI-14).

$$\frac{v^*}{E_0} = \frac{k_3k_5(B^*)(k_1k_4AP + k_1k_5A + k_4k_6PQ)}{(k_4P + k_5)(k_2k_4P + k_3k_5B + k_2k_5 + k_1k_4AP + k_1k_5A + k_4k_6PQ + k_3k_6BQ + k_2k_6Q + k_1k_3A} \qquad \text{(VI-14)}$$

It is of interest to note that the exchange equation derived using either assumption will be identical when the overall exchange for a reaction is measured, i.e., A$\longleftrightarrow$Q for a bireactant system, or if a partial exchange for a Ping Pong mechanism is considered. In the latter instance, the system will always be at chemical equilibrium.

In Table VI-2 are shown the usual bireactant mechanisms along with the rate equations derived for isotope exchange at equilibrium in the absence of abortive ternary complex formation. Although these equations show quantitatively which exchanges may or may not be altered when the other substrate-product pair is raised in an equilibrium ratio, the equations are too complex in most instances to permit evaluation of the various kinetic parameters.

Table VI-2. Isotope exchange rate equations for bireactant mechanisms at chemical equilibrium without abortive complexes

Mechanism	Exchange	Rate equation
Ordered Bi Uni	A⇌P	$R = \frac{V_1 (A)(B)}{\left[K_{ia}K_b + K_a(B)\right]\left[1 + \frac{A}{K_{ia}} + \frac{P}{K_{ip}}\right]}$
	B⇌P	$R = \frac{V_1 (B)}{K_b\left[1 + \frac{K_{ia}}{A} + \frac{K_{ia}(P)}{K_{ip}(A)}\right]}$
Random Bi Uni	All	See Random Bi Bi in Table VI-1 and delete terms containing K_{Iq}, K_{Ip}, and Q.
Ordered Bi Bi	A⇌Q	Use equations of Table VI-1 and delete terms containing K_{Ib} and K_{Ip}.
	A⇌P	
	B⇌P	
	B⇌Q	
Theorell-Chance	A⇌Q	$R = \frac{V_1 (A)(B)}{\left[K_a(B) + K_{ia}K_b + \frac{K_{iq}(P)}{K_{eq}}\right]\left[1 + \frac{A}{K_{ia}} + \frac{Q}{K_{iq}}\right]}$

Table VI-2 (continued)

Mechanism	Exchange	Rate equation
Theorell-Chance (continued)	$A \rightleftharpoons P$	$R = \dfrac{V_1(A)(B)}{\left[K_{ia}K_b + K_a(B)\right]\left[1 + \dfrac{A}{K_{ia}} + \dfrac{Q}{K_{iq}}\right]}$
	$B \rightleftharpoons Q$	$R = \dfrac{V_1(B)}{\left[K_b + \dfrac{K_{iq}(P)}{K_{ia}K_{eq}}\right]\left[1 + \dfrac{K_{ia}}{A} + \dfrac{K_{ia}(Q)}{K_{iq}(A)}\right]}$
	$B \rightleftharpoons P$	$R = \dfrac{V_1(B)}{K_b\left[1 + \dfrac{K_{ia}}{A} + \dfrac{K_{ia}(Q)}{K_{iq}(A)}\right]}$
Random Bi Bi [a]	All	See Table VI-1 and delete terms containing K_{Ib} and K_{Ip}.
Ping Pong	$A \rightleftharpoons Q$	$R = \dfrac{V_1(A)(B)}{\left[K_a(B) + \dfrac{K_{ia}K_b(P)}{K_{ip}}\right]\left[1 + \dfrac{A}{K_{ia}} + \dfrac{Q}{K_{iq}} + \dfrac{K_{ip}(A)}{K_{ia}(P)}\right]}$
	$A \rightleftharpoons P$	$R = \dfrac{V_1(A)}{K_a\left[1 + \dfrac{A}{K_{ia}} + \dfrac{Q}{K_{iq}} + \dfrac{K_{ip}(A)}{K_{ia}(P)}\right]}$

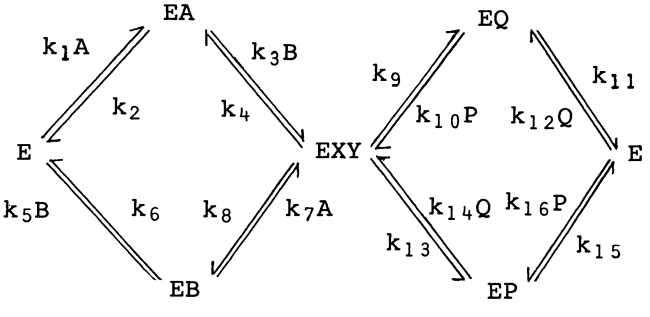
Table VI-2 (continued)

Mechanism	Exchange	Rate equation
Ping Pong (continued)	B⟷Q	$R = \dfrac{V_1(B)}{K_b\left[1 + \dfrac{B}{K_{ib}} + \dfrac{P}{K_{ip}} + \dfrac{K_{iq}(B)}{K_{ib}(Q)}\right]}$
Random Bi Bi	A⟷Q	$R = \dfrac{\left[k_{11}[k_9 + k_{13}] + k_{10}k_{13}(P)\right]\left[k_1k_3(A)(B) + [k_2k_7(A)(B) + k_3k_7(A)(B)^2]/K_{ib}\right]E_0}{k_2k_{11}\left[k_4 + k_8 + k_9 + k_{13}\right] + k_3k_{11}(B)[k_8 + k_9 + k_{13}] + k_2k_{10}(P)[k_4 + k_8 + k_{13}]}$
		$\left[+ k_3k_{10}(B)(P)[k_8 + k_{13}]\right]\left[1 + \dfrac{A}{K_{ia}}\left[1 + \dfrac{B}{K_b}\right] + \dfrac{B}{K_{ib}} + \dfrac{Q}{K_{iq}} + \dfrac{P}{K_{ip}}\right]$

[a] All steps are assumed to equilibrate rapidly relative to the interconversion of the ternary complexes.

3. Random Bi Bi

Derivation of the various exchange rate equations at equilibrium in the case of the Random Bi Bi mechanism in which the interconversion of the ternary complexes is not rate limiting is a formidable task. The rate equation for the mechanism described in Scheme VI-1 is presented in Table VI-2 for the A⟷Q exchange. The derivation requires that $R_{A\longleftrightarrow Q} = k_{11}(EQ^*) + k_{13}(EXY^*)$. The usual steady-state equations involving isotope are solved to permit EQ* to be expressed as a function of EXY* or *vice versa*. The labeled substrate A* may react with either E or EB; however, the latter complex does not exist in the steady-state. A substitution is therefore made for EB in terms of E from the expression $EB = \frac{(E)(B)}{K_{ib}}$. The solution of the rate equation is similar at this point to mechanisms that do not involve branched pathways.



Scheme VI-1

4. Theorell-Chance Mechanism

It has been stated that, in the case of Ordered Bi Bi mechanism, there will be an initial rise in the A⟷Q exchange as the B-P substrate-product pair is raised, followed by a depression of the exchange rate. It can be seen from Table VI-2, however, that in the case of the Theorell-Chance mechanism this may not be true. In the absence of abortive ternary complex formation, the A⟷Q exchange will not be inhibited as B and P are increased unless the ternary complexes, which are at low concentration in the Theorell-Chance mechanism, become kinetically significant. If these complexes do become important as B and P are increased, the A⟷Q exchange will decrease as it does in the case of the Ordered Bi Bi pathway.

C. Substrate Synergism

One of the interesting features of enzymes that exhibit Ping Pong mechanisms is their ability to catalyze half-exchange re-

actions. For example, when considering the mechanism of Scheme I-10, it is obvious that an A⇌P exchange reaction may occur in the absence of the other substrate-product pair, and *vice versa*. These partial exchange reactions are often quite slow relative to the overall chemical reaction, and until recently, the following conclusions were reached on the basis of such observations: the compounds undergoing exchange were contaminated by one or both of the other substrate-product pair, and the mechanism is really Sequential, a contaminating enzyme was responsible for the partial exchange, or the Ping Pong mechanism was not a significant feature of the primary kinetic mechanism. Recently, BRIDGER et al. (10) have suggested that such phenomena may be the result of substrate synergism; i.e., slow partial exchanges may become rapid in the presence of components of the nonexchanging substrate-product pair. BRIDGER et al. (10) have proposed one criterion for substrate synergism, and LUECK and FROMM (11) have presented another. The former proposal involves comparison of the partial exchange reactions in the presence and absence of the substrate-product pair not involved in the isotope exchange reaction. Ordinarily, it would be expected that the presence of B and Q would serve to decrease the A⇌P exchange of Scheme I-10. If the A⇌P exchange increases, on the other hand, synergism is probable.

When considering the Ping Pong mechanism, it is clear that the A⇌P exchange rate must be equal to or greater than the A⇌Q exchange rate. Similarly, the B⇌Q exchange rate must equal or exceed the A⇌Q exchange. Often a direct comparison is made between the partial exchange rate and the initial exchange rate, but no direct relationship between the two exists. For the mechanism of Scheme I-10, the rate of the A⇌P exchange is:

$$\frac{E_0}{R} = \frac{(k_2 + k_3)}{k_1 k_3}\left[\frac{k_1}{k_2} + \frac{k_1 k_3}{k_2 k_4 (P)} + \frac{1}{A}\right] \qquad \text{(VI-15)}$$

Clearly, this equation is not related directly to the initial rate equation for the Ping Pong mechanism either in the presence or absence of product. It is possible to obtain the maximal velocity for the A⇌P exchange from a plot of 1/R *versus* 1/A at different levels of P. The intercepts of the resulting family of parallel lines will equal $[(k_2 + k_3)/k_2 \cdot k_3 E_0] + [(k_2 + k_3)/k_2 k_4 (P)(E_0)]$, and a secondary plot of intercepts *versus* 1/P will yield the intercept or reciprocal maximal exchange velocity, $(k_2 + k_3)/k_2 k_3 (E_0)$.

The maximal velocity from initial rate studies for the Ping Pong mechanism, V_1, is $k_3 k_7 E_0/(k_3 + k_7)$. From this discussion, it is clear that the maximum velocity for the forward reaction is not related to the maximal velocity for the partial exchange reaction. Furthermore, depending upon whether k_2 is equal to, greater than, or less than k_7, the maximal exchange velocity may be equal to, greater than, or less than the maximal initial velocity. It is clear that no direct relationship exists between these two quantities, and thus, it is not valid to use a comparison of initial velocity and partial exchange velocity as a basis of substrate synergism.

Initial rates and exchange rates for the Ping Pong Bi Bi mechanism are related as follows:

$$\frac{1}{R_{max,\ A\rightleftharpoons P}} + \frac{1}{R_{max,\ B\rightleftharpoons Q}} = \frac{1}{V_1} + \frac{1}{V_2} \qquad \text{(VI-16)}$$

where $V_2 = k_2k_6E_0/(k_2 + k_6)$ and $R_{max,\ B\rightleftharpoons Q} = k_6k_7E_0/(k_6 + k_7)$.

From these considerations it is clear, as suggested by LUECK and FROMM (11), that one must evaluate all four parameters of Eq. (VI-16) before one can draw definitive conclusions regarding the importance of, and explanation for, slow partial exchange reactions relative to initial rates.

D. Calculation of Kinetic Parameters

Determination of kinetic parameters from isotope exchange experiments is not possible except in a few cases, e.g., the Ping Pong and Rapid Equilibrium Bi Bi mechanisms. In the former case, it will be necessary to do a series of kinetic studies on the partial reactions. It will not be possible to obtain exact values for kinetic parameters for the random pathway, only so-called upper and lower limits for certain kinetic constants.

1. The Ping Pong Bi Bi Mechanism

If the isotope exchange studies of the two partial reactions, $A\rightleftharpoons P$ and $B\rightleftharpoons Q$, are carried out, it is possible to evaluate the following parameters: K_a/V_1, K_p/V_1, K_{ia}, K_{ip}, K_b/V_1, K_q/V_1, K_{ib}, and K_{iq}. If the maximal velocity V_1 is known from initial rate experiments, the four Michaelis constants can then be calculated. It is also possible to calculate the maximal rate of isotope exchange (R_{max}) as already indicated.

When considering the $A\rightleftharpoons P$ exchange (see Table VI-2) in the absence of B and Q, plots of the reciprocal exchange rate *versus* 1/A will give a family of parallel lines at different fixed levels of P. The slope of these curves will be K_a/V_1 and the intercepts on the 1/exchange axis will have values of $\frac{K_a}{V_1K_{ia}}(1 + \frac{K_{ip}}{P})$. If the factor intercept/slope is plotted against 1/P, the intercept of the replots will be $1/K_{ia}$ and the slope K_{ip}/K_{ia}. If these experiments involved the $B\rightleftharpoons Q$ exchange in the absence of A and P, the parameters K_b/V_1, K_q/V_1, K_{ib}, and K_{iq} could be evaluated.

Table VI-2 lists the exchange rate for the conversion of A to P. The equation for the conversion of P to A is

$$R = \frac{V_2(P)}{K_p\left[1 + \frac{P}{K_{ip}} + \frac{K_{ia}(P)}{K_{ip}(A)}\right]}. \qquad \text{(VI-17)}$$

It can readily be shown that this expression is identical to the equation for the A⟷P exchange of Table VI-2, where B and Q = O. Equation (VI-17) allows calculation of K_p/V_2 by analogy with the procedure for determination of K_a/V_1.

2. The Random Bi Bi Mechanism (Rapid Equilibrium)

It can be shown from the equation for the rapid equilibrium Random Bi Bi mechanism of Table IV-2 that, when abortive ternary complexes do not form,

$$R = \frac{1}{\frac{1}{V_1} + \frac{1}{V_2}}. \qquad \text{(VI-18)}$$

when all substrates and products are at saturating concentrations. Thus R, or the apparent maximal exchange velocity, will represent a lower limit for V_1 and for V_2.

E. Experimental Protocol

Although measurements of substrate-product exchange reactions may be conducted at or away from thermodynamic equilibrium, we shall restrict this discussion to the techniques applying to the former. Moreover, there will be no treatment of kinetic isotope effects upon the rates of isotope exchange presented here, as we shall assume that the radioactive substrates and products behave identically to the nonradioactive substrates and products. For a discussion of such isotope effects in enzyme catalyzed reactions, the reader is referred to the excellent review by RICHARDS (12) and several reports from BOYER's laboratory (13, 14). Since any particular half-reaction catalzyed by those enzymes displaying Ping Pong kinetic mechanisms is also at equilibrium whenever the substrate and product of the respective half-reaction are present, the measurement of such exchanges does fall into the category to be described.

The basic equation relating the rate of isotope exchange, R, to the concentrations of the substrate-product pair can be given as (1):

$$R = -\left[\ln(1 - F)\right]\ (A)(P)/t\ \left[(A) + (P)\right] \qquad \text{(VI-19)}$$

where A and P are the substrate and product of the exchange reaction under consideration, and F is the fractional attainment

of isotopic equilibrium determined at time t[4] . Because isotopic equilibrium can be defined as the point at which the specific radioactivity of A is exactly equivalent to the specific radioactivity of P, F is measured by the ratio of total radioactivity in P at some time t to the total radioactivity in P after attainment of isotopic equilibrium[5]. The usual approach used to make a measurement of R is to permit the substrates and products to incubate in the presence of enzyme to ensure thorough equilibration, to then initiate the exchange of labeled material by the addition of a small aliquot of highly radioactive substrate (A*) or product (P*), and, finally, to quench the reaction at some time t and determine F after separating and counting the total radioactivity in the A and P pools. From t, F, (A), and (P), one may then readily evaluate R, which the reader should note has units of molarity $\cdot$ min^{-1}. Although this seems relatively straight forward, there are a number of factors which are implicit in Eq. (VI-19) to be considered before such measurements are actually undertaken. Moreover, there are a number of empirical considerations which can greatly simplify the task if properly mastered. Some of these implicit and empirical factors form the basis for the following discussion, and they should prove to be of interest to anyone attempting to carry out equilibrium exchange kinetic studies. It is advisable, however, for the reader to also consult a number of the references cited in this section to gain an appreciation of specific technical problems encountered with particular enzymic reactions. These references also contain a wealth of information on the preparation, purification, separation, and measurement of labeled compounds.

To conduct equilibrium exchange reactions, one must have some knowledge of the apparent equilibrium constant, K_{eq}, which is often referred to as the mass action ratio. It is really this apparent constant, and *not* the true thermodynamic constant, K, that is pertinent to such studies. For many reactions, K_{eq} depends upon the relative affinity of the substrates and products for a particular metal ion, or, more commonly, upon the hydrogen ion activity. For example, the hexokinase reaction equilibrium is strongly influenced by both these factors (15), and the pH and magnesium ion concentration should always be specified when K_{eq} is presented. For any two substrate-two product reaction, K_{eq} is given as:

$$K_{eq} = \frac{(P)(Q)}{(A)(B)}. \qquad \text{(VI-20)}$$

Since we must obtain an accurate value of P* and A* at isotopic equilibrium to obtain accurate estimates of F, choosing the

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[4] For the exchange reaction between substrate A and water, this expression reduces to: $R = -(A)[\ln(1 - F)]/t$.

[5] Although radioactive substrates and products are most generally employed in such experiments, nonradioactive isotopes such as deuterium, ^{18}O, or ^{15}N may also be used.

proper (P)/(A) and (Q)/(B) ratios to be used in the experiments is quite important. In general, the experimentally adjustible (P)/(A) ratio should fall in the range from 0.05 to 20. If the ratio lies outside this range, it may be quite difficult to obtain an accurate value of $P^*_{time} = t/P^*_{isotopic\ equilibrium}$ or the P*/A* ratios. It is also true that the presence of contaminating levels of P* in A* is most cruical when the (P)/(A) ratio is outside the recommended range. One should also choose the (P)/(A) ratio such that an accurate progress curve for the conversion of A* into P* can be obtained. Obviously, it is inadvisable to choose a ratio of 0.05 if the exchange rate were to be measured by the addition of radioactive A*, of which only about 5% would be converted into P at isotopic equilibrium. This would mean that F will be 0.5 when only 2.5% of A* is transferred into the P pool. It would clearly be more advantageous to arrange the experimental conditions such that a large fraction of A* appears in this product pool.

A compromising factor in the choice of the (P)/(A) ratio is, of course, the (Q)/(B) ratio which must be adjusted such that the product of these ratios satisfies K_{eq}. Although Q and B do not appear in Eq. VI-19, their concentration will determine the degree of saturation of the enzyme with respect to Q and B, and thereby affect R. It is therefore important to bear in mind that one can often alter K_{eq} as described earlier. It should be recognized, however, that changing the pH will also influence the apparent stability constants for metal-ligand complexes, which often serve as the actual substrates in the reaction. In any case, once the (P)/(A) and (Q)/(B) ratios are chosen, it is advisable to mix P and A or Q and B in these ratios at concentrations corresponding to the highest levels to be used in the rate experiments; this prevents errors in preparing the reaction mixtures, and by properly combining these solutions and making serial dilutions of them, one can conveniently set up an exchange experiment with minimal delay.

Another factor of some importance is the time at which the reaction is quenched. As shown in Fig. VI-3, the conversion of A* to P* obeys a simple first-order relationship as described by Eq. (VI-19). To minimize the error in measuring F, one should stop the exchange reaction when approximately 50% of the A* is consumed. Estimates obtained in the time interval corresponding to 0.5 to 3 times the half-life for the exchange generally give adequate values provided that the experimental error is less than 5%. From Fig. VI-3, it is also evident that the incubation time must exceed a period equal to approximately 5-6 times the reaction half-life to get accurate values of the distribution of total radioactivity at isotopic equilibrium. It is advisable to experimentally obtain this distribution as there are often concentration-dependent changes in the substrate/product ratios if a metal ion binds preferentially to one substrate or product.

At this point it may be of value to illustrate how isotope exchange experiments were carried out with yeast hexokinase to establish the participation of a random kinetic mechanism. The exchange measurements were carried out at pH 6.5 to decrease

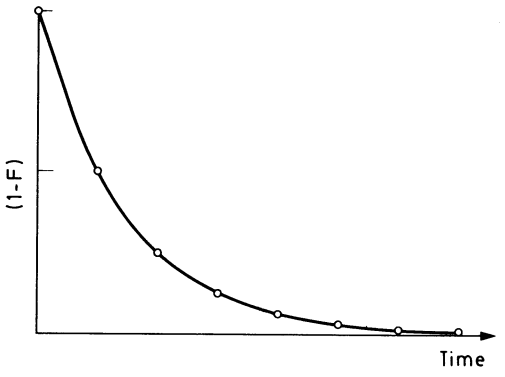

Fig. VI-3. Plot of (1-F) *versus* time after addition of isotope to a system at chemical equilibrium

the preponderance of ADP and glucose-6-P at equilibrium. The equilibrium constant in the presence of excess metal ion at this pH is 400, whereas at pH 8.0 the apparent equilibrium constant is roughly 12,700. In addition to buffer and magnesium ion, each reaction mixture contained a variable concentration of ADP and ATP, maintained at a constant (ADP)/(ATP) ratio of 19. Similarly, the glucose-6-P and glucose were maintained at a (glucose-6-P)/(glucose) ratio of 20, but their absolute concentrations were held at a fixed level which was saturating based upon the known values of their Michaelis constants from initial rate measurements. This can be easily accomplished by combining three solutions: Solution A, containing imidazole-nitrate buffer and magnesium ion; Solution B, containing the ADP and ATP in their proper ratio; and Solution C, containing the proper ratio of the sugar substrate and sugar-P. By making dilutions of Solution B, one may measure the $R_{\text{glucose-6-P}\rightleftharpoons\text{glucose}}$ or the $R_{\text{ADP}\rightleftharpoons\text{ATP}}$ as a function of the absolute levels of the nucleotides by introducing labeled glucose or ATP, respectively. Before the addition of labeled substrate or product, the reaction mixtures were permitted to fully equilibrate in the presence of yeast hexokinase, and the exchange reaction rates were then measured after the addition of labeled compound. The data for such an experiment, as well as for the companion experiment in which the levels of glucose-6-P and glucose were varied, are presented in Fig. VI-4. Lack of a depression in the exchange rate when all hexokinase substrates and products approach saturation excludes the participation of an ordered kinetic mechanism. It should be noted that the observed maximal ADP$\rightleftharpoons$ATP exchange rate is approximately 50% greater than the corresponding glucose$\rightleftharpoons$glucose-6-P exchange rate; the significance of this observation has already been discussed. Suffice it to say that the rate of substrate-product interconversion is not equivalent for each pathway in the random mechanism describing the hexokinase reaction, and this equilibrium exchange technique provides the strongest evidence for this phenomenon.

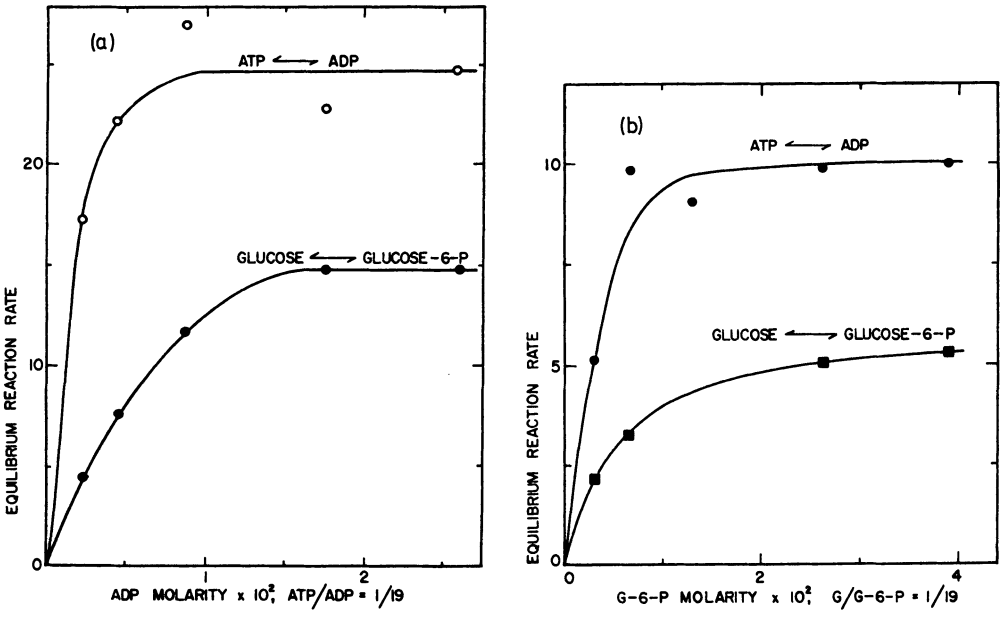

Fig. VI-4 a and b. (a) The effect of ATP and ADP concentrations on equilibrium reaction rates catalyzed by hexokinase. The reaction mixtures contained at 25°: 57.8 mM imidazole-NO_3, pH 6.5; 13 mM $Mg(NO_3)_2$; 2.5 mM glucose; 38.5 mM glucose-6-phosphate; 16.8 µg (29 Kunitz-McDonald units) of yeast hexokinase per ml; ATP and ADP as shown in the figure; and 0.34 mg of bovine serum albumin per ml. (b) Effect of glucose and glucose-6-phosphate concentration on equilibrium reaction rates catalyzed by hexokinase. The reaction mixtures contained, at 25°: 57.8 mM imidazole-NO_3, pH 6.5; 13 mM $Mg(NO_3)_2$; 0.99 to 2.2 mM ATP; 25.6 mM ADP, glucose, and glucose-6-phosphate as in figure; 7.83 µg (13.5 Kunitz-McDonald units) of yeast hexokinase per ml; and 0.624 mg of bovine serum albumin per ml

It is also possible to attempt to measure the initial rate of equilibrium exchange reactions as described by MORRISON and CLELAND (2). In this method, one adds a small aliquot of labeled substrate or product and follows the initial rate of transfer from the reactant to product pool. The initial rate of exchange is, of course, determined by the rapidity with which the substrate and product shuttle forth and back at equilibrium. During the early phase of the exchange measurement, the reverse reaction will occur, but the amount of P* formed from A* that returns to the substrate A pool will be negligible. Ultimately, the reverse reaction rate will become appreciable, and finally the distribution of label will approach isotopic equilibrium. In this respect, initial rates of equilibrium isotope exchange reactions are entirely analogous to kinetic studies of the *net* reaction rate. This method requires A* to be of higher specific radioactivity than the method previously described, and one now must measure the appearance of a substantially smaller fraction of P* such that less than 10-20% of A* is utilized. Because one should determine F in the former method at several time inter-

vals to insure valid measurements, both methods require about the same amount of effort. One inherent disadvantage of the initial rate approach, however, is that the same amount of tracer must be added to each reaction mixture; with the former method, one need not add identical amounts of tracer provided that the P*/A* ratio is evaluated at isotopic equilibrium, as should generally be done. An example of the use of the initial rate of isotope exchange technique in studies of rabbit skeletal muscle

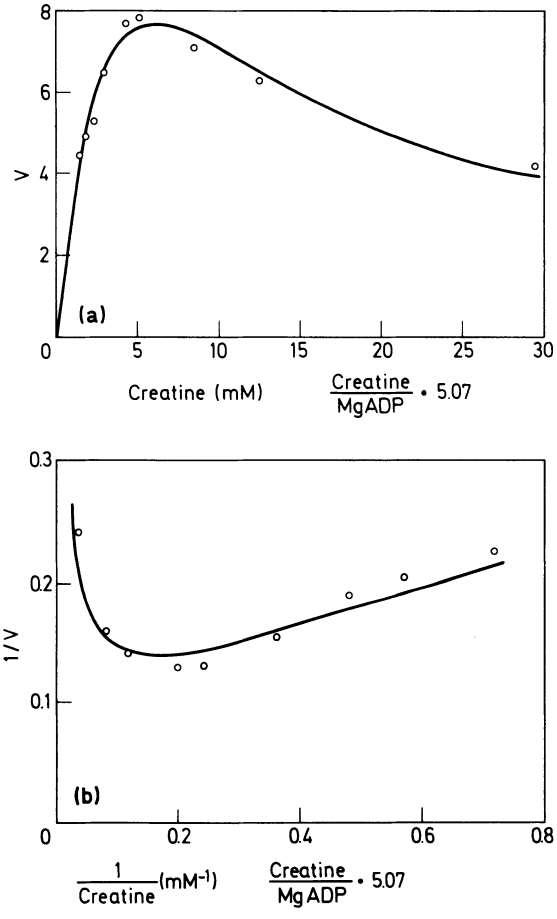

Fig. VI-5 a and b. (a) Effect of increasing concentrations of the MgADP-creatine pair on the initial velocity of the ATP-ADP exchange. (b) Comparison of the experimental data of Fig. VI-5(a), plotted in reciprocal form. The theoretical values for 1/v were multiplied by 1.55 and are represented by the solid lines. Basic reaction mixtures contained in 0.5 ml: 0.1 M triethanolamine-HCl buffer (pH 8.0), 0.01 mM EDTA, 3.85 mM ATP, 0.323 mM ADP, 5.52 mM $MgCl_2$, 1.39 mM creatine, 0.756 mM phosphocreatine, and 1 µg of creatine kinase. The concentrations of $MgADP^-$ and creatine were increased as indicated in the figure. The exchange reaction was started by the addition of 40 µl of ^{14}C-ATP (0.4 µC); temperature, 30°. Exchange rate (V) is expressed as millimicromoles per min per µg of enzyme

creatine kinase is shown in Fig. VI-5. The data indicate that raising the absolute concentrations of $MgADP^{1-}$ and creatine decreases the rate of the ATP⟷ADP exchange reaction as a result of the formation of an enzyme-MgADP-creatine abortive ternary complex. This experiment also illustrates a potential use of isotope exchange measurements to detect kinetically important abortive complexes.

Another means by which it may be possible to distinguish compulsory ordered and random addition mechanisms was recently described by WEDLER and BOYER (16). In this approach, the absolute levels of all substrates and products are varied while their relative concentrations are held constant. The basic idea is that, for cumpulsory ordered binding mechanisms, inhibition of the appropriate exchange rates will be observed as the absolute concentrations of reactants and products are increased sufficiently high. On the other hand, competitive effects between substrates and products observed in random mechanisms will not inhibit these exchange rates. This approach is illustrated for the *Escherichia coli* glutamine synthetase reaction in Fig. VI-6. In this case no depression in the exchange rate was observed, and the data are incompatible with an ordered addition of substrates.

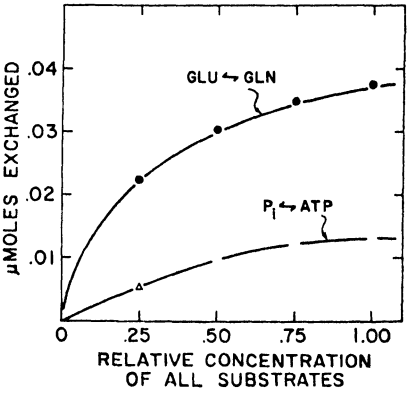

Fig. VI-6. The effects on equilibrium exchange rates of varying all substrate concentrations simultaneously and in constant ratio. Details of the experimental protocol may be found in Reference (16)

Finally, it was recently demonstrated that equilibrium exchange measurements may be useful in studies of kinetic properties at high enzyme concentrations (17). It was reasoned that the rates of equilibrium exchange reactions which are necessarily carried out in the presence of both reaction products may be considerably slower than the rates of the net reaction. For the latter, accurate initial reaction rates can only be obtained by the use of fast reaction instrumentation, generally of the stopped-flow type. Moreover, for reactions such as those catalyzed by many phosphotransferases, it is necessary to couple the production

of a product to a second enzyme system involving a chromophoric product, or to measure the rate of proton release by using a dye such as cresol red, which may bind to the enzyme itself. These approaches and their associated experimental difficulties are partially obviated by the isotope-exchange technique, which only requires a means for rapidly initiating and terminating the reaction. For example, it was possible to demonstrate that the maximal exchange rates of the hexokinase reaction are strictly proportional to the concentration of the yeast enzyme up to levels of 0.1 mg/ml. Furthermore, PURICH and FROMM (17) showed that the kinetic reaction mechanism at such concentrations was still random. The utility of this approach is further illustrated by the data presented in Table VI-3. Here, the enzyme concentrations tabulated represent that amount of enzyme that could be conviently studied by these techniques provided that the reaction time was 1 sec.

Table VI-3. Estimated enzyme concentrations at which isotope exchange measurements could be made provided that the reaction periods were 1.0 second

Enzyme and Reference Number	Exchange Reaction[a]	Estimated enzyme concentration (mg/ml)
Hexokinase (3)	Glucose-Glucose-6-P	22
	ADP-ATP	9.8
Maltodextrin Phosphorylase (18)	P_i-Glucose-1-P	3.7
	Dextrin-Glucose-1-P	4.4
Creatine Kinase (2)	Creatine-Creatine-P	5.9
	ADP-ATP	14
Galactokinase (19)	Galactose-Galactose-1-P	40
	ADP-ATP	19
Malate Dehydrogenase (20)	Oxaloacetate-Malate	1.6
	DPNH-DPN^+	0.3
Alcohol Dehydrogenase (21)	Ethanol-Acetaldehyde[b]	1.8
	DPNH-DPN^+, [b]	4.0

[a] For clarity, only the exchange reactions for the varied substrate-product pair are presented. Since the nonvaried substrate-product pair may be at any experimentally convenient concentration, it does not generally limit the method.

[b] Estimates for the alcohol dehydrogenase exchange rates were made from the values observed in the absence of imidazole.

F. Isotope-Trapping

In an attempt to gain insight into the mechanism of glutamine synthetase action, MEISTER and his coworkers (22) sought to identify the nature of the substrate bound to the enzyme using pulse-chase type experiments. They found that when ^{14}C-glutamate and ATP were incubated with enzyme and NH_2OH and a large excess of ^{12}C-glutamate added simultaneously, a significant amount of label was incorporated into the newly formed glutamine. Other combinations of preincubated substrates, e.g., ^{14}C-glutamate and NH_2OH, did not permit trapping of the label. It was finally concluded that only ^{14}C-glutamate and ATP form a complex with glutamine synthetase which precludes equilibration with added ^{12}C-glutamate when glutamine is formed.

ROSE et al. (23) in an elegant series of studies have attempted to quantitate and formalize the isotope-trapping procedure using hexokinase as a model. Scheme VI-2 outlines their approach to the calculation of k_2, the off constant for ^{14}C-glucose (G*) binding in the hexokinase reaction.

$$E + G^* \underset{k_2}{\overset{k}{\leftrightarrows}} E \cdot G^* \underset{k_4}{\overset{k_3(ATP)}{\rightleftharpoons}} E \cdot G^* \cdot ATP \xrightarrow{k_5} E + P^*$$

Scheme VI-2

The rate of conversion of E · G* to E + G* is $k_2(E \cdot G^*)$. Under the experimental conditions ATP and ^{12}C-glucose are added and the reaction rapidly quenched. The ^{14}C-glucose is diluted by ^{12}C-glucose to the extent that only a very small fraction of ^{14}C-glucose is carried to glucose-6-P after cold glucose addition. This small carry over could easily be accounted for by including ^{3}H-glucose in with the ^{12}C-glucose and subtracting the ^{3}H-glucose-6-P from the ^{14}C-glucose-6-P.

The rate of conversion of E · G* to P is equal to $(E \cdot G^*)k_3(ATP)k_5/(k_4 + k_5)$ where $k_5/(k_4 + k_5)$ represents the fraction of E · G* · ATP which is converted to P. At a certain level of ATP, designated $K_{1/2}$, half of the E · G* will be converted to G* and the other half to P*. Under these conditions,

$$k_2 = k_3 K_{1/2} k_5/(k_4 + k_5). \qquad \text{(VI-21)}$$

The fraction $k_3/(k_4 + k_5)$ is $1/K_{ATP}$ and k_5 is k_{cat}. Equation (VI-21) thus becomes

$$k_2 = \frac{K_{1/2} \cdot k_{cat}}{K_a}. \qquad \text{(VI-22)}$$

The terms k_{cat} and K_a are determined from initial velocity experiments where

$$k_{cat} = V_1/E_0. \qquad \text{(VI-23)}$$

In order to calculate k_2, it is necessary to determine $K_{1/2}$ from a pulse chase experiment and K_a and k_{cat} from initial rate experiments. In their experiments with hexokinase, ROSE et al. (23) graphed ^{14}C-glucose trapped against the concentration of ATP. The value of $K_{1/2}$ was then determined as the concentration of ATP required for 50% trapping.

The isotope-trapping approach does have certain limitations. It is important that the complex involving the labeled substrate (E · G* in Scheme VI-2) have enough radioactive substrate bound to it so as to provide enough labeled product for significant counting. This can be accomplished with high specific activity substrate, an enzyme that has a low dissociation constant for the labeled substrate, and large quantities of enzyme. ROSE et al. (23), for example, use a trypsin treated isozyme of hexokinase rather than the native enzyme, because the treated enzyme has a greater affinity (by approximately a factor of ten) for glucose. In their determination of $K_{1/2}$, the investigators used a 25 μM solution of enzyme; however, initial rate experiments with the native enzyme require about 1 nM enzyme (24). Experiments of this type are, of course, seriously compromised if the kinetic parameters such as k_{cat} and K_a are affected by alterations in enzyme concentration. This problem could be circumvented by measurement of inital rates at enzyme levels used in the isotope-trapping experiments. A stopped-flow device could be used for this purpose.

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Chapter VII

Isomerization Mechanisms and the Φ and Haldane Relationships

A. The Φ Relationships

In 1957 DALZIEL (1) described certain relationships that must be obeyed when initial rate experiments are carried out in both directions at a single pH for kinetically reversible bireactant systems. More recently, DALZIEL has extended this treatment to terreactant enzymes (2). The use of these relationships, called Φ relations, represents an important and powerful tool in enzyme kinetics for making a choice between certain kinetic mechanisms; however, its application requires very exact kinetic data and experimental reversibility. Another problem, which will be alluded to later, is that at least one type of kinetic mechanism, the rapid equilibrium Random Bi Bi mechanism, can in theory provide Φ relationships consistent with all those bireactant mechanisms that exhibit specific and unique Φ relationships.

In a historical context, certain experimentally unpredicted Φ relationships led MAHLER and his coworkers to propose the existence of the so-called isomechanisms (3). This most important contribution represents another example of serendipity that results when kinetic data do not adhere to predictable theory.

In the section below are described some of the Φ relationships for a variety of kinetic mechanisms for Uni reactant and Bi reactant systems. Many of these equations are taken from the studies of DALZIEL (1).

Table I-1 relates DALZIEL's (1) and CLELAND's (4) nomenclature. Expressing initial velocity equations in either form has certain distinct advantages. CLELAND's nomenclature for kinetic equations permits one to obtain a relatively large number of ways in which K_{eq} can be expressed in terms of kinetic parameters. On the other hand, DALZIEL's (1) method permits one to describe clusters of rate constants as coefficients, called Φ's. It is possible to gain insight into the sequence of enzyme and substrate interactions and certain isomerization steps from a comparison of the different Φ relationships.

Equations (VII-1) and (VII-2) describe initial rate equations for the Theorell-Chance (Mechanism I-9) and Ordered Bi Bi mechanisms (Mechanism I-8), respectively, in the absence of product in terms of Φ coefficients (1).

$$\frac{E_0}{v} = \frac{1}{k_5} + \frac{1}{k_1 A} + \frac{1}{k_3 B} + \frac{k_2}{k_1 k_3 (A)(B)} = \Phi_0 + \frac{\Phi_A}{A} + \frac{\Phi_B}{B} + \frac{\Phi_{AB}}{(A)(B)} \qquad \text{(VII-1)}$$

$$\frac{E_0}{v} = \left(\frac{1}{k_5} + \frac{1}{k_7}\right) + \frac{1}{k_1 A} + \frac{(k_4 + k_5)}{k_3 k_5 B} + \frac{k_2 (k_4 + k_5)}{k_1 k_3 k_5 (A)(B)} =$$

$$\Phi_0 + \frac{\Phi_A}{A} + \frac{\Phi_B}{B} + \frac{\Phi_{AB}}{(A)(B)} \qquad \text{(VII-2)}$$

It can be seen that, as in the case of CLELAND's nomenclature (4), the initial rate equations are identical for these two mechanisms.

DALZIEL's Φ relationships (1) represent a comparison between the Φ coefficients in one direction and those in the other direction; i.e., Φ_0 and $\frac{\Phi_P \Phi_Q}{\Phi_{PQ}}$ and Φ_0' and $\frac{\Phi_A \Phi_B}{\Phi_{AB}}$. In Eq. (VII-2) $\Phi_0 = \left(\frac{1}{k_5} + \frac{1}{k_7}\right)$ and thus for the reverse reaction $\Phi_0' = \left(\frac{1}{k_2} + \frac{1}{k_4}\right)$. The reader is referred to Chapter III for a review of procedures for substituting reverse for forward reaction rate constants for symmetrical mechanisms.

Table VII-1 compares the Φ relationships for a few bireactant kinetic mechanisms. A serious limitation in this approach involves the type of relationship to be expected for the rapid equilibrium Random Bi Bi mechanism. The following equalities hold for the mechanism of Scheme I-7:

$$\Phi_0 = \frac{1}{k_1}, \quad \Phi_A = \frac{K_a}{k_1}, \quad \Phi_B = \frac{K_b}{k_1}, \quad \Phi_{AB} = \frac{K_{ia} K_b}{k_1} \qquad \text{(VII-3)}$$

$$\Phi_0' = \frac{1}{k_2}, \quad \Phi_Q = \frac{K_q}{k_2}, \quad \Phi_P = \frac{K_p}{k_2}, \quad \Phi_{PQ} = \frac{K_{iq} K_p}{k_2} \qquad \text{(VII-4)}$$

If a comparison is made between $\Phi_0'\left(\frac{1}{k_2}\right)$ and $\frac{\Phi_A \Phi_B}{\Phi_{AB}}\left(\frac{K_a}{k_1 K_{ia}}\right)$ and $\Phi_0\left(\frac{1}{k_1}\right)$ and $\frac{\Phi_P \Phi_Q}{\Phi_{PQ}}\left(\frac{K_q}{k_2 K_{iq}}\right)$, it is clear that *any* Φ relationship may exist. Thus certain Φ relationships that seem to be unique (see Table VII-1) may also apply to the Random Bi Bi mechanism. These considerations point to the fact that one cannot use these relationships exclusively to make a choice of mechanism for bireactant systems. The procedure is obviously useful if the investigator can support a proposed mechanism which has been arrived at by other means, with the Φ relationships.

