Transients are all.

They being born must die and being dead
are glad to be at rest.

"John Barleycorn: the memoirs
of an alcoholic", by Jack London

PREFACE

Rapid reaction techniques are essential for the elucidation of the chemical mechanisms of enzyme reactions. The steady-state method can only lead to limited information about the rate constants of specific kinetic steps and cannot provide the rates of formation and breakdown of reaction intermediates. To obtain these, fast reaction techniques have to be used. However, the fast reaction techniques involving flow methods have proceeded up to now without an accompanying and unified theory of the transient-phase kinetics. This thesis provides such a theory in unified terms for model enzyme mechanisms. An attempt has been made to correlate some of the published data with the present theory. However, its full use has to come from more accurate and more careful experimental investigation, the importance of the relative concentrations of the various reagents being born in mind.

Part of the work described in this thesis has been published; the remainder is under preparation for publication:

1. Non-Steady-State Kinetics for a Double Intermediate Enzyme Mechanism: The Case of High Enzyme Concentrations, N. H. Hijazi and K. J. Laidler, Can. J. Chem., 50, 1440 (1972).
2. Transient-Phase and Steady-State Kinetics for Enzyme Activation, N. H. Hijazi and K. J. Laidler, Can. J. Bioch., 51, 806 (1973).
3. Transient-Phase and Steady-State Kinetics for Inhibited Enzyme Systems. I Single Intermediate Mechanisms, N. H. Hijazi and K. J. Laidler, Can. J. Bioch., 51, 815 (1973).
4. Transient-Phase and Steady-State Kinetics for Inhibited Enzyme Systems. II Double-Intermediate Mechanisms, N. H. Hijazi and K. J. Laidler, Can. J. Bioch., 51, 822 (1973).

5. Transient-Phase and Steady-State Kinetics for Enzyme Systems Involving Two Substrates, N. H. Hijazi and K. J. Laidler, Can. J. Bioch., 51, 832 (1973).

6. Influence of Premixing on Transient-Phase Kinetics for Two-Substrate Systems, N. H. Hijazi and K. J. Laidler, Biochim., Biophys. Acta, in press.

7. Kinetic Equations for Consecutive Reversible Reactions with Special Reference to Protein Denaturation, N. H. Hijazi and K. J. Laidler, J. Chem. Soc., Faraday Trans. I, 68, 1235 (1972).

8. Transient-Phase Equations for Enzyme Systems and the Analysis of Experimental Results, N. H. Hijazi and K. J. Laidler, Biochim. Biophys. Acta, submitted for publication.

9. Transient-Phase of the Hydrolysis of p-Nitrophenylacetate by α-Chymotrypsin, R. J. Maguire, N. H. Hijazi and K. J. Laidler, Biochim. Biophys. Acta, submitted for publication.

10. Transient-Phase Kinetics of Simple Mechanisms with Nucleophiles, N. H. Hijazi and K. J. Laidler, manuscript in preparation.

11. Transient-Phase of Two Systems Exhibiting Co-Operativity in the Steady-State, N. H. Hijazi and K. J. Laidler, manuscript in preparation.

12. Transient-Phase Kinetics for Enzyme Reactions Involving Two Competing Substrates, N. H. Hijazi and K. J. Laidler, Can. J. Bioch., submitted for publication.

During the course of his studies at the University of Ottawa, the author has also published:

13. Dynamics of Collinear A + BC Systems, N.H. Hijazi and K.J. Laidler, J. Chem. Phys., 58, 349 (1973).

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ABSTRACT

Chapter One

The limitations of the steady-state method are discussed in the introduction. The advantages of the transient-phase methods are pointed out and their importance in the elucidation of the mechanisms of enzyme action are also discussed. The mathematical technique, Laplace-Carson transformation technique, is briefly discussed.

A review of the theoretical treatments for the transient-phase kinetics of single and double intermediate mechanisms is presented in this chapter.

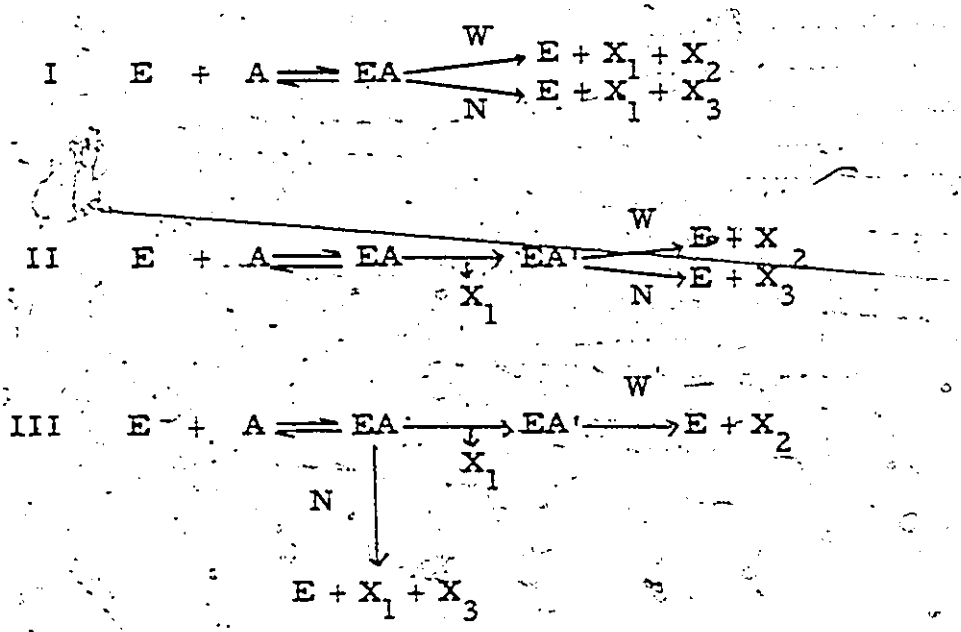
The Laplace-Carson transformation technique is used to solve the kinetic equations applicable to single and double intermediate mechanisms under two simplifying assumptions (1) $a_o \gg e_o$ (2) $e_o \gg a_o$. The results are analyzed and methods of calculating the specific rate constants are presented.

The mechanism of two competing substrates is also considered in this chapter, and its transient-phase kinetics analyzed.

Chapter Two

The single and double intermediate mechanisms in the presence of a nucleophile, N, can lead to three products instead of the usual two products in the presence of water, W, as the only nucleophile.

The transient-phase as well as the steady-state kinetics will be modified in the presence of the nucleophile. This chapter examines the transient-phase and steady-state kinetics of three basic mechanisms in the presence of an added nucleophile:



Methods of analyzing the data to calculate the specific rate constants are described.

It is found that a transient-phase investigation, in which a measurement is made of the induction period for any product, leads to an excellent criterion for distinguishing the three mechanisms.

Chapter Three

A non-steady-state analysis has been worked out for two mechanisms in which an activator Q can become attached to an enzyme-substrate complex EA, the species EAQ breaking down more rapidly than EQ. It is shown that if EAQ breaks down into EQ + product there can be no steady state. If, however, EAQ breaks down into E + Q + product, the transient phase is followed by a steady state in which the product vs. time curve is linear. A special case of this mechanism is when Q is the substrate (substrate activation). Some published kinetic data, on carboxypeptidase A, are analyzed with reference to the derived equations.

Chapter Four

Sigmoid kinetics in the steady state is acquiring great importance in terms of feedback inhibitions as well as co-operative phenomena in metabolic processes. It is sometimes difficult to distinguish the reaction mechanisms on the basis of steady-state kinetics. This chapter is an initial effort to study the sigmoidal kinetic phenomenon in the transient phase where the rates of formation and breakdown of intermediates can be detected.