Table VII-1. Φ Relationships for bireactant kinetic mechanisms

Mechanism	Relationship
Ordered Uni Bi (Mechanism I-5)	$\Phi_0 > \frac{\Phi_P\Phi_Q}{\Phi_{PQ}}$
Theorell-Chance (Mechanism I-9)	$\Phi_0 = \frac{\Phi_P\Phi_Q}{\Phi_{PQ}}$, $\Phi_o' = \frac{\Phi_A\Phi_B}{\Phi_{AB}}$
Ordered Bi Bi (Mechanism I-8)	$\Phi_o > \frac{\Phi_P\Phi_Q}{\Phi_{PQ}}$, $\Phi_o' > \frac{\Phi_A\Phi_B}{\Phi_{AB}}$
Ping Pong (Mechanism I-10)	None, Φ_{AB} and Φ_{PQ} equal 0.
Random Bi Bi (Mechanism I-7)	Any Φ relationship is possible.

DALZIEL (2) has presented Φ relationships that apply to certain terreactant systems, and the reader is referred to the original work for additional information on this subject.

The determination of the Φ parameters can be made by analogy to the kinetic parameters, K_{ia}, K_a, K_b, etc., which are considered in Chapter III. Figure VII-1 is a primary plot of E_0/v *versus* 1/A and it evaluates the intercept and slope of the double reciprocal plots in terms of Φ parameters and substrate B. Replots of the intercepts and slopes as a function of 1/B are depicted in

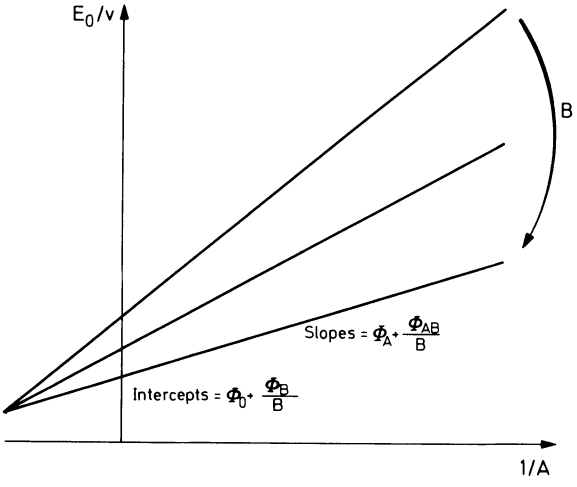


Fig. VII-1. Plot of E_0/v *versus* 1/A at three different fixed concentrations of substrate B. The values for the intercepts and slopes are shown on the graph

Figs. VII-2 (a) and VII-2 (b), respectively. These replots permit calculation of the four Φ parameters. The experimental protocol is identical whether one determines the Φ or CLELAND's (4) kinetic parameters.

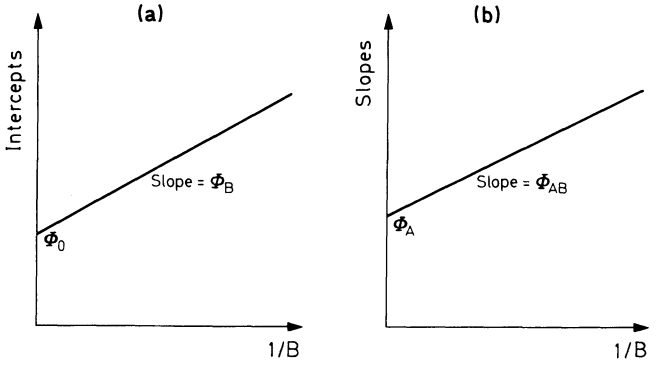


Fig. VII-2 a and b. (a) Secondary plot of intercepts *versus* 1/B from the primary plot of Fig. VII-1. The values for the intercepts and slopes of the secondary plot are shown on the graph. (b) Secondary plot of slopes *versus* 1/B from the primary plot of Fig. VII-1. The values for the intercepts and slopes of the secondary plot are shown on the graph

B. The Haldane Relationships

HALDANE (5) was the first to show that a relationship exists between certain kinetic parameters for a kinetic mechanism and the apparent equilibrium constant, K_{eq}. In the case of the mechanism depicted in Scheme I-2,

$$K_{eq} = \frac{k_1 k_3}{k_2 k_4}. \qquad \text{(VII-5)}$$

This equation may be expressed in terms of kinetic parameters in the following ways,

$$K_{eq} = \frac{V_1 K_p}{V_2 K_a} = \frac{K_{ip}}{K_{ia}}. \qquad \text{(VII-6)}$$

ALBERTY took advantage of these identities to extend the treatment to bireactant systems (6). He showed that a choice of mechanism could be made between the Theorell-Chance and the Ordered Bi Bi and rapid equilibrium Random Bi Bi mechanisms.

NORDLIE and FROMM (7) used the Haldane Relationship, as proposed by ALBERTY (6), to rule out the Theorell-Chance mechanism for the enzyme ribitol dehydrogenase (ribitol:NAD oxidoreductase; 1.1.1.c). They first determined K_{eq} from both the NAD^+ and NADH

sides of the reaction at pH 8.0 in Tris-Chloride buffer at 28°. Kinetic studies of both the forward and reverse reaction were also undertaken at this pH and temperature.

In order to derive Haldane Relationships, it is necessary to first get an expression for K_{eq} in terms of rate constants for a particular mechanism.

Three mechanisms, the Ordered Bi Bi, the rapid equilibrium Random Bi Bi, and the steady-state Random Bi Bi, will be used to indicate how the K_{eq} is obtained.

1. Ordered Bi Bi (Scheme I-8):

$$E + A \rightleftharpoons EA, \quad \frac{(EA)}{(E)(A)} = \frac{k_1}{k_2} \tag{VII-7}$$

$$EA + B \rightleftharpoons EXY, \quad \frac{(EXY)}{(EA)(B)} = \frac{k_3}{k_4} \tag{VII-8}$$

$$EXY \rightleftharpoons EQ + P, \quad \frac{(EQ)(P)}{(EXY)} = \frac{k_5}{k_6} \tag{VII-9}$$

$$EQ \rightleftharpoons E + Q, \quad \frac{(E)(Q)}{(EQ)} = \frac{k_7}{k_8} \tag{VII-10}$$

The expression EXY is taken to represent the central complexes. Multiplying the four equilibria gives

$$K_{eq} = (P)(Q)/(A)(B) = k_1k_3k_5k_7/k_2k_4k_6k_8. \tag{VII-11}$$

If the mechanisms are symmetrical and not branched, the rate constants will be related to K_{eq} in the following fashion:

$$K_{eq} = \frac{\text{product of odd numbered rate constants}}{\text{product of even numbered rate constants}}. \tag{VII-12}$$

2. Rapid Equilibrium Random Bi Bi (Scheme I-7):

$$\frac{(EA)}{(E)(A)} = \frac{1}{K_{ia}}; \quad \frac{(EAB)}{(EA)(B)} = \frac{1}{K_b}; \quad \frac{(E)(Q)}{(EQ)} = K_{iq}; \quad \frac{(EQ)(P)}{(EPQ)} = K_p \tag{VII-13}$$

$$E = \frac{K_{ia}K_b(EAB)}{(A)(B)} = \frac{K_{iq}K_p(EPQ)}{(P)(Q)} \tag{VII-14}$$

$$K_{eq} = \frac{(P)(Q)}{(A)(B)} = \frac{K_{iq}K_p(EPQ)}{K_{ia}K_b(EAB)} \tag{VII-15}$$

From the expressions $v_1 = k_1(EAB)$, $v_2 = k_2(EPQ)$, and $v_1 = v_2$ at equilibrium,

$$K_{eq} = \frac{k_1K_{iq}K_p}{k_2K_{ia}K_b} = \frac{V_1K_{iq}K_p}{V_2K_{ia}K_b} = \frac{V_1K_{ip}K_q}{V_2K_{ib}K_a} = \frac{V_1K_{iq}K_p}{V_2K_{ib}K_a} = \frac{V_1K_{ip}K_q}{V_2K_{ia}K_b} \quad \text{(VII-16)}$$

3. Steady-State Random Bi Bi (Scheme V-7):

$$\frac{(EA)}{(E)(A)} = \frac{k_1}{k_2}; \quad \frac{(EB)}{(E)(B)} = \frac{k_3}{k_4}; \quad \frac{(EAB)}{(EA)(B)} = \frac{k_5}{k_6}; \quad \frac{(EAB)}{(EB)(A)} = \frac{k_7}{k_8} \quad \text{(VII-17)}$$

$$\frac{(EQ)}{(E)(Q)} = \frac{k_{16}}{k_{15}}; \quad \frac{(EP)}{(E)(P)} = \frac{k_{18}}{k_{17}}; \quad \frac{(EPQ)}{(EQ)(P)} = \frac{k_{12}}{k_{11}}; \quad \frac{(EPQ)}{(EP)(Q)} = \frac{k_{14}}{k_{13}} \quad \text{(VII-18)}$$

$$\frac{(EAB)^2}{(E)^2(A)^2(B)^2} = \frac{k_1k_3k_5k_7}{k_2k_4k_6k_8}; \quad \frac{(EPQ)^2}{(E)^2(P)^2(Q)^2} = \frac{k_{12}k_{14}k_{16}k_{18}}{k_{11}k_{13}k_{15}k_{17}} \quad \text{(VII-19)}$$

$$E^2 = \frac{k_2k_4k_6k_8(EAB)^2}{k_1k_3k_5k_7(A)^2(B)^2} = \frac{k_{11}k_{13}k_{15}k_{17}(EPQ)^2}{k_{12}k_{14}k_{16}k_{18}(P)^2(Q)^2} \quad \text{(VII-20)}$$

$$\frac{k_1k_3k_5k_7k_{11}k_{13}k_{15}k_{17}(EPQ)^2}{k_2k_4k_6k_8k_{12}k_{14}k_{16}k_{18}(EAB)^2} = \left[\frac{(P)(Q)}{(A)(B)}\right]^2 \quad \text{(VII-21)}$$

at equilibrium $k_9(EAB) = k_{10}(EPQ)$ and

$$\left(\frac{k_9}{k_{10}}\right)^2 = \left(\frac{EPQ}{EAB}\right)^2 \quad \text{(VII-22)}$$

$$K_{eq} = \frac{k_9}{k_{10}}\left[\frac{k_1k_3k_5k_7k_{11}k_{13}k_{15}k_{17}}{k_2k_4k_6k_8k_{12}k_{14}k_{16}k_{18}}\right]^{1/2} \quad \text{(VII-23)}$$

One of the significant advantages of using CLELAND's nomenclature (4) is that it is capable of generating many more Haldane Relationships than the other nomenclatures currently in vogue. For example, using DALZIEL's Φ's (1) only one Haldane can be obtained for the Ordered Bi Bi mechanism (Eq. (VII-24)), whereas with CLELAND's nomenclature two Haldanes are possible (Eq. (VII-25)).

$$K_{eq} = \frac{\Phi_{PQ}}{\Phi_{AB}}. \quad \text{(VII-24)}$$

$$K_{eq} = \frac{V_1K_pK_{iq}}{V_2K_{ia}K_b} = \frac{(V_1)^2K_{ip}K_q}{(V_2)^2K_aK_{ib}}. \quad \text{(VII-25)}$$

According to CLELAND (4), the various kinetic parameters are related to K_{eq} in the following manner:

$$K_{eq} = \frac{(V_1)^n K_{(p)} K_{(q)} K_{(r)}}{(V_2)^n K_{(a)} K_{(b)} K_{(c)}} \qquad \text{(VII-26)}$$

where $K_{(a)}$ may be K_a or K_{ia}. It can be seen from Eq. (VII-25) that n equals either 1 or 2 for the Ordered Bi Bi mechanism. On the other hand, for the Theorell-Chance mechanism there are sixteen Haldane Relationships and now n can equal -1, 0, 1, 2, and 3. The *Appendix* contains certain of the Haldane Relationships along with the initial rate equations for certain kinetic mechanisms.

GARCES and CLELAND (8) investigated the kinetics of the yeast nucleoside diphosphate kinase reaction and found the mechanism to be Ping Pong Bi Bi. They also determined the equilibrium constant for the reaction at pH 8.0 in 0.1 M triethanolamine-acetate buffer in the presence of 10 μM ethylenediaminetetraacetate and 1 mM free Mg^{2+} at 30°. The kinetic studies were carried out under the same conditions, and an excellent correlation was found between the equilibrium constant and the kinetic parameters; i.e., the Haldane Relationship. Equation (VII-27) illustrates the Haldanes for the Ping Pong mechanism along with the values for the various expressions.

$$K_{eq} = \frac{K_{ip}K_{iq}}{K_{ia}K_{ib}} = \frac{V_1 K_{ip} K_q}{V_2 K_{ia} K_b} = \frac{V_1 K_p K_{iq}}{V_2 K_a K_{ib}} = \frac{(V_1)^2 K_p K_q}{(V_2)^2 K_a K_b} \qquad \text{(VII-27)}$$

1.28 1.28 1.31 1.26 1.28

GARCES and CLELAND (8) were also able to determine the equilibrium constants (K_{eq}) for the two partial reactions illustrated in Scheme I-10 for the Ping Pong Bi Bi mechanism:

$$\text{MgATP} + \text{E} \rightleftharpoons \text{F} + \text{MgADP} \qquad \text{(VII-28)}$$

$$K_{eq_1} = \frac{V_1 K_p}{V_2 K_a} = 0.188 \qquad \text{(VII-29)}$$

$$\text{MgUDP} + \text{F} \rightleftharpoons \text{E} + \text{MgUTP} \qquad \text{(VII-30)}$$

$$K_{eq_2} = \frac{V_1 K_q}{V_2 K_b} = 6.76 \qquad \text{(VII-31)}$$

The Haldane relationship is a useful adjunct in studies of isotope exchange at equilibrium as well as simple isotope exchange experiments. For example, let us assume that one is interested in measuring the P⟷A exchange in the presence of B and Q and let us further assume that the kinetic parameters are pH independent. If a proton is either utilized or liberated in the reaction, one may calculate the pH at which one could maximize

the exchange being measured to obtain experimentally meaningful results.

The Haldane expression has been used as an aid in determination of rate constants when studies of product inhibition are made in a single direction only (see Chapter V). If the rate constants are determined independently, the Haldane Relationship may be used as a check of the accuracy of these constants as illustrated for yeast nucleoside diphosphate kinase (8).

The Haldane expression has also proven to be of value in understanding why one isozyme may catalyze a thermodynamically reversible reaction, whereas another isozyme will not. This point may be illustrated by citing data with yeast and mammalian hexokinase (9). These enzymes, although not isozymes, do catalyze the same reaction and therefore the reactions exhibit the same K_{eq}. The yeast enzyme catalyzed reaction, is demonstrably reversible at pH values from 6.5 (10) to 7.6 (11). On the other hand, the enzyme from bovine brain catalyzes a reaction that can be reversed only with difficulty at acid pH (12). The Haldane Relationship for either the rapid equilibrium Random Bi Bi mechanism or the Ordered Bi Bi mechanism is

$$K_{eq} = \frac{V_1 K_{iq} K_p}{V_2 K_{ia} K_b} . \qquad \text{(VII-32)}$$

At pH 7.5 K_{eq} = 4,000 (13). In the case of the yeast system, V_1/V_2 is approximately 20, whereas in the case of the mammalian enzyme, this ratio is about 25,000. This relatively large difference between the two hexokinases is primarily a result of the fact that K_{iq} for glucose-6-P is about 1 μM for the brain enzyme and about 5 mM for the yeast enzyme catalyzed system.

The specific activity of yeast hexokinase is 200-600 μ moles/mg/min, whereas the specific activity for the brain enzyme is about 80 μ moles/mg/min. Thus an equivalent amount of yeast enzyme catalyzes the reverse hexokinase reaction at 3,000 to 10,000 times more rapidly than the brain enzyme. It is for this reason that the latter phosphotransferase seems "irreversible", and one need not be concerned that these enzymes may violate the concept of microscopic reversibility. The physiological implications involved in understanding such phenomena are obvious; not only does the K_{eq} affect the directionality of enzymatic reactions, but the kinetic parameters such as the Michaelis constants, dissociation constants, etc., also play an important role in this regard.

C. Isomerization Mechanisms

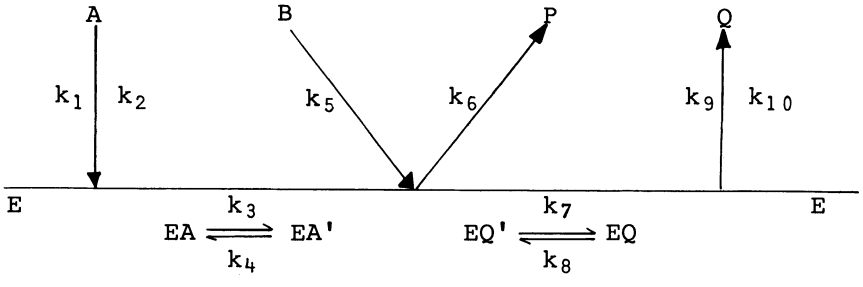
PELLER and ALBERTY (14) have rigorously shown that the maximal velocity cannot be greater than any unimolecular rate constant involved in the direction of substrate going to product. If cal-

culation of the rate constants for a mechanism leads to negative values, or if the numerical value of a constant is less than the maximal velocity (3) or if $\Phi_0' < \Phi_A\Phi_B/\Phi_{AB}$, then isomerization of one or more stable enzyme forms may be occuring (3). An alternative explanation for certain of these effects is the suggestion of MAHLER et al. (3) that an inactive binary complex may be formed which has a lower dissociation constant than the analogous active binary complex.

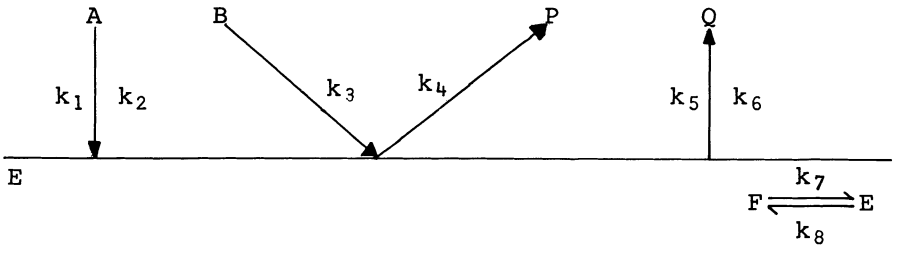
Experimental evidence has accumulated which indicates that a large number of enzyme systems exhibit "anomalous" kinetic parameters that can best be explained by invoking the idea that certain stable enzyme forms isomerize. ALBERTY and his coworkers (15) compiled a list of pyridine-linked anerobic dehydrogenase systems that quite probably undergo isomerization steps in their kinetic mechanisms. These include heart muscle lactate dehydrogenase and yeast alcohol dehydrogenase. Studies by WRATTEN and CLELAND (16) suggest that liver alcohol dehydrogenase exhibits an Iso Ordered Bi Bi mechanism, and data for muscle lactate dehydrogenase implicate an Iso Theorell-Chance mechanism (17).

In Chapter I it was pointed out that isomerization of stable enzyme forms leads to alterations in rate equations when compared with analogous mechanisms in which isomerizations do not occur. On the other hand, if a central complex form isomerizes, its effect will not be determinable from initial rate studies. In Chapter V it was shown that, for the Uni Uni mechanisms of Schemes I-3 and I-4, isomerization of the free enzyme affects the product inhibition patterns. It was also shown that, if the free enzyme isomerizes in the Ordered Bi Bi mechanism (Scheme V-6), there will be an alteration in the product inhibition patterns relative to the simple Ordered Bi Bi mechanism. Although it is clear that isomerization steps serve to compromise product inhibition studies and evaluation of rate constants from initial rate experiments, these effects, if recognized, give additional insight into the kinetic mechanism.

These points may be illustrated by reference to the two "Iso" Theorell-Chance mechanisms described in Schemes VII-1 and VII-2.



Scheme VII-1



Scheme VII-2

In the case of the mechanism of Scheme VII-1, which will be referred to as the Di-Iso Theorell-Chance mechanism,

$$\Phi_0 = \frac{1}{k_3} + \frac{1}{k_7} + \frac{1}{k_9} + \frac{k_8}{k_7 k_9} \qquad \text{(VII-33)}$$

$$\Phi_A = \frac{(k_2 + k_3)}{k_1 k_3}; \quad \Phi_B = \frac{(k_3 + k_4)}{k_3 k_5}; \quad \Phi_{AB} = \frac{k_2 k_4}{k_1 k_3 k_5} \qquad \text{(VII-34)}$$

The Φ values for the reverse reaction may be determined as suggested in Chapter II, Section A-4.

$$\Phi_{o'} = \frac{1}{k_2} + \frac{1}{k_4} + \frac{1}{k_8} + \frac{k_3}{k_2 k_4} \text{ and } \frac{\Phi_A \Phi_B}{\Phi_{AB}} = \frac{1}{k_2} + \frac{1}{k_3} + \frac{1}{k_4} + \frac{k_3}{k_2 k_4} \qquad \text{(VII-35)}$$

If $\Phi_0' > \frac{\Phi_A \Phi_B}{\Phi_{AB}}$ then it can readily be shown that $\Phi_0 < \frac{\Phi_P \Phi_Q}{\Phi_{PQ}}$. On the other hand, if $\Phi_0 > \frac{\Phi_P \Phi_Q}{\Phi_{PQ}}$ then $\Phi_0' < \frac{\Phi_A \Phi_B}{\Phi_{AB}}$. Reference to Table VII-1 indicates that one of these relationships is not comparable with either the Theorell-Chance or Ordered Bi Bi mechanism.

The Φ parameters for the mechanism described by Scheme VII-2 (Mono-Iso Theorell-Chance) are:

$$\Phi_0 = \frac{1}{k_5} + \frac{1}{k_7}; \quad \Phi_A = \frac{(k_7 + k_8)}{k_1 k_7}; \quad \Phi_B = \frac{1}{k_3}; \quad \Phi_{AB} = \frac{k_2 (k_7 + k_8)}{k_1 k_3 k_7} \qquad \text{(VII-36)}$$

$$\Phi_0' = \frac{1}{k_2} + \frac{1}{k_8}; \quad \Phi_Q = \frac{(k_7 + k_8)}{k_6 k_8}; \quad \Phi_P = \frac{1}{k_4}; \quad \Phi_{PQ} = \frac{k_5 (k_7 + k_8)}{k_4 k_6 k_8} \qquad \text{(VII-37)}$$

Table VII-2. Φ Relationships and product inhibition patterns for some Iso mechanisms

Mechanism and [rate equation]	Φ relationships	Product inhibition patterns [a] Product	Varied Substrate A	B	C
Mono-Iso Theorell-Chance (Mechanism VII-2) [ABQ, APQ] [c]	$\Phi_0' > \frac{\Phi_A\Phi_B}{\Phi_{AB}}$ $\Phi_0 > \frac{\Phi_P\Phi_Q}{\Phi_{PQ}}$	P	NC[b]	C	-
		Q	NC	NC	-
Di-Iso Theorell-Chance (Mechanism VII-1) [None] [c]	If $k_3 = k_8$, $\Phi_0' = \frac{\Phi_A\Phi_B}{\Phi_{AB}}$; $\Phi_0 = \frac{\Phi_P\Phi_Q}{\Phi_{PQ}}$	P	NC	C	-
	If $\Phi_0' > \frac{\Phi_A\Phi_B}{\Phi_{AB}}$ then $\Phi_0 < \frac{\Phi_P\Phi_Q}{\Phi_{PQ}}$ or *vice versa*	Q	C	NC	-
Mono-Iso Ordered Bi Bi [ABQ, APQ, ABPQ] [c]	Any Φ relationship may exist	P	NC	NC	-
		Q	NC	NC	-
Di-Iso Ordered Bi Bi [None] [c]	Any Φ relationship may exist	P	NC	NC	-
		Q	C	NC	-
Mono-Iso Ping Pong Bi Bi (Mechanism VII-4) [AQ, ABQ, APQ] [c]	None	P	NC	C	-
		Q	NC	NC	-

Di-Iso Ping Pong Bi Bi (Mechanism VII-5) [AQ, BP, ABP, ABQ, APQ, BPQ] [c]	None	P	NC	NC	-
		Q	NC	NC	-
Mono-Iso Uni Uni Bi Bi Ping Pong (Mechanism VII-6) [BCP, ABCP, BCPR, BPQR, BCPQR] [c]	None	P	NC	NC	NC
		Q	U	NC	NC
		R	C	U	U
Di-Iso Bi Uni Uni Bi Ping Pong (Mechanism VII-7) [CP, ACP, CPR, ABCP, ABCR, ABQR, APQR, CPQR, ABCQR, ABPQR] [c]	None	P	NC	NC	NC
		Q	U	U	NC
		R	NC	NC	U
Tri-Iso Hexa Uni Ping Pong (Mechanism VII-8) [ABR, ACQ, AQR, BCP, BPR, CPQ, ABCP, ABCQ, ABCR, ABQR, ACPQ, APQR, BCPR, BPQR, CPQR] [c]	None	P	NC	NC	U
		Q	U	NC	NC
		R	NC	U	NC

[a] These patterns do not take into account abortive complex formation.

[b] The abbreviations are: C(competitive); NC(noncompetitive); U(uncompetitive).

[c] Terms to be added to the denominator of the rate equations (Cleland form) in *Appendix* to analogous non-Iso mechanisms.

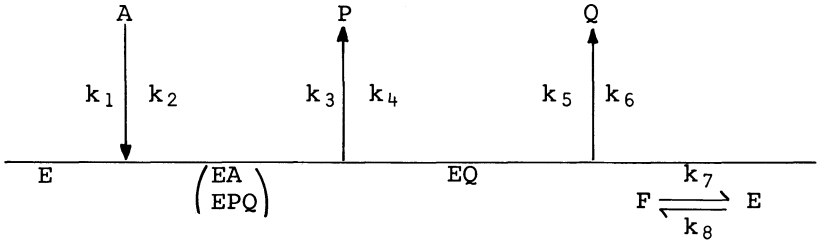
The two Φ relationships are:

$$\Phi_0' > \frac{\Phi_A\Phi_B}{\Phi_{AB}} \quad \text{and} \quad \Phi_0 > \frac{\Phi_P\Phi_Q}{\Phi_{PQ}} \qquad \text{(VII-38)}$$

These Φ relationships are identical to those obtained for the Ordered Bi Bi mechanism (Table VII-1). It is not possible to make a choice of mechanism using the Φ relationship as a criterion; however, the product inhibition patterns for the Mono-Iso mechanisms are unique. For the mechanisms described in Schemes I-4 and VII-2, the substrates and products react with different enzyme forms and are therefore not competitive inhibitors. This will be true for all Mono-Iso Ordered mechanisms when free enzyme isomerizes.

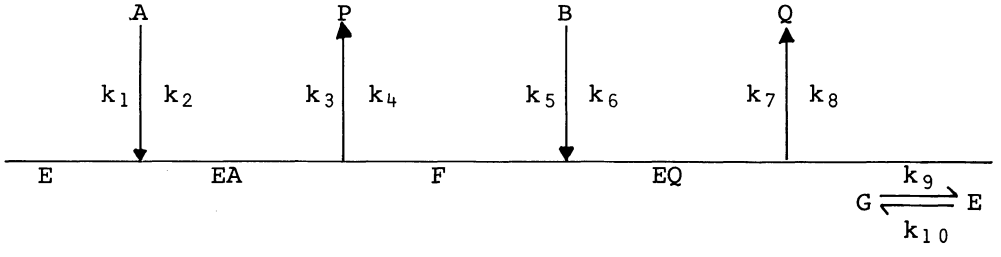
Cited below are a few additional Uni, Bi, and Terreactant Iso mechanisms. Table VII-2 illustrates some of the features of the Iso mechanisms that serve to distinguish them from mechanisms in which stable enzyme forms do not isomerize.

1. Iso-Uni Bi



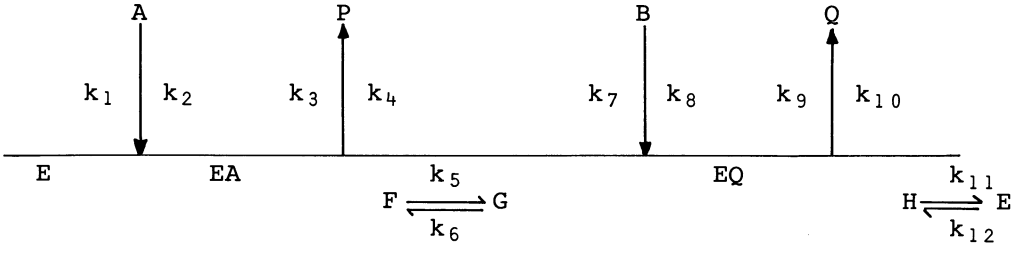
Scheme VII-3

2. Mono-Iso Ping Pong Bi Bi



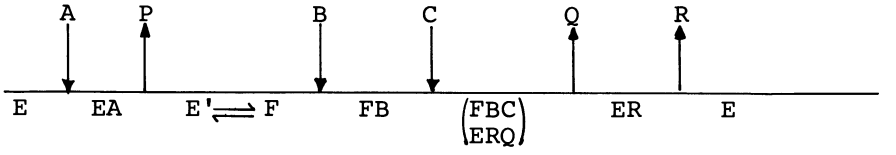
Scheme VII-4

3. Di-Iso Ping Pong Bi Bi



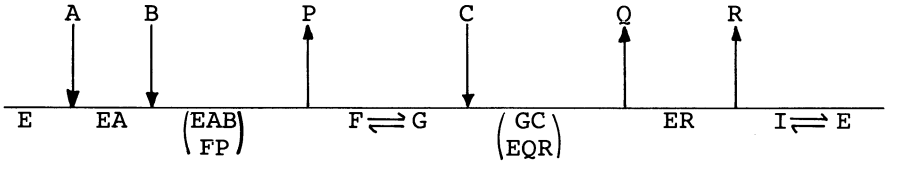
Scheme VII-5

4. Mono-Iso Uni Uni Bi Bi Ping Pong



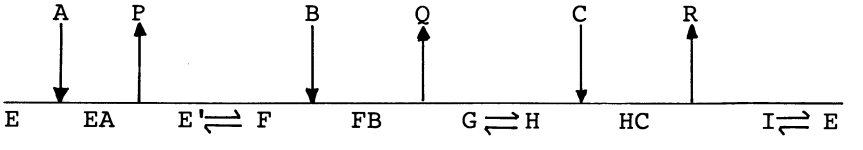
Scheme VII-6

5. Di-Iso Bi Uni Uni Bi Ping Pong



Scheme VII-7

6. Tri-Iso Hexa Uni Ping Pong



Scheme VII-8

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Chapter VIII

The Effect of Temperature and pH on Enzyme Activity

A. Effect of pH on Enzyme Kinetics *

It has long been recognized that enzyme catalysis is markedly influenced by alterations in the hydrogen ion concentration. MICHAELIS and DAVIDSOHN (1) in 1911 attempted to explain the characteristic bell-shaped velocity *versus* pH curve obtained for many enzyme catalyzed reactions. They proposed that the enzyme, which was assumed to be amphoteric, could exist in its acidic, basic or isoelectric form, and they suggested that it was this latter state of the enzyme that was catalytically active. Subsequent experimental studies of pH kinetics led MICHAELIS and ROTHSTEIN to propose in 1920 (2) that it was the ionization state of the enzyme substrate complex, rather than of the free enzyme, that caused changes in the rate of catalysis as pH is altered. However, it remained for HALDANE some ten years later to suggest that it was the charge distribution associated with certain functional groups on the enzyme, rather than the isoelectric point of the enzyme, that was responsible for the observed alterations in the rates of enzyme catalysis induced by changes in hydrogen ion concentration (3).

Investigations of how enzyme activity is affected by pH have given useful information on the mechanism of enzyme catalysis. It is now recognized that experiments involving pH kinetics may provide valuable insights into the nature of acidic and basic groups on the enzyme which are associated with catalysis. Such information may of course be obtained by a variety of more complex methods. For example, WINER and SCHWERT (4) found from relatively simple kinetic experiments that a histidyl residue in lactate dehydrogenase probably accepts a proton from lactate in the course of substrate oxidation. This suggestion has recently been supported by X-ray findings of ADAMS et al. (5). There are clearly serious inherent problems associated with pH kinetic studies of enzyme catalysis that limit the usefulness of this approach. These include: our inability at present to correlate particular pKs of free amino acids with amino acids residues as they exist in proteins; the possibility that rate limiting steps in a kinetic mechanism may change as the pH is altered; and finally, the possibility that a kinetically important pK may re-

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* In this chapter proton dissociation steps will be described by dissociation constants such as $K_{\bar{a}}$, $K_{\bar{b}}$, K_1, K_{Ea}, etc. For unireactant systems S, P, K_m, and V_m, will be substrate, product, Michaelis constant, and maximal velocity, respectively.

present a required conformational change of the enzyme rather than interaction of a group on the enzyme with the substrate. These limitations will be described in detail in the subsequent discussion.

1. pH Functions

If one considers the following scheme for the interaction of enzyme and protons, then two dissociations may be written,

$$EH_2^+ \underset{}{\overset{K_{\bar{a}}}{\rightleftharpoons}} EH \overset{K_{\bar{b}}}{\rightleftharpoons} E^-$$

Scheme VIII-1

$$K_{\bar{a}} = (EH)(H)/(EH_2^+) \quad \text{and} \quad K_{\bar{b}} = (E^-)(H)/(EH). \tag{VIII-1}$$

These expressions for the macroscopic constants, $K_{\bar{a}}$ and $K_{\bar{b}}$, may be substituted into the conservation of enzyme equation ($E_0 = E^- + EH + EH_2^+$) to yield three Michaelis pH functions (6).

$$EH = E_0/\left[1 + \frac{H}{K_{\bar{a}}} + \frac{K_{\bar{b}}}{H}\right] \tag{VIII-2}$$

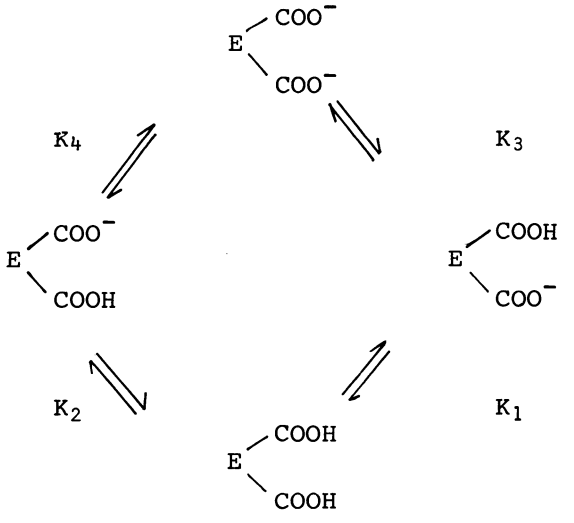
$$E^- = E_0/\left[1 + \frac{H}{K_{\bar{b}}} + \frac{H^2}{K_{\bar{a}}K_{\bar{b}}}\right] \tag{VIII-3}$$

$$EH_2^+ = E_0/\left[1 + \frac{K_{\bar{a}}}{H} + \frac{K_{\bar{a}}K_{\bar{b}}}{H^2}\right] \tag{VIII-4}$$

In Fig. VIII-1, EH of Eq. (VIII-2) is plotted against pH by making the assumptions that $E_0 = 1$, $K_{\bar{a}} = 10^{-6}$M, and $K_{\bar{b}} = 10^{-9}$M.

It may be readily demonstrated that, when $K_{\bar{a}}$ and $K_{\bar{b}}$ are widely separated, as they are in the example described by Fig. VIII-1, the plateau region will be relatively flat. A sharp maximum will occur when these values are close together. It can also be seen from Eqs. (VIII-3) and (VIII-4) that monovalent type titration curves may be obtained when E^- and EH_2^+ are plotted against pH. This results because in the acid region, the $K_{\bar{b}}/H$ term is small relative to one in the factor, $\frac{K_{\bar{a}}}{H}\left[1 + \frac{K_{\bar{b}}}{H}\right]$, in Eq. (VIII-4). A similar analogy holds in the basic pH region when considering Eq. (VIII-3).

If, in the preceeding discussion, it is assumed that there are two tautomers or isomers, rather than one for the intermediate form EH, the following scheme is obtained (7).



Scheme VIII-2

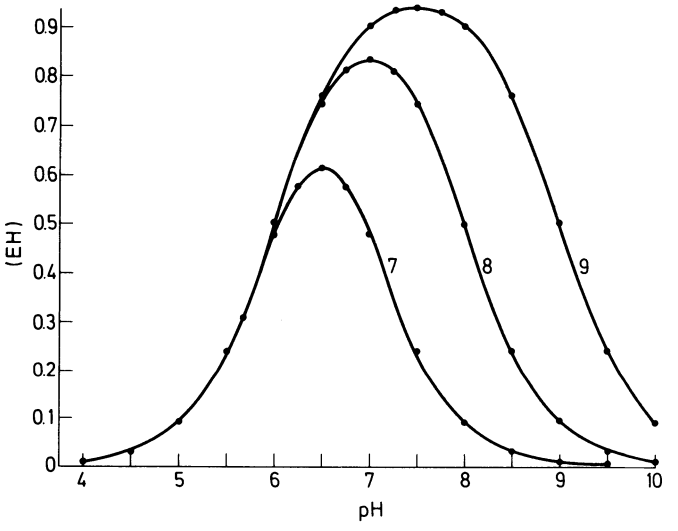


Fig. VIII-1. Plot of EH *versus* pH for the equation $EH = E_0/(1 + (H/K_a^-) + (K_b^-/H))$. E_0 is taken to be unity and $pK_a = 6$. The values 7, 8, and 9 indicate the pK_b values for each curve. Table VIII-1 illustrates the pK_a and pK_b values obtained from half-height estimations and those calculated using the procedure of ALBERTY and MASSEY (13)

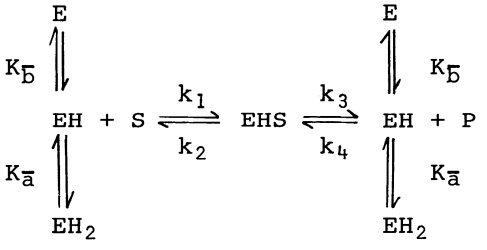
In this depiction, carboxyl and carboxylate groups are used; however, this outline is also applicable to cases in which the pKs are similar, but the functional groups different.

It can readily be shown, that $K_{\bar{a}} = (K_1 + K_2)$ and that $K_{\bar{b}} = \frac{1}{\frac{1}{K_3} + \frac{1}{K_4}}$. The microscopic constants, K_1, K_2, K_3, and K_4 are not independent but are related by the expression $K_1K_3 = K_2K_4$.

In studies of pH kinetics, it is the macroscopic constants, $K_{\bar{a}}$ and $K_{\bar{b}}$, that are experimentally determinable and not the micro constants. It can be seen that a knowledge of the macroscopic constants alone will not permit calculation of the microscopic constants, and another protocol, other than kinetic, will be required to give information on the two isomers described by Scheme VIII-2. Nevertheless, these relationships may be useful. If, for example, the microscopic constants are all equal, $K_{\bar{a}} = 4K_{\bar{b}}$. In the case of fumarase, studied by FRIEDEN and ALBERTY (8), dissociation constants were found to be 1.6 and 6.4 $\times$ 10^{-7} M suggesting that the microscopic constants are identical (3.2×10^{-7} M) for two groups at the active site.

2. The Effect of pH on Unireactant Models

In an attempt to explain the alteration of enzymatic activity as pH is varied, MICHAELIS and DAVIDSOHN (1) proposed the model formalized in Scheme VIII-3.



Scheme VIII-3

The rate equation for this mechanism can be derived by making either equilibrium or steady-state assumptions, or a combination of both. In the case of the Michaelis-Davidsohn mechanism, the form of the equation is the same regardless of the assumption made; however, depending upon the assumption made, K_m will either be a dissociation constant (equilibrium) or the Michaelis constant (steady-state). Shown below is the derivation assuming that the proton addition and removal steps are in rapid equilibrium relative to the other interconversions, which are in a steady-state.

The expression for velocity is $v = k_3(EHS)$, and the conservation of enzyme equation is $E_0 = E + EH_2 + EH + EHS$. What is required now is an equation for velocity, in terms of E_0 and S, which takes into account the various enzyme forms.

It is clear from earlier considerations that

$$d(EHS)/dt = k_1S(EH) - (k_2 + k_3)(EHS) = 0. \qquad \text{(VIII-5)}$$

We now have an expression for EH in terms of EHS; i.e.,

$$EH = \frac{K_m\ (EHS)}{S}. \qquad \text{(VIII-6)}$$

It is now only necessary to get the other two enzyme forms, E and EH_2 in terms of EH and, thus, in terms of EHS.

Remembering that we are dealing with equilibria and dissociation constants,

$$E = K_b^-(EH)/H \quad \text{and} \quad EH_2 = H(EH)/K_a^-. \qquad \text{(VIII-7)}$$

Equations (VIII-6) and (VIII-7) are now substituted into the conservation of enzyme expression to yield,

$$E_0 = EHS + \frac{K_m}{S}\left[1 + \frac{H}{K_a^-} + \frac{K_b^-}{H}\right](EHS). \qquad \text{(VIII-8)}$$

If it is recalled that $v = k_3(EHS)$, Eq. (VIII-8) may be divided through by EHS, and the appropriate substitution made for velocity to yield Eq. (VIII-9).

$$v = \frac{V_m}{1 + \frac{K_m}{S}\left[1 + \frac{H}{K_a^-} + \frac{K_b^-}{H}\right]} \qquad \text{(VIII-9)}$$

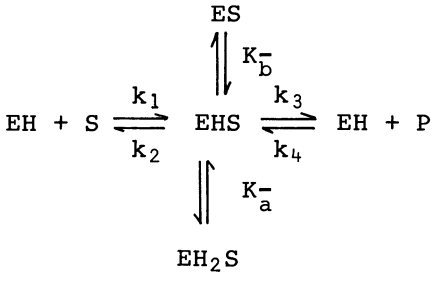
Equation (VIII-9) is the rate equation for the mechanism described by Scheme VIII-3 where $V_m = k_3E_0$. Equation (VIII-9) has two particularly interesting facets. First, if we allow $v = V_m/2$, we obtain an expression for K_m which is pH dependent; i.e.,

$$K_m,\ pH = K_m\left[1 + \frac{H}{K_a^-} + \frac{K_b^-}{H}\right]. \qquad \text{(VIII-10)}$$

Second, at infinite S, $v = V_m$; i.e., V_m is independent of pH and a plot of V_m *versus* pH will yield a line parallel to the abscissa. A graph of K_m *versus* pH will give rise to a typical bell-shaped curve.