The transient-phase kinetic equations applicable to two reaction schemes are presented. The two reaction mechanisms

I $$E + A \rightleftharpoons EA \longrightarrow E'A \begin{cases} \longrightarrow E' + X \\ \longrightarrow E' + A \end{cases}$$

II $$E + A \rightleftharpoons EA \xrightarrow{A} EA_2 \longrightarrow E + A + X$$

are known to exhibit co-operativity in the steady state. In this chapter, methods of calculating some of the specific rate constants are described on the basis of transient-phase experiments.

Chapter Five

Equations for the pre-steady state and the steady state are derived for enzyme systems in which enzyme E, substrate A and inhibitor Q are present, the enzyme concentration being lower than the substrate and inhibitor concentrations. It is assumed that the mechanism involves a single intermediate EA. Equations for competitive, anticompetitive and pure non-competitive inhibition are derived. When the inhibition is reversible the transient phase is followed by the establishment of a steady state. Analysis of experimental results is discussed for each type of inhibition. If the inhibition is irreversible, there is no steady state.

Chapter Six

Equations for the pre-steady state and the steady state are derived for enzyme systems in which the enzyme E, the substrate A and an inhibitor Q are present together, the enzyme concentration being much lower than the concentrations of A and Q. Various mechanisms are considered, all of them involving two intermediates EA and EA' (e.g. an acylenzyme). When the inhibition is reversible the transient phase is followed by the establishment of a steady state. It is shown how experimental pre-steady-state and steady-state results can be analyzed to obtain rate constants, including those for the binding of inhibitor. If the binding of inhibitor is irreversible there is no steady state.

Chapter Seven

The transient-phase and steady-state equations are derived for four enzyme mechanisms involving two substrates, namely (1) Theorell-Chance mechanism, (2) ping pong bi bi mechanism, (3) ordered ternary-complex mechanism, and (4) random ternary-complex mechanism. In each case, a discussion is presented of the way in which the individual rate constants can be separated on the basis of experimental transient-phase investigations.

Chapter Eight

Transient-phase kinetic equations are worked out for two-substrate enzyme reactions occuring by the Theorell-Chance, Ping Pong Bi Bi and ordered Ternary-Complex mechanisms (the equations for the Random Ternary-Complex mechanism are too complex to be useful). Two sets of premixing conditions were considered (i) E not premixed with the first substrate A, i.e. $E|A|B$, $E|A+B$ or $E+B|A$; and (ii) E premixed with the first substrate A, i.e. $E+A|B$. For each mechanism and premixing conditions two cases were treated: (a) enzyme concentration limiting, i.e. $e_o \ll a_o, b_o$, and (b) concentration of first substrate A limiting, $a_o \ll e_o, b_o$. In all cases (a) gives rise to a transient phase followed by a steady state, the transient phase being represented by two or more exponential terms. In all cases (b) gives no steady state; the concentrations of products X and Y rise to a final value of a_o in a manner represented by two or more exponential terms. Premixing of type (ii) leads to a more rapid initial rise than that of type (i), in cases (a) and (b). Some results on horse liver alcohol dehydrogenase are shown to be consistent with the equations derived for a Theorell-Chance mechanism; there is no evidence for the participation of the two types of active sites on the enzyme.

Chapter Nine

A general discussion of the transient-phase kinetic equations developed in the previous chapters is presented. A correlation rule has been proposed where the number of exponentials is equal to the number of intermediates in any enzyme-catalyzed reaction under two conditions (1) the enzyme concentration is limiting (2) one of the reaction products is being followed as a function of time. It is also proposed that the sum of exponents, $\sum_{i=1}^{\lambda} \lambda_i$, is equal to the sum of all kinetic steps in the mechanism:

$$\sum_{i=1}^{n} \lambda_i = \sum_i k_i C_i + \sum_j k_j$$

where $k_i C_i$ are the bimolecular steps and k_j are the unimolecular steps. A general discussion is presented for analysis of experimental results.

Some of the published data on the transient-phase kinetics of myosin, alkaline phosphatase and α-chymotrypsin are reinterpreted and correlated with the theoretical results.

Chapter Ten

The interconversion of the native (N) and denatured (D) form of a protein sometimes exhibits simple exponential kinetic behaviour consistent with the mechanism $N \rightleftharpoons D$. Sometimes, however, logarithmic plots of the variation of a property y with time are of a biphasic character, which requires that at least one additional species X is involved. Three alternative mechanisms are then possible:

I $N \rightleftharpoons X \rightleftharpoons D$

II $N \rightleftharpoons D \rightleftharpoons X$

III $X \rightleftharpoons N \rightleftharpoons D$

If a kinetic study is made of some change in a property, y, the variation of y with time shows a number of patterns of behaviour. The present work is concerned with an analysis of these patterns, which are classified with respect to the three mechanisms. It is shown that some patterns are consistent only with mechanism I, and some only with II, but that certain patterns can occur with either II or III.

RESUME OF TRANSIENT-PHASE KINETICS

The kinetic equations in the transient phase have been obtained for a variety of model enzyme mechanisms. The cases considered include simple single-substrate-single-enzyme reactions, reactions in the presence of modifiers (activators, inhibitors, and nucleophiles) and two-substrate-single-enzyme mechanisms.

The common aspect of all cases treated is the mathematical technique, the Laplace-Carson transformation technique, that has been used to integrate the differential equations applicable in each case. This technique hasproved to be of great value in the context of pre-steady-state kinetics of enzyme reactions.

The variation of product concentrations with time can only be obtained under the limiting cases of (a) the substrate concentration is in large excess of the enzyme concentration, $a_o >> e_o$ and (b) the enzyme concentration is in large excess of the substrate concentration, $e_o >> a_o$. It is to be noted that most enzyme reactions are studied experimentally under condition (a), $a_o >> e_o$.

A general result that has evolved from the theoretical equations nnder the conditions of $a_o >> e_o$, is that the variation of the product concentration with time is of the form

$$x = vt + \beta + \sum_{i=1}^{n} \beta_i e^{-\lambda_i t}$$

where v is the steady state rate and $\beta = \sum_{i=1}^{n} \beta_i$ is the intercept of the extrapolated steady-state line on the product axis of an x vs t plot. n is found to be equal to the number of intermediates in the mechanism in all cases considered. A plot of ln $(x-vt-\beta)$ against t will consist of n linear portions if the values of the λ_i's are not too close to one another. The variation of initial

concentration will affect the values of the λ_i's so that by this method one will be able to resolve the number of the λ_i's which is also the number of detectable intermediates. From the slopes of the linear portions of the ln $(x-vt-\beta)$ vs t plots, the numerical values of the λ_i's can be calculated. These values are not absolute and should be regarded as orders of magnitude. These numerical values are also subject to the errors of graphical analysis.

It is also found that the sum of the λ_i's is equal to the sum of all bimolecular terms and the sum of all unimolecular terms in the kinetcis mechanism

$$\sum_{i=1}^{n} \lambda_i = \sum k_i c_k + \sum k_j$$

where k_i is a second-order rate constant, c_i is the reagent involved in that bimolecular step and k_j is a first-order rate constant. The summation is over all steps for both bimolecular and unimolecular processes.

The variation of $\sum_{i=1}^{n} \lambda_i$ with initial c_i is linear and hence all k_i values can be calculated as well as $\sum k_j$. To separate the values of k_j's, plots of $\pi_{i=1}^{n} \lambda_i$ and $v \pi_{i=1}^{n} \lambda_i$ versus c_i can be used (π denotes the product of the λ_i's and v is the steady-state rate). These functions are given for all the kinetic mechanisms treated in this thesis.

It should be emphasized that the theoretical treatment presented in this thesis is intended to apply to certain clear-cut mechanistic schemes. Actual experimental results may in many cases involve complications that will have to be dealt with by extensions of the theoretical foundations with which this thesis has been primarily concerned.

CHAPTER ONE

TRANSIENT-PHASE AND STEADY-STATE KINETICS OF SIMPLE SYSTEMS

Introduction

Kinetic studies are a major tool towards the elucidation and understanding of the molecular mechanisms of enzyme reactions. The investigation of enzyme-catalyzed reactions is a part of man's attempts to reveal the nature of biological processes. The problem involved in enzyme reactions as well as in other chemical reactions, is the detection of possible intermediates in a reaction mechanism. The detection of an intermediate involves the determination of its lifetime as well as its mode of formation and transformation. The measurement of short lifetimes poses a special problem when the lifetime is so short that conventional kinetic techniques cannot be employed. Measurements of short lifetimes need fast reaction techniques, in which the resolution time of the instrument is shorter than the lifetime of the species to be detected.

Enzyme kinetics has been investigated largely in the steady-state region [1-10]. At low enzyme concentrations, the initial substrate concentrations are assumed to be constant during the initial part of the steady-state reaction. Theoretically, under steady-state conditions, the rate of change with time of the concentration of any transient intermediate in the reaction mechanism is equated to zero. Thus the rate equations, after imposition of the steady-state condition, can be solved to obtain the initial rate of the reaction and certain steady-state parameters. These parameters involve specific rate constants for individual reaction steps. To separate the individual rate constants and detect the intermediates, fast reaction techniques must be used. These techniques are: stopped-flow, pressure-jump, temperature-jump, NMR, electron paramagnetic resonance, electric-field jump and waves, and ultrasonics. The steady-state approximation is not valid for a time interval known

as the transient phase or pre-steady-state phase of the reaction. It is of interest and importance to have a theory of the kinetic behavior before the steady state is established, since modern experimental techniques have permitted measurements to be made during this early period. It is the purpose of this thesis to present a unified mathematical approach to the solution of the complicated differential equations obtained from different enzyme mechanisms under some simplifying assumptions which are easily attainable in practice. It is also the purpose of this thesis to cast the analytical solutions obtained into a form which could most easily be applied to experimental results, the rate constants being calculated preferably from linear plots. Comparison with experiment will be made whenever possible.

Theoretical

Several theoretical treatments of transient-phase kinetics have been given. Laidler [11] solved the single-substrate-single-intermediate-single-product mechanism, and Ouellet and Laidler [12] extended the treatment to the case of two intermediates. Later, Ouellet and Stewart [13] applied the treatment to the single-substrate-double-intermediate-double-product case, and they were concerned with the kinetics of formation of the proeuct that is released first. In these treatments, the assumption was made that the concentration

of substrate is greatly in excess of that of the enzyme. The converse case, in which the enzyme is in excess, was treated by Kasserra and Laidler [14] and by Hijazi and Laidler [15].

All the above treatments considered the reaction to be irreversible except for the binding of the substrate A, $E + A \rightleftharpoons EA$. Darvey [16] solved the completely reversible scheme, but his results are too complicated to be applied to experimental results.

All the theoretical treatments of the transient-phase [11-23] have used one assumption or another in order to solve the complex differential equations that arise even in the simplest Michaelis-Menten scheme. The mathematical approach to solving the differential equations has varied from simple integration of linear differential equations with the appropriate boundary conditions [11-15] to the use of the operational calculus [16] as well as numerical integration [20].

The main two assumptions that have been used to simplify (linearize) the differential equations so that they can be solved analytically are: (1) the substrate concentration is in great excess such that it is safe to assume that it does not change significantly during the transient phase or in the initial part of the steady state; (2) the enzyme concentration is in excess so that its concentration does not change much during the transient phase. Under this condition, it has been shown [14-15], and will be shown in this work, that there is no establishment of the steady state; the product concentration varies exponentially with time and reaches a limiting value at large values of time equal to the initial concentration of the limiting reagent, which is the substrate.

Under the above two simplifying assumptions the system behaves as a pseudo-first-order reaction and cross terms in the differential rate equations do not arise. This system of differential

equations is most conveniently solved by using the Laplace-Carson transformation technique. This technique is a standard method for solving linear differential equations and is described in standard texts on differential equations [24]. For applications of this method to kinetic problems see Rodiguin and Rodiguina [25] and Capellos and Bielski [26]. It is to be born in mind that specific initial conditions should be known because under differing initial conditions the procedure of solving linear differential equations remains essentially the same, except that the transformation is carried out in a different manner [25]. The essence of the method is to replace the differential, $\frac{d}{dt}$, of x by an operator p and then to treat p as a constant. One algebraically solves the simultaneous linear differential equations to obtain the unknown x, say, in a form called the transform of x. The conversion of the transform to the original, i.e. the solution of x as a function of t, is done most easily by using the extensive tables which have been compiled for the conversion of the Laplace-Carson transform to the original or vice versa. Appendix A of this thesis contains a short table of Laplace-Carson tranforms and their originals. In cases where a transform has not been tabulated, it is usually possible to decompose this transform into simpler terms which have been tabulated. The method extensively used is that of partial fractions [24-25] Sometimes, as will be seen later in this thesis, it will be convenient to use the Laplace-Carson transformation technique to obtain a solution for an unknown, and then to use simple integration of this solution to obtain the solution for another unknown.

In the following sections the simple Michaelis-Menten mechanism and the double-intermediate mechanism will be solved using Laplace-Carson transforms in order to compare the present method with other methods used in the literature, as well as to show the detailed application of the method. Furthermore a new approach

to the analysis of results will be presented which has not been previously reported. In this chapter the system of two non-interacting but competing substrates will also be solved; the solution for the latter system has not previously been given. In the following chapters more complex systems will be solved for the first time; these systems will involve activation, inhibition, and two-substrate enzyme systems.

Single-Intermediate Mechanism

The scheme of reactions applicable to the single-intermediate system, uncomplicated by the effects of activators and inhibitors, is as follows:

$$\underset{e_o - m}{E} + \underset{a_o - m - x}{A} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{m}{EA} \xrightarrow{k_2} \underset{e_o - m}{E} + \underset{x}{X}$$

The concentration of each species at any time t is indicated below it in the scheme; a_o, e_o are the initial concentrations of the substrate and enzyme, respectively.

The differential rate equations are:

[1] $$\dot{m} = k_1 (e_o - m)(a_o - m - x) - (k_{-1} + k_2) m$$

[2] $$\dot{x} = k_2 m$$

These equations cannot be solved exactly in analytical form unless one or more of certain conditions are satisfied. One such condition, which is easily realized in practice, is that $a_o \gg e_o$. When this condition is satisfied, the amount of product produced at the end of the transient phase is very much less than the initial substrate concentration. Since in addition m must also be very much

less than a_o, it follows that $a_o - m - x \approx a_o$. Another condition, which might prove to be more satisfactory for analysis of experimental results [14], is when $e_o \gg a_o$, when the approximation $e_o - m \approx e_o$ could be safely made. Equations [1] - [2] will be solved for the above two limiting cases in the following section.