It is also of interest to point out that in this mechanism, protonation of the free enzyme is required for catalysis, but more specifically for substrate binding. The enzyme form, EH, may represent a particular proton dependent conformation required for formation of the productive enzyme-substrate complex.

The postulation of MICHAELIS and ROTHSTEIN (2) that the enzyme-substrate complex, rather than the free enzyme, is amphoteric is depicted in Scheme VIII-4.



Scheme VIII-4

It is possible to invoke the rules described for the derivation of Eq. (VIII-9) to obtain an analogous expression for Scheme VIII-4. The rate equation for the Michaelis-Rothstein mechanism is

$$v = \frac{V_m}{\left[1 + \frac{H}{K_a^-} + \frac{K_b^-}{H}\right] + \frac{K_m}{S}}. \qquad \text{(VIII-11)}$$

When S is set equal to infinity, the following pH-dependent, maximal velocity expression is obtained,

$$V_m, pH = \frac{V_m}{\left[1 + \frac{H}{K_a^-} + \frac{K_b^-}{H}\right]}. \qquad \text{(VIII-12)}$$

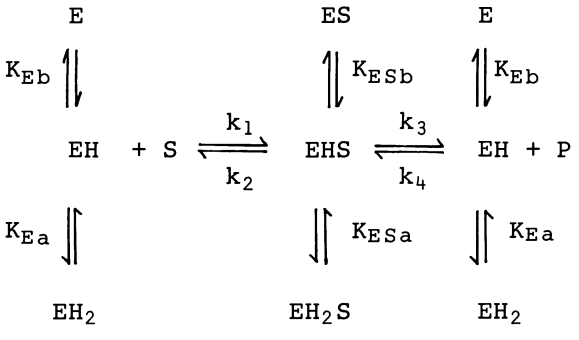
It may easily be shown by using the rules to obtain K_m, that

$$K_m, pH = \frac{K_m}{\left[1 + \frac{H}{K_a^-} + \frac{K_b^-}{H}\right]}. \qquad \text{(VIII-13)}$$

LAIDLER (9) has pointed out that, at low substrate concentration (i.e., where K_m/S is the dominant term in Eq. (VIII-11), the velocity expression will be independent of pH. It is also true that, for this mechanism, when $[V_m, pH/K_m, pH]$ is plotted against pH, the resulting curve will be a straight line parallel to the pH axis.

In the evaluation of contemporary concepts of pH kinetics, the details of which may be found elsewhere (10), VON EULER et al. (11) incorporated the ideas of MICHAELIS and DAVIDSOHN (1) and

MICHAELIS and ROTHSTEIN (2) into a single mechanism, which is shown in Scheme VIII-5.



Scheme VIII-5

Equation (VIII-14) illustrates the rate expression for this mechanism where $V_m = k_3E_0$ and $K_m = (k_2 + k_3)/k_1$. Equations (VIII-15) and (VIII-16) represent the pH-dependent V_m and K_m obtained from Eq. (VIII-14), respectively.

$$v = \frac{V_m}{\left[1 + \frac{H}{K_{ESa}} + \frac{K_{ESb}}{H}\right] + \frac{K_m}{S}\left[1 + \frac{H}{K_{Ea}} + \frac{K_{Eb}}{H}\right]} \qquad \text{(VIII-14)}$$

$$V_m,\ pH = \frac{V_m}{1 + \frac{H}{K_{ESa}} + \frac{K_{ESb}}{H}} \qquad \text{(VIII-15)}$$

$$K_m,\ pH = K_m \left[1 + \frac{H}{K_{Ea}} + \frac{K_{Eb}}{H}\right] \Big/ \left[1 + \frac{H}{K_{ESa}} + \frac{K_{ESb}}{H}\right] \qquad \text{(VIII-16)}$$

These last two equation indicate that, when either $[V_m, pH]$ or $[K_m, pH]$ is graphed as a function of pH, typical bell-shaped curves will be obtained. According to Eq. (VIII-15), only one pH optimum will be obtained when $[V_m, pH]$ is plotted against pH; however, a number of different curves may result from a graph of $[K_m, pH]$ *versus* pH for the mechanism of Scheme VIII-5. Depending upon the magnitude of the four dissociation constants, either one or no pH optimum may be obtained; however, when the ionization constants are widely separated, the data will approximate a nonsymmetrical bell-shaped curve with a single plateau. It is of interest to note that the $[V_m, pH]$ for the reverse reaction will contain the same denominator term as Eq. (VIII-15). Thus the pKs for this mechanism for the enzyme-substrate-proton complex should be the same for both the forward and reverse reactions.

It is possible to envision reaction of E and EH_2 with substrate in Scheme VIII-5. There will be no alteration in the basic rate

equation and kinetic parameters, if these new steps equilibrate rapidly and the complexes ES and EH_2S do not form product. Inclusion of these new pathways does not alter the conservation of enzyme equation. LAIDLER (9) has considered mechanisms in which steady-state conditions prevail and in which ES and EH_2S are productive complexes; however, the resulting rate expressions are too complicated to be of practical value. OTTOLENGHI (12) has indicated how certain of the complex steady-state rate equations can be simplified under certain limiting conditions.

3. Evaluation of Ionization Constants

Figure VIII-1 illustrates a series of bell-shaped curves of the type to be expected when some pH dependent kinetic parameter such as V_m is plotted against pH. If the pKs are widely spaced, e.g., two or more pK units apart, the pKs of the acidic and basic limbs of the curve may be estimated satisfactorily from the half-heights of the curves. On the other hand, a number of well defined procedures are currently in vogue for the determination of dissociation constants associated with enzyme catalysis. These methods involve the suggestions of ALBERTY and MASSEY (13) and DIXON (14)[6].

a) Procedure of Alberty and Massey (13)

The Alberty-Massey method for pK evaluation is an algebraic solution of graphical data obtained from kinetic experiments. The experimental protocol will be described in some detail later in this chapter.

In order to illustrate the procedure, it is necessary first to refer to Eq. (VIII-2). It is possible to obtain the hydrogen ion concentration, H_0, at the maximum point on the curve of a plot of EH *versus* pH. If one takes the first derivative of Eq. (VIII-2) and sets the resulting equation equal to zero, the following relationship is obtained,

$$(H_0)^2 = K_a^- K_b^- . \qquad \text{(VIII-17)}$$

If H_0 from Eq. (VIII-17) is substituted for H in Eq. (VIII-2), Eq. (VIII-18) is obtained

$$(EH)_{max} = \frac{E_0}{1 + 2\sqrt{K_b^-/K_a^-}} . \qquad \text{(VIII-18)}$$

Now, substituting for E_0 from Eq. (VIII-18) into Eq. (VIII-2) gives:

---

[6] It is important to note that for any of these analytical procedures to give valid pKs, the curves must be symmetrical.

$$\frac{(EH)}{(EH)_{max}}\left(1 + \frac{H}{K_a^-} + \frac{K_b^-}{H}\right) = \left(1 + 2\sqrt{K_b^-/K_a^-}\right). \qquad \text{(VIII-19)}$$

Under conditions where $(EH) = 1/2(EH)_{max}$ there will be two values for H, and Eq. (VIII-20) is obtained

$$H^2 + K_a^-K_b^- = HK_a^- + 4H\sqrt{K_a^-K_b^-} \quad \text{or} \quad H^2 - (K_a^- + 4H_0)(H) + H_0^2 = 0. \qquad \text{(VIII-20)}$$

DIXON and WEBB (15) have shown that this equation has two real roots and that their sum is equal to the factor $(K_a^- + 4H_0)$ in the quadratic equation. Thus

$$H_a + H_b = K_a^- + 4H_0 \qquad \text{(VIII-21)}$$

where H_a and H_b represent the hydrogen ion concentrations at $1/2(EH)_{max}$ on the acidic and basic limbs of the bell-shaped curve, respectively, and where H_0 is the hydrogen ion concentration at $(EH)_{max}$. K_b^- may be obtained from Eq. (VIII-17) and a knowledge of K_a^-.

Table VIII-1 illustrates the values for pK_a and pK_b obtained from Fig. VIII-1 by reading the pK values directly off the curves. It is obvious that, the farther apart pK_a and pK_b are, the better the pK estimate. It is clear from the data of Table VIII-1 that excellent estimates of pKs which are only one unit apart can be gotten using the protocol of ALBERTY and MASSEY (13).

Table VIII-1. Estimated and calculated pK values obtained from the data of Fig. VIII-1.

Theoretical pKs		Estimated pKs from 1/2 peak height		pKs calculated according to ALBERTY and MASSEY (13)	
pK_a	pK_b	pK_a	pK_b	pK_a	pK_b
6.00	7.00	5.68	7.33	6.06	6.95
6.00	8.00	5.87	8.13	6.09	7.98
6.00	9.00	5.95	9.04	5.99	9.09

b) Procedure of Dixon (14)

Although the ALBERTY-MASSEY (13) method for determination of acid and base dissociation constants has been in the literature almost as long as the graphical protocol of DIXON (14), the latter method has received the widest attention. DIXON's rules have been widely quoted and are as follows:

(a) The negative log of the kinetic parameter described by Eq. (VIII-2) will consist of straight-line sections (if

the pKs are sufficiently separated) joined by short curved parts.

(b) The straight portions have integral slopes; i.e., zero, one-unit or two-unit slopes. The latter two may be either positive or negative.

(c) Each bend indicates the pK of an ionizing group. The straight-line portions intersect at a pH corresponding to the pK.

(d) Each pK produces a change of one-unit in the slope.

(e) Each pK of a group in the ES complex produces a positive slope increase. Each pK of a group in either the free enzyme or free substrate produces a negative slope.

(f) The curvature at the bends is such that, at the intersection of the straight-line segments the intersection point is 0.3 unit above or below the graph. If two pKs occur together, the distance is 0.48.

(g) The slope of any straight-line segment is equal numerically to the change in charge when the enzyme-substrate complex dissociates to free enzyme and substrate.

Many of these rules can be understood by consideration of the following relationship:

$$f = \left[1 + \frac{H}{K_a^-} + \frac{K_b^-}{H} \right]. \qquad \text{(VIII-22)}$$

By taking the negative log of this equation and considering only the acid limb of the expression,

$$pf = - \log\left(1 + \frac{H}{K_a^-}\right). \qquad \text{(VIII-23)}$$

$$\text{If } \frac{H}{K_a^-} >> 1, \text{ then } pf = pH - pK_a, \qquad \text{(VIII-24)}$$

and it would be expected that a plot of pf *versus* pH will yield a straight line with a slope of +1. If one were to consider the pH in the region of K_b^-, the equation would be of the form, pf = -pH + pK_b, and a graph of pf *versus* pH gives a slope of -1. It is important to point out that these relationships are valid only when $\frac{H}{K_a^-} >> 1$ and $\frac{K_b^-}{H} >> 1$. If we assume that $\frac{H}{K_a^-}$ or $\frac{K_b^-}{H}$ must be 10 times greater than 1 for these relationships to hold, then it is clear that the tangent to the theoretical line of unit slope must be *at least* one pH unit below the pK_a or one pH unit above the pK_b. Unfortunately, this point is frequently overlooked by investigators in studies of pH kinetics.

Now considering Eq. (VIII-23), as the pH increases, $\frac{H}{K_a^-} < 1$, and at low hydrogen ion activity pf = 0. If pf = 0 is substituted into Eq. (VIII-24), we see that pH = pK_a; i.e., the pH equals the pK at the point of intersection of the two linear segments (Rule C).

Figures VIII-2 and VIII-3 depict the data of Fig. VIII-1 plotted in p (EH) *versus* pH form. It can be seen from Fig. VIII-2 that, when the pKs are widely separated (3 units apart), the horizontal straight-line segment is almost tangent to the calculated curve. On the other hand, when the pKs are not far apart (1 pK unit), the horizontal segment is not at all close to the pH curve (Fig. VIII-3). Fortunately, the position of the horizontal line can be

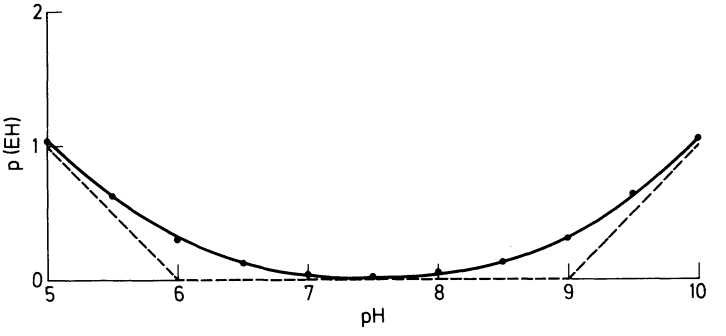


Fig. VIII-2. Graph of p(EH) *versus* pH of the data of Fig. VIII-1 for the curve with pK_a = 6 and pK_b = 9. The broken linear lines represent Dixon segments with slopes of +1, 0, and -1, intersection points at pH 6 and pH 9, and a vertical distance between the intersection point of the linear segments and the curve of 0.3 units

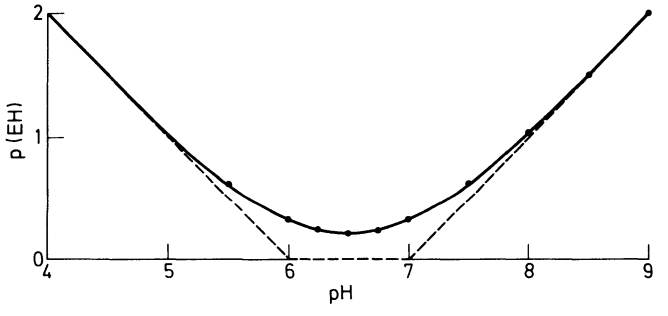


Fig. VIII-3. Graph of p(EH) *versus* pH of the data of Fig. VIII-1 for the curve with pK_a = 6 and pK_b = 7. The linear segments were drawn as indicated in the legend to Fig. VIII-2

estimated accurately: the distance between the experimental curve and the intersection of the two straight-line segments is 0.3. This can be seen by considering the acidic portion of Eq. (VIII-2),

$$EH = \frac{E_0}{\left[1 + \frac{H}{K_{\bar{a}}}\right]} . \qquad \text{(VIII-25)}$$

If E_0 equals 1 as illustrated in Figs. VIII-1 to VIII-3,

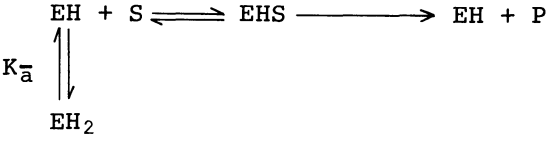
$$p(EH) = \log (1 + \frac{H}{K_a}). \qquad \text{(VIII-26)}$$

Remember that, where the two linear segments cross in Figs. VIII-2 and VIII-3, pK_a = pH and $H = K_a$. Thus, from Eq. (VIII-26), p (EH) = log 2 = 0.3. It is clear then that, at this intersection point, the vertical distance between the intersection point and the pH curve must be 0.3 unit.

This discussion suggests that the graphical procedure of DIXON (14) is a useful method for evaluation of the pKs associated with enzymic catalysis. Unfortunately, if the pKs are not widely separated, it becomes difficult to accurately evaluate the pKs. Although this inherent problem may exist in plots of log V_m *versus* pH, it is a more likely possibility when log K_m is graphed against pH. This can readily be appreciated when it is recognized from Eq. (VIII-15) that the parameter V_m contains two pKs, whereas K_m is associated with four pKs (Eq. (VIII-16)). It would seem more reasonable to plot $\log(K_m/V_m)$ and also log V_m against pH to obtain the most accurate estimates of the pKs associated with the enzymatic reaction.

It may be argued, and rightly so, that an undue emphasis has been placed upon an accurate estimation of pKs. For example, using Fig. VIII-3, if the horizontal line is drawn tangent to the curve at the minimum point, a pK_a of 5.8 rather than 6.0 will be obtained. Similarly, the pK_b will be too high by 0.2 unit. Although these deviations are probably within experimental error they are theoretically incorrect and in addition the error will be compounded as the pKs approach each other.

The problems outlined in determining the two pKs for a function of the type described by Eq. (VIII-22) are only partially relevant when only one pK is involved. This effect is frequently encountered and may represent the case where only two, rather than three (protonated, partially protonated, and unprotonated), enzyme forms exist. This may be illustrated as follows:



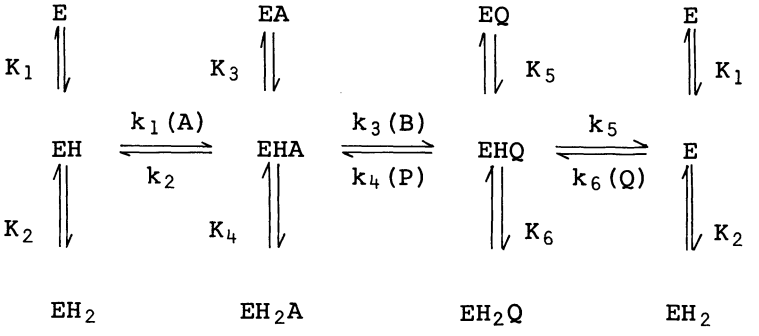
Scheme VIII-6

The expression for EH is now described by Eq. (VIII-25). If we again set E_0 = 1, at high pH, the limit of p(EH) will be zero and will coincide with the horizontal segment of the Dixon plot. It will still be necessary to obtain experimental data more than one pH unit below the pK_a to draw a tangent to that portion of the curve that is essentially linear. Again, the vertical distance between the experimental curve and the intersection of the linear drawn segments will be 0.3 unit.

4. Bisubstrate Systems

There are very few examples in the literature of studies of pH kinetics of bireactant systems. In these investigations, it must be assumed that neither the kinetic mechanism nor the rate-limiting step in the mechanism changes with change in pH.

The derivation of the rate equation for multireactant systems is similar to the cases described for unireactant mechanisms. One of the simpliest examples that might be considered is the Theorell-Chance mechanism in which the assumptions regarding acidic and basic forms of the enzyme are described in Scheme VIII-7:



Scheme VIII-7

The rate equation for this particular mechanism is:

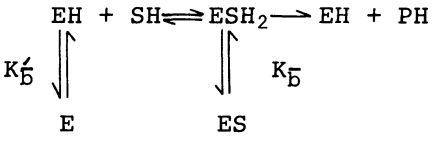
$$\frac{V_1}{v} = \left(1 + \frac{H}{K_6} + \frac{K_5}{H}\right) + \frac{K_a}{A}\left(1 + \frac{H}{K_2} + \frac{K_1}{H}\right) + \frac{K_b}{B}\left(1 + \frac{H}{K_4} + \frac{K_3}{H}\right) + \frac{K_{ia}K_b}{(A)(B)}\left(1 + \frac{H}{K_2} + \frac{K_1}{H}\right). \qquad \text{(VIII-27)}$$

It can be seen from Eq. (VIII-27) that plots of certain kinetic parameters against pH will give information on the proton association and dissociation for the various enzyme forms. It can be shown that, for the Theorell-Chance mechanism, a graph of $K_a/K_{ia}K_b$ against pH will yield a line that is independent of pH, provided that the rate constants k_2 and k_3 remain in a constant ratio. This will not be true for other mechanisms; e.g., the rapid equilibrium Random Bi Bi.

Presentation of rate equations for the usual bireactant systems does not seem warranted. These expressions may be simply derived if the assumption is made that the various protonated forms of the enzyme equilibrate rapidly relative to the other steps in the reaction sequence. It should be pointed out that, for the mechanisms presented thus far, it was assumed that only one enzyme-substrate complex form is enzymatically productive; however, *a priori*, there is no reason for this assumption to be correct.

5. Cooperative Proton Binding

The phenomenon of cooperative binding usually associated with substrate, product, and modifier binding to proteins may also occur with protons. A number of pathways may account for cooperative proton binding, and one example is listed in Scheme VIII-8:



Scheme VIII-8

The rate expression and $[V_m, pH]$ for the pathway shown in Scheme VIII-8 are described by Eqs. (VIII-28) and (VIII-29), respectively.

$$v = \frac{V_m}{\left(1 + \frac{K_b}{H^2}\right) + \frac{K_m}{SH}\left(1 + \frac{K'_b}{H}\right)} \qquad \text{(VIII-28)}$$

$$V_{m,pH} = \frac{V_m}{1 + \frac{K_b}{H^2}} \qquad \text{(VIII-29)}$$

In order to evaluate cooperative proton binding, a plot of Eq. (VIII-29) was made in which $[V_m, pH]$ is graphed as a function of pH. The solid curve in Fig. VIII-4 represents cooperative binding where $K_b = 10^{-8}$ M^2. The expression for normal proton binding was also included on the graph. This latter (broken line) curve

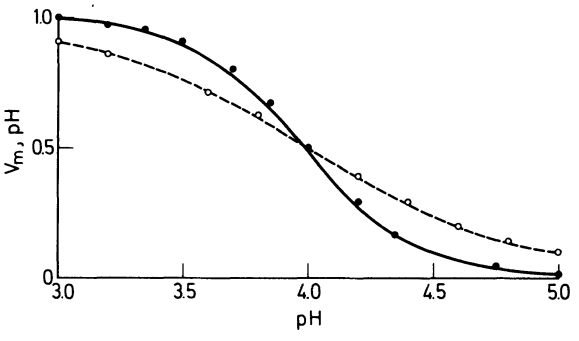


Fig. VIII-4. Plots of $[V_m, pH]$ *versus* pH for a cooperative binding system (solid line) and a noncooperative system (broken line). The cooperative curve was fit to the equation $[V_m, pH] = E_0/(1 + (K_b/H^2))$, $pK_b = 8$. The noncooperative binding equation was, $[V_m, pH] = E_0/(1 + (K_b/H))$, $pK_b = 4$. E_0 was taken to be unity in both equations

was generated from an expression analogous to Eq. (VIII-22), but for the basic limb of the titration curve assuming $K_5 = 10^{-4}$ M.

It can be seen from Fig. VIII-4 that both curves give an apparent pK of 4; however; the two curves differ in some significant respects. At one unit below its pK, the noncooperative species is 91% undissociated, whereas the cooperative species is 99% undissociated at 1 pK unit below its apparent pK. The distinction between these two cases can be seen even more clearly from Dixon plots. The cooperative two proton case will give a slope of two, whereas the noncooperative acid will exhibit a unit slope.

When considering Eq. (VIII-29), under conditions where $1 << K_5/H^2$, $p(V_m, pH) = 2pH - pK$ when V_m = unity. At the so-called pK_b, $K_5 = H^2$, and the vertical distance between the experimental curve and the linear and horizontal segments of the Dixon plot will be 0.3. The cooperative nature of the data, once recognized, permits calculation of the true pK.

SHUKUYA and SCHWERT (16) have presented spectral data for glutamate decarboxylase, which is strongly suggestive of cooperative binding according to the equation, $EH_4 \rightleftharpoons 4H^+ + E^{-4}$.

6. Identification of Amino Acid Residues from Studies of pH Kinetics

The ultimate aim of kinetic studies of pH effects is the identification of the groups at the active site of the enzyme. Table VIII-2 lists the pKs of a number of functional groups in small molecules and proteins.

Table VIII-2. Acid pKs of functional groups in small molecules and proteins

Group	Small molecule [a]	Protein [b]
α-Carboxyl	3.6	3.0-3.2
β- or γ-Carboxyl	4.6	3.0-4.7
Imidazole	6.0	5.6-7.0
α-amino	7.9	7.6-8.4
Sulfhydryl	9.2	8.3-8.6 [c]
Phenolic hydroxyl	9.8	9.8-10.4
ε-amino	< 10.5	9.4-10.6
guanidino	< 14	11.6-12.6

[a] GURD and WILCOX (17)

[b] COHN and EDSALL (18)

[c] BENESCH and BENESCH (19)

Unfortunately, in practice it is difficult to correlate the experimentally determined pK with a particular amino acid residue in the enzyme molecule. The rationale behind this problem is associated with two factors; the environment of the functional group and the effect of hydrogen bonding.

It is well established that, in most protein molecules there are regions of both hydrophobicity and hydrophilicity. In the former, neutral groups such as the carboxyl function will tend not to dissociate as readily as they might in a hydrophilic environment because of the relatively lower dielectric constant of the apolar environment. On the other hand, the pK of charged groups will not be greatly affected by alterations in the dielectric constant of the medium, e.g., $R - NH_3^+ \rightleftharpoons R\text{-}NH_2 + H^+$.

Hydrogen bonding of the potentially free proton of an acidic group, such as the carboxyl, will serve to raise the pK of the acid. Alternatively, there will be a decrease in the acidic pK of a basic group, if its free electron pair is involved in hydrogen bonding.

These effects point out the dangers inherent in ascribing a particular amino acid residue in a protein to an experimentally determined pK.

There are numerous examples in the literature of what might be called anomalous pK values of amino acid functions in proteins (20-24). One of the best examples of these concerns the pK of the ε amino group of lysine at the active site of acetoacetate decarboxylase (25). Investigations of JENCKS (26) suggest that the first step in the decarboxylation reaction is the formation of a Schiff base between an unprotonated ε amino lysyl residue on the enzyme and the keto function of the substrate. Plots of V_m and V_m/K_m *versus* pH indicate that a functional group on the enzyme with a pK in the range 5-7 is involved in the decarboxylation reaction (27). SCHMIDT and WESTHEIMER (25) used 2,4 dinitrophenyl propionate to acylate the amino groups of the decarboxylase and found that the rate of acylation is very similar to the rate of enzyme inactivation. Furthermore, they observed that the pH-rate profile for the acylation process is characterized by a monovalent type titration curve (Eq. (VIII-23)) with a $pK_a = 5.9$. They also demonstrated that the acylation reaction did not occur with enzyme inhibited by compounds that react with a lysyl residue at the active site on the decarboxylase. SCHMIDT and WESTHEIMER (25) concluded from these experiments that the ε amino group of lysine at the active site is more acidic, by 4 pK units, than usual ε amino lysine residues found in proteins. This contention is also supported by studies of FREY and WESTHEIMER (28) in which they found that the environment of the active site, as measured with a "reporter group", is capable of lowering the pK of the ε amino function of the essential lysyl residue to 5.9.

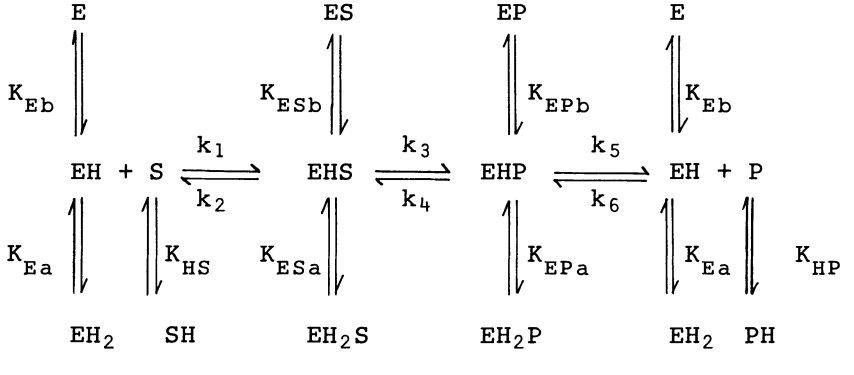
7. Some Limitations in the Study of pH Kinetics

a) Changes in the Mechanism

One of the assumptions tacitly made in studies of pH kinetics is that the mechanism governing the conversion of substrate to product is invariant. Some examples are currently available in the literature in which the reaction mechanism changes with alterations in the pH. Probably the best known example is the difference in mechanisms for the acid and base catalyzed hydrolysis of esters. With certain enzyme systems, the mechanism may also change, e.g., it may go from Random Bi Bi to Ordered Bi Bi. An alteration of this sort would obviously lead to erroneous conclusions regarding the pKs of functional groups involved in the catalytic process. The obvious method to use to circumvent, or at least recognize, this problem would be to study the kinetic mechanism of the system at a variety of pH values to insure that the mechanism is not pH dependent. In this context, SCHIMERLIK and CLELAND (29) have shown that the creatine kinase mechanism changes with pH alteration.

b) Changes in the Rate Limiting Step with pH Change

JENCKS (26) has cited numerous examples in nonenzymic systems where changes in pH change the nature of the rate limiting step. There is certainly evidence that similar effects may occur with enzyme systems (30). When this occurs, certain complications may arise in the determination of the pKs associated with the catalytic process. For example, when considering the Theorell-Chance mechanism illustrated in Scheme VIII-7, if the k_3 step becomes rate limiting such that the other steps equilibrate rapidly relative to it, it is possible that the first term in Eq. (VIII-27) will be absent. Another source of error in the determination of pKs can be seen to occur if the rate limiting step in the mechanism of Scheme VIII-9 changes.



Scheme VIII-9

The V_m for this pathway is described by Eq. (VIII-30).

$$V_m,pH = \frac{E_0}{\frac{1}{k_5}\left(1 + \frac{H}{K_{EPa}} + \frac{K_{EPb}}{H}\right) + \frac{(k_4 + k_5)}{k_3 k_5}\left(1 + \frac{H}{K_{ESa}} + \frac{K_{ESb}}{H}\right)}. \quad \text{(VIII-30)}$$

Now, if the rate limiting step of the mechanism should change such that $k_5 >> k_3, k_4$, the first term in the denominator of Eq. (VIII-30) *may* drop out. To carry this point one step further: if k_3 becomes rate limiting to the extent that the preceding steps equilibrate rapidly relative to it, then,

$$V_m,pH = \frac{E_0}{\frac{1}{k_3}\left(1 + \frac{H}{K_{ESa}} + \frac{K_{ESb}}{H}\right)}. \quad \text{(VIII-31)}$$

Thus the pKs will seem to change as the pH changes.

CLELAND (31) has indicated how alterations in the rate limiting step may also lead to erroneous pK evaluations in pH kinetic studies. If the $[V_m,pH]$ for a particular mechanism assumes the form described by Eq. (VIII-32)

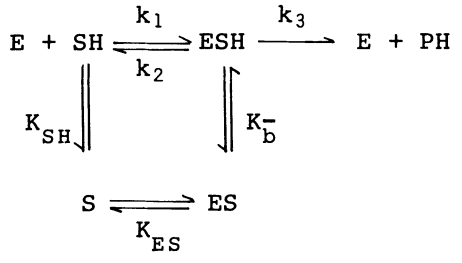
$$V_m,pH = \frac{k_1 k_2 E_0}{k_1\left(1 + \frac{K_b}{H}\right) + k_2}. \quad \text{(VIII-32)}$$

K_b may be calculated by the usual methods when $k_2 << k_1$. If k_1 becomes rate limiting because the k_2 step requires protonation of the enzyme, then k_2 may be much greater than k_1. When $k_2 = 100k_1$, the pK_b will be displaced too high by a factor of log 100 or 2 pH units.

c) Ionization of the Substrate with Change of pH

It has been assumed in the preceding discussion that ionization of the substrate with change of pH does not occur. In most cases the pKs for acidic or basic groups on the substrate are either known or can be determined with certainty. It is absolutely essential that the state of ionization of the substrate be known before pH kinetic experiments are undertaken. In many studies, it will be possible for the investigator to work in a pH range where the state of substrate protonation does not change with pH; e.g., with the amino acid glycine as substrate, the zwitter ion form of the amino acid will prevail in the pH range 3.5 - 9.5. It is unfortunate that there are so many examples in the literature in which v (initial velocity), not V_m, is plotted as a function of pH in the range where the substrate exists in more than one state of ionization. Complications may arise because only one substrate form may be active, but the possibility cannot be discounted that the nonactive substrate form may in fact act

as an inhibitor of the enzyme. This situation is illustrated in Scheme VIII-10.



Scheme VIII-10

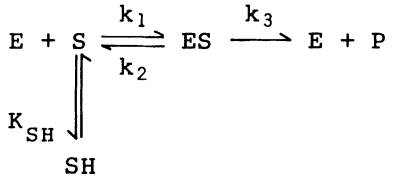
The $[V_m,pH]$ and $[K_m,pH]$ for this mechanism are:

$$V_m,pH = \frac{V_m}{1 + \frac{K_b^-}{H}} \qquad \text{(VIII-33)}$$

$$K_m,pH = \frac{K_m\left(1 + \frac{K_{SH}}{H}\right)}{1 + \frac{K_b^-}{H}} \qquad \text{(VIII-34)}$$

It is of interest that the $[V_m,pH]$ will be pH dependent. Obviously, saturation of the enzyme with substrate will not obviate this complication.

Now if we turn our attention to the situation in which the form of the substrate in the enzyme-substrate complex is unprotonated, a change in the ionization state of the substrate will not affect the $[V_m,pH]$. For example, in the pathway described in Scheme VIII-11.



Scheme VIII-11

The rate equation is

$$\frac{V_m}{v} = 1 + \frac{K_m\left(1 + \frac{H}{K_{SH}}\right)}{S_0} \qquad \text{(VIII-35)}$$

In Eq. (VIII-35), only the $[K_m,pH]$ will be affected by pH, and the pK determined from kinetic studies will be identical to the value one would obtain using any nonkinetic procedure. In this context then, the effect of pH on the mechanism outlined in Scheme VIII-11 can be placed in proper perspective.

8. Choosing a Buffer for Kinetic Experiments

An important decision must be made when choosing a buffer for kinetic studies. It is critical that the buffer does not inhibit or activate the system being studied. Ideally, then, its role should be that of a spectator substance. Because buffers are made up of anions or cations, substances that can bind to proteins, it is unrealistic to think in terms of an innocuous buffer. Thus a buffer that seems ideal at one pH or concentration may exhibit adverse effects when these parameters are altered.

In most studies of pH kinetics, the hydrogen ion concentration is varied 4 or 5 pH units. The usual effective buffer range for a monovalent buffer is 2 pH units - 1 unit above and 1 unit below the pK of the buffer. It will therefore be necessary to use a mixture of two buffers or, alternatively, a single buffer for a particular pH range and then another buffer in the region of its pK. In the latter case, the experimentalist will usually duplicate the kinetic studies by using the two different buffers in the same pH range to demonstrate that changing buffers does not effect the kinetic results. It is often useful to do a series of kinetic experiments with a variety of buffers in a particular range of pH to evaluate the effects of inhibition and activation. It will also be necessary to vary the amount of buffer to obtain a concentration range in which the system is not under the influence of buffer effects. For example, a particular buffer may exhibit adverse effects at 200 mM, but it may be perfectly innocuous at 25 mM. Finally, it must be remembered that, as the pH of the buffer is changed, there is a concomitant alteration in ionic strength, and it may be necessary to compensate for this change by the addition of some salt that does not influence the kinetics of the system under investigation. In this context, ALBERTY (32) has indicated how changes in ionic strength may alter the kinetic parameters for the enzyme fumarase. In enzyme reactions in which substrate concentration is in the millimolar range, and in which the substrates act as acids or bases, it will be necessary to make certain that the pH of the buffer is in fact the pH of the assay mixture. Thus, it may be necessary to compensate for ionic strength changes caused by the substrates themselves. This latter point is, of course, applicable to any system in which variation of substrate concentration leads to ionic strength effects that influence the kinetics of the system.

9. The pH Kinetics of the Fumarase Reaction

If one considers the mechanism outlined in Scheme VIII-5, it can readily be shown that the pKs determined from the V_m for the forward and reverse reactions must be the same. This follows from the fact that the only enzyme form associated with the V_m is EHS. In the study of fumarase by ALBERTY and his coworkers, the V_m and pKs were clearly different in the forward and reverse reactions (8). This point can readily be appreciated when considering the data for fumarase as shown in Figs. VIII-5 and VIII-6. FRIEDEN

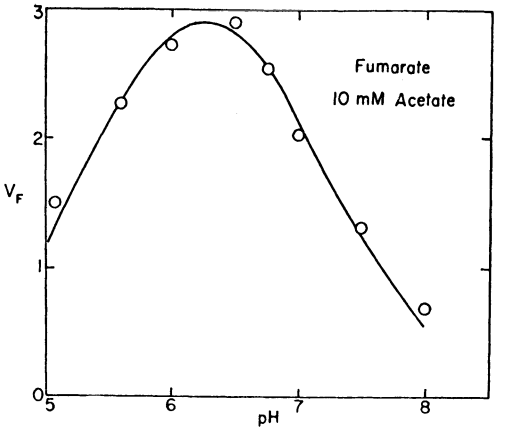


Fig. VIII-5. Plot of the maximal initial velocity for fumarase (V_f) as a function of pH with fumarate as substrate taken from the data of FRIEDEN and ALBERTY (8). The buffer was Tris-acetate, 10 mM, and the temperature 25°. The points are experimental and the solid line is a theoretical curve based upon Eq.(VIII-15)

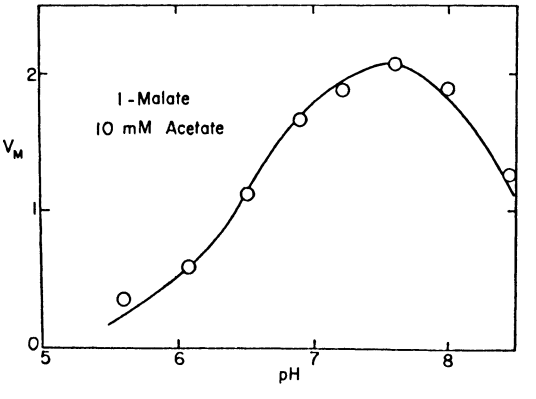


Fig. VIII-6. Plot of the maximal initial velocity for fumarase (V_M) as a function of pH with malate as substrate taken from the data of FRIEDEN and ALBERTY (8). Other conditions as outlined in the legend to Fig. VIII-5

and ALBERTY (8) assumed the mechanism shown in Scheme VIII-9 for fumarase on the basis of these findings. The data they obtained for the V_ms and K_ms could be fit to equations that are analogous to Eqs. (VIII-15) and (VIII-16) by replacing the K_{ESa} and K_{ESb} in Eqs. (VIII-15) and (VIII-16) with the apparent ionization constants K'_{ESa}, K'_{ESb}, K'_{EPa} and K'_{EPb}. As pointed out by ALBERTY (10), the relationships between the apparent ionization constants obtained from kinetic data and the dissociation constants described in Scheme VIII-9 are:

$$K'_{ESa} = \frac{(k_3 + k_4 + k_5)}{\frac{(k_4 + k_5)}{K_{ESa}} + \frac{k_3}{K_{EPa}}} \qquad \text{(VIII-36)}$$

$$K'_{ESb} = \frac{(k_4 + k_5)K_{ESb} + k_3K_{EPb}}{(k_3 + k_4 + k_5)} \qquad \text{(VIII-37)}$$

$$K'_{EPa} = \frac{(k_2 + k_3 + k_4)}{\frac{(k_2 + k_3)}{K_{EPa}} + \frac{k_4}{K_{ESa}}} \qquad \text{(VIII-38)}$$

$$K'_{EPb} = \frac{(k_2 + k_3)K_{EPb} + k_4K_{ESb}}{(k_2 + k_3 + k_4)} \qquad \text{(VIII-39)}$$

It can be seen from Eqs. (VIII-36) to (VIII-39) that the apparent dissociation constants lie somewhere between the ionization constants for the enzyme-fumarate and enzyme-malate complexes. It is not possible from these kinetic studies to evaluate the true ionization constants described in Scheme VIII-9; i.e., Eq. (VIII-36) is a single equation with two unknowns, K_{ESa} and K_{EPa}. Using the graphical procedure of ALBERTY and MASSEY (13), FRIEDEN and ALBERTY (8) calculated the four apparent ionization constants described in Eqs. (VIII-36) to (VIII-39) from the data of Figs. VIII-5 and VIII-6. It should be noted that the curves in these two figures were theoretical lines based upon the experimentally determined dissociation constants and Eq. (VIII-15).

It can be shown that, for the mechanism described by Scheme VIII-9,

$$K_{m,pH} = K_m \frac{\left(1 + \frac{H}{K_{Ea}} + \frac{K_{Eb}}{H}\right)\left(1 + \frac{H}{K_{HS}}\right)}{\left(1 + \frac{H}{K'_{ESa}} + \frac{K'_{ESb}}{H}\right)}. \qquad \text{(VIII-40)}$$

Knowing K_{HS} and K_{HP}, FRIEDEN and ALBERTY (8) graphed $[V_{m,pH}/K_{m,pH}]$ for both the forward and reverse reactions and determined K_{Ea} and K_{Eb} from the resulting bell-shaped curves. This procedure was not completely straight-forward and involved the use of the Haldane

Equation as well as a knowledge of the equilibrium constant for the reaction (K_{eq}) and the ionization constants for fumaric (K_{HS}) and malic (K_{HP}) acids.

It can be shown that the following relationships are valid for fumarase:

$$K_{app} = \frac{(\text{Malate})}{(\text{Fumarate})} = K_{eq} \frac{\left(1 + \frac{H}{K_{HP}}\right)}{\left(1 + \frac{H}{K_{HS}}\right)} = \frac{V_f K_M}{V_M K_f} \qquad \text{(VIII-41)}$$

where V_f, K_M, V_M, and K_f, represent maximal velocity forward reaction, Michaelis constant from the malate side of the reaction, maximal velocity reverse reaction, and Michaelis constant forward reaction. Taking K_{eq} to be 4.4, FRIEDEN and ALBERTY (8) plotted $V_f(1 + H/K_{HS})/4.4\ K_f$ and $V_M(1 + H/K_{HP})K_M$ *versus* pH. The results of these studies, which are shown in Fig. VIII-7, permitted evaluation of K_{Ea} and K_{Eb}. This can be recognized when one divides Eq. (VIII-15) by Eq. (VIII-16).

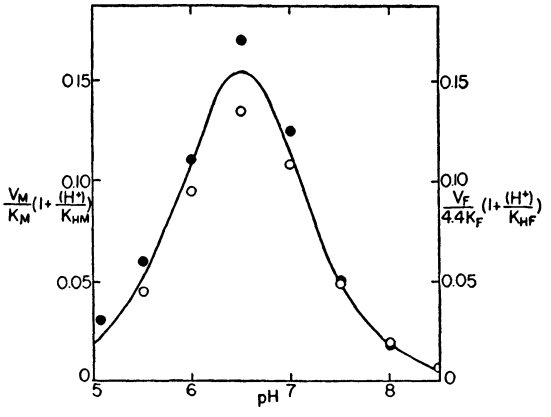


Fig. VIII-7. Plot of V_f (1 + (H/K_{HF})/4.4 K_F (●) and V_M (1 + (H/K_{HM})/K_M) (○) *versus* pH in 10 mM Tris-acetate buffer at 25° compared to the theoretical curve (solid line) calculated from Eq. (VIII-15) divided by Eq. (VIII-16). The graph is taken from the data of FRIEDEN and ALBERTY (8)

The variation of the kinetic parameters of fumarase as a function of pH in Tris-acetate buffer, at an ionic strength of 0.01, and at a number of temperatures, was reinvestigated by BRANT et al. (33). Table VIII-3 illustrates the results obtained at 21° by these investigators. If it is assumed that the more acidic group involved in the fumarase reaction is a carboxyl and the relatively more basic group, imidazole (34), some interesting conclusions can be obtained from the pKs shown in Table VIII-3.