Case 1: $a_o \gg e_o$; $a_o - m - x \approx a_o$

Under the above conditions, eqs. [1] - [2] become

[3] $$\dot{m} = k_1 a_o (e_o - m) - (k_{-1} + k_2) m$$

[4] $$\dot{x} = k_2 m$$

If the differentials are replaced by the operator p and terms in m are separated eq. [3] becomes

[5] $$m = \frac{k_1 a_o e_o}{p + k_1 a_o + k_{-1} + k_2}$$

Eq. [5] is called the transform for m. The original for eq. [5] (Appendix A) is

[6] $$m = \frac{k_1 a_o e_o}{k_1 a_o + k_{-1} + k_2} - \frac{k_1 a_o e_o}{k_1 a_o + k_{-1} + k_2} e^{-\lambda t}$$

where $\lambda = k_1 a_o + k_{-1} + k_2$

Substitution of eq. [6] into [4] and integration with the boundary conditions $t = 0$, $x = 0$, gives for x

[7] $$x = \frac{k_1 k_2 a_o e_o}{k_1 a_o + k_{-1} + k_2} t + \frac{k_1 k_2 a_o e_o}{(k_1 a_o + k_{-1} + k_2)^2} (e^{-\lambda t} - 1)$$

This is the same equation obtained by Laidler [11]. Analysis of eq. [7] to obtain the rate constants has been carried out in terms of the induction period; another method is by expanding the exponential and accepting only the first two terms (17), which simplifies eq. [7] to:

$$[8] \qquad x = \tfrac{1}{2} k_1 k_2 e_o a_o t^2$$

In eq. [8], k_2, e_o, a_o are usually known so that k_1 can be calculated. Since the Michaelis constant, $(k_{-1}+k_2)/k_1$, is known from steady-state methods, then k_{-1} can be calculated.

The variation of x with t, eq. [7] is shown schematically in Fig. 1. It is obvious that three quantities can be directly determined from an experimental plot: (1) the slope of the linear part, which is the initial steady-state rate, v. (2) the intercept on the time axis, which is the induction period $\tau = 1/(k_1 a_o + k_{-1} + k_2)$. (3) the intercept on the x axis which is $\beta = v/(k_1 a_o + k_{-1} + k_2)$. It is obvious that

$$[9] \qquad \tau = \beta/v = 1/(k_1 a_o + k_{-1} + k_2)$$

and

$$[10] \qquad 1/\tau = v/\beta = k_1 a_o + k_{-1} + k_2$$

A plot of $1/\tau$ or v/β against a_o is linear with a slope of k_1 and an intercept of $k_{-1} + k_2$. Since k_2 is usually known from steady-state methods, k_{-1} can easily be calculated.

Another method of analyzing eq. [7] is by writing the equation as

$$[11] \qquad x - vt + \beta = \beta e^{-\lambda t}$$

Fig. 1 Schematic plot of x against t (eq. [7]

A plot of $\ln (x - vt + \beta)$ against t is linear with a slope of $\lambda = k_1 a_o + k_{-1} + k_2$. This plot is preferable to plots of $1/\tau$ or v/β because it serves as a check that there is only one exponential term, which gives a single linear plot of $\ln (x - v + \beta)$ against t. If there were more than one exponential, then the semi-ln plots will consist of more than one linear region (see double-intermediate case) and the analysis of the induction period or v/β according to eq. [10] will be invalid.

Case 2: $e_o \gg a_o$; $e_o - m \approx e_o$

Under these conditions, eqs. [1] - [2] become

[12] $$\dot{m} = k_1 e_o (a_o - m - n) - (k_{-1} + k_2) m$$

[13] $$\dot{x} = k_2 m$$

Replacing the differentials by operators, p, and solving eqs. [12] - [13] algebraically gives

[14] $$m = \frac{k_1 e_o a_o p}{(p + \lambda_1)(p + \lambda_2)}$$

[15] $$x = \frac{k_1 k_2 e_o a_o}{(p + \lambda_1)(p + \lambda_2)}$$

where λ_1 and λ_2 are the negative roots of the quadratic

[16] $$p^2 + p (k_1 e_o + k_{-1} + k_2) + k_1 k_2 e_o = 0$$

Equations [14] - [15] are the transforms of m and x. The originals are (Appendix A)

[17] $$m = \frac{k_1 e_o a_o}{\lambda_2 - \lambda_1} e^{-\lambda_1 t} + \frac{k_1 e_o a_o}{\lambda_1 - \lambda_2} e^{-\lambda_2 t}$$

$$[18] \qquad x = \frac{k_1 k_2 e_o a_o}{\lambda_1 \lambda_2} - \frac{k_1 k_2 e_o a_o}{\lambda_1 (\lambda_2 - \lambda_1)} e^{-\lambda_1 t} - \frac{k_1 k_2 e_o a_o}{\lambda_2 (\lambda_1 - \lambda_2)} e^{-\lambda_2 t}$$

However $\lambda_1 \lambda_2 = k_1 k_2 e_o$ (eq. [16] so that

$$[19] \qquad x = a_o - \frac{k_1 k_2 e_o a_o}{\lambda_1 (\lambda_2 - \lambda_1)} e^{-\lambda_1 t} - \frac{k_1 k_2 e_o a_o}{\lambda_2 (\lambda_1 - \lambda_2)} e^{-\lambda_2 t}$$

Thus, the variation of product x with time, t, eq. [19], is a two-exponential rise which approaches the value a_o, the initial concentration of the substrate. Analysis of eq. [19] can be carried out by plotting ln x against t. This plot will consist of two linear regions, and is referred to as biphasic. The slopes of the two regions correspond to the values of $-\lambda_1$ and $-\lambda_2$. However from eq. [16],

$$[20] \qquad \lambda_1 \lambda_2 = k_1 k_2 e_o$$

$$[21] \qquad (\lambda_1 + \lambda_2) = k_1 e_o + k_{-1} + k_2$$

Hence, a plot of $\lambda_1 \lambda_2$ against e_o gives $k_1 k_2$ and a plot of $(\lambda_1 + \lambda_2)$ against e_o gives k_1 as a slope and $k_{-1} + k_2$ as an intercept. Thus the two plots can lead to all individual rate constants without the need to use values of k_2 or K_m from steady-state analysis.

Double-Intermediate Mechanism

In most enzyme mechanisms, there is a second intermediate, such as an acyl enzyme:

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA \xrightarrow{k_2} EA' \xrightarrow{k_3} E + \begin{matrix} Y \\ y \end{matrix}$$

$$\begin{matrix} e_o - m - n & a_o - m - n - y & m & x & n \end{matrix}$$

where the concentrations of all species at time t are indicated below them; e_o and a_o are the initial concentrations of enzyme and substrate, respectively.

The differential rate equations governing the system again cannot be integrated for the general case, and simplifying assumptions have to be used for obtaining analytical solutions for the species involved. The two cases which can be realized experimentally are (1) $a_o \gg e_o$ and consequently $a_o - m - n - y \approx a_o$; (2) $e_o \gg a_o$ and consequently $e_o - m - n \approx e_o$. The above system will be solved for these two limiting cases.

Case 1: $a_o \gg e_o$; $a_o - m - n - y \approx a_o$

The differential rate equations are

$$[22] \qquad \dot{m} = k_1 a_o (e_o - m - n) - (k_{-1} + k_2) m$$

$$[23] \qquad \dot{n} = k_2 m - k_3 n$$

$$[24] \qquad \dot{x} = k_2 m$$

$$[25] \qquad \dot{y} = k_3 n$$

Replacing differentials by the operator, p, and algebraic solution leads to the following transforms for m and n:

$$[26] \qquad m = \frac{k_1 e_o a_o (p + k_3)}{(p + \lambda_1)(p + \lambda_2)}$$

$$[27] \qquad n = \frac{k_1 k_2 a_o e_o}{(p + \lambda_1)(p + \lambda_2)}$$

where λ_1 and λ_2 are the negative roots of the quadratic equation

$$[28] \qquad p^2 + p(k_1 a_o + k_{-1} + k_2 + k_3) + k_3 (k_{-1} + k_2) + k_1 a_o (k_2 + k_3) = 0$$

Conversion of eqs. [26] and [27] to their original forms leads to the final solution for the variation of the intermediates m and n with time, t:

$$[29] \qquad m = \frac{k_1 k_3 a_o e_o}{\lambda_1 \lambda_2} - \frac{k_1 a_o e_o (k_3 - \lambda_1)}{\lambda_1 (\lambda_2 - \lambda_1)} e^{-\lambda_1 t} - \frac{k_1 a_o e_o (k_3 - \lambda_2)}{\lambda_2 (\lambda_1 - \lambda_2)} e^{-\lambda_2 t}$$

$$[30] \qquad n = \frac{k_1 k_2 a_o e_o}{\lambda_1 \lambda_2} - \frac{k_1 k_2 a_o e_o}{\lambda_1 (\lambda_2 - \lambda_1)} e^{-\lambda_1 t} - \frac{k_1 k_2 a_o e_o}{\lambda_2 (\lambda_1 - \lambda_2)} e^{-\lambda_2 t}$$

Substitution of eqs. [29] and [30] into [24] and [25] respectively and integration with the boundary conditions: t = 0, x = 0, y = 0 leads to the solutions for x and y as functions of time, t (the value of $\lambda_1 \lambda_2 = k_3 (k_{-1} + k_2) + k_1 a_o (k_2 + k_3)$, eq. [28], has been substitute):

$$[31] \qquad x = \frac{k_1 k_2 k_3 a_o e_o}{k_1 a_o (k_2 + k_3) + k_3 (k_{-1} + k_2)} t + \frac{k_1 k_2 a_o e_o (k_3 - \lambda_1)}{\lambda_1^2 (\lambda_2 - \lambda_1)} (e^{-\lambda_1 t} - 1) + \frac{k_1 k_2 a_o e_o (k_3 - \lambda_2)}{\lambda_2^2 (\lambda_1 - \lambda_2)} (e^{-\lambda_2 t} - 1)$$

$$[32] \qquad y = \frac{k_1 k_2 k_3 a_o e_o}{k_1 a_o (k_2 + k_3) + k_3 (k_{-1} + k_2)}\, t$$

$$+ \frac{k_1 k_2 k_3 a_o e_o}{\lambda_1^2 (\lambda_2 - \lambda_1)} (e^{-\lambda_1 t} - 1)$$

$$+ \frac{k_1 k_2 k_3 a_o e_o}{\lambda_2^2 (\lambda_1 - \lambda_2)} (e^{-\lambda_2 t} - 1)$$

From eqs. [31] and [32] it is seen that the variation of x and y with time, t, is a biphasic, two-exponential, approach to a steady-state which is linear in t. The variation of a product according to the general form of eqs. [31] and [32] with time is shown schematically in Fig. 2.

The analysis of results can be carried out in the following manner. Eq. [31] can be written as

$$[33] \qquad x = v t + \beta + \beta_1 e^{-\lambda_1 t} + \beta_2 e^{-\lambda_2 t}$$

where $-\beta = (\beta_1 + \beta_2)$

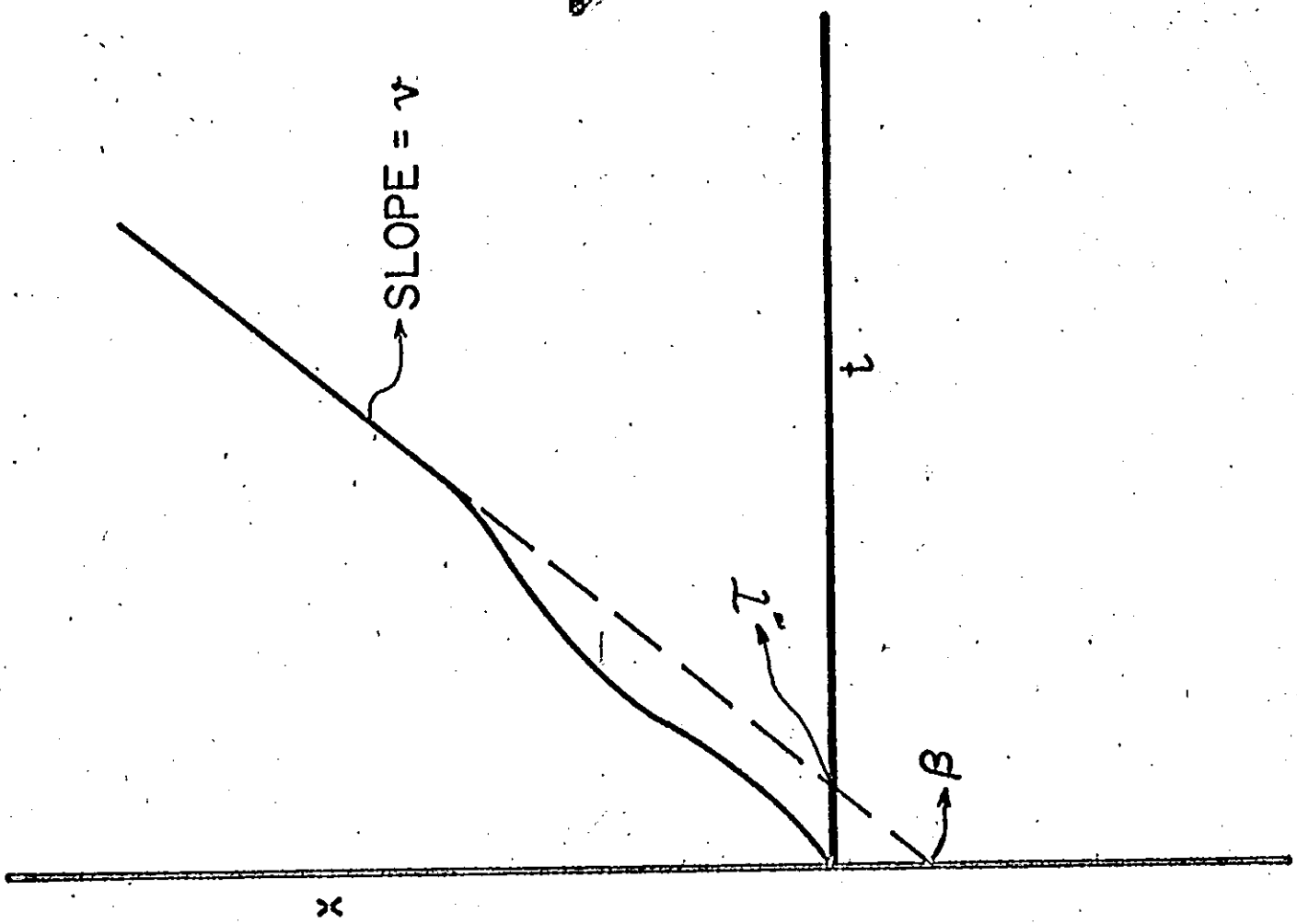
From Fig. 2, three quantities can be determined: (1) the slope of the linear part, v, which corresponds to the initial steady-state rate. (2) The intercept on the product-axis, β. (3) The intercept on the time-axis, τ. It is easily shown that

$$[34] \qquad \beta/v = \tau = -\frac{1}{k_3} + \frac{k_1 a_o + k_{-1} + k_2 + k_3}{k_3 (k_{-1} + k_2) + k_1 a_o (k_2 + k_3)}$$

A plot of t against a_o is hyperbolic with

$$[35] \qquad \lim_{a_o \to \infty} \tau = \tau_\infty = -k_2/(k_2 + k_3) k_3$$

Fig. 2 Schematic plot of x against t (eq. [31]).