It appears that when malate binds to fumarase, there is a four-fold decrease in the proton binding strength of the carboxyl group, whereas when fumarate binds, there is an eight-fold increase in the proton binding strength. On the other hand, malate decreases the proton binding strength of the imidazole function by a factor of one hundred, whereas the acid-base properties of histidine at the active site are not altered when fumarate binds to fumarase. It is possible to conclude from these results that malate prevents ionization and that fumarate may in fact promote ionization of the more acidic group on the enzyme.

Table VIII-3. pKs for enzyme (E), enzyme • malate (EM), and enzyme • fumarate (EF) at 21° in 0.01 M Tris-acetate buffer (33).

pK_{Ea}	pK_{Eb}	pK_{EMa}	pK_{EMb}	pK_{EFa}	pK_{EFb}
5.8	7.1	6.4	9.1	4.9	7.0

B. The Effect of Temperature on Enzyme Catalyzed Reactions

The fact that enzyme catalyzed reactions are markedly temperature dependent has been long recognized (35). When inital reaction velocity is graphed against temperature, a bell-shaped curve of the type described in Fig. VIII-8, is obtained. It is now recognized that at least two factors control the shape of this curve: the effect of temperature on the rate constants of the reaction, and the effect of temperature on the enzyme[7]. As might be expected, increasing temperature increases the rate of the reaction; however, there is a concomitant inactivation of the enzyme which

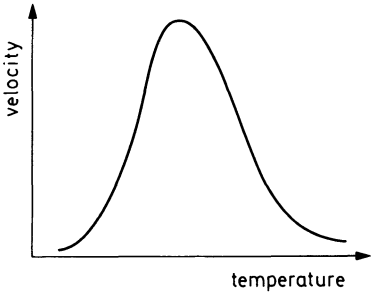


Fig. VIII-8. Plot of initial velocity *versus* temperature for an enzyme catalyzed reaction

[7] The assay method is important in this regard as the descending limb of the curve represents an irreversible change and is time dependent.

serves to decrease the initial velocity. These two opposing effects are largely responsible for the initial velocity-temperature profile shown in Fig. VIII-8. In general the temperature coefficient for the chemical reaction is less than the temperature coefficient for enzyme inactivation.

The basic purpose of undertaking experiments on the effect of temperature on enzyme kinetics is to provide a pictorial model of the interactions of the enzyme and substrate at the various kinetically significant steps in the enzymic reaction. Because the rate-temperature data cannot be unequivocally interpreted, the model arrived at is more qualitative than quantitative. This point which cannot be over-emphasized has led to many unwarranted conclusions on the mechanism of enzyme catalysis.

In order to indicate how one may build a pictorial model from studies of temperature effects on enzyme catalysis, it is necessary to briefly review certain of the concepts of classical thermodynamics and of absolute rate theory.

1. Collision Theory and the Arrhenius Equation

ARRHENIUS was among the first to attempt a quantitative formulation of the dependence of the rate constant for a chemical reaction on temperature. The Arrhenius equation, which was arrived at empirically, is:

$$k = Ae^{-Ea/RT}. \qquad \text{(VIII-42)}$$

The constants A and Ea are referred to as the frequency factor and the activation energy, respectively. These constants may be evaluated from a graph of log k *versus* 1/T in which the slope of the resulting straight line is $-Ea/2.303R$ where R is the universal gas constant, $8.31 Jmole^{-1}K^{-1}$. The frequency factor may be determined from the log k axis intercept.

An appreciation of Ea may be obtained from the energy level-reaction coordinate diagram of Fig. VIII-9 when it is remembered that

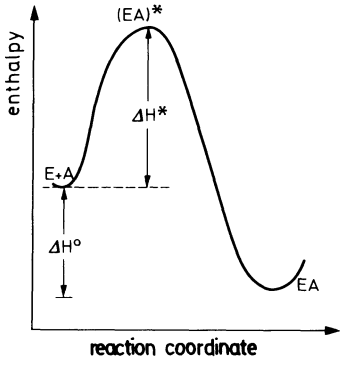


Fig. VIII-9. Plot of the enthalpy *versus* the extent of reaction for the system: E + A = EA

$$Ea = \Delta H^* + RT. \qquad \text{(VIII-43)}$$

For the chemical reaction, E + A = EA, it is possible to determine ΔH^o from a Van't Hoff plot; i.e., from the equation

$$\log K_{ia} = \frac{\Delta H^o}{2.303R}\left(\frac{1}{T}\right) + C \qquad \text{(VIII-44)}$$

where K_{ia} is the dissociation constant for the reaction under consideration. ΔH^o, which is the enthalpy of the reaction when all components of the system are at unit activity, is obtained graphically analogously to Ea. In Fig. VIII-9, the reaction as indicated is exothermic (i.e., heat is liberated), whereas the reverse reaction is endothemic (ΔH^o is +). In Fig. VIII-9, (EA)* is the transition state, and ΔH^* is the potential energy that the reactants must possess in order to achieve the transition state.

In order for molecules to react chemically, they must collide, and it is apparent that only a small fraction of molecules that collide react. Thus, the rate of a chemical reaction is equal to the collision frequency multiplied by the fraction of the molecules that possess enough energy to react. If this latter constraint were not placed upon colliding molecules, all reactions would approximate the rates of diffusion controlled processes. The concept of reaction rates in terms of collision theory is

$$\text{rate} = (\text{collision frequency})(\text{probability of effective collision}). \qquad \text{(VIII-45)}$$

For a bimolecular reaction the rate may be expressed as

$$k = Ze^{-Ea/RT} \qquad \text{(VIII-46)}$$

where k is the bimolecular rate constant, and Z the number of collisions per second in 1 c.c. between the substrate molecules.

In Eq. (VIII-46), the factor $e^{-Ea/RT}$ represents the probability that a collision will be effective. Equation (VIII-46) has been found to be reasonably valid for reactions of atoms and small molecules in the gas phase; however, this equation is not in harmony with data obtained with large molecules, ions and dipolar molecules. The basic inconsistency between experimental findings for gas reactions and Eq. (VIII-46) is a result of the fact that the collision theory model does not consider the orientation of the colliding reactants to be a factor in the reaction rate. This problem has been partially corrected by introducing a steric factor, P, which is taken to represent the fraction of effective collisions from the standpoint of proper molecular orientation (9); i.e.,

$$k = PZe^{-Ea/RT}. \qquad \text{(VIII-47)}$$

LAIDLER (9) has pointed out that marked deviations from collision theory are to be expected when electrostatic forces exist between reacting molecules in aqueous solution. For example, not only will the collision frequency be reduced by mutual repulsion of like charged molecules, but the resulting complex, once formed, will be of greater charge than the individual molecules. Water would be expected to be more strongly bound to the complex than to the individual molecules. LAIDLER (9) refers to this effect as "electroconstriction" and indicates that it will serve to decrease the frequency factor, which is equivalent to PZ in Eq. (VIII-47). On the other hand, A is expected to increase when two oppositely charged ions shed their water of hydration when they form a complex.

2. Transition-State Theory

Although attempts have been made at various times to modify the kinetic theory of reaction rates and thus circumvent many of its inherent inadequacies, these efforts have largely been abandoned in recent years. The studies of PELZER and WIGNER (36) and EYRING and his coworkers (37-39) have led to the transition-state or absolute reaction rate theory, which is currently the simplest and most widely used hypothesis for explaining rates of chemical reactions. The theory assumes that, in going from substrates to products, the reactants assume an intermediate configuration called the transition state.

Transition-state theory is explained by considering, as an example, the reaction,

$$A - B + C \rightleftharpoons A - C + B. \qquad \text{(VIII-48)}$$

If it is assumed that the atoms are on a straight line, the position of each atom can be expressed in terms of two coordinates; i.e., the A-B distance (R_{AB}) and the A-C distance (R_{AC}). It is then possible to plot the potential energy as a function of the two coordinates, or a contour map can be made. Fig. VIII-10 illustrates a "typical" contour surface diagram for this system. In going from reactants to products in Eq. (VIII-48), a pathway called the "reaction coordinate" is followed. The valleys followed by the reaction coordinate join at a "pass" or "saddle point", which is the so-called transition state. The valleys seen in Fig. VIII-10 do not join because, as C approaches A-B and as B approaches A-C, B and C repel each other and there is an increase in potential energy. If this repulsion did not occur, reactions would be essentially diffusion controlled. In the transition state or activated complex, all the characteristics of a normal molecule have been preserved except one; i.e., a vibrational degree of freedom has been transformed into a translation along the reaction coordinate leading to product.

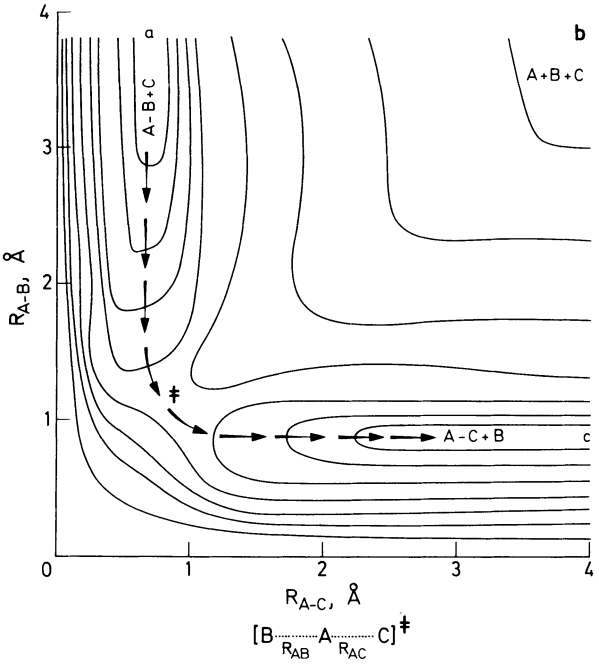


Fig. VIII-10. Variation of the potential energy surface for the reaction A - B + C = A - C + B. The contour diagram indicates the energy surface. The valleys represent the initial and final states and meet at (‡), which is the saddle point or transition state. The broken line with arrows represents the reaction coordinate. Points "a" and "c" are potential energy minima whereas "b" is a maximum

Figure VIII-11 shows how the potential energy varies as a function of the reaction coordinate assuming that the lowest energy requirement is followed. Chemical reactions do not necessarily

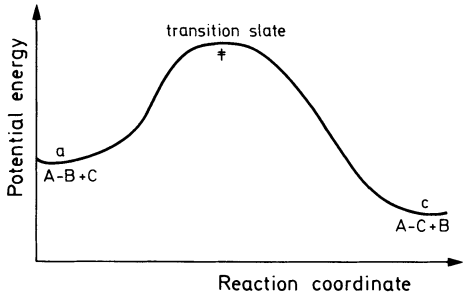


Fig. VIII-11. Plot of potential energy as a function of the reaction coordinate. The symbols, "a", ‡, and "c" are defined in the legend to Fig. VIII-10

follow the lowest energy pathway, as illustrated in Figs. VIII-10 and VIII-11, but may in fact zigzag; however, for the purposes of this discussion, the outline provided is reasonably consistent with current transition-state theory. A more exact and detailed description of this concept may be found in the book by FROST and PEARSON (40).

In some hydrogen transfer reactions, the hydrogen may *tunnel* through a narrow potential barrier rather than pass over it (41). This effect is often used to explain the marked differences in primary isotope effects, which cannot be explained simply on differences in zero point energies between hydrogen and deuterium.

In terms of transition-state theory, the reaction rate is equal to the concentration of molecules in the transition-state multiplied by the rate at which they pass over the energy barrier required to reach product. If it is assumed that the molecules in the activated complex are in equilibrium (really a pseudoequilibrium) with nonactivated molecules, it is relatively simple to calculate the concentration of molecules in the former energy state. For the reaction,

$$A + B \xrightarrow{k_1} (AB)^* \xrightarrow{k_2} \text{products} \qquad \text{(VIII-49)}$$

$$K^* = (AB)^*/(A)(B). \qquad \text{(VIII-50)}$$

According to transition-state theory,

$$v = (AB)^* \text{ (rate of transversing barrier)} \qquad \text{(VIII-51)}$$

and thus,

$$v = K^* (A)(B)(\text{rate of transversing barrier}). \qquad \text{(VIII-52)}$$

The rate at which molecules pass over the activated complex barrier and decompose to products is K_BT/h, where K_B, T, and h equal the Boltzmann constant, absolute temperature, and Planck's constant, respectively. From these considerations,

$$v = K^*(A)(B)K_BT/h = k_1(A)(B) \qquad \text{(VIII-53)}$$

and finally,

$$k_1 = K^*K_BT/h. \qquad \text{(VIII-54)}$$

To be precise, the right-hand portion of Eq. (VIII-54) should be divided by the transmission coefficient, which predicts whether the activated complex will form products or dissociate back into reactants. In most cases the transmission coefficient will approximate unity.

It has been assumed that the activated complex is a normal, stable molecule in equilibrium with reactants, and thus thermodynamic laws may be applied to the initial and final (transition) states; e.g.,

$$\Delta G^* = -RT \ln K^*. \qquad \text{(VIII-55)}$$

ΔG^* is taken to be in its standard state of unit concentration.

It is possible to get the rate constant for a reaction in terms of the free energy of activation (ΔG^*), the entropy of activation (ΔS^*), and the enthalpy of activation (ΔH^*) as follows:

$$k_1 = \frac{K_B T}{h} e^{-\Delta G^*/RT} = \frac{K_B T}{h} e^{-\Delta H^*/RT} \cdot e^{\Delta S^*/R} . \qquad \text{(VIII-56)}$$

The relationship between the energy of activation, Ea, and ΔH^* in solution is provided by Eq. (VIII-43). It is possible, therefore, by studying the effect of temperature on k for a reaction, to use an Arrhenius plot to determine Ea and then to obtain the activation parameters ΔG^* and ΔS^* from Eq. (VIII-56).

The usual thermodynamic parameters are only indirectly related to the activation parameters. Figure VIII-12 illustrates one such relationship. This diagram illustrates why a highly exergonic reaction may occur slowly, if at all, in a kinetic sense. The equilibrium portion of the reaction may be very favorable in terms of product to substrate concentrations; however, the ΔG^* may be high enough to preclude the reaction from taking place in a measurable time frame. The function of the enzyme, or indeed of any catalyst, is to lower the energy of activation, or raise the ΔS^*, or both.

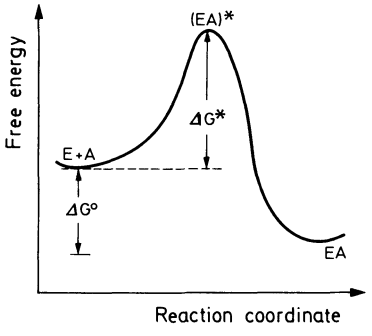


Fig. VIII-12. Plot of the free energy *versus* the extent of the reaction for the system: E + A = EA

3. Significance of Activation Enthalpy and Activation Entropy

Enzymes exhibit turnover numbers (number of moles substrate converted to product/min/active site) in the range 10^2 to 10^6. These extremely high catalytic efficiencies have been attributed to activation entropy (ΔS^*) effects. The significance of ΔS^* is that it gives information on the nature of the transition state. Data of this type are obviously necessary for an understanding of enzyme catalysis. Because ΔS^* is, among other things, a measure of the orientation of substrates in the transition state relative to

unassociated substrate molecules, a knowledge of ΔS^* sometimes permits the investigator to make an educated guess regarding the nature of the catalytic mechanism. A number of other factors that are known to affect ΔS^* are: unmixing of solvent and substrates, loss of translational entropy (i.e., freezing of the substrate when it binds the enzyme), relative orientation of solvent molecules on the substrate and the enzyme-substrate complex, simple order of the reaction (i.e., a bimolecular reaction forms a complex that acts as a single molecule), and conformational changes in the enzyme itself. Entropy increases are suggestive of disordering of structure, whereas a decrease in entropy is indicative of systems being more ordered in going from the initial to the final state. Unfortunately, any measured ΔS^* is a composite of many effects and the resulting activation entropy cannot usually be attributed to a single factor or even a small number of factors.

When the substrate in solution (1M) is brought in contact with an enzyme, there is a loss of a solute species in the transition state and the ΔS^* for this process is -7 to -8 e.u. This effect is sometimes referred to as the entropy of unmixing (9). If the enzyme and substrate are hydrophobic or form hydrogen bonds with each other, the ΔS^* may be positive.

When a substrate is bound to an enzyme and an activated complex is formed, there is a loss of rotational and translational degrees of freedom. This effect of "bond freezing" may represent approximately 6 e.u. per bond (42, 43).

LAIDLER (9) has considered the ΔS^* for ionic interactions which he refers to as electrostatic interactions. He has shown that when two charged ions, Z_A and Z_B, form a complex,

$$\Delta S^* = -10 Z_A Z_B \text{ e.u.} \qquad \text{(VIII-57)}$$

In the case of $Z_A = +2$ and $Z_B = -1$, $Z_A Z_B$ would equal -2 and $\Delta S^* = 20$ e.u. If both ions are positive, $\Delta S^* = -20$ e.u. LAIDLER has pointed out that very serious deviations from Eq. (VIII-57) may arise based upon salt effects, the assumption that the solvent is a continuous dielectric, the assumption that the complex formed is a double-sphere structure, and the assumption of the size of the activated complex.

Reaction of neutral molecules with other neutral molecules should lead to ΔS^* values near zero; however, the activated complex may bind water strongly leading to negative entropies.

Another important type of entropy effect involves the enzyme itself. It is now well recognized that when enzymes bind substrates, conformational changes may result in the enzyme. If the enzyme structure is loosened, the activation entropy will be positive whereas a more compact structure will give a negative ΔS^*. Unfortunately, one cannot know what effect substrate interaction with the enzyme will have on ΔS^*.

It should be clear from this discussion that very many phenomena give rise to the value of ΔS^* which is arrived at experimentally. It becomes very difficult then, to fish out an effect or effects

that lead to a particular value for ΔS^*. A number of other limitations come to mind when attempting studies of this type. Some of these will be immediately obvious experimentally, whereas others will be quite subtle. If either the kinetic mechanism or the rate limiting step should change with temperature, the results may be impossible to interpret. Furthermore, Arrhenius plots may show discontinuities and this effect may arise from changes in the heat capacity of the solvent or sharp temperature dependent changes in the enzyme. The pH of the reaction mixtures may also be expected to change with temperature, if ionization of the buffer varies appreciably with temperature.

Although it is clearly difficult to interpret activation entropy effects, it is currently believed that entropy effects are of primary importance in enzyme catalysis. WESTHEIMER (44) looks upon the enzyme as being an entropy trap. He argues that the enzyme uses electrostatic and van der Waals forces to compensate for translational entropy when the substrate binds. This process is then reversed when the product is lost - all with a net reduction in the energy of activation.

LINDERSTRØM-LANG and SCHELLMAN (45) have suggested that the enzyme raises the ΔS^* by having the catalytic groups on a single molecule simultaneously, as opposed to having them on separate molecules. They consider the case of three molecules of 0.1 M concentration (two catalytic molecules and one substrate) and calculate that the unmixing activation entropy is about -25 e.u. In addition they suggest that the ΔS^* will be lowered by an additional 10-15 e.u. because of orientation effects. It can readily be seen that for an enzyme, two of the molecules are combined into a single entity, thereby increasing ΔS^*.

These calculations seem to ignore two factors. If the enzyme and substrate can shed their bound water when forming the enzyme-substrate complex ($E\cdots OH_2 + A\cdots H_2O \rightarrow EA + 2H_2O$), the entropy change may be positive. In addition, hydrophobic regions of the enzyme and substrate cause water to assume an ice-like structure around them which is highly ordered. Complex formation lowers the exposed surface and frees this water to become less ordered. The entropy change in this case may also be positive.

HAMMES has suggested that enzymes may enhance reaction rates by decreasing the energy of activation in a rather interesting manner (46). He shows that when a chemical bond is broken, and this bond breaking is coupled to bond formation, there is a decrease in Ea relative to bond breaking alone. He refers to this effect as "energy compensation". To cite one of many examples, in the reaction, $H_2 \longrightarrow 2H$, Ea = 418 KJ/mole, whereas in the reaction $H_2 + I_2 \longrightarrow 2HI$, Ea = 170 KJ/mole. HAMMES suggests that bonds on the enzyme, but not at the active site, may play this role of energy compensation. Finally, he proposes that although there is an energy requirement for this enzyme-substrate bond formation, the overall activation energy for the reaction is lowered by this process of energy compensation. HAMMES suggests that compensation effects may also be important in entropic considerations.

4. Application of Transition-State Theory to the α-Chymotrypsin Reaction

BENDER and his coworkers have studied the thermodynamic and activation parameters of the α-chymotrypsin reaction extensively (47). The kinetic model for the reaction which is Uni Bi and involves an acyl-enzyme intermediate (EQ) is as follows:

$$E + A \underset{}{\overset{K_{ia}}{\rightleftharpoons}} EA \xrightarrow{k_2} EQ \xrightarrow[+\ P]{k_3} E + Q. \qquad \text{(VIII-58)}$$

BENDER et al. (47) studied the deacylation of the enzyme as a function of temperature. By using a variety of different covalently bound substrates, at a pH in which the pK for the deacylation reaction was pH independent, these workers were able to determine the activation parameters for the various reactions. The tabulated activation parameters are shown in Table VIII-4. The enzyme-substrate compounds are listed in decreasing order of specificity. This kinetic specificity is clearly not a function of ΔH^* which does not vary with the various acyl-enzyme derivatives. On the other hand, the ΔG^* increases as the specificity decreases, and this effect is essentially entropic in nature.

Table VIII-4. The activation parameters of the deacylation of some acyl-α-chymotrypsins [a]

acyl-enzyme	ΔG^* KJ/mole	ΔH^* KJ/mole	ΔS^* e.u.
N-acetyl-L-tyrosyl-	59.8	43.1	-13.4
N-acetyl-L-tryptophanyl-	74.8	50.2	-19.8
trans-cinnamoyl-	84.0	46.8	-29.6
acetyl-	85.3	40.5	-35.9

[a] From BENDER et al. (47).

BENDER et al. (47) have suggested that in highly specific structures such as N-acetyl-L-tyrosyl-α-chymotrypsin, the ground state is very similar in configuration to the transition state, whereas with non-specific substrates, e.g., acetyl-α-chymotrypsin, the substrate has more degrees of freedom of association on the enzyme before reaching the transition state. On the basis of these suggestions, it would follow that there would be a greater loss of rotational entropy when the non-specific acyl substrate assumes the transition state than in the case of the specific substrate. BENDER and his coworkers (47) suggest that the difference in the ΔS^* between acetyl and N-acetyl-L-tyrosyl-α-chymotrypsin, some 23 e.u., may be due to the freezing of rotation of four bonds in the ground state of the latter compound relative to N-acetyl-α-chymotrypsin. They do not, however, ex-

clude the possibility that conformational changes in the enzyme with the various acyl derivatives might account for the different entropic effects.

Another explanation for these observations is that the tyrosyl and tryptophanyl complexes have a greater hydrophobic exposure and when they fit into specific pockets, which are filled with organized water, there is a release of some structured water. This possibility would also result in a more positive ΔS^*.

It was possible, using the rate expression for the mechanism of Eq. (VIII-58) to evaluate K_{ia}, k_2 and k_3 as a function of temperature. With this information in hand, BENDER and his associates were able to plot ΔG^* as a function of the reaction coordinate for different covalent substrates of α-chymotrypsin. The reader is referred to this work and to studies of WESTLEY (48), LUMRY (49), LINDERSTRØM-LANG and SCHELLMAN (45), and LAIDLER (9) for a comprehensive review of this area of research.

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Chapter IX

Cooperativity and Allostery

The problem of enzyme regulation and control has commanded a great deal of attention in recent years. There are many facets to this general area of enzymology; however, this discussion will be limited to two regulatory phenomena: allostery and cooperativity. A certain amount of confusion has arisen regarding the relationship between enzyme cooperativity and allostery. Suffice it to say, cooperativity and allostery are separate events; however, a given enzyme system may exhibit either one or both phenomena.

The purpose of cooperativity seems to be twofold. First, it permits an enzyme to remain inactive even in the presence of substrate, thus preventing the accumulation of unwanted metabolic products. Second, when the enzyme does respond to substrate, the response occurs (relative to noncooperative enzymes) over a narrow range of substrate.

By definition, enzymes which are allosteric have, in addition to the active catalytic site, which is common to all enzymes, a second and topologically distinct or allosteric site. These enzymes may be either monomeric or oligomeric, and when certain modulators or effectors bind at the allosteric site, the catalytic properties at the active site are altered. These effects may be manifested as alterations in the Michaelis constants (or dissociation constants) of substrates and products (called K systems), the maximal velocity (called V systems), or both.

It is often stated that, for an enzyme to exhibit cooperativity, it must be oligomeric; however, this is not true. A number of kinetic models have been presented which show that cooperative kinetics may be obtained with monomeric enzyme systems. Bovine serum albumin is an example of a monomeric protein that exhibits cooperative ligand binding. In the case of oligomeric systems, the subunits interact in some manner when substrate or effector binding occurs. If the presence of one substrate molecule on the enzyme facilitates binding of the next substrate molecule, the effect is positive cooperativity. On the other hand, if substrate binding inhibits association of additional substrate and enzyme, the effect is negative cooperativity. It should be pointed out that in this discussion reference is made to substrates of the same species. Interactions between similar ligands are referred to as homotropic interactions and between different ligands as heterotropic interactions.

In studies of cooperativity and allostery, attempts are often made to correlate experimental ligand binding and kinetic data with equations for particular models by the procedure of curve

fitting. It is often observed, however, that good fits of the experimental data are obtained for a variety of models because of the large number of independent variables present in many of the equations used to describe the postulated models. Investigators are sometimes able to circumvent this problem by correlating physical and chemical changes that accompany ligand binding with particular models of allostery and cooperativity.

The purpose of Chapter IX is to summarize the state of allostery and cooperativity as viewed by this writer. It is difficult to obtain a clear picture of these processes because the various models depend, to a large degree, on states of protein structure that are reasonably well understood in only a few cases. This limitation will obviously be eliminated in the future, and a more exact treatment of allostery and cooperativity will be possible. The attainment of this end will provide both the protein chemist and the kineticist with an enormous challenge.

A. Cooperativity

1. The Hill Equation

In 1910 A.V. HILL (1) attempted to explain oxygen binding to hemoglobin with the aid of a mathematical model. Figure IX-1 represents the type of data obtained when binding of oxygen

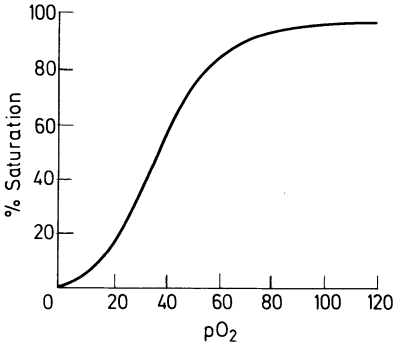


Fig. IX-1. Oxygen-hemoglobin saturation curve. Plot of % saturation *versus* the partial pressure of O_2 (pO_2) in mm Hg

by hemoglobin is plotted as a function of the partial pressure of oxygen. HILL assumed that the protein exists either free or totally associated with ligand as suggested by Eq. (IX-1)

$$E + nA = EA_n. \qquad \text{(IX-1)}$$

The dissociation constant, K, for this reaction is

$$K = \frac{(E)\,(A)^n}{EA_n}. \qquad \text{(IX-2)}$$

Implicit in this treatment is the assumption that there are no intermediates.

$$E_0 = E + EA_n \qquad \text{(IX-3)}$$

Substitution of Eq. (IX-3) into Eq. (IX-2) yields

$$\bar{\nu} = \frac{EA_n}{E_0} = \frac{A^n}{K + A^n}. \qquad \text{(IX-4)}$$

Inspection of Eq. (IX-4) reveals that, when A = 0, $EA_n/E_0 = 0$, and that, when $A \to \infty$, $EA_n/E_0 = 1$. The slope of the curve described by Eq. (IX-4) is $d\bar{\nu}/dA = nKA^{n-1}/(K + A^n)^2$, and the coordinates of the single point of inflection are $\left(\sqrt[n]{\frac{K(n-1)}{(n+1)}}, \frac{(n-1)}{2n}\right)$.

It can be seen that, at finite values of A, the slope of the curve changes continuously, and it is therefore difficult to evaluate n and K. Equation (IX-4) may be expressed in linear form as shown in Eq. (IX-5). It is possible to evaluate n, the Hill coefficient, from the graph shown in Fig. IX-2 as well as the dissociation constant, K.

$$\log \frac{\bar{\nu}}{(1 - \bar{\nu})} = n \log A - \log K \qquad \text{(IX-5)}$$

As already stated, in the derivation of the Hill equation, it is assumed that the intermediates EA_1, EA_2, ..., EA_{n-1} do not

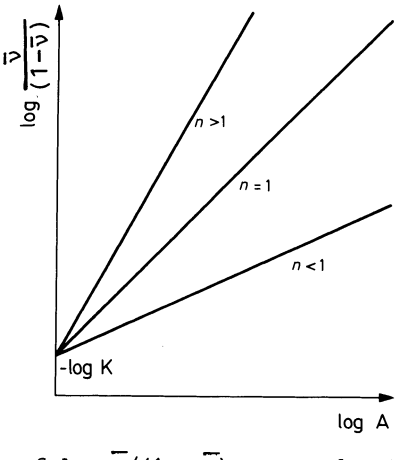


Fig. IX-2. A Hill plot of log $\bar{\nu}/(1 - \bar{\nu})$ *versus* log A. The Hill coefficient n for n > 1 (positive cooperativity), n = 1 (noncooperativity), and n < 1 (negative cooperativity) is obtained from the slope of the straight line

exist. This concept is difficult to accept in a chemical sense and suggests that the system is 100% cooperative.

It is important to point out at this time that Hill plots (log $\bar{\nu}/(1 - \bar{\nu})$ *versus* log A) can be made of models other than that described by the Hill equation. When n > 1, the system exhibits positive cooperativity. For n < 1 the system displays negative cooperativity, whereas if n = 1, the system follows a normal binding isotherm. These points are illustrated in Fig. IX-2.

It has been suggested (2) that, if Hill plots are made as illustrated in Fig. IX-3, it is possible to calculate the average interaction energy involved in cooperative binding. WEBER (3) has shown that the interaction energy may be determined from the differences in free energies of ligand binding and that Hill plots are not required for this determination.

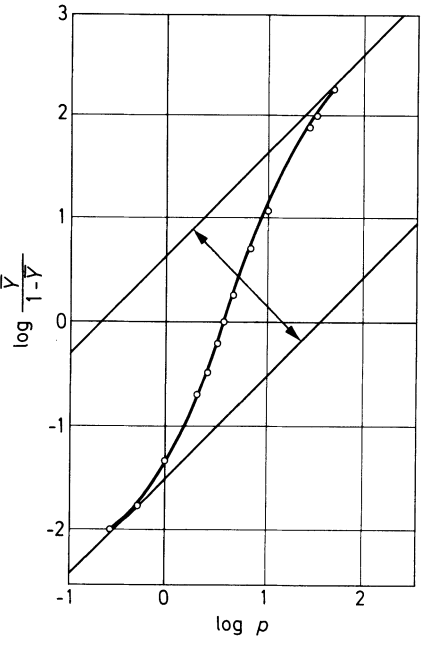


Fig. IX-3. A Hill plot of the oxygen equilibrium of sheep hemoglobin according to the method of WYMAN (2). The perpendicular (arrows) to the linear lines is used to calculate the interaction energy

MONOD et al. (4) have shown that it is possible to convert Eq. (IX-5) into one involving kinetic rather than thermodynamic parameters by assuming the involvement of a quasi-equilibrium. Equation (IX-4) defines $\bar{\nu}$ as $\bar{\nu} = EA_n/E_0$. If the numerator and denominator of Eq. (IX-4) are multiplied by the rate constant k involved in the reaction

$$E + nA = EA_n \xrightarrow{k} E + \text{product} \qquad \text{(IX-6)}$$

then

$$\bar{\nu} = \frac{k(EA_n)}{kE_0} = \frac{v}{V_1}. \qquad \text{(IX-7)}$$

Equation (IX-4), which was derived for experiments involving binding measurements, may now be used as suggested by MONOD et al. (4) for initial rate studies.

$$\log \frac{v}{(V_1 - v)} = n \log A - \log K. \qquad \text{(IX-8)}$$

PURICH and FROMM (5) have pointed out that, although the velocity form of the Hill equation (Eq. (IX-8)) may be used for one-substrate systems, its use must be attended with caution when studying multisubstrate systems. Consider, for example, the simple ordered binding model presented in Scheme IX-1.

$$E + A = EA, \; K_{ia}$$

$$EA + B = EAB, \; K_b$$

$$EAB + A = EA_2B, \; K_a$$

$$EA_2B \xrightarrow{k} E + \text{Products}$$

Scheme IX-1

According to Eq. (IX-9)

$$\bar{\nu}_a = \frac{EA + EAB + 2EA_2B}{E_0} \qquad \text{(IX-9)}$$

where

$$\bar{\nu} = \frac{\text{moles of ligand bound to protein}}{\text{total moles of protein}} = \frac{\sum_{i=1}^{n} i\, EA_i}{E + \sum_{i=1}^{n} EA_i} \qquad \text{(IX-10)}$$

By analogy with the substitution made in Eq. (IX-7), if a similar manipulation is made for Eq. (IX-9),

$$\bar{\nu}_a = \frac{k\left[EA + EAB + 2EA_2B\right]}{kE_0} = \frac{v}{V_1}. \qquad \text{(IX-11)}$$

The kinetic expression for the mechanism described by Scheme IX-1, assuming all steps equilibrate rapidly relative to the breakdown of the quarternary complex, is

$$v = \frac{V_1}{1 + \frac{K_a}{A} + \frac{K_aK_b}{(A)(B)} + \frac{K_{ia}K_aK_b}{(A)^2(B)}} \qquad \text{(IX-12)}$$

where $V_1 = kE_0$.

Equation (IX-11) may now be transformed as follows:

$$v = \frac{V_1\left[1 + \frac{B}{K_b}\left(1 + \frac{2(A)}{K_a}\right)\right]}{\left[1 + \frac{K_{ia}}{A} + \frac{B}{K_b}\left(1 + \frac{A}{K_a}\right)\right]} \quad . \qquad \text{(IX-13)}$$

It is clear that the velocity expression for Eq. (IX-13) is not equivalent to the analogous initial rate equation for the mechanism of Scheme IX-1, i.e., Eq. (IX-12).

Even when B is saturating, the two expressions differ, and it is thus reasonable to conclude that, although it may be valid to make the substitution described by Eq. (IX-7) for one substrate systems, similar manipulations may not be valid when considering multisubstrate systems.

With all its limitations, the virtue of the Hill equation when used properly is that it does give the experimentalist some insight into the question of whether the system is cooperative or noncooperative.

2. The Adair Equation

Although the Hill equation gave a reasonably good fit to the oxygen saturation curve of hemoglobin, the results were explained more satisfactorily by ADAIR's thermodynamic description of multiple equilibria (6). The Adair equation for tetrameric hemoglobin does not identify binding sites but rather binding steps as follows:

$$E + A = EA, \ \bar{K}_1$$

$$EA + A = EA_2, \ \bar{K}_2$$

$$EA_2 + A = EA_3, \ \bar{K}_3$$

$$EA_3 + A = EA_4, \ \bar{K}_4$$

Scheme IX-2

In Scheme IX-2, E, A, and $\bar{K}$ are taken to be hemoglobin, oxygen, and an equilibrium constant, respectively.

In the derivation of the Adair equation, the four equilibria of Scheme IX-2 are expressed in terms of unliganded enzyme, E, and substituted into Eq. (IX-10).

$$EA = (E)\bar{K}_1(A); \ EA_2 = (E)\bar{K}_1\bar{K}_2(A)^2; \ EA_3 = (E)\bar{K}_1\bar{K}_2\bar{K}_3(A)^3;$$

$$EA_4 = \bar{K}_1\bar{K}_2\bar{K}_3\bar{K}_4(A)^4 \qquad \text{(IX-14)}$$

$$\bar{\nu} = \frac{\bar{K}_1(A) + 2\bar{K}_1\bar{K}_2(A)^2 + 3\bar{K}_1\bar{K}_2\bar{K}_3(A)^3 + 4\bar{K}_1\bar{K}_2\bar{K}_3\bar{K}_4(A)^4}{1 + \bar{K}_1(A) + \bar{K}_1\bar{K}_2(A)^2 + \bar{K}_1\bar{K}_2\bar{K}_3(A)^3 + \bar{K}_1\bar{K}_2\bar{K}_3\bar{K}_4(A)^4} . \quad \text{(IX-15)}$$

In the general case of an n-mer, the Adair equation is

$$\bar{\nu} = \frac{\bar{K}_1(A) + 2\bar{K}_1\bar{K}_2(A)^2 + \cdots\cdots + n\bar{K}_1\bar{K}_2\cdots\cdots\bar{K}_n(A)^n}{1 + \bar{K}_1(A) + \bar{K}_1\bar{K}_2(A)^2 + \cdots\cdots + \bar{K}_1\bar{K}_2\cdots\cdots\bar{K}_n(A)^n} . \quad \text{(IX-16)}$$

The Adair equation generates sigmoidal data when $\bar{\nu}$ is plotted as a function of A only if $\bar{K}$ increases as n increases. Inspection of Eq. (IX-16) reveals that, when A = 0, $\bar{\nu}$ = 0 and that, as $A \to \infty$, $\bar{\nu}$ = n. If binding of additional substrate molecules to the enzyme is enhanced by substrate already bound, binding is positively cooperative; i.e., $\bar{K}_1 < \bar{K}_2 < \bar{K}_3 < \bar{K}_4$. Negative cooperativity occurs when the reverse relationship pertains[8]. Finally, it is not unreasonable to expect that both positive and negative cooperativity may occur for a particular system; i.e., $\bar{K}_1 < \bar{K}_2 < \bar{K}_3 > \bar{K}_4$.

In the discussion of positive and negative cooperativity, it is assumed that the sites are equivalent in the absence of ligand. For example, titration of an amino acid where the amino and carboxyl groups are intrinsically different is not an example of negative cooperativity, whereas titration of carbonic acid is.

If the ligand binding sites on a protein are identical and do not affect each other when ligand is bound, the various binding constants are related by a single equilibrium constant as shown in Eq. (IX-17).

$$\bar{K}_i = \frac{\bar{K}(n - i + 1)}{i} \quad \text{(IX-17)}$$

It should be pointed out that it is not essential that the sites be identical, but rather that the thermodynamic macroscopic constants be the same. In Eq. (IX-17) the binding constants are assumed to be identical ($\bar{K}$), and n and K_i refer to be the total number of ligand binding sites and the binding constant for the i-th ligand bound, respectively.

When Eq. (IX-17) is substituted into Eq. (IX-16) (i.e., when the binding constants of Scheme IX-2 are taken to be identical), the following expression is obtained, where K is a dissociation constant,

$$\bar{\nu} = \frac{n\bar{K}(A)}{1 + \bar{K}(A)} = \frac{n(A)}{K + A} . \quad \text{(IX-18)}$$

---

[8] The differences between the binding constants must be greater than the statistical differences predicted by Eq. (IX-17).

It can be seen from Eq. (IX-18) that plots of $\bar{\nu}$ *versus* A are hyperbolic and not sigmoidal. Thus, it is clear that the sigmoidal nature of the Adair equation results from the fact that the thermodynamic constants that describe ligand binding differ for each site. The binding constants themselves tell very little about the binding mechanism, and other procedures are required to obtain information on how the processes of positive and negative cooperativity occur.

Equation (IX-16) is one of the most general models for sigmoidicity. FERDINAND (7) and LAIDLER and BUNTING (8) have shown that sigmoidal binding will result in the case of a dimer described by Eq. (IX-19).

$$\bar{\nu} = \frac{i(A) + j(A)^2}{k + \ell(A) + m(A)^2} \tag{IX-19}$$

if

$$\frac{k}{\ell} > \frac{i}{j}. \tag{IX-20}$$

It can be seen that this limitation is satisfied by the Hill equation where $i = \ell = 0$.

It is of interest to note that for a dimeric system that can be described by the Hill equation, a plot of $\log (\bar{\nu}/1 - \bar{\nu})$ *versus* $\log (A)$ would not be expected to be linear. This can be seen from Eq. (IX-21).

$$\log \frac{\bar{\nu}}{(1 - \bar{\nu})} = \log \frac{\bar{K}_1(A) + 2\bar{K}_1\bar{K}_2(A)^2}{\left[1 - \bar{K}_1\bar{K}_2(A)^2\right]}. \tag{IX-21}$$

Equation (IX-21) may be reduced to the Hill equation by assuming $\bar{K}_2(A) >> 1$ and $1 >> \bar{K}_1\bar{K}_2(A)^2$.

It is important to note that, although a model may seem to conform to Eq. (IX-19), a plot of $\bar{\nu}$ or v *versus* A will not be sigmoidal if the relationship described by Eq. (IX-20) is not valid. Thus in the case of two different enzymes, E_1 and E_2 catalyzing the same reaction (9),

$$v_0 = v_1 + v_2 = \frac{V_1 A}{K_1 + A} + \frac{V_1' A}{K_1' + A} \tag{IX-22}$$

$$v_0 = \frac{\left[\bar{V}_1 K_1' + V_1' K_1\right](A) + \left[\bar{V}_1 + V_1'\right](A)^2}{K_1 K_1' + \left[\bar{K}_1 + K_1'\right](A) + (A)^2}. \tag{IX-23}$$

It can readily be shown that, although Eq. (IX-23) resembles the general Adair expression (Eq. (IX-19)), the condition described by Eq. (IX-20) is not satisfied. A plot of v_0 *versus* A will not be sigmoidal. Also note that when $\bar{K}_1 > \bar{K}_2$ (negative cooperativity) for a dimer, sigmoidicity is not predicted by the Adair equation.