[36] $\text{Lim}_{a_o = 0} \tau = \tau_o = 1/(k_{-1} + k_2)$

However if one plots ln $(x - vt - \beta)$ against t (cf. eq. [31]) two straight regions are obtained. The slopes of the two regions are the exponents $-\lambda_1$ and $-\lambda_2$. From eq. [28]:

[37] $$(\lambda_1 + \lambda_2) = k_1 a_o + k_{-1} + k_2 + k_3$$

[38] $$\lambda_1 \lambda_2 = k_3 (k_{-1} + k_2) + k_1 (k_2 + k_3) a_o$$

A plot of $(\lambda_1 + \lambda_2)$ against a_o gives k_1 as the slope of the linear plot and $(k_{-1} + k_2 + k_3)$ as the intercept. Also a plot of $\lambda_1 \lambda_2$ against a_o gives $k_1 (k_2 + k_3)$ as the slope and $k_3 (k_{-1} + k_2)$ as the intercept. From this slope $(k_2 + k_3)$ can be calculated, and from the intercept of $(\lambda_1 + \lambda_2)$ plots, k_{-1} can be calculated. Furthermore, from k_{-1} and τ_o, k_2 can be calculated and consequently k_3. Maguire, Hijazi and Laidler [27] applied these methods to the α-chymotrypsin-catalyzed reaction of p-nitrophenyl acetate, and a sample plot of ln $(x - vt - \beta)$ against t is shown in Fig. 3.

It is seen that all the rate constants can be calculated from transient-phase experiments without resort to values calculated from conventional steady-state methods. It is to be noted that various other plots can be devised.

For a different, but approximate, approach to the analysis of data, it is worth consulting the work of Laidler et al.[10, 12, 13].

Analysis of results when the production of y is monitored can be carried out along the same lines as for x. It is to be noted, however, that for y, the β/v relation is as follows:

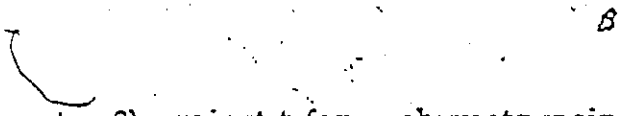
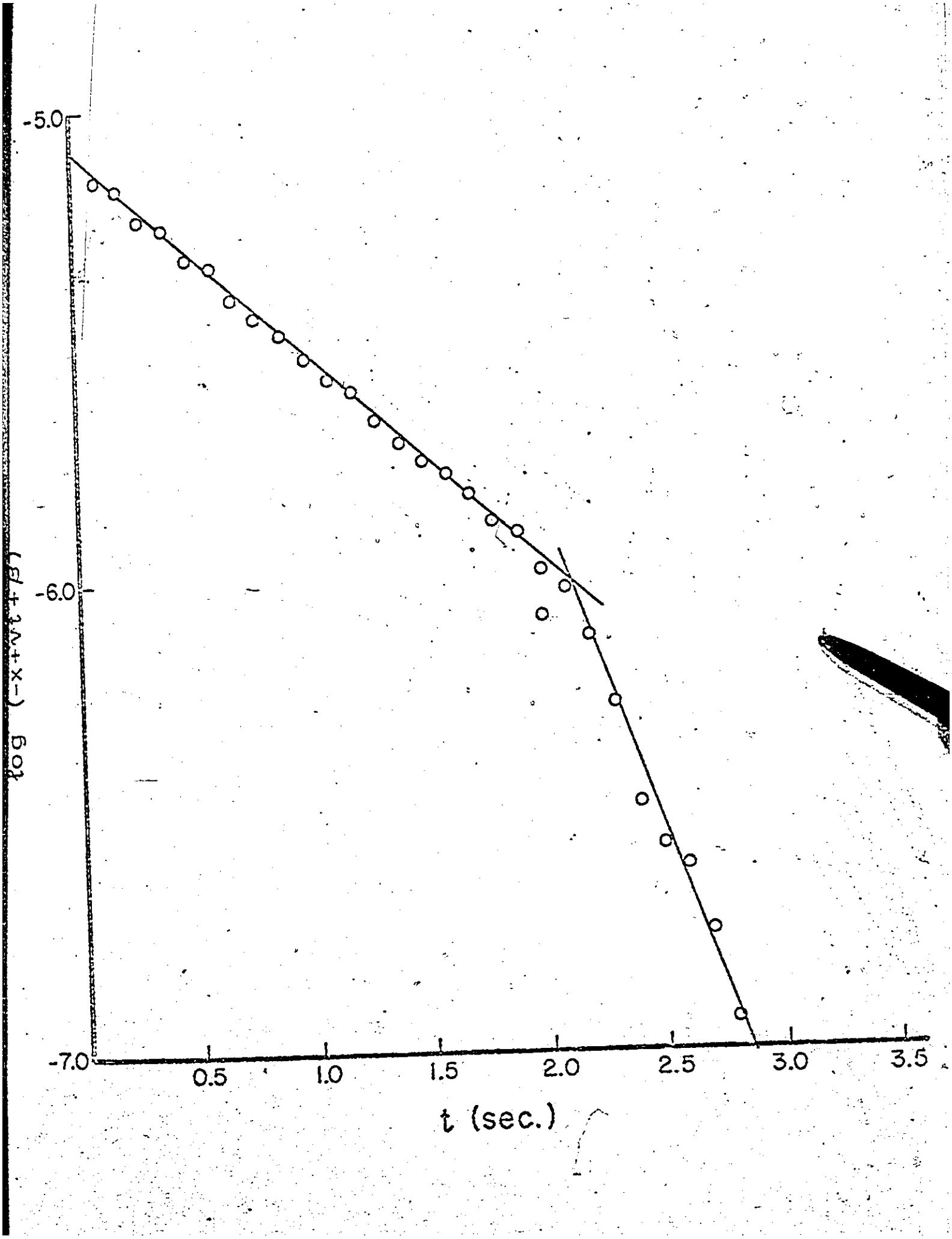


Fig. 3 A plot of ln $(x - vt - \beta)$ against t for α-chymotrypsin catalyzed hydrolysis of p-nitrophenyl acetate. Data of Maguire et al.[27]


-5.0
-6.0
-7.0
log (β/+1V+x-)
0.5
1.0
1.5
2.0
2.5
3.0
3.5
t (sec.)

$$[39] \qquad \beta/v = \tau = \frac{k_1 a_o + k_{-1} + k_2 + k_3}{k_3 (k_{-1} + k_2) + k_1 a_o (k_2 + k_3)}$$

The other relations, $\lambda_1 \lambda_2$ and $\lambda_1 + \lambda_2$, are exactly the same as for x.

Case 2: $e_o \gg a_o$; $e_o - m - n \sim e_o$

Under these conditions the differential rate equations can be written as

$$[40] \qquad \dot{m} = k_1 e_o (a_o - m - n - y) - (k_{-1} + k_2) m$$

$$[41] \qquad \dot{n} = k_2 m - k_3 n$$

$$[42] \qquad \dot{x} = k_2 m$$

$$[43] \qquad \dot{y} = k_3 n$$

Replacing the differentials by the operator, p, and solution leads to the following transforms for m, n, x and y:

$$[44] \qquad m = \frac{k_1 e_o a_o p}{(p + \lambda_1)(p + \lambda_2)}$$

$$[45] \qquad n = \frac{k_1 k_2 e_o a_o p}{(p + \lambda_1)(p + \lambda_2)}$$

$$[46] \qquad x = \frac{k_1 k_2 a_o e_o}{(p + \lambda_1)(p + \lambda_2)}$$

$$[47] \qquad y = \frac{k_1 k_2 k_3 a_o e_o}{(p + \lambda_1)(p + \lambda_2)(p + \lambda_3)}$$

where $\lambda_3 = k_3$ and λ_1 and λ_2 are the negative roots of the quadratic

$$[48] \qquad p^2 + p (k_1 e_o + k_{-1} + k_2) + k_1 k_2 e_o = 0$$

Eqs. [44] - [47] can be replaced by their originals, and thus solutions for all species as functions of time will have been

achieved. In this study we are interested in the variation of products X and Y with time t. Thus eqs. [46] - [47] give

$$[49] \qquad x = a_o - \frac{k_1 k_2 a_o e_o}{\lambda_1 (\lambda_2 - \lambda_1)} e^{-\lambda_1 t} - \frac{k_1 k_2 a_o e_o}{\lambda_2 (\lambda_1 - \lambda_2)} e^{-\lambda_2 t}$$

$$[50] \qquad y = a_o - \frac{k_1 k_2 k_3 a_o e_o}{\lambda_1 (\lambda_2 - \lambda_1)(\lambda_3 - \lambda_1)} e^{-\lambda_1 t} - \frac{k_1 k_2 k_3 a_o e_o}{\lambda_2 (\lambda_1 - \lambda_2)(\lambda_3 - \lambda_2)} e^{-\lambda_2 t}$$

$$- \frac{k_1 k_2 k_2 a_o e_o}{\lambda_3 (\lambda_1 - \lambda_3)(\lambda_2 - \lambda_3)} e^{-\lambda_3 t}$$