3. The Scatchard Plot

Binding data are frequently expressed in terms of a Scatchard plot of $\bar{\nu}/A$ *versus* $\bar{\nu}$ (10). The Scatchard equation is obtained by rearranging Eq. (IX-18) into the following form:

$$\frac{\bar{\nu}}{A} = \frac{n}{K} - \frac{\bar{\nu}}{K}. \qquad \text{(IX-24)}$$

Figure IX-4 illustrates how the Scatchard plot may be used to evaluate both n and K where Eq. (IX-24) is obeyed (curve a). A straight line is obtained only when the ligand binding sites are identical and noninteracting. When cooperativity is either positve or negative, the Scatchard plots are nonlinear, and it is not possible to accurately determine values for n and K. If the Adair equation is rearranged into the form shown by Eq. (IX-24), it can be seen that both the intercepts and slopes are functions of the ligand A, and the resulting Scatchard plots are nonlinear. An example of positive and negative cooperativity data as described by KOSHLAND (11) is shown in Fig. IX-4.

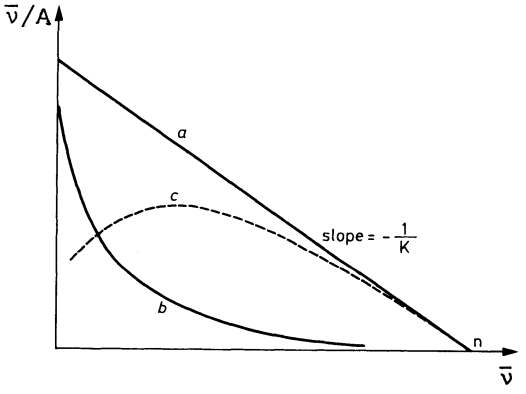


Fig. IX-4. A Scatchard plot of $\bar{\nu}/A$ *versus* $\bar{\nu}$ for (a) a noncooperative system and systems which exhibit negative (b) and positive (c) cooperativity

B. Molecular Models

Even though mathematical models such as the Adair equation are capable of describing sigmoidal binding and, in some cases, velocity data with certain enzymes reasonably well, this treatment does not provide a conceptual understanding of the processes that cause the effects observed. A number of molecular models have been presented in recent years in an attempt to explain cooperativity and allostery. Some of these have received a large degree of acceptance and will be outlined very briefly.

1. The Monod Model (MWC) (12)

The molecular model proposed by MONOD et al. (MWC) (12) to explain cooperativity and allostery is based upon a number of postulates. They include the following assumptions:

a) The enzymes are oligomeric, and the oligomers are made up of identical protomers or subunits.

b) The oligomers exist in two or more different conformational states (R and T), which are in equilibrium with each other and related by the equilibrium constant L.

c) The various conformations differ in their affinity for ligands.

d) An alteration in the conformation of any subunit of an oligomer alters the conformation of all the subunits within either the R or T state; i.e., the conformation changes are concerted. In other words, the molecular symmetry of the entire oligomer is maintained and hybrid states do not exist.

The Monod model may be formulated as follows where F is taken to be a ligand:

$$R_0 \overset{L}{\rightleftharpoons} T_0$$

$$R_0 + F \rightleftharpoons R_1, \; K_{R1} \qquad\qquad T_0 + F \rightleftharpoons T_1, \; K_{T1}$$

$$R_1 + F \rightleftharpoons R_2, \; K_{R2} \qquad\qquad T_1 + F \rightleftharpoons T_2, \; K_{T2}$$

$$\vdots \qquad\qquad\qquad\qquad \vdots$$

$$R_{n-1} + F \rightleftharpoons R_n, \; K_{Rn} \qquad\qquad T_{n-1} + F \rightleftharpoons T_n, \; K_{Tn}$$

Scheme IX-3

Let us consider first how an expression may be obtained which relates the saturation function, $\bar{\nu}$, to the ligand concentration for the model depicted in Scheme IX-3 in the case of a tetramer. $\bar{\nu}$ may be expressed in terms of R and T as follows, if it is assumed that ligand binding is exclusive to the R state of the protein.

$$\bar{\nu} = \frac{R_1 + 2R_2 + 3R_3 + 4R_4}{R_0 + R_1 + R_2 + R_3 + R_4 + T_0}. \qquad \text{(IX-25)}$$

The Monod model assumes that the binding constants are all equal for ligand binding to a particular protein conformation. Thus, by using the equilibrium expression in Scheme IX-3 along with Eq. (IX-17), the following identities are obtained.

$$R_1 = R_0\bar{K}_1(F) = 4R_0\bar{K}_r(F) \qquad \text{(IX-26)}$$

$$R_2 = R_0\bar{K}_1\bar{K}_2(F)^2 = 6R_0(\bar{K}_r)^2(F)^2 \qquad \text{(IX-27)}$$

$$R_3 = R_0\bar{K}_1\bar{K}_2\bar{K}_3(F)^3 = 4R_0(\bar{K}_r)^3(F)^3 \qquad \text{(IX-28)}$$

$$R_4 = R_0\bar{K}_1\bar{K}_2\bar{K}_3\bar{K}_4(F)^4 = R_0(\bar{K}_r)^4(F)^4 \qquad \text{(IX-29)}$$

Substituting Eqs. (IX-26) to (IX-29) into Eq. (IX-25), but using dissociation rather than association constants, gives

$$\bar{\nu} = \frac{4F/K_r \left[1 + 3F/K_r + 3(F)^2/(K_r)^2 + (F)^3/(K_r)^3\right]}{\left[1 + 4F/K_r + 6(F)^2/(K_r)^2 + 4(F)^3/(K_r)^3 + (F)^4/(K_r)^4 + T_0/R_0\right]} =$$

$$\frac{4F/K_r\,(1 + F/K_r)^3}{(1 + F/K_r)^4 + T_0/R_0}. \qquad \text{(IX-30)}$$

Equation (IX-30) may be expressed in more general terms,

$$\bar{\nu} = \frac{n\alpha(1 + \alpha)^{n-1}}{(1 + \alpha)^n + L} \qquad \text{(IX-31)}$$

where n = number of sites, $\alpha = F/K_r$, and L, the allosteric constant (so defined by MONOD et al. (12)) equals T_0/R_0.

It is also possible to obtain an expression that describes the fraction of sites bound by ligand. This expression, the saturation function $\bar{Y}$, is defined as, $\bar{Y}_F = \frac{\bar{\nu}}{n}$ = fraction of sites bound by ligand F.

Equation (IX-31) was derived assuming essentially exclusive ligand binding to the R conformer; however, Eq. (IX-32) describes the case in which the ligand binds to both the R and T states, but with different affinities (Scheme IX-4). In this representation the protomers of one state are shown as squares and of the other state as circles.

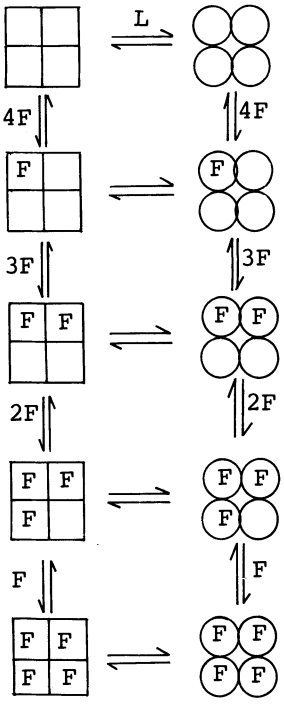
$$\bar{Y}_F = \frac{\alpha(1 + \alpha)^{n-1} + LC\alpha(1 + C\alpha)^{n-1}}{(1 + \alpha)^n + L(1 + C\alpha)^n} \qquad \text{(IX-32)}$$

In the derivation, $C = K_r/K_T$ where K_T is the dissociation constant for ligand binding to T.

MONOD et al. (12) have presented theoretical plots of $\bar{Y}$ *versus* α for different values of L and C. Figure IX-5 indicates the effect of the allosteric constant L on the cooperativity of a system in which C = 0. Figure IX-6 shows how C affects the cooperative response of a system to changes in α at a large fixed value for L.

In these figures, the homotropic effect of F is clearly a function of the allosteric constant L and the ratio of the dissociation constants for ligand binding to the R and T conformers. According to the MWC model (12), heterotropic effects are due to displacement of the equilibrium between the R and T states. In Fig. IX-5 where C = 0, $K_T >> K_R$ and ligand binding is said to be exclusive. Nevertheless, sigmoidicity is observed at values of L in excess of L = 1.

Examination of Eq. (IX-32) reveals that, when ligand binding is not exclusive (i.e., when $C \neq 0$), the MWC equation takes the



Scheme IX-4

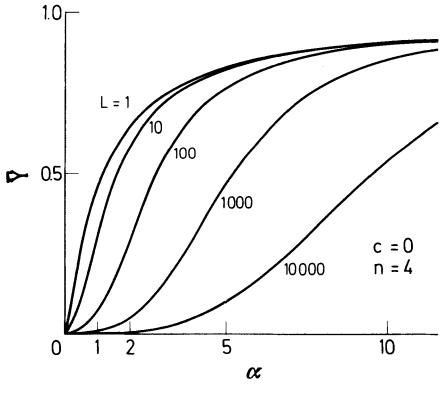


Fig. IX-5. Theoretical curves of the saturation function $\bar{Y}$ drawn to various values of the constants L and C, with n = 4 (12) according to Eq. (IX-32)

form of the Michaelis-Menten equation under certain conditions. These include the cases where L = 0 and where C = 1.

It is important to note that, for the MWC model, the binding constants are identical, and thus negative cooperativity is not a feature of this mechanism.

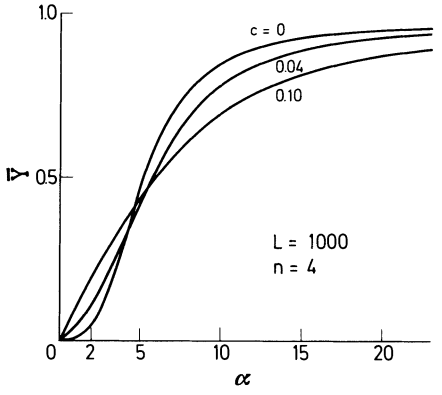
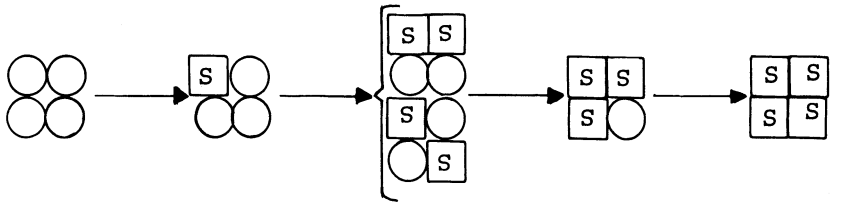


Fig. IX-6. Theoretical curves of the saturation function $\bar{Y}$ drawn to various values of the constants L and c, with n = 4 (12) according to Eq. (IX-32)

2. The Adair-Koshland Model (13)

KOSHLAND and his coworkers have attempted to explain cooperativity and allostery in molecular terms by using the Adair model of multiple equilibria (13). According to the Adair-Koshland (AK) model, enzymes exist as oligomers. Ligand binding induces a conformational change in the subunit to which it is bound, and this in turn causes alterations in the subunit-subunit interactions. The AK model is similar to the MWC model in that the enzyme is thought to exist in two conformational states. On the other hand, the AK model assumes that the microscopic constants describing ligand binding are different. The AK model, like the original Adair approach, provides for negative cooperativity, a feature missing in the MWC model. In addition, the AK model requires the existence of hybrid protein conformational states. Scheme IX-5 illustrates the square system (13) of the AK model. Other molecular models such as the linear, tetragonal, and concerted model (similar to the MWC model) have been suggested (13).

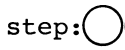


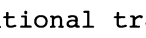
Scheme IX-5

The following symbols are used in the derivation of the AK model for a dimer,

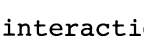
E = ◯◯; ES_1 = [S]◯ or ◯[S]; ES_2 = [S][S] (IX-33)

The sequence of events leading to ES_2 is then

Dissociation step:  (The binding constant K_{AA} is expressed as a dissociation constant and is set to unity) (IX-34)

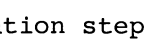
Conformational transition:  (IX-35)

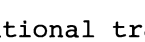
Binding: (IX-36)

Subunit interaction:  (IX-37)

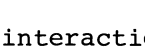
Sum of the four steps: , $\frac{ES_1}{(E)(S)} = \frac{2K_{tAB}K_{SB}K_{AB}}{K_{AA}} = K_1$

The factor 2 in the equation for K_1 arises statistically from the fact that the ligand can bind to either subunit. The second ligand adds to the dimer in a similar fashion; however, statistically it can add to only one of the subunits.

Dissociation step:  (IX-38)

Conformational transition:  (IX-39)

Binding: (IX-40)

Subunit interaction:  (IX-41)

Sum of the last four steps: , $\frac{ES_2}{(ES_1)(S)} =$

$$\frac{K_{tAB}K_{SB}K_{BB}}{K_{AB}} = K_2.$$

The terms ES_1 and ES_2 are now substituted into the equation for $\bar{Y}$:

$$\bar{Y} = \frac{\bar{\nu}}{n} = \frac{\bar{\nu}}{2} = \frac{K_{tAB}K_{SB}K_{AB}(S) + K_{tAB}^2 K_{SB}^2 K_{BB}(S)^2}{1 + 2K_{tAB}K_{SB}K_{AB}(S) + K_{tAB}^2 K_{SB}^2 K_{BB}(S)^2}. \quad \text{(IX-42)}$$

Cooperativity will either be positive or negative depending upon the ratio of $K_{tAB}K_{SB}K_{AB}$:$K_{tAB}^2 K_{SB}^2 K_{BB}$. If this ratio is less than 1, cooperativity will be positive. If cooperativity is negative, the ratio will be greater than 1.

In the derivation of Eq. (IX-42), only homotropic interactions of enzymes and ligands are considered. KOSHLAND (11) has discussed in detail how heterotropic effects may be expressed within the context of the AK model. The heterotropic effectors presumably induce conformational changes in the subunits of the oligomer, which may enhance or inhibit formation of the subunit structure to which the substrate binds. The effector may therefore either facilitate substrate binding and therefore catalysis,

or alternatively, in the case of a negative modulator, an opposite effect will be achieved.

It should be noted that Eq. (IX-42) like the original Adair equation will reduce to the case that provides for a hyperbolic, rather than a sigmoidal, response to ligand when certain assumptions are made regarding the various binding constants (14).

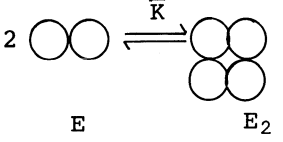
The enzyme glyceraldehyde-3-P dehydrogenase (D-glyceraldehyde-3-phosphate: NAD oxidoreductase (phosphorylating), (EC 1.2.1.12) catalyzes the simultaneous oxidation and phosphorylation of glyceraldehyde-3-P to 1, 3 diphosphoglycerate with the concomitant reduction of NAD^+ to NADH. The rabbit muscle enzyme is kown to be a tetramer with four identical subunits (15). The dehydrogenase seems to exhibit normal Michaelis-Menten kinetics (16, 17); however, a large body of evidence shows that NAD^+ binding is cooperative. CONWAY and KOSHLAND (18) have demonstrated that glyceraldehyde-3-P dehydrogenase exhibits negative cooperative binding of NAD^+; i.e., the binding of each molecule of NAD^+ makes it more difficult for binding of the next molecule. These workers observed that protein conformational changes as measured by sulfhydryl group reactivity and viscosity measurements attend binding of at least one mole of NAD^+ per mole of enzyme. Temperature-jump experiments (19) and low angle X-ray scattering studies (20) support the proposal of alterations in the enzyme conformation as NAD^+ becomes associated with the protein. All these observations are best reconciled with the Adair-Koshland model of cooperativity. The MWC model is excluded because it makes no provision for negative cooperativity.

The observation that glyceraldehyde-3-P dehydrogenases displays normal initial rate kinetics and negative cooperativity for NAD^+ binding may indicate that, in the presence of all substrates, the enzyme is locked into a conformation (or conformations) that permits all active sites to act identically and independently.

3. Subunit-Subunit Polymerization

One of the proposals suggested to explain sigmodicity involves molecular models that provide for subunit polymerization and depolymerization. NICOL et al. (21) and FRIEDEN (22) have described examples of subunit association and dissociation that give rise to equations that are very similar in form to those described by the MWC model.

Consider, for example, the reaction described by Scheme IX-6, which involves a rapid equilibrium between a dimeric and tetrameric form of an enzyme.



Scheme IX-6

The following equilibria are to be expected if ligand binds to each subunit.

$$E + L = EL,\ \bar{K}_{EL};\quad EL + L = EL_2,\ \bar{K}_{EL_2} \tag{IX-43}$$

$$E_2 + L = E_2L,\ \bar{K}_{E_2L};\ E_2L + L = E_2L_2,\ \bar{K}_{E_2L_2};\quad E_2L_2 + L = E_2L_3,\ \bar{K}_{E_2L_3};\ E_2L_3 + L = E_2L_4,\ \bar{K}_{E_2L_4} \tag{IX-44}$$

$$\bar{\nu} = \frac{EL + 2EL_2 + E_2L + 2E_2L_2 + 3E_2L_3 + 4E_2L_4}{E + EL + EL_2 + E_2 + E_2L + E_2L_2 + E_2L_3 + E_2L_4}. \tag{IX-45}$$

Substituting from Eq. (IX-44) into Eq. (IX-45) gives

$$\bar{\nu} = \frac{\bar{K}_{EL}(E)(L) + 2\bar{K}_{EL}\bar{K}_{EL_2}(E)(L)^2 + \bar{K}_{E_2L}(E_2)(L) + 2\bar{K}_{E_2L}\bar{K}_{E_2L_2}(E_2)(L)^2 + 3\bar{K}_{E_2L}\bar{K}_{E_2L_2}\bar{K}_{E_2L_3}(E_2)(L)^3 + 4\bar{K}_{E_2L}\bar{K}_{E_2L_2}\bar{K}_{E_2L_3}\bar{K}_{E_2L_4}(E_2)(L)^4}{(E)\left[1 + \bar{K}_{EL}(L) + \bar{K}_{EL}\bar{K}_{EL_2}(L)^2\right] + (E_2)\left[1 + \bar{K}_{E_2L}(L) + \bar{K}_{E_2L}\bar{K}_{E_2L_2}(L)^2 + \bar{K}_{E_2L}\bar{K}_{E_2L_2}\bar{K}_{E_2L_3}(L)^3 + \bar{K}_{E_2L}\bar{K}_{E_2L_2}\bar{K}_{E_2L_3}\bar{K}_{E_2L_4}(L)^4\right]} \tag{IX-46}$$

If it is assumed that the ligand binding sites on the dimer are independent and noninteracting and that the same condition prevails for the tetramer,

$$\bar{\nu} = \frac{2\bar{K}_E(L)\left[1 + \bar{K}_E(L)\right] + 4\bar{K}(E)\bar{K}_{E_2}(L)\left[1 + \bar{K}_{E_2}(L)\right]^3}{\left[1 + \bar{K}_E(L)\right]^2 + \bar{K}(E)\left[1 + \bar{K}_{E_2}(L)\right]^4}. \tag{IX-47}$$

It is of interest to note that Eq. (IX-47) is similar in form to Eq. (IX-32), which was derived for the MWC model involving nonexclusive binding.

The basis for the cooperativity phenomenon of the subunit-subunit polymerization model is the equilibrium between the two different oligomers. If only one protein form existed, ligand binding would not be sigmoidal. The equilibrium described by Scheme IX-6 could be shifted by dilution and by a number of different perturbants such as changes in temperature, pH, ionic strength, and homotropic and heterotropic effectors.

A situation that involves exclusive binding to one of the protein forms is illustrated in the case of lamprey hemoglobin, which exists predominantly as monomers when oxygenated and as oligomers when deoxygenated (23). This characterization of lamprey hemoglobin may be described according to Eqs. (IX-48) and (IX-49) where E, E_2, EA, and A represent monomer, dimer, oxygenated monomer, and oxygen, respectively.

$$2E \rightleftharpoons E_2,\ \bar{K}_E \tag{IX-48}$$

$$E + A \rightleftharpoons EA,\ \bar{K}_{ia} \tag{IX-49}$$

If

$$E_0 = E + 2E_2 + EA \tag{IX-50}$$

and

$$\bar{\nu} = \bar{y} = EA/E_0 \tag{IX-51}$$

then

$$\left[\frac{\bar{y}E_0}{\bar{K}_{ia}(A)}\right]^2 = \frac{E_2}{\bar{K}_E}. \tag{IX-52}$$

If one finds as BRIEHL did with lamprey hemoglobin that $E_2 >> E$, then

$$\left[\frac{\bar{y}E_0}{\bar{K}_{ia}(A)}\right]^2 = \frac{E_0(1 - \bar{y})}{2\bar{K}_E}. \tag{IX-53}$$

Equation (IX-53) may be plotted in modified Hill form; i.e., $\log \frac{\bar{y}^2}{(1 - \bar{y})}$ *versus* log A. For this particular model, n = 2, and the apparent dissociation constant (K_E in Eq. (IX-53)) will vary with protein concentration as determined from the modified Hill plot. This effect is not to be expected in systems in which the protein does not dissociate into subunits because of factors such as dilution or ligand binding.

Ligand binding to a protein or enzyme may induce either polymerization or depolymerization, which in turn may give rise to sigmoidicity. This possibility is illustrated in Eqs. (IX-54) and (IX-55).

$$E + A = EA_1,\ \bar{K}_{EA} \tag{IX-54}$$

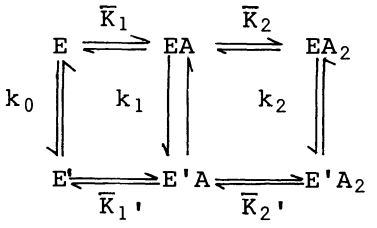
$$2EA_1 = E_2A_2,\ \bar{K}_{E_2A_2} \tag{IX-55}$$

The binding expression for this mechanism is a function of enzyme concentration.

$$\bar{\nu} = \frac{\bar{K}_{EA}(A) + 2(\bar{K}_{EA})^2\bar{K}_{E_2A_2}(E)(A)^2}{1 + \bar{K}_{EA}(A) + (\bar{K}_{EA})^2\bar{K}_{E_2A_2}(E)(A)^2} \tag{IX-56}$$

4. Protein Isomerization

In 1965 WEBER (24) proposed that, if proteins tautomerize and ligand can bind to the tautomers, cooperativity may be in evidence. WEBER's model, which assumes that all the protein tautomers are in rapid equilibrium, is shown in Scheme IX-7.



Scheme IX-7

According to Eq. (IX-10),

$$\bar{\nu} = \frac{EA + E'A + 2\left[EA_2 + E'A_2\right]}{E + E' + EA + E'A + EA_2 + E'A_2} . \qquad \text{(IX-57)}$$

Equation (IX-57) may be expressed in terms of equilibrium constants and the isomerization constant k_0 as follows:

$$\bar{\nu} = \frac{\left[\bar{K}_1 + k_0\bar{K}_1'\right](A) + 2\left[\bar{K}_1\bar{K}_2 + k_0\bar{K}_1'\bar{K}_2'\right](A)^2}{\left[1 + k_0\right] + \left[\bar{K}_1 + k_0\bar{K}_1'\right](A) + \left[\bar{K}_1\bar{K}_2 + k_0\bar{K}_1'\bar{K}_2'\right](A)^2} . \qquad \text{(IX-58)}$$

Equation (IX-58) is similar in form to the Adair equation for a dimer (Eq. (IX-16)), and the mechanism outlined in Scheme IX-7 may give rise to cooperative ligand binding.

WEBER has recently described ligand binding to proteins and ligand interactions after binding in terms of free energy changes (3). In the case of Scheme IX-7, two apparent equilibrium constants may be written for dissociation of A from the different protein tautomers,

$$K_{app}(1) = \frac{(E + E')(A)}{(EA + E'A)} \qquad \text{(IX-59)}$$

and

$$K_{app}(2) = \frac{(EA + E'A)(A)}{(EA_2 + E'A_2)} . \qquad \text{(IX-60)}$$

It is possible to describe the various enzyme expressions in Eqs. (IX-59) and (IX-60) in terms of dissociation constants depicted in Scheme IX-7. WEBER (3) finally relates the apparent dissociation constants to apparent free energies of binding. This treatment has also been extended to heterotropic ligand binding and subunit interactions that result from ligand binding.

The apparent free energy of binding of a ligand will include the free energy involved in the conformation change of the protein. A similar determination may be made with a different ligand species and finally with both ligands together. The difference between the apparent free energy of binding of both ligands and the sum of the apparent free energies of binding of each ligand

alone is referred to as the free energy of coupling. The free energy of coupling has been found to be very small (e.g., 4.18 - 6.27 KJ/mole), and is related to cooperativity. When the coupling free energy between the ligands is negative, cooperativity is positive, whereas negative cooperativity is observed when the coupling free energy is positive. When the free energy coupling is zero, the system does not exhibit cooperativity. WEBER's description includes treatment of the binding of allosteric modifiers and the effect of ligands on subunit-subunit interactions.

C. Kinetic Models

A relatively large number of kinetic models have been proposed in recent years to account for cooperativity. These models may be divided into two classes; those that assume subunit-subunit interaction and those that assume alternative pathways for enzyme-substrate interaction. Although many of these models seem to account adequately for sigmoidal kinetics, some of them seem to be unrealistic in a chemical sense.

1. Kinetic Models Involving Subunit-Subunit Interaction

Probably the simplest kinetic model that assumes some type of subunit-subunit interaction is one analogous to the Adair model. Consider for example a pathway of the type depicted in Scheme IX-2, but for a dimer. If it is assumed that both the EA and EA_2 complexes are capable of product formation, the initial rate equation is,

$$v = \frac{V_1 \left[K_2(A) + (A)^2\right]}{K_1K_2 + K_2(A) + (A)^2}. \qquad \text{(IX-61)}$$

In Eq. (IX-61) the K's are dissociation constants and the maximal velocity, V_1, is equal to $k(E_0)$ where k is the unimolecular rate constant for the breakdown of EA and EA_2 to products.

If only the ternary complex gives rise to product, the kinetic equation is

$$v = \frac{V_1(A)^2}{K_1K_2 + K_2(A) + (A)^2}. \qquad \text{(IX-62)}$$

Equations (IX-61) and (IX-62) were derived assuming that all steps in the kinetic mechanism equilibrate rapidly relative to the decay of the productive binary and ternary complexes.

Equations (IX-61) and (IX-62) give rise to sigmoidal kinetics within the constraints implied by Eq. (IX-20). It should be noted that only when it is assumed that both EA and EA_2 form

product does the kinetic equation (Eq. (IX-61)) resemble the Adair expression (Eq. (IX-19)).

Equation (IX-4) can be plotted in double reciprocal form as shown in Fig. IX-7. The slopes of the plots described by Fig. IX-7 have been analyzed in detail by DALZIEL and ENGEL (25).

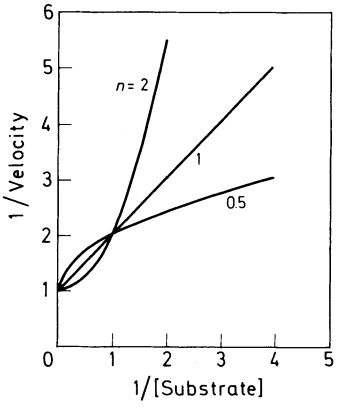


Fig. IX-7. Double reciprocal plot of 1/velocity *versus* 1/substrate concentration for the Hill equation. V_1 and K are taken to be 1, and n = 0.5, 1, and 2 (11)

It is possible to treat the mechanism for a dimer by using steady-state rather than equilibrium assumptions. Consider the mechanism described by Scheme IX-7 in this context.

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \xrightarrow{k_5} E + P$$

$$EA + A \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EA_2 \xrightarrow{k_6} EA + P$$

Scheme IX-8

The kinetic equation for this mechanism, Eq. (IX-63), is similar in form to Eq. (IX-61)

$$v = \frac{[k_1k_5(k_4 + k_6)(A) + k_1k_3k_6(A)^2]E_0}{[k_2 + k_5][k_4 + k_6] + [k_1k_4 + k_1k_6 + k_3k_6](A) + k_1k_3(A)^2} \quad \text{(IX-63)}$$

If the binary complex shown in Scheme IX-8 does not form product (i.e., $k_5 = 0$), the initial rate expression becomes

$$v = \frac{k_1k_3k_6(A)^2E_0}{k_2(k_4 + k_6) + [k_1k_4 + k_1k_6 + k_3k_6](A) + k_1k_3(A)^2} \quad \text{(IX-64)}$$

Equation (IX-64) is very similar in form to the analogous kinetic equation (Eq. (IX-62)) derived with equilibrium assumptions. Equations (IX-63) and (IX-64) will yield sigmoidal kinetics provided the various rate constants support the relationship described by Eq. (IX-20).

WORCEL et al. (26) have found that reduced nicotinamide adenine dinucleotide oxidase exhibits positive cooperative kinetics when 1/velocity is plotted as a function of 1/NADH concentrations. Furthermore, good fits to the initial rate data were obtained with Eq. (IX-64).

FRIEDEN (27) has suggested mechanisms that may give rise to sigmoidal kinetics in which the substrate may bind to two different loci on the enzyme. One such mechanism is illustrated in Scheme IX-9.

$$E + A = EA, K_1 \qquad EA \xrightarrow{k_1} E + P$$

$$E + A = AE, K_2 \qquad AE \xrightarrow{k_2} E + P$$

$$EA + A = EA_2, K_3 \qquad EA_2 \xrightarrow{k_3} EA + P$$

$$AE + A = EA_2, K_4 \qquad EA_2 \xrightarrow{k_3} AE + P$$

Scheme IX-9

If it is assumed that all steps in Mechanism IX-9 equilibrate rapidly relative to the breakdown of the active binary and ternary complexes to product, Eq. (IX-65) is obtained:

$$v = \frac{[(k_1K_3 + k_2K_4)(A) + k_3(A)^2]E_0}{K_1K_3 + (K_3 + K_4)(A) + (A)^2}. \qquad \text{(IX-65)}$$

Although Eq. (IX-65) is of the same form as the Adair equation, the relationships described by Eq. (IX-20) must apply if this equation is to exhibit sigmoidal kinetics.

2. Kinetic Models Involving Alternative Pathways of Substrate Addition and Enzyme Isomerization

A number of kinetic mechanisms have been proposed in an attempt to explain cooperativity in terms of alternative pathways of substrate addition to enzymes. Only a few representative models will be presented to provide some insight into the type of mechanism that may lead to sigmoidicity.

The steady-state Random Bi Bi mechanism gives rise to a rate expression (Eq. (V-37)) of the type that will give sigmoidal kinetics within certain limits (Eq. (IX-20)). If the concentration of one of the substrates is held constant, Eq. (V-37) will take the form of Eq. (IX-19). An extensive analysis of this equation has been provided by FERDINAND (7), DALZIEL and ENGEL (25), and LAIDLER and BUNTING (8). It should be noted that the

kinetic equation for the steady-state Random Bi Bi mechanism is identical with the Adair equation, and whereas the Adair and Koshland models are based upon unireactant mechanisms, this equation is applicable to bireactant systems.

a) Enzyme Isomerization Mechanisms

WEBER (24) has suggested that cooperativity may occur if proteins can isomerize and reach equilibrium slowly compared to equilibration of the ligand with the various isomeric protein species. An extension of this suggestion is the proposal of RABIN who has presented a kinetic model to explain cooperativity based upon a substrate induced enzyme isomerization (28). Rabin's mechanism is presented in Scheme IX-10 along with the applicable rate equation (Eq. (IX-66)).

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \xrightarrow{k_3} EA' \xrightarrow{k_7} E' + P$$

$$EA' \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E' + A$$

$$E' \xrightarrow{k_8} E$$

Scheme IX-10

$$v = \frac{k_7\left[k_8(A) + k_6(A)^2\right]E_0/(k_5 + k_7)}{\frac{k_8(k_2 + k_3)}{k_1k_3} + \left[1 + \frac{k_8}{k_3} + \frac{k_8}{(k_5 + k_7)}\right](A) + \frac{k_6(A)^2}{(k_5 + k_7)}} \qquad \text{(IX-66)}$$

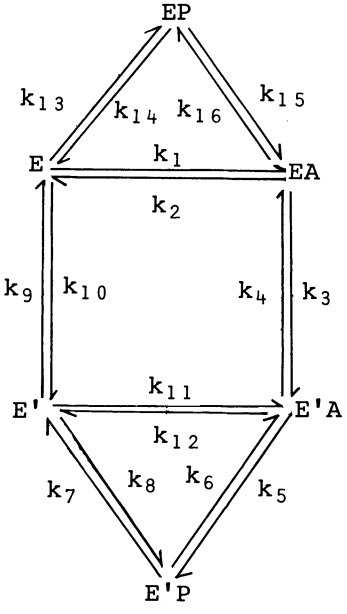
When the k_3 step is made reversible (28), there is no change in the form of the resulting rate equation.

It was pointed out in Chapter VII that isomerization of enzyme-substrate complexes is not at all unusual. It is this type of reaction that provides the basis for the mechanism of Scheme IX-10.

HATFIELD et al. (29, 30), FRIEDEN (22), and AINSLIE et al. (31) have shown that, if the isomerization of enzyme forms is relatively slow compared with substrate binding and catalysis, lags and bursts in product production with time may be observed. The lag, or hysteretic effect described in Fig. III-3 is very probably a consequence of the slow enzyme isomerization as first proposed by WEBER (24). FRIEDEN (22) describes hysteretic enzymes as those that respond slowly to rapid changes in ligand concentrations.

The studies of AINSLIE et al. (31) will be used as an example of a system that may exhibit both hysteresis and cooperativity.

Scheme IX-11 describes a very simple mechanism that may give rise to these phenomena.



Scheme IX-11

The initial rate equation for this mechanism is in the form described by Eq. (IX-61), and thus sigmoidicity may result when velocity is plotted as a function of substrate concentration. AINSLIE et al. (31) have used the digital computer to evaluate cooperativity for the mechanism of Scheme IX-11. They have found, for example, that the steps governed by the rate constants k_3, k_4, k_9, and k_{10} must be slow relative to other steps for cooperativity to occur. Although these slow isomerization steps are required for cooperativity, normal kinetics may be observed under other conditions; i.e., if the substrate binding steps (k_1/k_2 or k_{11}/k_{12}) are at equilibrium. It should be pointed out that both positive and negative cooperativity may be accounted for with the model described in Scheme IX-11.

The model of Scheme IX-11 also predicts both lags and bursts in product formation with time under certain conditions. In order to understand how such events may occur, it is necessary to obtain kinetic expressions for the steady-state and pre-steady-state velocity; i.e., velocity where time (t) = 0.

Examination of the mechanism of Scheme IX-11 reveals that it is composed of two catalytic cycles joined together by two different steps involving slow isomerizations. The velocity for this model is

$$\frac{dP}{dt} = v = k_7(E'P) + k_{14}(EP). \qquad \text{(IX-67)}$$

If the two cycles produce product at different rates, a shift with time in going from the rapid cycle to the slow cycle will produce a velocity burst. The reverse effect will result in a lag in product formation in going from the pre-steady-state to the steady-state.

In order to derive equations that account for hysteretic and burst phenomena, three simplifying assumptions are made (31):

a) That each cycle reaches an internal steady-state before a steady-state is reached between the two cycles;

b) Product inhibition does not occur; and

c) Substrate concentration does not change.

The concentrations of the two cycles (C_1 and C_2) are related to each other and to the various enzyme forms in the following way:

$$C_0 = C_1 + C_2 \qquad \text{(IX-68)}$$

$$C_1 = E + EA + EP \quad \text{and} \quad C_2 = E' + E'A + E'P \qquad \text{(IX-69)}$$

$$\frac{dC_1}{dt} = k_9(E') + k_4(E'A) - k_{10}(E) - k_3(EA). \qquad \text{(IX-70)}$$

The enzyme forms E', E'A, E and EA may be expressed in terms of C_1 and C_2 as follows:

$$\frac{dC_1}{dt} = \frac{[k_9(E') + k_4(E'A)]C_2}{C_2} - \frac{[k_{10}(E) + k_3(EA)]C_1}{C_1}. \qquad \text{(IX-71)}$$

The determinants for the various enzyme species may be obtained as suggested in Chapter II. Equation (IX-71) may be gotten into a form that is amenable to integration by using the relationship of Eq. (IX-68).

$$\frac{dC_1}{dt} = hC_0 - C_1(h + i). \qquad \text{(IX-72)}$$

The coefficients h and i are defined by AINSLIE et al. (31) and consist of rate constants and substrate terms, and Eq. (IX-72) may be integrated between the limits t = 0 and t. In order to eliminate the constant of integration, $C_{1,0}$ the relationship $C_1/C_2 = k_9/k_{10}$ is used and the expression for C_1 obtained,

$$C_1 = C_0\left[\frac{h}{(h + i)} - \left(\frac{h}{h + i} - \frac{k_9}{k_9 + k_{10}}\right) e^{-(h + i)t}\right]. \qquad \text{(IX-73)}$$

It is possible to evaluate C_2 with a knowledge of C_1 from Eq. (IX-68). The velocity for the reaction of Eq. (IX-67) can be described in another way, e.g.,

$$\frac{dP}{dt} = \frac{k_7(E'P)C_2}{\left[E' + E'A + E'P\right]} + \frac{k_{14}(EP)C_1}{\left[E + EA + EP\right]} \quad . \qquad \text{(IX-74)}$$

When values for the determinants of the various enzyme forms, along with the identities for C_1 and C_2 (Eqs. (IX-68) and (IX-73)) are substituted into Eq. (IX-74), the expression for the product-time relationship for the model described by Scheme IX-11 is obtained. This expression will take the form

$$\frac{dP}{dt} = aC_0 + C_0(b-a)\left[\frac{h}{(h+i)} - \left(\frac{h}{(h+i)} - \frac{k_9}{(k_9+k_{10})}\right) e^{-(h+i)t}\right] \qquad \text{(IX-75)}$$

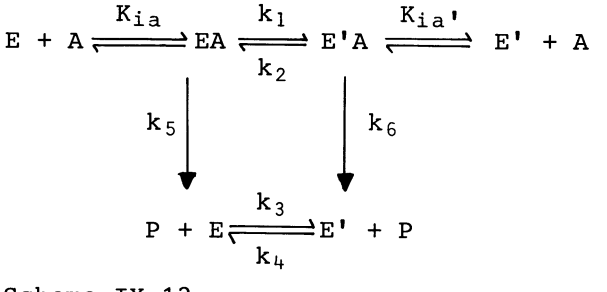
where a and b represent the coefficients associated with C_2 and C_1, respectively, of Eq. (IX-74).

The coefficient of t in Eq. (IX-75) represents the rate constant for the transition in going from C_1 to C_2. The half-time of the transition is (31)

$$t1/2 = \frac{0.693}{(h+i)} \quad . \qquad \text{(IX-76)}$$

It can be shown that in Eq. (IX-75) when t becomes large, the exponential term is eliminated, and this expression becomes identical in form to Eq. (IX-61) (31). Equation (IX-61) represents the steady-state velocity phase of the reaction described by the model of Scheme IX-11; i.e., where all steps in the reaction have reached a steady-state. Figure IX-8 describes a plot of product formation as a function of t/t1/2 for a variety of computer simulated conditions for the model of Scheme IX-11. This graph indicates that the model does indeed accomodate both burst and lag phenomena.

FRIEDEN (22) has presented a number of models that explain hysteresis. They are based on mechanisms of the type shown in Scheme IX-12.



Scheme IX-12

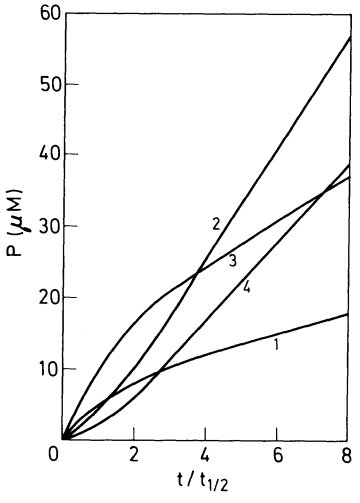


Fig. IX-8. Progress curves (product *versus* time) of the slow isomerization mechanism (Scheme IX-11). Different enzyme concentrations and rate constants were used by AINSLIE et al. (31) to generate the simulations shown on the graph

In the mechanism described in Scheme IX-12, the substrate binding steps equilibrate rapidly relative to the slow interconversion of the different enzyme species. The product-time relationship for this mechanism is the same as Eq. (IX-75), but is expressed by FRIEDEN (22) as

$$\frac{dP}{dt} = v_t = v_f + (v_0 - v_f)e^{-at}. \qquad \text{(IX-77)}$$

In Eq. (IX-77), v_t, v_f, and v_0 represent velocity at time t, velocity at $t \longrightarrow \infty$, and velocity at $t = 0$, respectively. The complex constant a is defined as:

$$a = \frac{k_1A + k_3K_{ia}}{K_{ia} + A} + \frac{k_2A + k_4K_{ia}'}{K_{ia}' + A}. \qquad \text{(IX-78)}$$

When Eq. (IX-77) is integrated, an expression for P is obtained

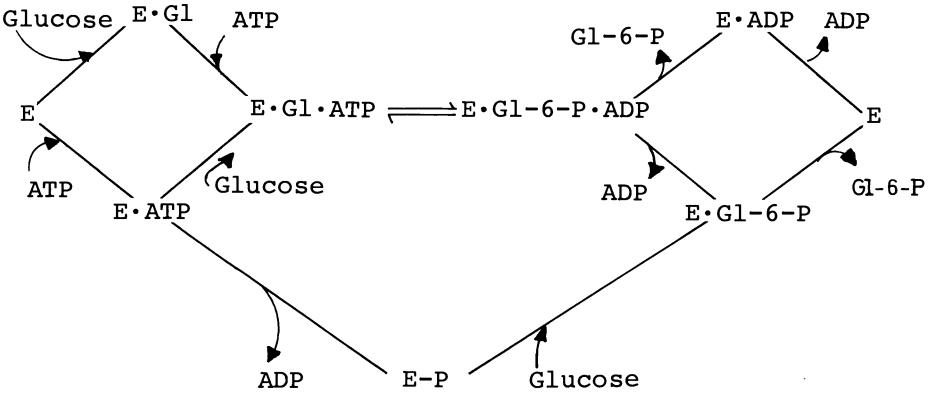
$$P_t = v_f t - (v_f - v_0)(1 - e^{-at})/a. \qquad \text{(IX-79)}$$

This last expression indicates that the amount of product produced in the hysteretic system is equal to the amount of product expected in the absence of the lag phase $v_f t$, minus the amount of product not produced because of the lag phase of the reaction. The last term in Eq. (IX-79) represents the magnitude of the lag phase in terms of product.

FRIEDEN (22) has presented evidence, indicating that frog phosphorylase a exhibits a hysteretic response after addition of substrate, which is in accord with Eq. (IX-77).

b) Alternative Pathway Mechanisms

WONG and HANES (32) were the first to propose that alternative pathway mechanisms lead to rate equations that give rise to sigmoidal kinetics. The hypothetical mechanism for hexokinase outlined in Scheme IX-13 incorporates the features of a Random Bi Bi Sequential mechanism and a Ping Pong Bi Bi mechanism. The initial rate expression according to WONG and HANES is of the form described by Eq. (IX-19)



Scheme IX-13

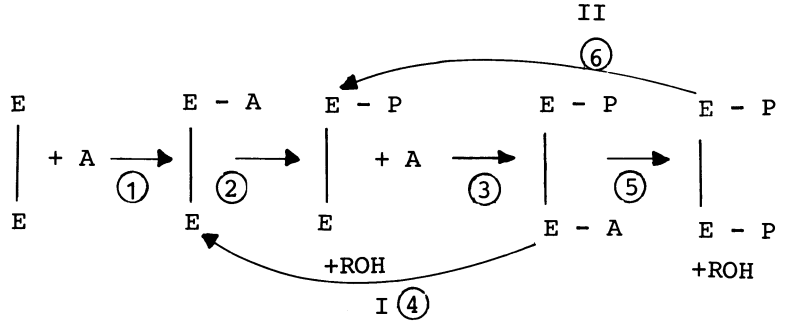
SWEENY and FISHER (33) and GRIFFIN and BRAND (34) have presented a number of different models involving alternative pathways that give rise to sigmoidal kinetics. These mechanisms will not be considered here, and the reader is referred to the original articles for additional information on this subject.

c) Half-Site Reactivity and Negative Cooperativity

A large number of enzymes studied recently seem to exhibit negative cooperativity as determined from ligand binding experiments and, at the same time, show normal Michaelis-Menten kinetics (35). These enzymes include alkaline phosphatase, malate dehydrogenase, glyceraldehyde-3-P dehydrogenase, horse liver alcohol dehydrogenase, *E. coli* CTP synthetase, and liver glutamate dehydrogenase. An interesting common feature of these enzymes is that they are proteins with identical subunits. LAZDUNSKI and associates used the term Flip-Flop mechanism in an attempt to explain the very interesting but anomalous behavior of these enzymes (36). A brief summary of experiments with calf intestine alkaline phosphatase serves to illustrate the Flip-Flop mechanism (37).

The catalytic mechanism of alkaline phosphatase is known to involve the participation of a phosphoryl enzyme intermediate. Ligand binding experiments with inorganic orthophosphate (P_i)

reveal two nonequivalent binding sites. One binds P_i strongly and the other, loosely at pH 8.0 with the characteristics of negative cooperativity. Stopped-flow experiments reveal that substrates phosphorylate both sites at different rates at acid pH, whereas only one site is phosphorylated at pH 8.0. At alkaline pH only half of the sites are reactive at any time. These observations are summarized in the mechanism shown in Scheme IX-14.



Scheme IX-14

In Scheme IX-14, A is taken to represent the substrate and E - A, a Michaelis complex, whereas E - P represents covalently bound P_i. The details of the mechanism are as follows:

1. Binding of substrate at step ① precludes additional substrate binding.

2. Phosphorylation of a seryl residue occurs at the active site with desorption of the alcohol portion of the substrate.

3. The phosphoryl enzyme next allows binding of a second substrate molecule.

4. This hybrid enzyme form, which contains both a Michaelis complex and phosphoryl enzyme, may either liberate P_i ④ or form the diphosphoryl enzyme intermediate ⑤ plus the alcohol product. In the first instance substrate binding ③ is coupled to enzyme dephosphorylation ④. The authors refer to this as a Flip-Flop mechanism (37). Alternatively, enzyme phosphorylation ⑤ may be coupled with dephosphorylation ⑥.

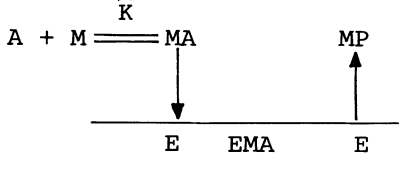
It can be seen from the alkaline phosphatase model of Scheme IX-14 that if mechanism I is followed, only step ① appears in the pre-steady-state, whereas steps ②, ③, and ④ are part of the steady-state. By analogy, in the case of mechanism II, the pre-steady-state steps are ① and ② and the steady-state steps are ③, ⑤, ⑥. Step ⑥ is not a part of mechanism I, nor is step ④ a part of mechanism II. Either of these mechanisms leads to Michaelis-Menten kinetics.

The idea that horse liver alcohol dehydrogenase exhibits half-site reactivity (37a) has been challenged (37b). BERNHARD et al. (37a), using a rapid-mixing stopped-flow spectrophotometer, observed a transient accumulation of product when aromatic aldehydes were reduced by NADH that was equivalent to one half the enzyme site concentration; however, site equivalence was found with acetaldehyde as substrate (37c). TATEMOTO (37b) found that transient formation of enzyme-NAD^+ from NADH and benzaldehyde corresponded to the total site concentration when a rapid-mixing stopped-flow spectrophotometer or fluorimeter was used. In the reverse reaction TATEMOTO observed less than full-site reactivity; however, he attributed this finding to formation of a significant amount of nonfluorescent productive ternary complex.

It is not at all clear at this time what advantage, if any, half-site reactivity provides to the cell in the context of regulation. On the other hand, the various conformational changes involved in Flip-Flop mechanisms may serve to facilitate catalysis.

d) Pseudocooperativity in Transphosphorylase Enzymes

It was pointed out in Chapter III that sigmoidal kinetic may arise in transphosphorylase systems, if an incorrect calculation is made for the concentration of the metal-substrate complex. This usually occurs when the assumption is made that the concentration of the metal binding species represents the true substrate concentration. It must be born in mind that in virtually all cases studied,the active substrate is the metal-substrate complex, rather than the free substrate. Figure IX-9 represents theoretical data obtained for the mechanism illustrated in Scheme IX-15 under three different conditions (5).



Scheme IX-15

For example, the theoretical velocity-response curves presented in Fig. IX-9 show how the normal hyperbolic dependence (curve A) can give sigmoidal substrate-saturation curves (curves B and C). For curve A, the stability constant for the complex is assumed to be sufficiently great so that $[A_{total}]$ is equivalent to the concentration of the metal-substrate complex. Alternatively, one may assume that the stability constant is lower but that there is a sufficient concentration of free metal ion to force the substrate into combination with the metal ion. The responses in curves B and C were computed assuming a stability constant of 10000 M^{-1}. For curve B it is assumed that the free uncomplexed substrate is noninhibitory, whereas for curve C it is assumed that free substrate acts as a competitive inhibitor.

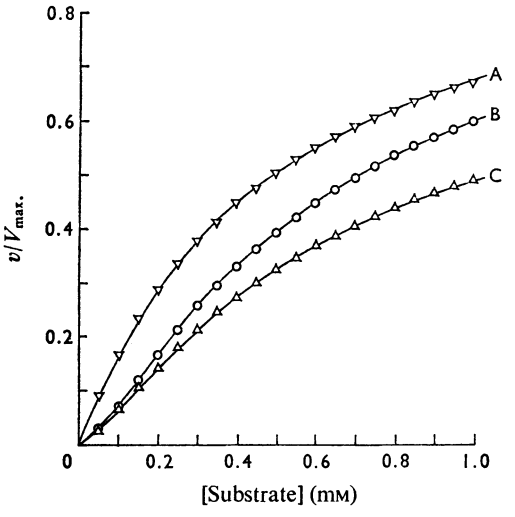


Fig. IX-9. Plot of v/V_{max} *versus* the millimolar concentration of total substrate for a model one-substrate enzyme (5). The concentrations of A_{free} and the metal-A complex were estimated assuming a stability constant of 10000 M^{-1}. The K_m and K_I for metal metal-A complex and A_{free}, respectively, were assumed to be 0.5 mM. The ratio v/V_{max} was calculated from the rate expression for a simple one-substrate enzyme obeying Michaelis-Menten kinetics. Curve A (▽) represents the case where total [substrate] = [metal-A complex]; curve B (○) is where the velocity is strictly a function of the metal-A complex concentration, as determined by using the stability constant; curve C (△) is where velocity is dependent upon metal-A complex concentration as in curve B, but also accounts for competitive inhibition by A_{free} relative to the metal-A complex. The total metal ion to total substrate ratio was maintained at 1.0

In the derivation of the equations used to calculate the data shown in Fig. IX-9, the kinetic model of Scheme IX-15 gives the following velocity-substrate relationships,

$$v = \frac{V_1 (MA)}{K_{MA} + MA}. \qquad \text{(IX-80)}$$

The concentration of MA may be calculated as follows:

$$M + A = MA \qquad \text{(IX-81)}$$

$$\overline{K} = \frac{MA}{(M)(A)} = \frac{MA}{[M_0 - MA][A_0 - MA]}. \qquad \text{(IX-82)}$$

Equation (IX-82) is expanded and the quadratic equation solved for MA:

$$MA = \frac{[A_0 + M_0 + K] - [(A_0 + M_0 + K)^2 - 4M_0A_0]^{1/2}}{2}. \qquad \text{(IX-83)}$$

Note that $\overline{K}$ (Eq. (IX-82)) is a formation constant, whereas K (Eq. (IX-83)) is a dissociation constant.

Equation (IX-83) permits calculation of the true substrate (MA) concentration.

Curve C of Fig. IX-9 was calculated assuming that free uncomplexed substrate (A) is a competitive inhibitor of MA; i.e.,

$$v = \frac{V_1 (MA)}{K_{MA}\left[1 + \frac{A}{K_I}\right] + MA}. \qquad \text{(IX-84)}$$

It should be noted that the conditions described give rise to sigmoidicity, and in addition, this effect is magnified if the uncomplexed substrate acts as a competitive inhibitor of the system. A more complete analysis of these effects can be found in an article by PURICH and FROMM (5).

D. Allostery

Some enzymes contain, in addition to the active site, a topologically distinct second, or allosteric site. Although the allosteric site is devoid of catalytic activity, it is capable of affecting catalysis at the active site. Like the active site, the allosteric site exhibits varying degrees of specificity. In this discussion, allostery will be restricted to physiological effectors. It is clear that buffer ions and protons not directly involved in catalysis will fit the definition of allosteric effectors.

The most widely accepted explanation of allostery holds that, when certain ligands bind at the allosteric site, a conformational change occurs that either enhances or inhibits catalysis. These effects are manifested through alterations in the kinetic parameters for the enzyme; i.e., the Michaelis constants (K systems) or the maximal velocity (V systems). In the case of systems that exhibit cooperative kinetics, sigmoidicity may either be exaserbated or eliminated. Enzymes that display normal Michaelis-Menten kinetics may exhibit sigmoidicity, inhibition, or activation in the presence of the allosteric effector.

There are many examples in the literature of oligomeric allosteric enzymes and a few well-documented cases of allostery associated with monomeric enzymes. In the former cases, the allosteric site may be on the same subunit as the active site, or alternatively, on a separate subunit.

By definition, heterotropic effects will describe interactions between different ligands, whereas homotropic effects will describe interactions between similar ligands (12).

1. Nonsigmoidal Systems

a) Noncompetitive Inhibition

Probably the simplest example of allostery is the case of noncompetitive inhibition in a unireactant system (Scheme IV-2). By definition, the noncompetitive inhibitor (negative modifier or effector) binds at a site other than the active site (the allosteric site) and alters the properties of the active site.

b) Partial Competitive Inhibition

Another example of enzyme inhibition or activation that may be accomplished with heterotropic effectors involves partial competitive inhibition (Chapter IV, Scheme IV-2). The difference between noncompetitive inhibition and partial competitive inhibition is associated with the fate of the enzyme-substrate-modifier complex and the affinity of the enzyme for the substrate and modifier. The former type of inhibition assumes the ternary complex is inactive, whereas in partial competitive inhibition (or activation) this complex is active. A more complete treatment of this facet of allostery is provided in Chapter IV and by the theoretical treatment of FRIEDEN (27).

c) Mutually Exclusive Binding

Competitive inhibition of enzyme action (Scheme IV-1) is sometimes described as an inhibitor-modulated increase in the Michaelis constant for the substrate. An analogous effect by inorganic orthophosphate (P_i) is believed to occur in the case of bovine brain hexokinase. Here, however, the modulator serves to activate the enzyme by causing dissociation of the inhibitor.

It is now well established that the kinetic mechanism of bovine brain hexokinase action is approximated by the rapid equilibrium Random Bi Bi mechanism (38-41). The enzyme is markedly inhibited by its products, ADP, and glucose-6-P. Inhibition by ADP is noncompetitive with respect to either substrate, and available experimental evidence suggests that ADP binds at an allosteric site as well as at the active site (39). The rationale behind this suggestion was made on the basis that ADP is not a linear noncompetitive inhibitor of the hexokinase reaction (Fig. IX-10).

Inhibition by glucose-6-P is well recognized to be linear-competitive with respect to ATP and linear-noncompetitive relative to glucose (42, 43). It has been long recognized that P_i reverses the inhibitory effect of glucose-6-P (44); however, P_i has no effect on the kinetic parameters of the hexokinase reaction in the absence of glucose-6-P (45). Both ATP and P_i are competitive with respect to glucose-6-P in kinetic and binding experiments; however, ATP and P_i do not compete with each other for the same site on hexokinase (46, 47). The mechanism shown in Scheme IX-16

was proposed to account for these and other findings (46, 47) based upon reports that the enzyme is monomeric (48, 49).

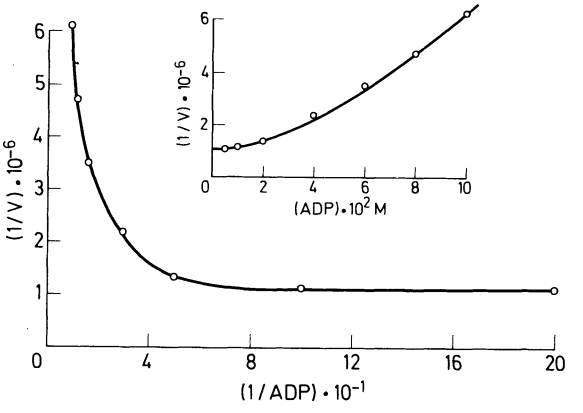
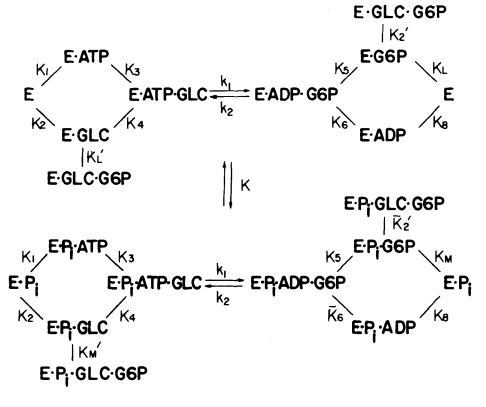


Fig. IX-10. Plot of the reciprocal of the initial forward reaction velocity *versus* the reciprocal of the concentration of ADP (39). In the inset 1/v is plotted against ADP. ATP and glucose were maintained at K_a and $2K_a$, respectively

The mechanism of deinhibition of glucose-6-P inhibited hexokinase assumes that the enzyme may exist either as the P_i associated enzyme or as the free enzyme.



Scheme IX-16

These two enzyme conformations are proposed to be in rapid equilibrium with each other. When P_i is bound to hexokinase, the dissociation constant for glucose-6-P is markedly elevated and deinhibition occurs. Equation (IX-85) describes the initial rate expression which seems to account for the currently available binding and kinetic data for the model depicted in Scheme IX-16.

$$\frac{V_1}{v} = 1 + \frac{K_G}{(\text{Glucose})} + \frac{K_{ATP}}{(\text{ATP})}\left[1 + \frac{\text{G6P}}{K_L' f'(P_i)}\right] + \frac{K_{iATP} \cdot K_{Gl}}{(\text{ATP})(\text{Glucose})}\left[1 + \frac{\text{G6P}}{K_L f(P_i)}\right] \qquad \text{(IX-85)}$$

where $f'(P_i) = \dfrac{K + P_i}{K + \dfrac{K_L'}{K_M'}(P_i)}$ and $f(P_i) = \dfrac{K + P_i}{K + \dfrac{K_L}{K_M}(P_i)}$ and

K_L' and K_M' are the constants for glucose-6-P dissociation from E·glucose·glucose-6-P and E·P_i·glucose·glucose-6-P, respectively. Brain hexokinase seems, then, to be an example of an allosteric enzyme that does not display cooperative kinetics and does not involve subunit interaction.

2. The Monod Model (12)

Another feature of the MWC model is that it is capable of explaining heterotropic effects. Consider, for example, a system capable of binding three different ligands at three topologically distinct sites. If one of these ligands is the substrate (S), which binds almost exclusively to the R state, and the other two ligands are: a) an inhibitor (I), which binds to the T state, and b) an activator (A), which binds to the R state, an expression analogous to Eq. (IX-31) may be derived, which accounts for binding of the various ligands. Equation (IX-86) describes these effects when using the MWC model where $\bar{Y}_s$ is the saturation function for the substrate.

$$\bar{Y}_s = \frac{\alpha(1 + \alpha)^{n-1}}{\dfrac{L(1 + \beta)^n}{(1 + \gamma)^n} + (1 + \alpha)^n}. \qquad \text{(IX-86)}$$

In Eq. (IX-86), β and γ represent I/K_I and A/K_A, respectively, where K_I and K_A are the microscopic dissociation constants for binding of A and I.

It can be seen from Eq. (IX-86) that, when β and γ approach zero, Eq. (IX-31) is obtained. Figure IX-11 shows how β and γ affect the homotropic interactions of the substrate. Activation serves to eliminate the homotropic interactions of the substrate, whereas the heterotropic inhibitor serves to increase substrate cooperactivity. All the ligands, S, A, and I exhibit cooperative homotropic effects.

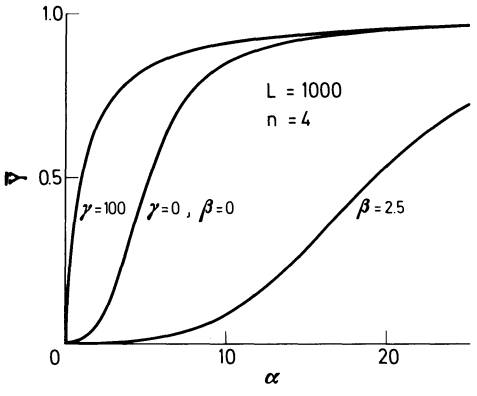


Fig. IX-11. Theoretical curves showing the heterotropic effects of an allosteric activator (γ) or inhibitor (β) upon the shape of the saturation function for substrate (α) based upon Eq. (IX-86) according to MONOD et al. (12)

Aspartate transcarbamylase (carbamyl phosphate: L-aspartate transcarbamylase, EC 2.1.3.2) (ATCase) from *E. coli* is an example of an enzyme system that seems to closely approximate the MWC model of cooperativity in some respects. ATCase is the first committed step in pyrimidine nucleotide biosynthesis and mediates the synthesis of N-carbamylaspartate from carbamylphosphate and aspartate.

$$\text{L-aspartate} + \text{carbamylphosphate} \rightleftharpoons \text{N-carbamylaspartate} + \text{phosphate} \qquad \text{(IX-87)}$$

The enzyme is susceptable to feedback inhibition by CTP (50). ATP acts as an activator of ATCase and serves to reverse the effects of CTP (50). The enzyme itself is composed of six regulatory subunits and six catalytic subunits arranged as indicated in Fig. IX-12 (51, 52). The larger catalytic monomers are grouped together as two trimers around a three-fold axis of symmetry (vertical axis). These two trimers are arranged around a two-fold axis of symmetry (horizontal axis). The six smaller regulatory polypeptides exist as dimers between the catalytic trimers.

When binding experiments are carried out with succinate, a competitive inhibitor of aspartate, in the presence of carbamylphosphate, sigmoidal binding is observed (53). A Scatchard plot of these findings indicates positive cooperativity (Fig. IX-4) (54). The Hill plot for succinate binding results in a nonlinear function with a maximum value for n of 1.6 (Fig. IX-13) (54). Sigmoidicity also appears when velocity is plotted as a function of L-aspartate as shown in Fig. IX-14 (55). It can be seen from Fig. IX-14 that CTP exaggerates the sigmoidicity whereas ATP serves to eliminate it. It is of interest to note that the maximal velocity of all of the progress curves generated in Fig. IX-14 is the same. ATCase is referred to as a "K" system as

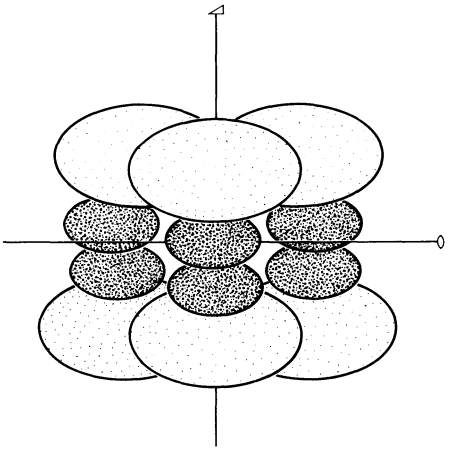


Fig. IX-12. Proposal for the arrangement of the polypeptide chains in aspartate transcarbamylase (51)

opposed to enzymes whose maximal velocities are altered by the modifier (referred to as "V" systems).

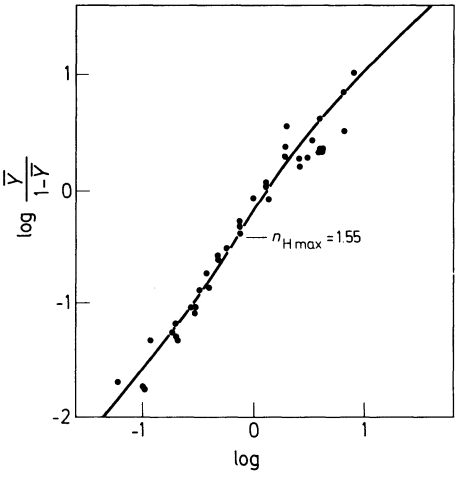


Fig. IX-13. Hill plot of the fractional saturation ($\bar{Y}$) of native ATCase by succinate. The line was computed from Eq. (IX-32) for n = 4, L = 4, and $C \leq 10^{-3}$. The maximum slope $N_{Hmax} = 1.55$ (54)

The catalytic and regulatory subunits of ATCase can be dissociated with various reagents (55). In the absence of the regulatory

subunits, the catalytic subunits are enzymatically active; however, they do not exhibit cooperativity. The regulatory subunits are catalytically inactive but bind CTP.

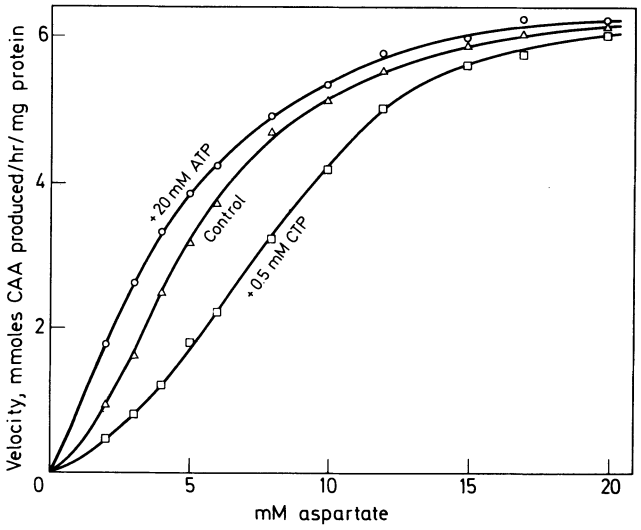


Fig. IX-14. The response of ATCase to the activator ATP, and inhibitor, CTP (55). The nucleotides were present in concentrations sufficient to produce their maximal effects

CHANGEUX and RUBIN (54) have found that succinate binding to ATCase could be explained quite well by using the MWC model; i.e., good fits to the equations of the concerted mechanism were obtained with the experimental data. They have determined the ratio of enzyme in both the R and T states (L) and C, the ratio of the ligand binding constants. Their findings indicate that the free energy involved in the R-T transition is very small.

HAMMES and WU, using the temperature-jump technique, also concluded that succinate binding to ATCase could best be explained by invoking the MWC model for cooperativity (56).

The feedback inhibitor CTP seems to exhibit negative cooperativity when it binds ATCase (57). Scatchard plots for CTP binding suggest a value of 5.8 sites per ATCase molecule. WINLUND and CHAMBERLIN (57) have proposed two models in an attempt to explain CTP binding to ATCase. One proposal is that the folding of the polypeptide chain in the regulatory dimer produces two sites with different microscopic binding constants. Alternatively, the possibility exists that the ligand binding sites are identical but the CTP molecules themselves interfere with each others binding. It seems that the MWC model (12) is not a viable mechanism for CTP binding to ATCase based upon the observation of negative cooperativity.

It has already been indicated that the nucleotides ATP and CTP produce very different effects on the sigmoidal substrate binding

isotherm (Fig. IX-14). WU and HAMMES (58) investigated activation of ATCase with an analog of ATP (6-mercapto-9-β-D-ribofuranosylpurine-5'-triphosphate). They studied binding of the analog to the catalytic and regulatory subunits and to the native enzyme. The ATP analog and CTP both seem to bind by a similar mechanism. It is proposed that effector binding results in the following reactions:

$$E + ATP \rightleftharpoons X_1 \rightleftharpoons X_2 \qquad \text{(IX-88)}$$

$$E + CTP \rightleftharpoons X_2 \rightleftharpoons X_1 \qquad \text{(IX-89)}$$

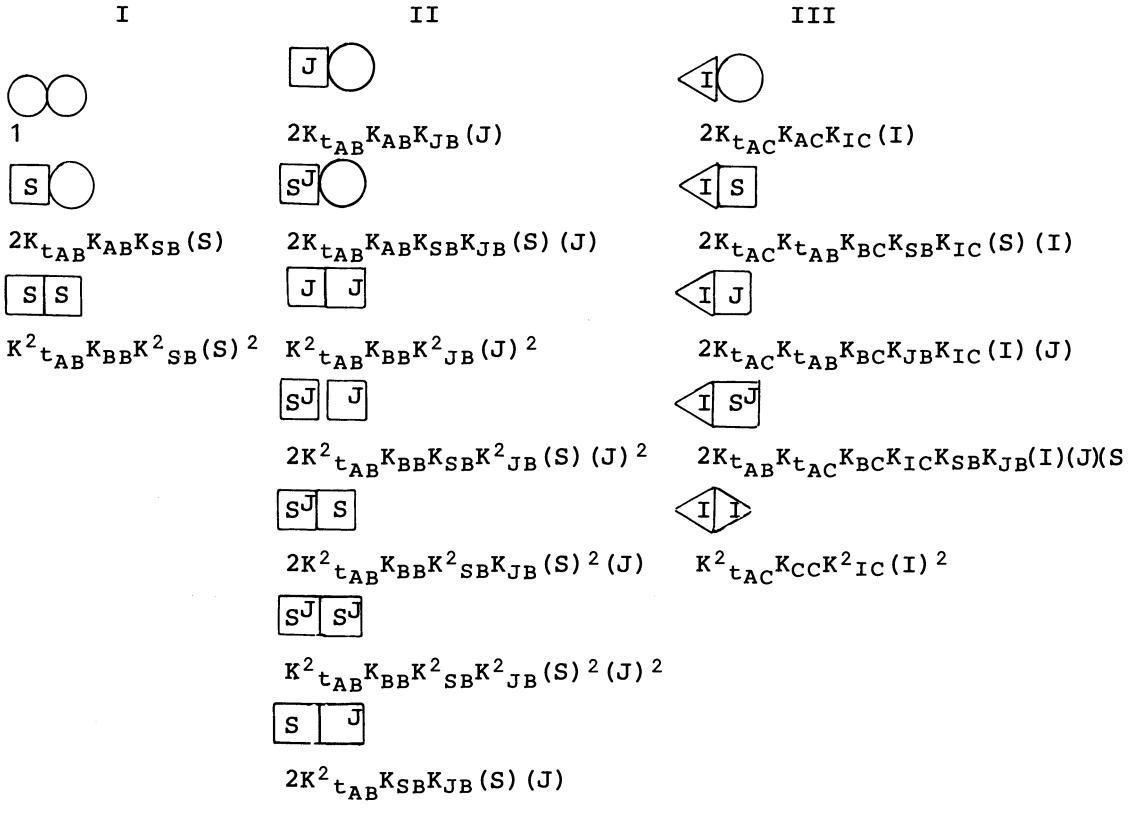
where X_1 and X_2 represent different conformational states of ATCase. WU and HAMMES (58) further suggest that substrates and ATP form the X_2 state of ATCase preferentially, whereas CTP preferentially forms the X_1 state.

3. The Adair-Koshland Model (13)

The Adair Koshland model of allostery, unlike the MWC proposal, provides for both positive and negative homotropic effects. Equations (IX-33) to (IX-41) illustrate the basic features of the Adair-Koshland model involving a homotropic effector, the substrate. It will be remembered that, for the dimer used as a point of illustration, the enzyme existed in an initial state (○○), a final state ([S][S]), and intermediate hybrid conformations. This same model may be enlarged to include both positive (J) and negative (I) effectors. Scheme IX-17 illustrates the various forms of the enzyme that might be expected to occur in the presence of S, J, and I for a system that exhibits both homotropic and heterotropic effects (11).

It can be seen that the activator, J, induces the same conformational states that exist when the dimer binds the substrate, S. On the other hand, the inhibitor, I, induces a completely different enzyme conformation when it is bound. The various steps involved in the formation of any conformer illustrated in Scheme IX-17 can be obtained by analogy with Eqs. (IX-33) to (IX-41).

A saturation equation for $\bar{Y}$ may be written involving Scheme IX-17. When J = I = O, this expression will reduce to Eq. (IX-42). It has been suggested that the expression for $\bar{\nu}$ (Eq. (IX-42)) could be converted to the analogous velocity expression by multiplying the [S]○ term by k_1 and [S][S] by k_2 (14). It has been further suggested that a similar manipulation is possible in the case of an essential activator, J, for the model of Scheme IX-17 (14). In this case the numerator will contain the following enzyme forms: $[S^J]\bigcirc$, $[S^J][J]$, $[S^J][S]$, and $[S^J][S^J]$, all multiplied by k_{cat}. Here it is assumed that the rate constant for the breakdown of each complex is the same. It has already been pointed out that the transformation of a binding equation to a velocity equation requires much more than a simple substitution of v for $\bar{\nu}$.



Scheme IX-17

4. Enzyme Isomerization Mechanisms

AINSLIE et al. (31) and FRIEDEN (22) have described how modifiers, both positive and negative, could affect enzyme isomerization mechanisms that give rise to cooperativity, burst, and hysteretic phenomena. Consider, for example, the mechanism described by Scheme IX-11 and how modifiers may alter its kinetic properties. A modifier may combine with certain enzyme forms and affect the values for the various rate constants by causing them to either increase or decrease. These heterotropic effects will appear to be either positive or negative depending upon which particular constant is changed and the direction of the change.

Threonine deaminase (Threonine hydro-lyase, deaminating, EC 4.2.1.16) from *Bacillus subtilis* is an example of an enzyme system that displays both cooperativity and hysteresis (29, 30). Initial rate kinetics are normal for the enzyme; however, in the presence of isoleucine, the threonine kinetics exhibit sigmoidicity (30). In addition, the system shows a hysteretic effect in the presence of isoleucine. These observations may be explained

by assuming a conformational transition between two forms of threonine deaminase as proposed by HATFIELD and UMBARGER (30), AINSLIE et al. (31), and FRIEDEN (22).

5. Kinetic Models

RABIN (28) has indicated how the kinetic model illustrated in Scheme IX-10 could provide for the effect of allosteric modifiers. If a modifier reacts with the enzyme so as to transform the term ($k_8 + k_6A$) to k_8 by permitting $k_8 >> k_6A$, cooperativity will be abolished. Other assumptions regarding relationships between the rate constants, when modifiers bind the various enzyme forms, will either accelerate or inhibit the reaction.

The steady-state Random Bi Bi mechanism might be expected to give either cooperative or noncooperative velocity responses to substrate depending upon the magnitude of the various rate constants (see Chapter V). Computer simulation of the steady-state Random Bi Bi mechanism shown in Scheme V-7 indicates that, when the rate constants k_2, k_4, k_{15}, and k_{17} are smaller than k_9, substrate activation is predicted (59). It might be inferred that a shift in the magnitude of certain rate constants caused by binding of heterotropic modifiers might have some influence in changing the response of an enzyme with the mechanism of Scheme V-7 from cooperative to noncooperative.

DALZIEL (60) has derived initial rate equations to explain both homotropic and heterotropic effects within the context of the MWC (15) nonexclusive ligand binding model of MONOD et al. (15). DALZIEL assumes that only the ligand-free R and T states of the enzyme are in equilibrium, that each enzyme-substrate complex may break down to product, but at different rates, and that the catalytic activities of the two enzyme states are different. The rate expression he obtained is similar in form to Eq. (IX-32) and is presented in Eq. (IX-90).

$$v = \frac{nE_0\left[k_2\alpha(1 + \alpha)^{n-1} + L\ell_2\alpha C(1 + C\alpha)^{n-1}\right]}{(1 + \alpha)^n + L(1 + C\alpha)^n} \qquad \text{(IX-90)}$$

All of the terms in Eq. (IX-90) have been defined in the discussion of Eq. (IX-32) except k_2 and ℓ_2 which represent the unimolecular rate constants for the breakdown of R state enzyme-substrate complexes and the T state enzyme-substrate complexes to product, respectively.

DALZIEL (60) points out that like the saturation curves obtained with nonreactive ligands, the rate curves will exhibit their most pronounced homotropic interactions when K_T and K_r are most different (and K_T is smaller) and when L is large.

Under conditions in which $K_r = K_T$, Eq. (IX-90) reduces to Eq. (IX-91) which does not incorporate either homotropic or heterotropic effects.

$$v = \frac{nE_0(F)(k_2 + L\ell_2)}{(F + K_r)(1 + L)} \qquad \text{(IX-91)}$$

If a modifier binds either the R or T state of the enzyme so that the value for L changes, the rate of the system described by Eq. (IX-91) would change (V system).

Heterotropic effects may be incorporated into Dalziel's modification of the MWC model by allowing additional interactions of modifiers (M) with both forms of the enzyme. When this is done, L in Eq. (IX-90) is replaced by L', where

$$L' = \frac{L(1 + M/K_{TM})^n}{(1 + M/K_{RM})^n}. \qquad \text{(IX-92)}$$

In Eq. (IX-92), K_{TM} and K_{RM} represent the microscopic dissociation constants for modifier binding to the T and R states of the enzyme, respectively.

When $K_{RM} < K_{TM}$ the modifier will serve to activate the system. On the other hand, the modifier will act like a heterotropic inhibitor when $K_{RM} > K_{TM}$.

If in Dalziel's model, binding of the substrate, F, is exclusive to the R state of the enzyme (C = 0), Eq. (IX-90) reduces to

$$v = \frac{nk_2E_0(F)}{K_r\left[1 + \alpha + \frac{L}{(1 + \alpha)^{n-1}}\right]}. \qquad \text{(IX-93)}$$

When Eq. (IX-92) is substituted for L in Eq. (IX-93) it can be seen that the modifier will not alter the maximal velocity (nK_2E_0). Under conditions where $K_{RM} > K_{TM}$ there is an increase in homotropic interactions, whereas these interactions decreases when $K_{RM} < K_{TM}$. The modifier will serve as an activator when the latter relationship holds as L' will decrease and the system will exhibit normal Michaelis-Menten kinetics.

E. Product Effects

Although enzymologists in their studies of enzyme kinetics do not as a rule consider the effects of enzyme, product, substrate, activator, and inhibitor levels in the cell, these factors do markedly influence the expression of enzyme activity. The following is a very brief and simple description of how product may alter cooperativity for a dimer that may be characterized by the Adair equation (Eq. (IX-16)) (61).

If the product P can compete with the substrate so that binding is mutually exclusive,

$$\bar{Y}_A = \frac{\bar{K}_1(A) + 2\bar{K}_1\bar{K}_2(A)^2}{1 + \bar{K}_1(A) + \bar{K}_1\bar{K}_2(A)^2 + \bar{K}_3(P) + \bar{K}_3\bar{K}_4(P)^2}. \qquad \text{(IX-94)}$$

The formation constants $\bar{K}_3$ and $\bar{K}_4$ are obtained from the expressions

$$\bar{K}_3 = EP/(E)(P) \text{ and } \bar{K}_4 = EP_2/(EP)(P). \qquad \text{(IX-95)}$$

Figure IX-15 illustrates how $\bar{Y}$ is affected in the absence and presence of different concentrations of product. It can be seen from the simulations that product should have a marked effect on

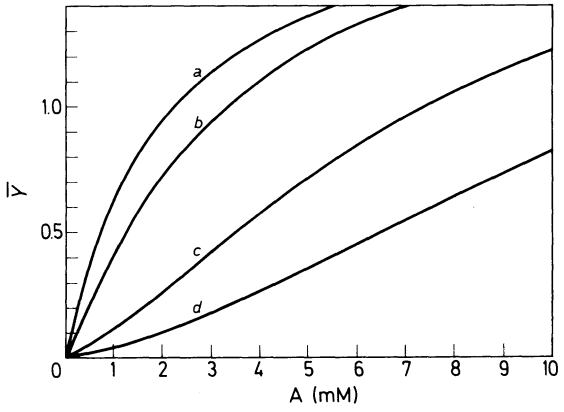


Fig. IX-15. Plot of the computed fraction of total substrate sites occupied, $\bar{Y}$, *versus* the millimolar concentration of substrate A in the absence and presence of product (61). The curves were generated utilizing Eq. (IX-94) by assuming that $\bar{K}_1$, $\bar{K}_2$, $\bar{K}_3$, and $\bar{K}_4$ were 1000 M^{-1}, 200 M^{-1}, 1000 M^{-1}, and 200 M^{-1} respectively. Substrate was varied in the concentration range from 0 to 10.0 mM, and product concentrations were none (curve a), 1.0 mM (curve b), 5.0 mM (curve c), and 10.0 mM (curve d)

the response of cooperative systems. Figure IX-16 describes the effect of the product fumarate on the sigmoidal kinetics of the enzyme aspartase (aspartate ammonia-lyase, EC 4.3.1.1) from *E. coli* (61).

If binding by substrate and product is not exclusive and if a ternary complex involving enzyme, A, and P can form, it is possible to derive an expression analogous to Eq. (IX-94). This equation will be similar in form to Eq. (IX-94). Expressions that use the MWC assumptions may also be derived to take into account the effects of products.

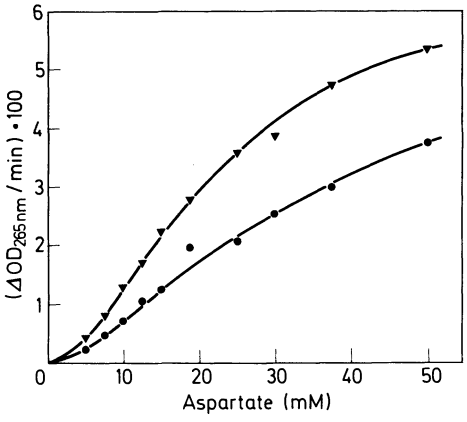


Fig. IX-16. Plot of the initial velocity of the *Escherichia coli* aspartase reaction *versus* L-aspartate in the absence (▼) and presence (●) of 1.46 mM fumarate (61)

References

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Appendix I

Rate Equations, Determinants, and Haldane Expressions for Some Common Kinetic Mechanisms

Rate equations, determinants, and Haldane expressions from some common mechanisms presented in the text which adhere to the steady-state assumption. K_{eq} is taken to be the apparent equilibrium constant.