Eqs. [49] and [50] are the same as obtained in a different way by Kasserra and Laidler [14] and by Hijazi and Laidler [15]. The above analyzed their results by using approximations; analysis with eqs. [49] and [50] to calculate the rate constants will now be carried out without any approximation.

Analysis of eq. [49] is the same as that for a single-intermediate case and thus will not be repeated here.

Analysis of eq. [50] is as follows: a plot of ln x against t is triphasic, three linear regions with different slopes, and the slopes of these regions are $-\lambda_1$, $-\lambda_2$ and $-\lambda_3$. Thus plots of ln x against t for different enzyme concentrations alters λ_1 and λ_2, while $\lambda_3 = k_3$ remains constant. Thus k_3 is easily calculated. On the other hand λ_1 and λ_2 obey the following relations:

$$\lambda_1 + \lambda_2 = k_1 e_o + k_{-1} + k_2$$

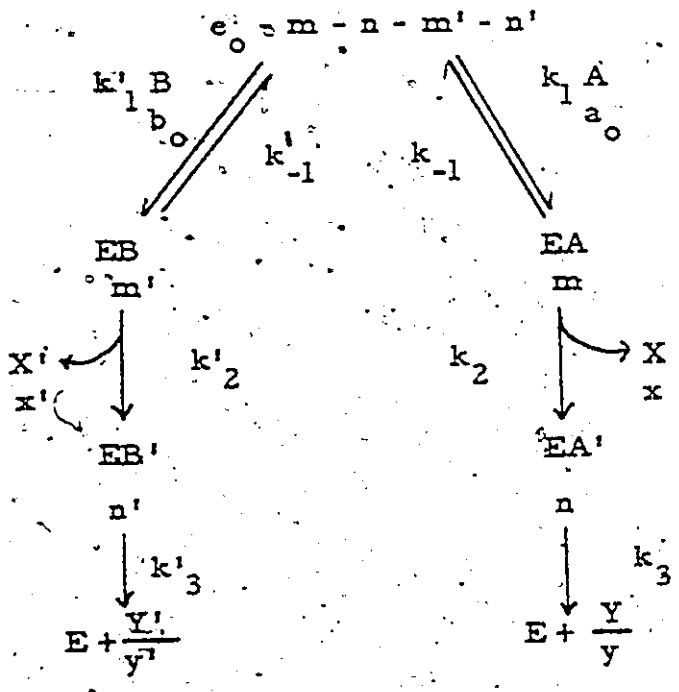
$$\lambda_1 \lambda_2 = k_1 k_2 e_o$$

Analysis of the above two relations has been carried out for the single-intermediate mechanism and no further details will be given here.

Reactions of Two Competing Substrates

Many enzymes catalyze the reaction of more than one substrate, and it is of interest to consider the case in which an enzyme is catalyzing the reaction of two substrates. This case is to be distinguished from the case of two substrates that are reacting with one another. An example of the case under consideration is the action of an esterase on a mixture of the D- and L- forms of an ester substrate.

If the two substrates react at different sites on the enzymes, then each substrate is described by its own Michaelis-Menten behavior. If, however, the two substrates react at the same site on the enzyme they will complete with one another. A simple situation, involving two intermediates, is shown below.



This mechanism was found to apply to the chymotrypsin-catalyzed hydrolysis of mixtures of acetyl-L tryptophanamide and acetyl-L-tyrosinamide [28].

It is assumed that the concentrations of both substrates, A and B, are in great excess of enzyme concentration so that it can be safely assumed that the concentrations of A and B are not significantly changed in the short transient phase of the reaction and in the initial steady state.

The differential rate equations are:

$$[51] \quad \dot{m} = k_1 a_o (e_o - m - m' - n - n') - (k_{-1} + k_2) m$$

$$[52] \quad \dot{x} = k_2 m$$

$$[53] \quad \dot{n} = k_2 m - k_3 n$$

$$[54] \quad \dot{y} = k_3 n$$

$$[55] \quad \dot{m}' = k'_1 b (e_o - m - m' - n - n') - (k'_{-1} + k'_2) m'$$

$$[56] \quad \dot{x}' = k'_2 m'$$

$$[57] \quad \dot{n}' = k'_2 m' - k'_3 n'$$

$$[58] \quad \dot{y}' = k'_3 n'$$

Eqs. [51] - [58] can be integrated by the Laplace-Carson transformation technique as described in the previous sections.

The transform for m is

$$[59] \quad \bar{m} = \frac{k_1 a_o e_o (p + k_3) (p + k'_3) (p + k'_{-1} + k'_2)}{(p + \lambda_1)(p + \lambda_2)(p + \lambda_3)(p + \lambda_4)}$$

and the original for m is

$$[60] \quad m = \frac{k_1 k_3 k'_3 (k'_{-1} + k'_2) a_o e_o}{\lambda_1 \lambda_2 \lambda_3 \lambda_4} - \sum_{i=1}^{4} \frac{k_1 a_o e_o (k_3 - \lambda_i)(k'_3 - \lambda_i)(k'_{-1} + k'_2 - \lambda_i)}{\lambda_i (p - \lambda_i)} e^{-\lambda_i t}$$

where the λ_i's are the roots of the equation

[61] $$p^4 + Mp^3 + Np^2 + Lp + O = 0$$

and

[62] $$M = k_1 a_o + k'_1 b_o + k_{-1} + k'_{-1} + k_2 + k'_2 + k_3 + k'_3$$

[63] $$N = k_1 k_2 a_o + k_3 (k_1 a_o + k_{-1} + k_2) + k'_1 k'_2 b_o + k'_3 (k'_1 b_o + k'_{-1} + k'_2) + k_1 a_o (k'_{-1} + k'_2 + k'_3) + (k_{-1} + k_2 + k_3)(k'_1 b_o + k'_{-1} + k'_2 + k'_3)$$

[64] $$L = k'_1 b_o (k_{-1} + k_2 + k_3)(k'_2 + k'_3) + k_1 a_o (k'_{-1} + k'_2 + k'_3) (k_2 + k_3) + k_3 (k_{-1} + k_2)(k'_1 b_o + k'_{-1} + k'_2 + k'_3) + k'_3 (k'_{-1} + k'_2)(k_1 a_o + k_{-1} + k_2 + k_3)$$

[65] $$O = k_1 k'_3 a_o (k'_{-1} + k'_2)(k_2 + k_3) + k'_1 k_3 b_o (k_{-1} + k_2)(k'_2 + k'_3) + k_3 k'_3 (k_{-1} + k_2)(k'_{-1} + k'_2)$$