Uni Uni Mechanism (Scheme II-1)

Rate Equation:

$$v = \frac{(k_1k_3k_5A - k_2k_4k_6P)E_0}{E + EA + EP}$$

In Cleland Form:

$$v = \frac{V_1V_2(A - \frac{P}{K_{eq}})}{V_2K_a + V_2A + \frac{V_1P}{K_{eq}}}$$

Determinants:

$$E = k_2(k_4 + k_5) + k_3k_5$$

$$EA = k_1A(k_4 + k_5) + k_4k_6P$$

$$EP = k_6P(k_2 + k_3) + k_1k_3A$$

Haldanes:

$$K_{eq} = \frac{k_1k_3k_5}{k_2k_4k_6} = \frac{V_1K_p}{V_2K_a}$$

Kinetic Parameters:

$$V_1 = k_3k_5E_0/(k_3 + k_4 + k_5), \quad V_2 = k_2k_4E_0/(k_2 + k_3 + k_4)$$

$$K_a = \frac{(k_2k_4 + k_2k_5 + k_3k_5)}{k_1(k_3 + k_4 + k_5)}, \quad K_p = \frac{(k_2k_4 + k_2k_5 + k_3k_5)}{k_6(k_2 + k_3 + k_4)}$$

$$K_{ia} = k_2/k_1 , \qquad K_{ip} = k_5/k_6$$

Uni Uni Mechanism (Scheme I-3)

Rate Equation:

$$v = \frac{(k_1k_3A - k_2k_4P)E_0}{E + EA}$$

In Cleland Form:

$$v = \frac{V_1 V_2 (A - \frac{P}{K_{eq}})}{V_2 K_a + V_2 A + \frac{V_1 P}{K_{eq}}}$$

Determinants:

$$E = k_2 + k_3$$

$$EA = k_1 A + k_4 P$$

Haldanes:

$$K_{eq} = \frac{k_1 k_3}{k_2 k_4} = \frac{V_1 K_p}{V_2 K_a} = \frac{K_{ip}}{K_{ia}}$$

Kinetic Parameters:

$$V_1 = k_3 E_0, \quad V_2 = k_2 E_0$$

$$K_a = \frac{(k_2 + k_3)}{k_1}, \quad K_p = \frac{(k_2 + k_3)}{k_4}$$

$$K_{ia} = k_2/k_1 , \quad K_{ip} = k_3/k_4$$

Iso Uni Uni Mechanism (Scheme I-4)

Rate Equation:

$$v = \frac{(k_1 k_3 k_5 A - k_2 k_4 k_6 P) E_0}{E + EA + F}$$

In Cleland Form:

$$v = \frac{V_1 V_2 (A - \frac{P}{K_{eq}})}{V_2 K_a + V_2 A + \frac{V_1 P}{K_{eq}} + \frac{V_2 AP}{K_{iip}}}$$

Determinants:

$$E = k_5 (k_2 + k_3) + k_2 k_4 P$$

$$EA = k_1 k_5 A + k_4 k_6 P + k_1 k_4 AP$$

$$F = k_6 (k_2 + k_3) + k_1 k_3 A$$

Haldanes:

$$K_{eq} = \frac{V_1 K_p}{V_2 K_a} = \frac{V_1 K_{iip}}{V_2 K_{iia}}$$

Kinetic Parameters:

$$V_1 = k_3 k_5 E_0/(k_3 + k_5), \quad V_2 = k_2 k_6 E_0/(k_2 + k_6)$$

$$K_a = \frac{(k_2 + k_3)(k_5 + k_6)}{k_1(k_3 + k_5)}, \quad K_p = \frac{(k_2 + k_3)(k_5 + k_6)}{k_4(k_2 + k_6)}$$

$$K_{iia} = (k_2 + k_6)/k_1 , \quad K_{iip} = (k_3 + k_5)/k_4$$

$$K_{ia} = k_2/k_1, \quad K_{ip} = k_5/k_6$$

Ordered Uni Bi Mechanism (Scheme I-5)

Rate Equation:

$$v = \frac{(k_1 k_3 k_5 A - k_2 k_4 k_6 PQ) E_0}{E + (EA + EPQ) + EQ}$$

In Cleland Form:

$$v = \frac{V_1 V_2 (A - \frac{PQ}{K_{eq}})}{V_2 K_a + V_2 A + \frac{V_1 K_q P}{K_{eq}} + \frac{V_1 K_p Q}{K_{eq}} + \frac{V_1 PQ}{K_{eq}} + \frac{V_2 AP}{K_{ip}}}$$

Determinants:

$$E = k_5(k_2 + k_3) + k_2 k_4 P$$

$$(EA + EPQ) = k_1 k_5 A + k_1 k_4 AP + k_4 k_6 PQ$$

$$EQ = k_6 Q(k_2 + k_3) + k_1 k_3 A$$

Haldanes:

$$K_{eq} = \frac{k_1 k_3 k_5}{k_2 k_4 k_6} = \frac{V_1 K_{ip} K_q}{V_2 K_{ia}} = \frac{V_1 K_p K_{iq}}{V_2 K_a}$$

Kinetic Parameters:

$$V_1 = k_3 k_5 E_0/(k_3 + k_5) ; \qquad V_2 = k_2 E_0$$

$$K_a = \frac{k_5(k_2 + k_3)}{k_1(k_3 + k_5)}; \quad K_p = \frac{(k_2 + k_3)}{k_4}; \quad K_q = \frac{k_2}{k_6}; \quad K_{ip} = \frac{(k_3 + k_5)}{k_4}$$

$$K_{ia} = k_2/k_1 \ , \quad K_{iq} = k_5/k_6$$

Ordered Bi Bi Mechanism (Scheme I-8)

Rate Equation:

$$v = \frac{(k_1k_3k_5k_7AB - k_2k_4k_6k_8PQ)E_0}{E + EA + (EAB + EPQ) + EQ}$$

In Cleland Form:

$$v = \frac{V_1V_2(AB - \frac{PQ}{K_{eq}})}{V_2K_{ia}K_b + V_2K_bA + V_2K_aB + V_2AB + \frac{V_1K_qP}{K_{eq}} + \frac{V_1K_pQ}{K_{eq}} + \frac{V_1PQ}{K_{eq}} + \frac{V_1K_qAP}{K_{ia}K_{eq}} + \frac{V_2K_aBQ}{K_{iq}} + \frac{V_2ABP}{K_{ip}} + \frac{V_1BPQ}{K_{ib}K_{eq}}}$$

Determinants:

$$E = k_2k_7(k_4 + k_5) + k_3k_5k_7B + k_2k_4k_6P$$

$$EA = k_1k_7A(k_4 + k_5) + k_1k_4k_6AP + k_4k_6k_8PQ$$

$$(EAB + EPQ) = k_1k_3k_7AB + k_2k_6k_8PQ + k_1k_3k_6ABP + k_3k_6k_8BPQ$$

$$EQ = k_2k_8Q(k_4 + k_5) + k_1k_3k_5AB + k_3k_5k_8BQ$$

Haldanes:

$$K_{eq} = \frac{k_1k_3k_5k_7}{k_2k_4k_6k_8} = \frac{V_1K_pK_{iq}}{V_2K_{ia}K_b} = \frac{V_1^2K_{ip}K_q}{V_2^2K_aK_{ib}}$$

Kinetic Parameters:

$$V_1 = k_5k_7E_0/(k_5 + k_7), \quad V_2 = k_2k_4E_0/(k_2 + k_4)$$

$$K_a = \frac{k_5k_7}{k_1(k_5 + k_7)}, \quad K_q = \frac{k_2k_4}{k_8(k_2 + k_4)}$$

$$K_b = \frac{k_7(k_4 + k_5)}{k_3(k_5 + k_7)}, \quad K_p = \frac{k_2(k_4 + k_5)}{k_6(k_2 + k_4)}$$

$$K_{ib} = \frac{(k_2 + k_4)}{k_3}, \quad K_{ip} = \frac{(k_5 + k_7)}{k_6}$$

$$K_{ia} = k_2/k_1 \ , \quad K_{iq} = k_7/k_8$$

Theorell-Chance Mechanism (Scheme I-9)

Rate Equation:

$$v = \frac{(k_1k_3k_5AB - k_2k_4k_6PQ)E_0}{E + EA + EQ}$$

In Cleland Form:

$$v = \frac{V_1V_2(AB - \frac{PQ}{K_{eq}})}{V_2K_{ia}K_b + V_2K_bA + V_2K_aB + V_2AB + \frac{V_1K_qP}{K_{eq}} + \frac{V_1K_pQ}{K_{eq}} + \frac{V_1PQ}{K_{eq}} + \frac{V_1K_qAP}{K_{ia}K_{eq}} + \frac{V_2K_aBQ}{K_{iq}}}$$

Determinants:

$$E = k_2k_5 + k_3k_5B + k_2k_4P$$

$$EA = k_1k_5A + k_1k_4AP + k_4k_6PQ$$

$$EQ = k_2k_6Q + k_1k_3AB + k_3k_6BQ$$

Haldanes:

$$K_{eq} = \frac{k_1k_3k_5}{k_2k_4k_6} = \frac{V_1K_{ip}K_q}{V_2K_aK_{ib}} = \frac{V_1K_pK_{iq}}{V_2K_aK_{ib}} = \frac{V_1K_pK_{iq}}{V_2K_{ia}K_b} = \frac{V_1K_{ip}K_q}{V_2K_{ia}K_b} = \frac{V_1K_{ip}K_{iq}}{V_2K_aK_b}$$

$$= \frac{V_1K_{ip}K_{iq}K_{ib}}{V_2K_bK_{ia}K_b} = \frac{V_2K_{ip}K_{iq}}{V_1K_{ib}K_{ia}} = \frac{K_{ip}K_{iq}}{K_aK_{ib}} = \frac{V_1{}^2K_qK_p}{V_2{}^2K_aK_{ib}} = \frac{V_1{}^2K_qK_{ip}}{V_2{}^2K_aK_b}$$

$$= \frac{V_1{}^2K_qK_p}{V_2{}^2K_{ia}K_b} = \frac{V_1{}^2K_{iq}K_p}{V_2{}^2K_aK_b} = \frac{K_{ip}K_{iq}}{K_{ia}K_b} = \frac{K_{ip}{}^2K_q}{K_{ia}K_bK_p} = \frac{K_{ip}{}^2K_q}{K_aK_{ib}K_p} = \frac{V_1{}^3K_pK_q}{V_2{}^3K_aK_b}$$

Kinetic Parameters:

$$V_1 = k_5E_0 \qquad V_2 = k_2E_0$$

$$K_a = \frac{k_5}{k_1} \qquad K_q = \frac{k_2}{k_6}$$

$$K_b = \frac{k_5}{k_3} \qquad K_p = \frac{k_2}{k_4}$$

$$K_{ib} = \frac{k_2}{k_3} \qquad K_{ip} = \frac{k_5}{k_4}$$

$$K_{ia} = k_2/k_1 \qquad K_{iq} = k_5/k_6$$

Ping Pong Bi Bi Mechanism (Scheme I-10)

Rate Equation:

$$v = \frac{(k_1k_3k_5k_7AB - k_2k_4k_6k_8PQ)E_0}{E + (EA + FP) + F + (FB + EQ)}$$

In Cleland Form:

$$v = \frac{V_1V_2(AB - \frac{PQ}{K_{eq}})}{V_2K_bA + V_2K_aB + V_2AB + \frac{V_1K_qP}{K_{eq}} + \frac{V_1K_pQ}{K_{eq}} + \frac{V_1PQ}{K_{eq}} + \frac{V_1K_qAP}{K_{ia}K_{eq}} + \frac{V_2K_aBQ}{K_{iq}}}$$

Determinants:

$$E = k_5k_7B(k_2 + k_3) + k_2k_4P(k_6 + k_7)$$

$$(EA + FP) = k_1k_5k_7AB + k_1k_4AP(k_6 + k_7) + k_4k_6k_8PQ$$

$$F = k_1k_3A(k_6 + k_7) + k_6k_8Q(k_2 + k_3)$$

$$(FB + EQ) = k_1k_3k_5AB + k_5k_8BQ(k_2 + k_3) + k_2k_4k_8PQ$$

Haldanes:

$$K_{eq} = \frac{k_1k_3k_5k_7}{k_2k_4k_6k_8} = \frac{K_{ip}K_{iq}}{K_{ia}K_{ib}} = \frac{V_1K_{ip}K_q}{V_2K_{ia}K_b} = \frac{V_1K_pK_{iq}}{V_2K_aK_{ib}} = \frac{V_1{}^2K_pK_q}{V_2{}^2K_aK_b}$$

Kinetic Parameters:

$$V_1 = k_3k_7E_0/(k_3 + k_7); \qquad V_2 = k_2k_6E_0/(k_2 + k_6)$$

$$K_a = \frac{k_7(k_2 + k_3)}{k_1(k_3 + k_7)}; \qquad K_q = \frac{k_2(k_6 + k_7)}{k_8(k_2 + k_6)}$$

$$K_b = \frac{k_3(k_6 + k_7)}{k_5(k_3 + k_7)}; \qquad K_p = \frac{k_6(k_2 + k_3)}{k_4(k_2 + k_6)}$$

$$K_{ib} = \frac{k_6}{k_5}; \qquad K_{ip} = \frac{k_3}{k_4}$$

$$K_{ia} = k_2/k_1; \qquad K_{iq} = k_7/k_8$$

Ordered Ter Ter Mechanism (Scheme I-12)

Rate Equation:

$$v = \frac{(k_1k_3k_5k_7k_9k_{11}ABC - k_2k_4k_6k_8k_{10}k_{12}PQR)E_0}{E + EA + EAB + (EABC + EPQR) + EQR + ER}$$

In Cleland Form:

$$v = \frac{V_1V_2(ABC - \frac{PQR}{K_{eq}})}{V_2K_{ia}K_{ib}K_c + V_2K_{ib}K_cA + V_2K_{ia}K_bC + V_2K_cAB + V_2K_bAC + V_2K_aBC}$$

$$+ V_2ABC + \frac{V_1K_{ir}K_qP}{K_{eq}} + \frac{V_1K_{iq}K_pR}{K_{eq}} + \frac{V_1K_rPQ}{K_{eq}} + \frac{V_1K_qPR}{K_{eq}} + \frac{V_1K_pQR}{K_{eq}}$$

$$+ \frac{V_1PQR}{K_{eq}} + \frac{V_1K_qK_{ir}AP}{K_{ia}K_{eq}} + \frac{V_2K_{ia}K_bCR}{K_{ir}} + \frac{V_1K_qK_{ir}ABP}{K_{ia}K_{ib}K_{eq}} + \frac{V_1K_rAPQ}{K_{ia}K_{eq}} + \frac{V_2K_aBCR}{K_{ir}}$$

$$+ \frac{V_2K_{ia}K_bCQR}{K_{iq}K_{ir}} + \frac{V_1K_{ir}K_qABCP}{K_{ia}K_{ib}K_{ic}K_{eq}} + \frac{V_1K_{ip}K_rABCQ}{K_{ia}K_{ib}K_{ic}K_{eq}} + \frac{V_1K_rABPQ}{K_{ia}K_{ib}K_{eq}} + \frac{V_2K_aBCQR}{K_{iq}K_{ir}}$$

$$+ \frac{V_2K_aK_{ic}BPQR}{K_{ip}K_{iq}K_{ir}} + \frac{V_2K_{ia}K_bCPQR}{K_{ip}K_{iq}K_{ir}} + \frac{V_1K_rABCPQ}{K_{ia}K_{ib}K_{ic}K_{eq}} + \frac{V_2K_aBCPQR}{K_{ip}K_{iq}K_{ir}}$$

Determinants:

$$E = k_2k_4k_9k_{11}(k_6 + k_7) + k_2k_5k_7k_9k_{11}C + k_3k_5k_7k_9k_{11}BC$$
$$+ k_2k_4k_6k_8k_{11}P + k_2k_4k_6k_8k_{10}PQ$$

$$EA = k_1k_4k_9k_{11}A(k_6 + k_7) + k_1k_5k_7k_9k_{11}AC + k_1k_4k_6k_8k_{11}AP$$
$$+ k_1k_4k_6k_8k_{10}APQ + k_4k_6k_8k_{10}k_{12}PQR$$

$$EAB = k_1k_3k_9k_{11}AB(k_6 + k_7) + k_1k_3k_6k_8k_{11}ABP + k_1k_3k_6k_8k_{10}ABPQ$$
$$+ k_2k_6k_8k_{10}k_{12}PQR + k_3k_6k_8k_{10}k_{12}BPQR$$

$$(EABC + EPQR) = k_1k_3k_5k_9k_{11}ABC + k_1k_3k_5k_8k_{11}ABCP +$$
$$k_2k_4k_8k_{10}k_{12}PQR + k_1k_3k_5k_8k_{10}ABCPQ +$$
$$k_2k_5k_8k_{10}k_{12}CPQR + k_3k_5k_8k_{10}k_{12}BCPQR$$

$$EQR = k_2k_4k_{10}k_{12}QR(k_6 + k_7) + k_1k_3k_5k_7k_{11}ABC + k_2k_5k_7k_{10}k_{12}CQR$$
$$+ k_1k_3k_5k_7k_{10}ABCQ + k_3k_5k_7k_{10}k_{12}BCQR$$

$$ER = k_2k_4k_9k_{12}R(k_6 + k_7) + k_2k_5k_7k_9k_{12}CR + k_2k_4k_6k_8k_{12}PR + k_1k_3k_5k_7k_9ABC + k_3k_5k_7k_9k_{12}BCR$$

Haldanes:

$$K_{eq} = \frac{k_1k_3k_5k_7k_9k_{11}}{k_2k_4k_6k_8k_{10}k_{12}} = \frac{K_{ip}K_{iq}K_{ir}}{K_{ia}K_{ib}K_{ic}} = \frac{V_1K_pK_{iq}K_{ir}}{V_2K_{ia}K_{ib}K_c}$$

Kinetic Parameters:

$$V_1 = k_7k_9k_{11}E_0/(k_7k_9 + k_7k_{11} + k_9k_{11});$$

$$V_2 = k_2k_4k_6E_0/(k_2k_4 + k_2k_6 + k_4k_6)$$

$$K_a = \frac{k_7k_9k_{11}}{k_1(k_7k_9 + k_7k_{11} + k_9k_{11})}; \quad K_q = \frac{k_2k_4k_6}{k_{12}(k_2k_4 + k_2k_6 + k_4k_6)}$$

$$K_b = \frac{k_7k_9k_{11}}{k_3(k_7k_9 + k_7k_{11} + k_9k_{11})}; \quad K_p = \frac{k_2k_4k_6}{k_{10}(k_2k_4 + k_2k_6 + k_4k_6)}$$

$$K_c = \frac{k_9k_{11}(k_6 + k_7)}{k_5(k_7k_9 + k_7k_{11} + k_9k_{11})}; \quad K_r = \frac{k_2k_4(k_6 + k_7)}{k_8(k_2k_4 + k_2k_6 + k_4k_6)}$$

$$K_{ia} = k_2/k_1, \quad K_{ib} = k_4/k_3, \quad K_{ic} = k_6/k_5, \quad K_{ip} = k_7/k_8$$

$$K_{iq} = k_9/k_{10}, \quad K_{ir} = k_{11}/k_{12}$$

Ordered Ter Bi Mechanism (Similar to Scheme I-12 but with R omitted)

Rate Equation:

$$v = \frac{(k_1k_3k_5k_7k_9ABC - k_2k_4k_6k_8k_{10}PQ)E_0}{E + EA + EAB + (EABC + EPQ) + EQ}$$

In Cleland Form:

$$v = \frac{V_1V_2(ABC - \frac{PQ}{K_{eq}})}{V_2K_{ia}K_{ib}K_c + V_2K_{ib}K_cA + V_2K_{ia}K_bC + V_2K_cAB + V_2K_bAC + V_2K_aBC + V_2ABC + \frac{V_1K_pQ}{K_{eq}} + \frac{V_1K_qP}{K_{eq}} + \frac{V_1PQ}{K_{eq}} + \frac{V_1K_qAP}{K_{ia}K_{eq}} + \frac{V_2K_{ia}K_bCQ}{K_{iq}} + \frac{V_1K_qABP}{K_{ia}K_{ib}K_{eq}} + \frac{V_2K_aBCQ}{K_{iq}}}$$

$$+\frac{V_2K_aK_{ic}BPQ}{K_{ip}K_{iq}}+\frac{V_2K_{ia}K_bCPQ}{K_{ip}K_{iq}}+\frac{V_1K_qABCP}{K_{ia}K_{ib}K_{ic}K_{eq}}+\frac{V_2K_aBCPQ}{K_{ip}K_{iq}}$$

Determinants:

$$E = k_2k_4k_9(k_6 + k_7) + k_2k_5k_7k_9C + k_2k_4k_6k_8P + k_3k_5k_7k_9BC$$

$$EA = k_1k_4k_9A(k_6 + k_7) + k_1k_5k_7k_9AC + k_1k_4k_6k_8AP + k_4k_6k_8k_{10}PQ$$

$$EAB = k_1k_3k_9AB(k_6 + k_7) + k_2k_6k_8k_{10}PQ + k_1k_3k_6k_8ABP + k_3k_6k_8k_{10}BPQ$$

$$(EABC + EPQ) = k_2k_4k_8k_{10}PQ + k_1k_3k_5k_9ABC + k_2k_5k_8k_{10}CPQ + k_1k_3k_5k_8ABCP + k_3k_5k_8k_{10}BCPQ$$

$$EQ = k_2k_4k_{10}Q(k_6 + k_7) + k_2k_5k_7k_{10}CQ + k_1k_3k_5k_7ABC + k_3k_5k_7k_{10}BCQ$$

Haldanes:

$$K_{eq} = \frac{k_1k_3k_5k_7k_9}{k_2k_4k_6k_8k_{10}} = \frac{K_{ip}K_{iq}}{K_{ia}K_{ib}K_{ic}} = \frac{V_1K_pK_{iq}}{V_2K_{ia}K_{ib}K_c}$$

Kinetic Parameters:

$$V_1 = k_7k_9E_0/(k_7 + k_9); \qquad V_2 = k_2k_4k_6E_0/(k_2k_4 + k_4k_6 + k_2k_6)$$

$$K_a = \frac{k_7k_9}{k_1(k_7 + k_9)}; \qquad K_p = \frac{k_2k_4(k_6 + k_7)}{k_8(k_2k_4 + k_4k_6 + k_2k_6)}$$

$$K_b = \frac{k_7k_9}{k_3(k_7 + k_9)}; \qquad K_q = \frac{k_2k_4k_6}{k_{10}(k_2k_4 + k_4k_6 + k_2k_6)}$$

$$K_c = \frac{k_9(k_6 + k_7)}{k_5(k_7 + k_9)}$$

$$K_{ia} = k_2/k_1\ ,\ K_{ib} = k_4/k_3\ ,\ K_{ic} = k_6/k_5\ ,\ K_{ip} = k_7/k_8\ ,$$

$$K_{iq} = k_9/k_{10}$$

Hexa Uni Ping Pong Mechanism (Scheme I-17)

Rate Equation:

$$v = \frac{(k_1k_3k_5k_7k_9k_{11}ABC - k_2k_4k_6k_8k_{10}k_{12}PQR)E_0}{E + EA + E' + E'B + E'' + E''C}$$

In Cleland Form:

$$v = \frac{V_1V_2(ABC - \frac{PQR}{K_{eq}})}{V_2ABC + V_2K_cAB + V_2K_bAC + V_2K_aBC + \frac{V_2K_cK_{ib}AQ}{K_{iq}} + \frac{V_2K_aK_{ic}BR}{K_{ir}} + \frac{V_2K_bK_{ia}CP}{K_{ip}} + \frac{V_2K_cK_{ia}K_{ib}PQ}{K_{ip}K_{iq}} + \frac{V_2K_aK_{ib}K_{ic}QR}{K_{ip}K_{ir}} + \frac{V_2K_bK_{ia}K_{ic}PR}{K_{ip}K_{ir}} + \frac{V_1PQR}{K_{eq}} + \frac{V_2K_cABQ}{K_{iq}} + \frac{V_2K_bACP}{K_{ip}} + \frac{V_2K_cK_{ib}APQ}{K_{ip}K_{iq}} + \frac{V_2K_aK_{ic}BQR}{K_{iq}K_{ir}} + \frac{V_2K_aBCR}{K_{ir}} + \frac{V_2K_bK_{ia}CPR}{K_{ip}K_{ir}}}$$

Determinants:

$$E = k_5k_7k_9k_{11}(k_2 + k_3)BC + k_2k_4k_9k_{11}(k_6 + k_7)CP + k_2k_4k_6k_8(k_{10} + k_{11})PQ$$

$$EA = k_1k_5k_7k_9k_{11}ABC + k_1k_4k_9k_{11}(k_6 + k_7)ACP + k_1k_4k_6k_8(k_{10} + k_{11})APQ + k_4k_6k_8k_{10}k_{12}PQR$$

$$E' = k_1k_3k_9k_{11}(k_6 + k_7)AC + k_1k_3k_6k_8(k_{10} + k_{11})AQ + k_6k_8k_{10}k_{12}(k_2 + k_3)QR$$

$$E'B = k_1k_3k_5k_9k_{11}ABC + k_1k_3k_5k_8(k_{10} + k_{11})ABQ + k_5k_8k_{10}k_{12}(k_2 + k_3)BQR + k_2k_4k_8k_{10}k_{12}PQR$$

$$E'' = k_1k_3k_5k_7(k_{10} + k_{11})AB + k_5k_7k_{10}k_{12}(k_2 + k_3)BR + k_2k_4k_{10}k_{12}(k_6 + k_7)PR$$

$$E''C = k_1k_3k_5k_7k_9ABC + k_5k_7k_9k_{12}(k_2 + k_3)BCR + k_2k_4k_9k_{12}(k_6 + k_7)CPR + k_2k_4k_6k_8k_{12}PQR$$

Haldanes:

$$K_{eq} = \frac{k_1k_3k_5k_7k_9k_{11}}{k_2k_4k_6k_8k_{10}k_{12}} = \frac{K_{ip}K_{iq}K_{ir}}{K_{ia}K_{ib}K_{ic}} = \frac{V_1K_{ip}K_{iq}K_r}{V_2K_{ia}K_{ib}K_c} = \frac{V_1K_pK_{iq}K_{ir}}{V_2K_aK_{ib}K_{ic}}$$

$$= \frac{V_1K_{ip}K_qK_{ir}}{V_2K_{ia}K_bK_{ic}} = \frac{V_1{}^2K_pK_qK_{ir}}{V_2{}^2K_aK_bK_{ic}} = \frac{V_1{}^2K_pK_{iq}K_r}{V_2{}^2K_aK_{ib}K_c} = \frac{V_1{}^2K_{ip}K_qK_r}{V_2{}^2K_{ia}K_bK_c} = \frac{V_1{}^3K_pK_qK_r}{V_2{}^3K_aK_bK_c}$$

Kinetic Parameters:

$$V_1 = k_3k_7k_{11}E_0/(k_3k_7 + k_3k_{11} + k_7k_{11})$$

$$V_2 = k_2k_6k_{10}E_0/(k_2k_6 + k_2k_{10} + k_6k_{10})$$

$$K_a = \frac{k_7k_{11}(k_2 + k_3)}{k_1(k_3k_7 + k_3k_{11} + k_7k_{11})}; \quad K_p = \frac{k_6k_{10}(k_2 + k_3)}{k_4(k_2k_6 + k_2k_{10} + k_6k_{10})}$$

$$K_b = \frac{k_3k_{11}(k_6 + k_7)}{k_5(k_3k_7 + k_3k_{11} + k_7k_{11})}; \quad K_q = \frac{k_2k_{10}(k_6 + k_7)}{k_8(k_2k_6 + k_2k_{10} + k_6k_{10})}$$

$$K_c = \frac{k_3k_7(k_{10} + k_{11})}{k_9(k_3k_7 + k_3k_{11} + k_7k_{11})}; \quad K_r = \frac{k_2k_6(k_{10} + k_{11})}{k_{12}(k_2k_6 + k_2k_{10} + k_6k_{10})}$$

$$K_{ia} = k_2/k_1, \quad K_{ib} = k_6/k_5, \quad K_{ic} = k_{10}/k_9, \quad K_{ip} = k_3/k_4,$$

$$K_{iq} = k_7/k_8, \quad K_{ir} = k_{11}/k_{12}$$

Ordered Bi Uni Uni Bi Ping Pong (Scheme I-18)

Rate Equation:

$$v = \frac{(k_1k_3k_5k_7k_9k_{11}ABC - k_2k_4k_6k_8k_{10}k_{12}PQR)E_0}{E + EA + EAB + E' + E'C + ER}$$

In Cleland Form:

$$v = \frac{V_1V_2(ABC - \frac{PQR}{K_{eq}})}{V_2K_{ia}K_bC + V_2K_cAB + V_2K_bAC + V_2K_aBC + V_2ABC}$$

$$+ \frac{V_1K_{ir}K_qP}{K_{eq}} + \frac{V_1K_qK_{ir}AP}{K_{ia}K_{eq}} + \frac{V_2K_{ia}K_bCR}{K_{ir}}$$

$$+ \frac{V_1K_rPQ}{K_{eq}} + \frac{V_1K_qPR}{K_{eq}} + \frac{V_1K_pQR}{K_{eq}} + \frac{V_1K_qK_{ir}ABP}{K_{ia}K_{ib}K_{eq}}$$

$$+ \frac{V_1K_rK_{ip}ABQ}{K_{ia}K_{ib}K_{eq}} + \frac{V_1K_rAPQ}{K_{ia}K_{eq}} + \frac{V_2K_aK_{ic}BQR}{K_{iq}K_{ir}}$$

$$+ \frac{V_2K_aBCR}{K_{ir}} + \frac{V_2K_{ia}K_bCQR}{K_{iq}K_{ir}} + \frac{V_1PQR}{K_{eq}} + \frac{V_1K_rK_{ip}ABCQ}{K_{ia}K_{ib}K_{ic}K_{eq}}$$

$$+ \frac{V_1K_rABPQ}{K_{ia}K_{ib}K_{eq}} + \frac{V_2K_aBCQR}{K_{iq}K_{ir}} + \frac{V_2K_{ic}K_aBPQR}{K_{ip}K_{iq}K_{ir}}$$

Determinants:

$$E = k_2k_7k_9k_{11}C(k_4 + k_5) + k_2k_4k_6k_{11}P(k_8 + k_9)$$
$$+ k_3k_5k_7k_9k_{11}BC + k_2k_4k_6k_8k_{10}PQ$$

$$EA = k_1k_7k_9k_{11}AC(k_4 + k_5) + k_1k_4k_6k_{11}AP(k_8 + k_9)$$
$$+ k_1k_4k_6k_8k_{10}APQ + k_4k_6k_8k_{10}k_{12}PQR$$

$$EAB = k_1k_3k_7k_9k_{11}ABC + k_1k_3k_6k_{11}ABP(k_8 + k_9) + k_2k_6k_8k_{10}k_{12}PQR$$
$$+ k_1k_3k_6k_8k_{10}ABPQ + k_3k_6k_8k_{10}k_{12}BPQR$$

$$E' = k_1k_3k_5k_{11}AB(k_8 + k_9) + k_2k_8k_{10}k_{12}QR(k_4 + k_5)$$
$$+ k_1k_3k_5k_8k_{10}ABQ + k_3k_5k_8k_{10}k_{12}BQR$$

$$E'C = k_1k_3k_5k_7k_{11}ABC + k_2k_7k_{10}k_{12}CQR(k_4 + k_5) + k_2k_4k_6k_{10}k_{12}PQR$$
$$+ k_1k_3k_5k_7k_{10}ABCQ + k_3k_5k_7k_{10}k_{12}BCQR$$

$$ER = k_2k_7k_9k_{12}CR(k_4 + k_5) + k_2k_4k_6k_{12}PR(k_8 + k_9) +$$
$$k_1k_3k_5k_7k_9ABC + k_3k_5k_7k_9k_{12}BCR$$

Haldanes:

$$K_{eq} = \frac{k_1k_3k_5k_7k_9k_{11}}{k_2k_4k_6k_8k_{10}k_{12}} = \frac{K_{ip}K_{iq}K_{ir}}{K_{ia}K_{ib}K_{ic}} = \frac{V_1K_{ip}K_qK_{ir}}{V_2K_{ia}K_{ib}K_c} = \frac{V_1K_pK_{iq}K_{ir}}{V_2K_{ia}K_bK_{ic}}$$
$$= \frac{V_1^2K_pK_qK_{ir}}{V_2^2K_{ia}K_bK_c}$$

Kinetic Parameters:

$$V_1 = k_5k_9k_{11}E_0/(k_5k_9 + k_5k_{11} + k_9k_{11})$$

$$V_2 = k_2k_4k_8E_0/(k_2k_4 + k_2k_8 + k_4k_8)$$

$$K_a = \frac{k_5k_9k_{11}}{k_1(k_5k_9 + k_5k_{11} + k_9k_{11})}; \quad K_r = \frac{k_2k_4k_8}{k_{12}(k_2k_4 + k_2k_8 + k_4k_8)}$$

$$K_b = \frac{k_9k_{11}(k_4 + k_5)}{k_3(k_5k_9 + k_5k_{11} + k_9k_{11})}; \quad K_q = \frac{k_2k_4(k_8 + k_9)}{k_{10}(k_2k_4 + k_2k_8 + k_4k_8)}$$

$$K_c = \frac{k_5k_{11}(k_8 + k_9)}{k_7(k_5k_9 + k_5k_{11} + k_9k_{11})}; \quad K_p = \frac{k_2k_8(k_4 + k_5)}{k_6(k_2k_4 + k_2k_8 + k_4k_8)}$$

$K_{ia} = k_2/k_1$, $K_{ib} = k_4/k_3$, $K_{ic} = k_8/k_7$, $K_{ip} = k_5/k_6$,
$K_{iq} = k_9/k_{10}$, $K_{ir} = k_{11}/k_{12}$

Ordered Uni Uni Bi Bi Ping Pong Mechanism (Scheme I-19)

Rate Equation:

$$v = \frac{(k_1k_3k_5k_7k_9k_{11}ABC - k_2k_4k_6k_8k_{10}k_{12}PQR)E_0}{E + EA + E' + EB + EBC + ER}$$

In Cleland Form:

$$v = \frac{V_1V_2(ABC - \frac{PQR}{K_{eq}})}{V_2K_cK_{ib}A + V_2K_cAB + V_2K_bAC + \frac{V_2K_cK_{ib}AP}{K_{ip}} + \frac{V_1K_rK_{ip}AQ}{K_{ia}K_{eq}} + V_2K_aBC}$$

$$+ V_2ABC + \frac{V_2K_bACP}{K_{ip}} + \frac{V_1K_rAPQ}{K_{ia}K_{eq}} + \frac{V_2K_cK_{ia}K_{ib}P}{K_{ip}} + \frac{V_2K_bK_{ia}CP}{K_{ip}}$$

$$+ \frac{V_1K_rPQ}{K_{eq}} + \frac{V_2K_cK_{ia}K_{ib}PR}{K_{ip}K_{ir}} + \frac{V_1PQR}{K_{eq}} + \frac{V_2K_aK_{ib}K_{ic}QR}{K_{iq}K_{ir}}$$

$$+ \frac{V_2K_aK_{ic}BQR}{K_{iq}K_{ir}} + \frac{V_2K_aBCR}{K_{ir}} + \frac{V_2K_bK_{ia}CPR}{K_{ip}K_{ir}} + \frac{V_1K_rABCQ}{K_{iq}K_{ir}}$$

$$+ \frac{V_2K_aBCQR}{K_{iq}K_{ir}} + \frac{V_2K_bK_{ia}CPQR}{K_{ip}K_{iq}K_{ir}} + \frac{V_1K_rK_{ic}ABQ}{K_{iq}K_{ir}}$$

Determinants:

$$E = k_2k_4k_6k_{11}(k_8 + k_9)P + k_5k_7k_9k_{11}(k_2 + k_3)BC$$
$$+ k_2k_4k_7k_9k_{11}CP + k_2k_4k_6k_8k_{10}PQ$$

$$EA = k_1k_4k_6k_{11}(k_8 + k_9)AP + k_1k_5k_7k_9k_{11}ABC + k_1k_4k_7k_9k_{11}ACP$$
$$+ k_1k_4k_6k_8k_{10}APQ + k_4k_6k_8k_{10}k_{12}PQR$$

$$E' = k_1k_3k_6k_{11}(k_8 + k_9)A + k_1k_3k_7k_9k_{11}AC + k_1k_3k_6k_8k_{10}AQ$$
$$+ k_6k_8k_{10}k_{12}(k_2 + k_3)QR$$

$$EB = k_1k_3k_5k_{11}(k_8 + k_9)AB + k_1k_3k_5k_8k_{10}ABQ +$$
$$k_5k_8k_{10}k_{12}(k_2 + k_3)BQR + k_2k_4k_8k_{10}k_{12}PQR$$

$$EBC = k_1k_3k_5k_7k_{11}ABC + k_1k_3k_5k_7k_{10}ABCQ + k_5k_7k_{10}k_{12}(k_2 + k_3)BCQR$$
$$+ k_2k_4k_7k_{10}k_{12}CPQR + k_2k_4k_6k_{10}k_{12}PQR$$

$$ER = k_2k_4k_6k_{12}(k_8 + k_9)PR + k_1k_3k_5k_7k_9ABC$$
$$+ k_5k_7k_9k_{12}(k_2 + k_3)BCR + k_2k_4k_7k_9k_{12}CPR$$

Haldanes:

$$K_{eq} = \frac{k_1k_3k_5k_7k_9k_{11}}{k_2k_4k_6k_8k_{10}k_{12}} = \frac{K_{ip}K_{iq}K_{ir}}{K_{ia}K_{ib}K_{ic}} = \frac{V_1K_qK_{ip}K_{ir}}{V_2K_cK_{ia}K_{ib}} = \frac{V_1K_pK_{iq}K_{ir}}{V_2K_aK_{ib}K_{ic}}$$
$$= \frac{V_1^2K_pK_qK_{ir}}{V_2^2K_aK_cK_{ib}}$$

Kinetic Parameters:

$$V_1 = k_3k_9k_{11}E_0/(k_3k_9 + k_3k_{11} + k_9k_{11})$$

$$V_2 = k_2k_6k_8E_0/(k_2k_6 + k_2k_8 + k_6k_8)$$

$$K_a = \frac{k_9k_{11}(k_2 + k_3)}{k_1(k_3k_9 + k_3k_{11} + k_9k_{11})}; \quad K_p = \frac{k_6k_8(k_2 + k_3)}{k_4(k_2k_6 + k_2k_8 + k_6k_8)}$$

$$K_b = \frac{k_3k_9k_{11}}{k_5(k_3k_9 + k_3k_{11} + k_9k_{11})}; \quad K_q = \frac{k_2k_6(k_8 + k_9)}{k_{10}(k_2k_6 + k_2k_8 + k_6k_8)}$$

$$K_c = \frac{k_3k_{11}(k_8 + k_9)}{k_7(k_3k_9 + k_3k_{11} + k_9k_{11})}; \quad K_r = \frac{k_2k_6k_8}{k_{12}(k_2k_6 + k_2k_8 + k_6k_8)}$$

$$K_{ia} = k_2/k_1, \quad K_{ib} = k_6/k_5, \quad K_{ic} = k_8/k_7, \quad K_{ip} = k_3/k_4,$$

$$K_{iq} = k_9/k_{10}, \quad K_{ir} = k_{11}/k_{12}$$

Appendix II

ENZ-EQ

A Computer Program for Deriving Enzyme Rate Equations

Program Description: ENZ-EQ

The determinant procedure described by HURST [Can. J. Biochem. 45, 2015 (1967)] has been adapted for use with the IBM/360 computer in the PL/1 language. The determinant method is based on the solution of n-1 of n equations describing a steady-state enzyme system. The steady-state equations describing each enzyme form are input into the program and a determinant is evaluated which gives an algebraic value for the concentration of each enzyme form in the total reaction under steady-state conditions. The method is, in general terms, to generate a description of each determinant, to expand the determinant while eliminating zero terms, and finally to substitute rate constants and concentration terms into the expanded determinant to give the final expression.

Although the problem and output are algebraic in nature, the program's processing is entirely numeric except for the output of the final answers. This fact makes the program easily convertible to FORTRAN computer language. A numeric approach is possible because the non-numeric symbols needed are limited to a given set, namely a given number of rate constants and concentrations. Thus, these symbols are stored in two arrays and a reference to a rate constant or a concentration is the subscript of an element in an array. An algebraic term is represented by an array containing one or more such subscripts. An algebraic expression is represented by a linear list of pairs of arrays (one for rate constants and another for concentrations), together with a coefficient and a pointer, the latter referring to the next element in the list. The end of a list is indicated by a zero pointer. The terms (elements) in each list are kept in a specific order so that algebraically similar terms will be kept next to each other and easily combined.

Since the terms from which the determinants are composed form a (2 to n, 1 to n) array, a particular determinant can be represented by specifying which terms are in it. And, since each determinant contains n-1 columns of the array of terms and all of the terms in those columns, it sufficies to specify the column numbers. Furthermore, each term of an expanded determinant contains one factor from each row of the determinant (and hence of the original array) and one from each column. If the factors in a term are ordered according to row number, it will again suffice to specify only the column numbers. Thus, e.g., if the original array is designated "A", then (2,4,3) represents both $\begin{vmatrix} A_{22} & A_{24} & A_{23} \\ A_{32} & A_{34} & A_{33} \\ A_{42} & A_{44} & A_{43} \end{vmatrix}$ and $A_{22}\ A_{34}\ A_{43}$. Finally, these two repre-

sentations can be combined so that (2,4,3) also represents

$$A_{22}\begin{vmatrix} A_{34} & A_{33} \\ A_{44} & A_{43} \end{vmatrix} \text{ and } A_{22}\ A_{34}\ \begin{vmatrix} A_{43} \end{vmatrix}.$$

Thus, various stages in the expansion of a determinant may be represented by an array of column numbers. The expansion of the determinants is carried out by use of minors.

Input Deck for Program ENZ-EQ

The desired mechanism is set up in steady-state d(EX)/dt form as described for the following mechanism:

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA$$

$$EA + B \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EAB$$

$$EAB \underset{k_6}{\overset{k_5}{\rightleftharpoons}} EP + Q$$

$$EP \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E + P$$

$$d(EA)/dt = k_1A(E) - (k_2 + k_3B)(EA) + k_4(EAB)$$

$$d(EAB)/dt = k_3B(EA) - (k_4 + k_5)EAB + k_6Q(EP)$$

$$d(EP)/dt = k_5(EAB) - (k_6Q + k_7)(EP) + k_8P(E)$$

The d(E)/dt term need not be set up as it is not used. The other equations completely describe the d(E)/dt term.

The steady-state equations are then set up in a table of coefficients as described by HURST.

E	EA	EAB	EP	n + 1
1	1	1	1	1
k_1A	$-(k_2+k_3B)$	k_4	0	0
0	k_3B	$-(k_4+k_5)$	k_6Q	0
k_8P	0	k_5	$-(k_6Q+k_7)$	0

The data deck is then coded from this table. Only positions containing non-zero terms are coded, and the first row is already in the program.. A rate constant is represented by a ± XY where XY is the subscript and the + or - depends on the sign of the rate constant in the table. For example, $-k_4$ would be -04. Three spaces are required for each rate constant description. The substrates A, B, C are represented by -01, -02, -03, respectively, and products P, Q, R are represented by +01, +02, +03, respectively. When no substrate or product term appears in the coefficient, 000 is used.

Each coefficient is referred to by its row and column location. k_1A is in row two, column one and is designated by 0201. The first two numbers represent the row, the second two, the column.

Each coefficient is described on a single data card as follows: The location is described in columns 1 to 4 on the card, then the complete coefficient is described in six column sets. For k_1A in row two, column one, the data card would read:
0 2 0 1 + 0 1 - 0 1 where 0201 represents the location, +01 re-
1 2 3 4 5 6 7 8 9 1o
presents k_1, and -01 represents A; while for the other coefficients the following code would be used:

$-(k_2 + k_3B)$ in row 2, column 2 ≡ 0202-02000-03-02

k_4 in row 2, column 3 ≡ 0203+04000

k_3B in row 3, column 2 ≡ 0302+03-02

$-(k_4 + k_5)$ in row 3, column 3 ≡ 0303-04000-05000

k_6Q in row 3, column 4 ≡ 0304+06+02

k_8P in row 4, column 1 ≡ 0401+08+01

k_5 in row 4, column 3 ≡ 0403+05000

$-(k_6Q + k_7)$ in row 4, column 4 ≡ 0404-06+02-07000

A lead card is used which has the value of N (the number of enzyme species) and NPS (the maximum number of substrate and product terms in any one term of the final determinants) in columns 1-3 and 4-6 respectively. For the mechanism above the lead card would be 004003. NPS is a dimensioning number and may have to be determined by trial and error. It should not be made larger than necessary to conserve space.

The final complete deck which consists of the lead card, the data cards describing the coefficients, and a blank card at the end to terminate input is placed after the //Go Gards DD* card.

Output from ENZ-EQ

The output consists of the individual determinants for each enzyme species E(1)---E(n) in order that they were input and the

total sum of all the determinants. The terms may be either all + or - but never both. If they are mixed, an error has been made in the input. The terms are ordered in terms of increasing rate constants. The output for the mechanism described above is as follows:

$$EX(1)\ [(E)] = k_2k_4k_6Q + k_2k_4k_7 + k_2k_5k_7 + k_3k_5k_7B$$

$$EX(2)\ [(EA)] = k_1k_4k_6AQ + k_1k_4k_7A + k_1k_5k_7A + k_4k_6k_8PQ$$

$$EX(3)\ [(EAB)] = k_1k_3k_6ABQ + k_1k_3k_7AB + k_2k_6k_8PQ + k_3k_6k_8BPQ$$

$$EX(4)\ [(EP)] = k_1k_3k_5AB + k_2k_4k_8P + k_2k_5k_8P + k_3k_5k_8BP$$

EX(0) is the sum of all the determinants and is output last.

ENZ-EQ: Flowchart Notes

1) N is the number of enzyme forms in the given system; NPS is the maximum number of concentration factors which may appear in any term of the calculations. (NPS may have to be determined by trial and error.)

5) I is zero when a blank card (which terminates input) is read.

8) If A (I, J) is not zero, then the Jth term of the Ith equation has already been read in.

9) I1 is the subscript in the array KS(50) of a rate constant; I2 is the subscript in the array PSS (-4:4) of a concentration. I2 is optional since concentration terms do not always appear.

10) UU is the subscript of the next unused element. If UU is zero, then all available storage has been used.

12) (a) If I1 is zero, then the last field on the card has been read.
(b) If I1 is less than zero, then the term is negative.

18-23) Unused

25) I indexes the determinants to be expanded.

28) The Ith determinant consists of all but the Ith column of the A array.

30) J indexes the level of expansion of the determinants.

31) L refers to successive terms of the expansion of the Ith determinant; L2 refers to successive terms of the *next* level of expansions; L3 is a flag which indicates whether or not the signs of the terms will need correction due to the method of expansion which changes signs of determinants of odd order.

32) I1 indexes the new terms created from one term in the present expansion.

33) Zero terms are eliminated immediately, not during the evaluation process.

34) If J=N-2, then the order of the minor being expanded is two and both terms must be checked for zero values now (blocks 33 and 35) since order 1 minors are not expanded and hence not checked.

46) I indexes the expanded determinants through the evaluation process.

47) The partial sum array is a device use to equalize the lengths of the expressions added together in order to increase the efficiency of the ADD subroutine.

48) L refers to successive terms in the expansion of the Ith determinant.

49) I1 is a partial product in multiplying out the factors of the term being evaluated.

50) J indexes the remaining factors of the term.

51) I2 refers to successive terms in the value of the Jth factor.

52) L2 is the new partial product.

ENZ-EQ Flowchart Notes: ADD Subroutine

1) On entry A1 refers to the expression to which the expression referred to by A2 is to be added; if F1 is one, then the A2 expression is to be saved; if F1 is zero, then the A2 expression may be destroyed. On exit, A1 refers to the sum.

2) L1 refers to the next term of the A1 expression, L2 to that of the A2 expression and L3 to the last term of the sum.

ENZ-EQ Flowchart Notes: MULT Subroutine

1) On entry, M1 refers to the multiplicand expression and M2 to the multiplier term (a single element). If F is one, then the multiplicand must be saved; if F is zero, then the multiplicand may be destroyed. On exit, P refers to the product.

2) L1 refers to the next term in the multiplicand; L3 refers to the last term in the product.

12) and 22) I refers to the next factor of the current multiplicand term, J to that of the multiplier, and L to the last term of the new product term.

```
1     ENZ_EQ: PROC OPTIONS(MAIN);
       /* THIS PROGRAM SOLVESTHE RATE EQUATIONS FOR COMPLEX ENZYME SYSTEMS.
          THE SOLUTIONS ARE ALGEBRAIC AND ARE IN TERMS OF RATE CONSTANTS AND
          CONCENTRATIONS OF ENZYMES,PRODUCTS AND REACTANTS. NO OTHER EXPRES-
          SIONS OR VALUES ARE ALLOWED. THE CONCENTRATIONS ARE REPRESENTED BY
          THE PRESENCE OF THEIR SUBSCRIPTS IN LISTS OF TERMS.
       */
2         DCL BULK(11) CHAR (16383) STATIC EXT;
3         DCL (XI,XN,XM) FIXED BIN(15);
                                                          /*   1   */
4         GET FILE (CARDS) EDIT (XN,XM) (2 F(3));
5         XI = 90000/(XN+XM+2);
                                                          /*   2   */
6         BEGIN;
       /*
          DECLARATIONS:
       */
7         N = XN;
8         NPS = XM;
9        DCL 1 EL(XI)   DEF BULK ,
                  2 (COEF,NXT) FIXED BIN(15),
                2 K(XN) FIXED BIN(15), /* HOLDS SUBSCRIPTS OF RATE CONST.*/
                  2 PS(XM) FIXED BIN(15); /* HOLDS SUBSCRIPTS OF PRODUCT
              AND REACTANT CONCENTRATIONS: POS FOR PROD; NEG FOR REACT. */
10        DCL (ERR,UU,I,J,L,I1,I2,L1,L2,PRSM(0:17),NMR,EXO) FIXED BIN(16);
11       DCL (L3,M1,M2,N,NPS) FIXED BIN(16);
12        DCL (NU1,NU2,NUTST) FIXED BIN(16);
13        DCL CARDS FILE STREAM INPUT;
14        DCL (NT(XN),EX(XN),SYN(0:XN),A(2:XN,XN)) FIXED BIN(16);
15       DCL LABEL CHARACTER(4);
16       DCL KS(50) CHAR(3) STATIC INIT('K1','K2','K3','K4','K5','K6','K7',
               'K8','K9','K10','K11','K12','K13','K14','K15','K16','K17',
               'K18','K19','K20','K21','K22','K23','K24','K25','K26','K27',
               'K28','K29','K30','K31','K32','K33','K34','K35','K36','K37',
               'K38','K39','K40','K41','K42','K43','K44','K45','K46','K47',
               'K48','K49','K50') VARYING;
17    DCL PSS(-5:5) CHAR(1)STATIC INIT('M','D','C','B','A',*,'P','Q','R','S'
          ,'I');
18        DCL P7 CHARACTER(5);
       /*
          INITIALIZATIONS:
       */
                                                          /*   3   */
19        PUT EDIT ('THE ARRAY OF STRUCTURES EL HAS BEEN ALLOCATED AS EL(',
                  XI,')')  (SKIP,A,F(5),A);
20       I = XI;
21        UU=1;  /* UU IS SUBSCRIPT OF FIRST UNUSED ELEMENT. */
22        DO J=1 TO I-1;
23             NXT(J)=J+1;  /*  CHAIN UNUSED ELEMENTS TOGETHER  */
24        END;
25        NXT(I)=0;
26        COEF=0;
```

ENZ_EQ: PROC OPTIONS(MAIN);

```
27        A=0; /* A(I,J) IS SUBSCR OF 1ST TERM OF I,J ELEMENT OF DETERM. */
28        NT=0;  /* NT IS NUMBER OF TERMS IN THE EXPANSION A DETERMINANT */
29        ERR=0; J=1;
31        DO I=0 TO N;
32             SYN(I)=J;
33             J=-J;
34        END;
       /*
          INPUT:
       */
35    IN_LOOP:
                                                          /*   4   */
          GET SKIP FILE(CARDS) EDIT(I,J) (2 F(2)); /* ONE CARD FOR EACH ELM*/
                                                          /*   5   */
36        IF I=0 THEN  /*  SIGNAL END OF INPUT WITH BLANK CARD */
37        GO TO DO_IT;
                                                          /*   6   */
38        IF I<2|I>N|J<1|J>N THEN
                                                          /*   7   */
39    ER: DO;
40             ERR=1;
41             GO TO IN_LOOP;
42        END;
                                                          /*   8   */
43        IF A(I,J)¬=0 THEN
44        GO TO ER;
                                                          /*   9   */
45    F_L:GET FILE(CARDS) EDIT(I1,I2) (2 F(3));
                                                          /*  10   */
46        IF UU=0 THEN
47        DO;
                                                          /*  11   */
48           GO TO OVERFLOW;
49        END;
                                                          /*  12A  */
50        IF I1=0 THEN   /*  I1 IS SUBSCR OF K FACTOR */
51        GO TO IN_LOOP;
                                                          /*  12B  */
52        IF I1<0 THEN
                                                          /*  13   */
53        DO;
54             COEF(UU)=-1;
55             I1=-I1;
56        END; ELSE
                                                          /*  14   */
57        COEF(UU)=1;
                                                          /*  15   */
58        IF I1 > 50|I2 < -5|I2 > 5   THEN
59        GO TO ER;
                                                          /*  16   */
60        K(UU,1)=I1;
61        K(UU,2)=0;
```

```
ENZ_EQ: PROC OPTIONS(MAIN);

        PS(UU,1)=I2;  /* I2 IS SUBSCR OF PROD OR REACT FACTOR(IF ANY) */
        PS(UU,2)=0;
                                                              /*  17  */
        IF A(I,J)=0 THEN
        A(I,J)=UU; ELSE
        NXT(L)=UU;
        L=UU;
        UU=NXT(UU);
        NXT(L)=0;  /*  MAY BE SEVERAL TERMS ON EACH CARD. */
        GO TO F_L;
     /*
        SUBROUTINES:
     */
        DCL ADD ENTRY(FIXED BIN(16),FIXED BIN(16),FIXED BIN(16));
                                                              /*   1  */
    ADD: PROC(A1,F1,A2);  /* ADDS A2 TO A1; RESULT 'IN' A1  */
        DCL (A1,F1,A2)      FIXED BIN(16);
        DCL (L1,L2,L3,L,F,C,I) FIXED BIN(16);
                                                              /*   2  */
        L1=A1;
        L2=A2;
        L3=0;
                                                              /*   3  */
        IF F1=0
                                                              /*   4  */
        THEN A2=0;
    ADD_LOOP:
                                                              /*   5  */
        IF L2=0 THEN
                                  DO;
                                  GO TO A30;
                                  END;
                                                              /*   6  */
        IF L1=0 THEN
        DO;
                                                              /*   7  */
             IF L3=0 THEN
                                                              /*   8  */
             IF F1 = 0 THEN
             DO;
                                                              /*   9  */
                A1 = L2;
                                  GO TO A30;
             END; ELSE
             DO;
                                                              /*  10  */
                  A1=0;
                  GO TO COPY;
             END; ELSE
                                                              /*  15  */
             IF F1 = 0 THEN
             DO;
```

```
ENZ_EQ: PROC OPTIONS(MAIN);

                                                              /*  16  */
                NXT(L3)=L2;
                                  GO TO A30;
             END; ELSE
             DO;
                                                              /*  17  */
                L1 = L3;
                PUT EDIT ('SORRY WRONG NUMBER')  ( SKIP,A);
                GO TO COPY;
             END;
        END;
        DO I=1 TO N;
                                                              /*  18  */
             IF K(L1,I)¬=K(L2,I) THEN
                                                              /*  26  */
             IF K(L1,I)<K(L2,I) THEN
             GO TO MOVE_L1; ELSE
             GO TO MOVE_L2;
             IF K(L1,I)=0 THEN
             GO TO PS_LOOP;
        END;
    PS_LOOP:
        DO I=1 TO NPS;
                                                              /*  18  */
             IF PS(L1,I)¬=PS(L2,I) THEN
                                                              /*  26  */
             IF PS(L1,I)<PS(L2,I) THEN
             GO TO MOVE_L1; ELSE
             GO TO MOVE_L2;
             IF PS(L1,I)=0 THEN
             GO TO EQUAL;
        END;
    EQUAL:  /* COMBINE ALGEBRAICALLY SIMILAR TERMS */
                                                              /*  19  */
        C=COEF(L1)+COEF(L2);
                                                              /*  20  */
        IF F1=0 THEN  /*  IF F1 IS ZERO, A2 IS NOT SAVED  */
                                                              /*  21  */
        DO;
             I=NXT(L2);
             NXT(L2)=UU;
             UU=L2;
             L2=I;
        END; ELSE
                                                              /*  22  */
        L2=NXT(L2);
                                                              /*  23  */
        IF C=0 THEN
                                                              /*  24  */
        DO;
             I=NXT(L1);
             NXT(L1)=UU;
```

```
ENZ_EQ: PROC OPTIONS(MAIN);

136                UU=L1;
137                L1=I;
138                IF L3=0 THEN
139                IF C ¬= 0 THEN
140                A1,L3=I; ELSE A1 = L1; ELSE
142                NXT(L3)=I;
143           END; ELSE
                                                        /*  25  */
144           DO;
145                COEF(L1)=C;
146                L3=L1;
147                L1=NXT(L1);
148           END;
149           GO TO ADD_LOOP;
150      COPY:     DO;
                                                        /*  11  */
151                DO WHILE(L2 ¬= 0);
                                                        /*  12  */
152                     IF UU=0 THEN
153           DO;
                                                        /*  13  */
154              GO TO OVERFLOW;
155           END;
                                                        /*  14  */
156                     L=NXT(UU);
157                     EL(UU)=EL(L2);
158                     L3=NXT(L2);
159                     NXT(UU)=0;
160                     IF A1=0 THEN
161                     A1=UU; ELSE
162                     NXT(L1) = UU;
163                     L1 = UU;
164                     L2=L3;
165                     UU=L;
166                END;
167                                        GO TO A30;
168                END;
169      MOVE_L1:
                                                        /*  27  */
           L3=L1;
170        L1=NXT(L1);
171        GO TO ADD_LOOP;
172      MOVE_L2:  /*  MERGE TERM OF A2 INTO A1  */
                                                        /*  28  */
           IF F1=0 THEN
                                                        /*  32  */
173        L=L2; ELSE
174        DO;
                                                        /*  29  */
175             IF UU=0 THEN
176        DO;
                                                        /*  30  */
```

```
ENZ_EQ: PROC OPTIONS(MAIN);

177             GO TO OVERFLOW;
178        END;
                                                        /*  31  */
179             L=UU;
180             UU=NXT(UU);
181             EL(L)=EL(L2);
182        END;
                                                        /*  33  */
183        L2=NXT(L2);
184        NXT(L)=L1;
185        IF L3=0 THEN
186        A1=L; ELSE
187        NXT(L3)=L;
188        L3=L;
189        GO TO ADD_LOOP;
190                          A30: ;
191      END ADD;
192        DCL MULT ENTRY(FIXED BIN(16),FIXED BIN(16),FIXED BIN(16),
                        FIXED BIN(16));
                                                        /*   1  */
193      MULT: PROC(M1,F,M2,P);  /* MULTIPIES EXPRESSION M1 BY TERM M2; */
194        DCL (M1,F,M2,P) FIXED BIN(16);   /*  RESULT  'IN' P */
195        DCL (L1,L3,I,J,L) FIXED BIN(16);
                                                        /*   2  */
196        L1=M1;
                                                        /*   3  */
197        IF F=0 THEN
                                                        /*   4  */
198        M1=0;
                                                        /*   5  */
199        L3=0;
200        IF M2=0 THEN
201        DO;
                                                        /*   6  */
202             P=0;
203             RETURN;
204        END;
205      MULT_LOOP:
                                                        /*   7  */
           IF L1=0 THEN
206        DO;
                                                        /*   8  */
207             IF L3=0 THEN
                                                        /*   9  */
208             P=0;
209             RETURN;
210        END;
                                                        /*  10  */
211        IF UU=0 THEN
212        DO;
                                                        /*  11  */
213           GO TO OVERFLOW;
```

```
ENZ_EQ: PROC OPTIONS(MAIN);

214         END;                                                    /*  12  */
215         I,J=1;
216         L=0;
217     FAC_LOOP1:                                                  /*  13  */
            IF I>N THEN
218         GO TO X; ELSE
219         IF K(L1,I)=0 THEN                                       /*  20  */
220      X: DO;
221               DO J=J TO N WHILE(K(M2,J)¬=0);
222                    L=L+1;
223                    IF L>N THEN
224                                   DO;
225                                   PUT EDIT ('228 ') (A);
226                    GO TO ERROR;
227                                   END;
228                    K(UU,L)=K(M2,J);
229               END; IF L<N THEN K(UU,L+1)=0;
232               GO TO PS_S;
233         END;                                                    /*  14  */
234         IF J>N THEN
235         GO TO Y; ELSE
236         IF K(M2,J)=0 THEN                                       /*  21  */
237      Y: DO;
238               DO I=I TO N WHILE(K(L1,I)¬=0);
239                    L=L+1;
240                    IF L>N THEN
241                                   DO;
242                                   PUT EDIT ('242 ') (A);
243                    GO TO ERROR;
244                                   END;
245                    K(UU,L)=K(L1,I);
246               END;
247               IF L<N THEN
248               K(UU,L+1)=0;
249               GO TO PS_S;
250         END;                                                    /*  15  */
251         IF L>N THEN
252                                   DO;
253                                   PUT EDIT ('250 ') (A);
254         GO TO ERROR;
255                                   END;                          /*  16  */
256         L=L+1;                                                  /*  17  */
257         IF K(L1,I)>K(M2,J) THEN                                 /*  18  */
```

```
ENZ_EQ: PROC OPTIONS(MAIN);

258         DO;
259              K(UU,L)=K(M2,J);
260              J=J+1;
261         END; ELSE                                               /*  19  */
262         DO;
263              K(UU,L)=K(L1,I);
264              I=I+1;
265         END;
266         GO TO FAC_LOOP1;
267      PS_S:                                                      /*  22  */
            I,J=1;
268         L=0;
269      FAC_LOOP2:                                                 /*  23  */
            IF I>NPS THEN
270         GO TO Z; ELSE
271         IF PS(L1,I)=0 THEN                                      /*  30  */
272      Z: DO;
273               DO J=J TO NPS WHILE(PS(M2,J)¬=0);
274                    L=L+1;
275                    IF L>NPS THEN
276                                   DO;
277                                   PUT EDIT ('271 ') (A);
278                    GO TO ERROR;
279                                   END;
280                    PS(UU,L)=PS(M2,J);
281               END;
282               IF L<NPS THEN
283               PS(UU,L+1)=0;
284               GO TO PROD;
285         END;                                                    /*  24  */
286         IF J>NPS THEN
287         GO TO W; ELSE
288         IF PS(M2,J)=0 THEN                                      /*  31  */
289      W: DO;
290               DO I=I TO NPS WHILE(PS(L1,I)¬=0);
291                    L=L+1;
292                    IF L>NPS THEN
293                                   DO;
294                                   PUT EDIT ('283 ') (A);
295                    GO TO ERROR;
296                                   END;
297                    PS(UU,L)=PS(L1,I);
298               END;
299               IF L<NPS THEN
300               PS(UU,L+1)=0;
301               GO TO PROD;
```

```
302         END;                                                          /*  25  */
303         IF L>N THEN
304                                 DO;
305                                 PUT EDIT ('293 ') (A);
306         GO TO ERROR;
307                                 END;
                                                                          /*  26  */
308         L=L+1;
                                                                          /*  27  */
309         IF PS(L1,I)>PS(M2,J) THEN
                                                                          /*  28  */
310         DO;
311              PS(UU,L)=PS(M2,J);
312              J=J+1;
313         END; ELSE
                                                                          /*  29  */
314         DO;
315              PS(UU,L)=PS(L1,I);
316              I=I+1;
317         END;
318         GO TO FAC_LOOP2;
                                                                          /*  32  */
319    PROD: COEF(UU)=COEF(L1)*COEF(M2);
                                                                          /*  33  */
320         IF L3=0 THEN
321         P=UU; ELSE
322         NXT(L3)=UU;
323         L3=UU;
324         UU=NXT(UU); NXT(L3)=0;
                                                                          /*  34  */
326         IF F=0 THEN
                                                                          /*  35  */
327         DO;
328              L=NXT(L1);
329              NXT(L1)=UU;
330              UU=L1;
331              L1=L;
332         END; ELSE
                                                                          /*  36  */
333         L1=NXT(L1);
334         GO TO MULT_LOOP;
335    ERROR:
                                                                          /*  37  */
         PUT EDIT('TOO MANY FACTORS IN A TERM') (SKIP(2),A);
336         STOP;
337    END MULT;
       /*
          PROCESSING:
       */
338    DO_IT:
                                                                          /*  24  */
```

ENZ_EQ: PROC OPTIONS(MAIN);

```
339         IF ERR = 1 THEN
            GO TO FIN;
                                                                          /*  25  */
340         DO I=1 TO N;  /* THIS LOOP GENERATES DESCRIPTIONS OF THE TERMS IN*/
                                                                          /*  26  */
341              IF UU=0 THEN    /* THE EXPANSION OF THE DETERMINANT */
342         DO;
                                                                          /*  27  */
343            GO TO OVERFLOW;
344         END;
                                                                          /*  28  */
345              EX(I)=UU;
346              NT(I)=1;
347              COEF(UU)=SYN(I-1);
348              I1=0;
349              DO J=I TO N-1;
350                   IF J=I THEN
351                   I1=I1+1;
352                   I1=I1+1;
353                   K  (UU,J)=I1;
354              END;
                                                                          /*  29  */
355              L=UU;
356              UU=NXT(UU);
357              NXT(L)=0;
                                                                          /*  30  */
358              DO J=1 TO N-2;
                                                                          /*  31  */
359                   L=EX(I);
360                   L2=0;
361                   L3=SYN(N-J-2);
                                                                          /*  32  */
362    EXPAND_LOOP:   DO I1=1 TO N-J;
                                                                          /*  33  */
363                        IF A(J+1,K(L,J+I1-1))¬=0 THEN
                                                                          /*  34  */
364                        IF J=N-2 THEN
                                                                          /*  35  */
365                        IF A(N,K(L,N-I1))¬=0 THEN
366                        GO TO EXPAND; ELSE; ELSE
368    EXPAND:             DO;
                                                                          /*  36  */
369                             IF UU=0 THEN
370         DO;
                                                                          /*  37  */
371            GO TO OVERFLOW;
372         END;
                                                                          /*  38  */
373                        IF L2=0 THEN
374                        EX(I)=UU; ELSE
375                        NXT(L2)=UU;            /*NEW ELEMENT */
376                        L2=UU;               /*LAST ELEMENT IN LIST */
```

```
ENZ_EQ: PROC OPTIONS(MAIN);

                        UU=NXT(UU);
          NXT(L2)=0;
                        NT(I)=NT(I)+1;
                                                          /*  39   */
                        DO I2=1 TO J-1;              /*COPIES UNCHNGD*/
                             K  (L2,I2)=K  (L,I2);
                        END;
                                                          /*  40   */
                        COEF(L2)=COEF(L)*SYN(I1-1);
                        IF L3=-1 THEN
                        COEF(L2)=COEF(L2)*SYN(I1);
                                                          /*  41   */
                        L1=I1+J-1;
                        DO I2=J TO N-1;
                             IF L1>N-1 THEN         /*DOES PERMUTING*/
                             L1=J;
                             K  (L2,I2)=K  (L,L1);
                             L1=L1+1;
                        END;
                    END EXPAND;
               END EXPAND_LOOP;
                                                          /*  42   */
               L1=NXT(L);                    /*PUT ORIGINAL ELEMENT BACK */
               NXT(L)=UU;
               UU=L;
               L=L1;
               NT(I)=NT(I)-1;
                                                          /*  43   */
               IF L¬=0 THEN
               GO TO EXPAND_LOOP;
           END;
      END;                        /*END OF DESCRIPTION OF DETERMINANTS */
                                                          /*  44   */
      DO I=1 TO N;
          PUT EDIT ('EX(',I,')=') (SKIP,A,F(1),A);
          L=EX(I);
          DO WHILE(L ¬= 0);
           IF COEF(L)<0 THEN
           PUT EDIT('-') (SKIP,A); ELSE
           PUT EDIT('+') (SKIP,A);
            PUT EDIT (('A(',J+1,',',K(L,J),') ' DO J=1 TO N-1))
                     (A,F(1),A,F(1),A);
            L=NXT(L);
          END;
      END;
   /*
      EVALUATE DETERMINANTS.
   */
                                                          /*  45   */
      IF UU = 0 THEN
      DO;
         GO TO OVERFLOW;
```

```
ENZ_EQ: PROC OPTIONS(MAIN);

      END;
      M1 = UU;
      UU = NXT(UU);
      EL(M1) = 0;
      COEF(M1) = -1;
                                                          /*  46   */
      DO I=1 TO N;
                                                          /*  47   */
           PRSM=0;
           L=EX(I);
                                                          /*  48   */
           DO WHILE(L¬=0);
                                                          /*  49   */
                I1=0;
                I2=A(2,K(L,1));   /*GETS 1ST FACTOR OF TERM */
                CALL ADD(I1,1,I2);
                                                          /*  50   */
                DO J=2 TO N-1;                          /* MULT TERMS */
                                                          /*  51   */
                     I2=A(J+1,K(L,J));
                          L2=0;
                                                          /*  52   */
                     DO WHILE(I2¬=0);                   /* MULTI A TERM */
                          CALL MULT(I1,1,I2,L1);
                          CALL ADD(L2,0,L1);
                          I2=NXT(I2);
                     END;
                     L1=I1;
                                                          /*  53   */
END_LIST_LOOP1:      I2=NXT(L1);
                     IF I2¬=0 THEN
                     DO;
                          L1=I2;
                          GO TO END_LIST_LOOP1;
                     END;
                     NXT(L1)=UU;
                     UU=I1;
                     I1=L2;
                END;
                                                          /*  54   */
           IF COEF(L) < 0 THEN
                                                          /*  55   */
           CALL MULT(I1,0,M1,I1);
                                                          /*  56   */
                PRSM(0)=I1;
                DO J=1 TO 17 WHILE(PRSM(J)¬=0);
                     CALL ADD(PRSM(J),0,PRSM(J-1));
                END;
                PRSM(J)=PRSM(J-1);
                PRSM(J-1)=0;
                                                          /*  57   */
                L1=NXT(L);
```

```
ENZ_EQ: PROC OPTIONS(MAIN);


458                     NXT(L)=UU;
459                     UU=L;
460                     L=L1;
461                END;
462                L1=0;

463                DO J=1 TO 17;
464                     IF PRSM(J)¬=0 THEN
465                     CALL ADD(L1,0,PRSM(J));
466                END;
467                EX(I)=L1;

468                CALL PRNT('EX('||I||')=',EX(I));
469           END;
470           EXO=EX(1);                         /*DENOMINATOR */
471           DO I=2 TO N;
472                CALL ADD(EXO,0,EX(I));
473           END;
474           CALL PRNT('EXO',EXO);
475          IF P7 = 'PUNCH' THEN CALL PUNCH(EXO);
477           GOTO FIN;
478      OVERFLOW:PUT EDIT ('OVER')(A);
479           GOTO FIN;
480          DCL PRNT ENTRY (CHAR(*),FIXED BIN(16));
481          PRNT: PROC(LABEL,PTR);
482          DCL LABEL CHAR(*), (J,L,PTR)FIXED BIN(16);
483               PUT EDIT  (LABEL)       (SKIP(3),A,F(1),A);
484          L = PTR;
```

/* 58 */

/* 59 */

```
485               DO WHILE (L ¬= 0);
486                PUT EDIT(COEF(L),' ') (SKIP,X(4),F(3),A);
487                 DO K11 = 1 TO N WHILE (K(L,K11) ¬= 0);
488                 PUT EDIT (KS(K(L,K11))) (X(1),A);
489                 END;
490                 DO K12 = 1 TO NPS WHILE(PS(L,K12) ¬= 0);
491                 PUT EDIT (PSS(PS(L,K12)))  (X(1),A);
492                 END;
493                 L=NXT(L);
494               END;
495          END PRNT;
496          DCL PUNCH ENTRY (FIXED BIN(16));
497          PUNCH:PROC(PTR);
498          DCL (J,L,PTR) FIXED BIN(16);
499          L = PTR;
500          DO WHILE (L ¬= 0);
501          PUT FILE(OUT) EDIT('+') (SKIP,COL(6),A);
502          DO K1=1 TO N WHILE (K(L,K1) ¬= 0);
503          IF K1 > 1 THEN PUT FILE(OUT) EDIT('*') (A);
505          PUT FILE(OUT) EDIT (KS(K(L,K1))) (A);
506          END;
507          DO K2 = 1 TO NPS WHILE(PS(L,K2) ¬= 0);
508          PUT FILE(OUT)  EDIT('*',PSS(PS(L,K2)),'(I)') (A,A,A);
509          END;
ENZ_EQ: PROC OPTIONS(MAIN);
510          PUT FILE(OUT) EDIT(' + ') (A);
511          L=NXT(L);
512          END;
513          END PUNCH;
514        FIN: PUT SKIP LIST('THE END');  END ENZ_EQ;
```

Appendix III

Plotting and Statistical Analysis of Kinetic Data Using the OMNITAB Program

Initial rate data may be analyzed according to the usual method of weighted least squares by a computer program written in the OMNITAB language. This language, developed by the National Bureau of Standards (1), is well suited to the analysis of kinetic data because of its extreme simplicity in programming (considerably easier then Fortran, though not so general or powerful) and the ease with which the model equation used to fit the data can be changed to forms other than the simple Michaelis-Menten case.

OMNITAB operations are carried out on an imaginary worksheet with 49 columns and 101 rows using commands in English in much the same way one would use to explain the problem to someone who was to do the calculations on a desk calculator. For example, to add two columns of numbers one can use the command,

ADD COLUMN 12 TO COL. 15 AND STORE THE RESULTS IN COL. 4.

OMNITAB actually "sees" only the words ADD, 12, 15, and 4 while the other words are comments there only for the convenience of the programmer. Using similar, easily understood commands, one reads in the initial velocities together with the substrate and inhibitor concentrations, computes the reciprocal velocities and relevant substrate or inhibitor expressions according to the model under consideration, storing each new variable in separate columns.

The command used for fitting the data has the form "FIT Y IN COL. 1, WEIGHTS IN 2, X IN 3,4,5, STORE THE COEFFICIENTS IN 6 AND THE RESIDUALS IN COL. 7," where Y is the dependent variable (reciprocal velocities) and "X" is the set of independent variables previously computed (and stored in columns 3,4,5 in this example). The best least-squares coefficients of the independent variables together with their standard deviations and the goodness of fit are stored in column 6 and are also automatically printed out together with other information on the fit (t and F statistics, means and other data for analysis of variance). This single FIT command produces two pages of output describing results.

One can, of course, use any of the several alternative linear forms for the model equation provided that the weights are properly treated. The weight, W_i, of the ith data point is given given by (2):

$$W_i = \frac{1/\sigma_i^2}{\frac{1}{N}\sum_{i=1}^{N}(1/\sigma_i^2)} \qquad (1)$$

where σ_i is the standard deviation of the dependent variable and N is the number of points. The standard deviation should, strictly speaking, be measured for each data point but this is rarely done because of the rather large number of measurements this would require. In order to have some basis for assigning the weights other than intuition, one can measure the standard deviation of the velocities at just two velocities (one on the high side, one on the low) by replicated measurements. The variance is then assumed to follow an expression of the form

$$\sigma^2(v_i) = c \cdot v_i^{\alpha} , \tag{2}$$

where c and α are constants determined from the two measured standard deviations. The weights take the form for the reciprocal expression

$$W(1/v_i) = \frac{N \cdot 1/\sigma^2(1/v_i)}{\sum_i 1/\sigma^2(1/v_i)} = \frac{N \cdot v_i^4/\sigma^2(v_i)}{\sum_i v_i^4/\sigma^2(v_i)}. \tag{3}$$

Substitution of (2) into (3) gives finally

$$W(1/v_i) = \frac{N \cdot v_i^{4-\alpha}}{\sum_i v_i^{4-\alpha}}. \tag{4}$$

For equal weights in the reciprocal plot one would use $\alpha = 4$ and for equal weights in the direct plot, $\alpha = 0$. In order to test these equations all of the fits using each of these values for α as well as experimentally determined values for α are made. The usual graphical analysis by hand may also be carried out in order to obtain some notion about the sensitivity of the kinetic parameters to the different weighting schemes. The values for each of the kinetic parameters and their standard deviations are calculated from the coefficients and their standard deviations obtained by the program, using the standard formulas (3) for the propagation of errors, assuming they are uncorrelated. Another consideration is that the errors in the dependent variable should be normally distributed about their mean. To examine this problem one should make 30 or more repeated measurements of the initial velocity under the same conditions. One can then find the standard deviation of the velocity of the mean, and the normalized third and fourth moment about the mean, β_1 and β_2. The values for a normal distribution are 0.0 and 3.0, respectively. The values found may therefore be judged as to whether they are sufficiently close to the values for the normal distribution so that the method of least squares is not seriously jeopardized.

A simple OMNITAB program for plotting and analyzing initial rate data is illustrated below. In order to obtain the computer weighted best fit to the experimental data for a particular model, the computer calculated coefficients may be used to plot the curves by hand. Alternatively, it may be desirable to write a plotting program in Fortran.

The ONITAB Computer Program. The most general model for reversible inhibition in a Sequential Bi Bi Mechanism which is first order with respect to each substrate is

$$\frac{1}{v} = \frac{1}{V_1}\left[\left(1 + \frac{I}{K_i}\right) + \frac{K_a}{A}\left(1 + \frac{I}{K_{ii}}\right) + \frac{K_b}{B}\left(1 + \frac{I}{K_{iii}}\right) + \frac{K_{ia}K_b}{AB}\left(1 + \frac{I}{K_{iv}}\right)\right] \tag{5a}$$

In the OMNITAB program shown here Eq. (5a) may be expressed in terms of independent and dependent variables, and coefficients where A is the varied substrate and B is held constant as follows:

$$\frac{1}{v} = \left[C_1(1) + C_2(I) + C_3\left(\frac{1}{A}\right) + C_4\left(\frac{I}{A}\right)\right] \tag{5b}$$

where

$$C_1 = \frac{1}{V_1}\left(1 + \frac{K_b}{B}\right) \tag{5c}$$

$$C_2 = \frac{1}{V_1}\left(\frac{1}{K_i} + \frac{K_b}{K_{iii}B}\right) \tag{5d}$$

$$C_3 = \frac{1}{V_1}\left(K_a + \frac{K_{ia}K_b}{B}\right) \tag{5e}$$

$$C_4 = \frac{1}{V_1}\left(\frac{K_a}{K_{ii}} + \frac{K_{ia}K_b}{K_{iv}B}\right) \tag{5f}$$

In order to change the model, only the "FIT" command need be altered. Thus, to FIT the data to Eq. (5b), the FIT statement would read: "FIT Y IN 11, WTS IN 20, X IN 5, 6, 12, 35, COEF IN 22, RES IN 23". The coefficients (5b - 5f) are exhibited in the computer printout.

In the case of a competitive inhibitor for A, reference to Column 5 would be deleted from the FIT statement for noncompetitive inhibition and coefficient C_2 would be zero.

In the absence of inhibitor in Eq. (5a) (I = 0), the FIT statement would read: "FIT Y IN 11, WTS IN 20, X IN 6, 12, 13, 14, COEF IN 22, RES IN 23". Coefficients C_1, C_2, C_3, and C_4 would be $1/V_1$, K_a/V_1, K_b/V_1, and $K_{ia}K_b/V_1$, respectively. The data entered in Column 3 (the concentration of B), would of course, vary in this example.

The basic uninhibited Sequential Bi Bi program may also be modified to accommodate the Ping Pong Bi Bi rate equation, by listing 6, 12, 13 after "X IN" the FIT statement. It is also

possible to use the basic program for unireactant equations by simply omitting reference to B terms in the fit statement.

Models involving power terms; e.g. A^2, can be conveniently incorporated into the basic OMNITAB program. This can be accomplished with a statement such as "RAISE COL ++ TO THE + 2.0 POWER AND STORE IN COL +++", followed by a command to take the reciprocal of COL +++ and store it in COL +; i.e., "DIVIDE 1.0 BY COL +++ AND STORE IN COL +". Col + will then appear in X in the FIT statement.

Two measures for the goodness of the fit of the data used to obtain the kinetic parameters of the assumed model are the average absolute % difference between the observed and calculated values for 1/v, <% diff>, and the standard deviation of the fit, defined as

$$s = \left\{ \frac{1}{\nu} \sum_{i=1}^{N} W_i \left[\left(\frac{1}{v_i} \right)_{\text{measured}} - \left(\frac{1}{v_i} \right)_{\text{calculated}} \right]^2 \right\}^{1/2} \qquad (6)$$

where $\nu = N - n - 1$ is the number of degrees of freedom left after fitting the N data points to the n + 1 adjustable parameters in the model. The values for s and <% diff> cited when an inhibitor is used are those for the data fitted by the model with no constraints on the other kinetic parameters. The values of the kinetic parameters are obtained in a manner analogous to that used in the traditional reciprocal plot method, i.e., the values for K_a, K_b, and $K_{ia}K_b$ are obtained from the data taken in the absence of an inhibitor by using model 5a and then they are used to obtain the inhibition constants through the analysis of data taken in the presence of an inhibitor.

In addition to the t-values of the coefficients and the measures, S^2 and <% diff>, of the goodness of fit of the data to the proposed model, the program carries out a very extensive analysis of the residuals which can also be used to detect any systematic variation of the error with velocity and substrate concentration. This information can be used to make more reliable and quantifiable rigorous judgements about the important question of the appropriateness of an hypothesized kinetic model to explain the data than is possible by any graphical procedure.

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```
     OMNITAB SEQUENTIAL BI BI PROGRAM
     $
     $      H.J. FROMM
     $         IOWA STATE UNIVERSITY
     $            JANUARY 1975
     $
READ THE FOLLOWING DATA INTO COLS 1,2,3,5                    $(V,A,B,I)
0.20E-06    0.04E-03    0.04E-03    0.00
0.25E-06    0.05E-03    0.04E-03    0.00
0.25E-06    0.07E-03    0.04E-03    0.00
0.33E-06    0.10E-03    0.04E-03    0.00
0.33E-06    0.20E-03    0.04E-03    0.00
0.07E-06    0.04E-03    0.04E-03    2.0E-03
0.09E-06    0.05E-03    0.04E-03    2.0E-03
0.11E-06    0.07E-03    0.04E-03    2.0E-03
0.14E-06    0.10E-03    0.04E-03    2.0E-03
0.18E-06    0.20E-03    0.04E-03    2.0E-03
READ ALPHA INTO COL 4
1.89
     $
     $  FOR UNIT WTS. IN RECIPROCAL PLOTS PUT ALPHA = 4
     $  FOR UNIT WTS. IN DIRECT PLOTS PUT ALPHA = 0
     $  OTHERWISE DETERMINE ALPHA EXPERIMENTALLY
     $
     $
     $  CALCULATE THE INDEPENDENT VARIABLES
     $
ADD 1.0 TO 0.0 AND STORE IN COL 6                            $(COEF 1 IN RATE EQ)
DIVIDE 1.0 BY COL 1 AND STORE IN COL 11                      $(1/V(EXP))
DIVIDE 1.0 BY COL 2 AND STORE IN COL 12                      $(1/A)
DIVIDE 1.0 BY COL 3 AND STORE IN COL 13                      $(1/B)
MULTIPLY COL 12 BY COL 13 AND STORE IN COL 14                $(1/A*B)
MULTIPLY COL 12 BY COL 5 AND STORE IN COL 35                 $(I/A)
MULTIPLY COL 13 BY COL 5 AND STORE IN COL 36                 $(I/B)
MULTIPLY COL 14 BY COL 5 AND STORE IN COL 37                 $(I/A*B)
     $
     $  CALCULATE THE WEIGHTS
     $
CHANGE THE SIGN OF THE ELEMENT IN COL 4                      $(-ALPHA)
RAISE COL 1 TO THE +4.0 POWER AND STORE IN COL 15            $(V**4)
RAISE COL 1 TO THE *1.4* POWER AND STORE IN COL 16           $(V**(-ALPHA))
MULTIPLY COL 15 BY COL 16 AND STORE IN COL 17                $(V**(4-ALPHA))
DIVIDE 1.0 BY COL 17 AND STORE IN COL 18                     $(1/V**(4-ALPHA))
AVERAGE OF COL 17 AND STORE IN COL 19                        $(WEIGHTS)
DIVIDE COL 17 BY COL 19 AND STORE IN COL 20                  $(NORMALIZED WEIGHTS)
     $
     $  ELIMINATE VARIABLES NOT IN MODEL FROM FIT STATEMENT
     $
     $  I IN COL 5, 1 IN COL 6, 1/A IN COL 12, 1/B IN COL 13, 1/A*B IN
     $  COL 14, I/A IN COL 35, I/B IN COL 36, I/(A*B) IN COL 37
     $
FIT Y IN 11, WTS IN 20, X IN 5,6,12,35, COEF IN 22, RES IN 23
     $
     $  CALCULATE % DIFFERENCE
     $
ADD COL 23 TO COL 11 AND STORE IN COL 25
RAISE COL 25 TO THE -1.0 POWER AND STORE IN COL 26
SUBTRACT COL 26 FROM COL 1 AND STORE IN COL 27
DIVIDE COL 27 BY COL 1 AND STORE IN COL 28
MULTIPLY COL 28 BY 100.0 AND STORE IN COL 29
ABS COL 29 AND STORE IN COL 30
AVERAGE COL 30 AND STORE IN COL 31
     $
     $  PRINT OUT THE RESULTS
     $
HEAD COL 1/ V
HEAD COL 2/ A
HEAD COL 3/ B
HEAD COL 5/ I
HEAD COL 11/ 1/V(EXP)
HEAD COL 20/ WEIGHTS
HEAD COL 23/ RESIDUALS
HEAD COL 29/ % DIFFERENCE
PRINT COLS 1,2,3,5,20,23,29,11
FORMAT A(//,3X,'AVG. % DIFFERENCE IS ',F9.4)
ABRIDGE A ROW 1 OF COL 31
     $
     $  DO A COMPLETE ANALYSIS OF THE RESIDUALS IN ORDER TO DETECT ANY
     $  SYSTEMATIC ERROR IN THE DATA OR MISFIT DUE TO AN INAPPROPRIATE
     $  MODEL.
STATISTICAL ANALYSIS OF COL 23
     $
     $  CREATE THE PLOTS.
     $
TITLEX 1/A
TITLEY 1/V
PLOT COL 11 VS COL 12
TITLEX 1/B
PLOT COL 11 VS COL 13
TITLEY RESIDUALS
TITLEX V
PLOT COL 23 VS COL 1
STOP
```

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