Substitution of eq. [60] into eq. [52], and integration with the boundary conditions: $x = 0$ at $t = 0$, gives for the first product as a function of time:

[66] $$x = v_s t + \sum_{i=1}^{4} \sum^{4} \frac{k_1 k_2 a_o e_o (k_3 - \lambda_i)(k'_3 - \lambda_i)(k'_{-1} + k'_2 - \lambda_i)}{\lambda_i^2 (p - \lambda_i)} (e^{-\lambda_i t} - 1)$$

where v_s is the initial steady state of production of x [the same as obtained from a strictly steady-state assumption (10)].

$$[67] \qquad v_s = \frac{k_2 a_o e_o}{\frac{k_{-1} + k_2}{k_1} + \frac{k_2 + k_3}{k_3} a_o + \frac{k'_1 (k_{-1} + k_2)(k'_2 + k'_3)}{k_1 (k'_{-1} + k'_2) k'_3} b_8}$$

If the variation of the second product, y, with time is being followed, then the equation is derived from the concentration of intermediate n as follows. The transform for n is

$$[68] \qquad n = \frac{k_1 k_2 a_o e_o (p + k'_3)(p + k'_{-1} + k'_2)}{(p + \lambda_1)(p + \lambda_2)(p + \lambda_3)(p + \lambda_4)}$$

The original for n is

$$[69] \qquad n = \frac{k_1 k_2 k'_3 (k'_{-1} + k'_2) a_o e_o}{\lambda_1 \lambda_2 \lambda_3 \lambda_4} - \sum_{i=1}^{4} \frac{k_1 k_2 a_o e_o (\lambda_i - k_3)(\lambda_i - [k'_{-1} + k'_2])}{\lambda_i (p - \lambda_i)} e^{-\lambda_i t}$$

Substitution of eq. [69] into eq. [54] and integration with the boundary condition $t = 0$, $y = 0$, gives:

$$[70] \qquad y = v_s t + \sum_{i=1}^{4} \frac{k_1 k_2 k_3 a_o e_o (\lambda_i - k'_3)(\lambda_i - [k'_{-1} + k'_2])}{\lambda_1^2 (p - \lambda_i)} (e^{-\lambda_i t} - 1)$$

where v_s is the same as in eq. [67] and the λ_i's are the same as in eq. [61].

Since the system is symmetrical in both branches of reaction and A and B are dummy symbols, the variation of x' and y' are of the same form as for x and y with the rate constants changed to the primed rate constants and a_o changed to b_o. Accordingly

$$[71] \qquad x' = v'_s t + \sum_{i=1}^{4} \frac{k'_1 k'_2 b_o e_o (k_3 - \lambda_i)(k'_3 - \lambda_i)(k_{-1} + k_2 - \lambda_i)}{\lambda_i^2 (p - \lambda_i)} (e^{-\lambda_i t} - 1)$$

$$[72] \qquad y' = v'_s t + \sum_{i=1}^{4} \frac{k'_1 k'_2 b_o e_o (\lambda_i - k_3)(\lambda_i - [k_{-1} + k_2])}{\lambda_i^2 (p - \lambda_i)} (e^{-\lambda_i t} - 1)$$

where

$$[73] \qquad v'_s = \frac{k'_2 b_o e_o}{\frac{k'_{-1} + k'_2}{k'_1} + \left(\frac{k'_2 + k'_3}{k'_3}\right) b_o + \frac{k_1 (k'_{-1} + k'_2)(k_2 + k_3)}{k'_1 (k_{-1} + k_2) k_3} a_o}$$

and the λ_i's are the same as before.

Analysis.

The analysis of results will only be in terms of the product x, but similar considerations apply to products Y, X' and Y'.

The variation of x with t, eq. [66], is composed of a four-exponential rise that approaches a linear steady-state variation. The slope of the linear part is equal to v_s, the initial steady-state rate given in eq. [67]. Extension of the linear part of the curve would give β, an intercept on the x-axis.

If a plot of v_s / e_o against a_o is used then, at high a_o, $v_s(\max) = k_2 k_3 (k_2 + k_3)$.

Equation [64] can be written as

$$[74] \qquad x - v_s t + \beta = \sum_{i=1}^{4} \beta_i e^{-\lambda_i t}$$

Hence a plot of $\ln (x - v_s t + \beta)$ against t will consist of four linear regions. The slopes of those regions correspond to the values of

$-\lambda_1, -\lambda_2, -\lambda_3, -\lambda_4$. However the λ_i's follow two important relations :

$$\prod_{i=1}^{4} \lambda_i = \lambda_1 \lambda_2 \lambda_3 \lambda_4 = k_1 k'_3 (k'_{-1} + k'_2)(k_2 + k_3) a_o + k'_1 k_3 (k_{-1} + k_2)(k'_2 + k'_3) b_o + k_3 k'_3 (k_{-1} + k_2)(k'_{-1} + k'_2)$$

$$\sum_{i=1}^{4} \lambda_i = k_1 a_o + k'_1 b_o + k_{-1} + k_2 + k_3 + k'_{-1} + k'_2 + k'_3$$

Hence, plots of $\sum_{i=1}^{4} \lambda_i$ against a_o and b_o give k_1, k'_1, and $(k_{-1} + k_2 + k_3 + k'_{-1} + k'_2 + k'_3)$. Also plots of $\prod_{i=1}^{4} \lambda_i$ against a_o and b_o give $k'_3 (k'_{-1} + k'_2)(k_2 + k_3)$, $k_3 (k_{-1} + k_2)(k'_2 + k'_3)$ and $k_3 k'_3 (k_{-1} + k_2)(k'_{-1} + k'_2)$. From the last three terms, by their division, one can calculate the relations

$$\frac{(k_2 + k_3)}{k_3 (k_{-1} + k_2)} \quad \text{and} \quad \frac{k'_2 + k'_3)}{k'_3 (k'_{-1} + k'_2)}$$

Another useful relation is

$$v_s \prod_{i=1}^{4} \lambda_i = k_1 k_2 k_3 k'_3 (k'_{-1} + k'_2) a_o e_o$$

Hence plots of $v_s \prod_{i=1}^{4} \lambda_i$ against a_o give $k_2 k_3 k'_3 (k'_1 + k'_2)$ since e_o and k_1 are known. Also a similar relation exists where

$$v'_s \prod_{i=1}^{4} \lambda_i = k'_1 k'_2 k'_3 k_3 (k_{-1} + k_2) a_o e_o$$

and determination of $k'_2 k'_3 k_3 (k_{-1} + k_2)$ is also possible.

Extensive algebraic analysis of the combinations of rate constants that could be measured experimentally could lead to some separation of rate constants.

If one is only interested in determining the rate constants, then a simpler procedure could be used. Provided that it is possible experimentally to have an enzyme concentration in large excess of substrate A and substrate B, a transient experiment under these conditions would effectively make the mechanism that of two completely separate double-intermediate mechanisms. Solution of these two mechanisms is the same as the one discussed under the double-intermediate mechanism in the case of $e_o \gg a_o$. Analysis of results to calculate the rate constants is the same as described in that section.

CHAPTER TWO

TRANSIENT PHASE KINETICS OF SIMPLE MECHANISMS INVOLVING NUCLEOPHILES

Introduction

Recently, there has been increasing interest in the investigation of enzymic reactions in the presence of added nucleophiles (29-31). The purpose of these investigations is to elucidate the reaction mechanism.

Hinberg and Laidler[30] have developed the steady-state kinetic equations for various models of enzyme mechanisms in the presence of nucleophiles. The authors were interested in classifying the different patterns of dependence of kinetic parameters on the nucleophile concentration. Hinberg and Laidler[30-31] applied their results to experimental data on alkaline phosphatase.

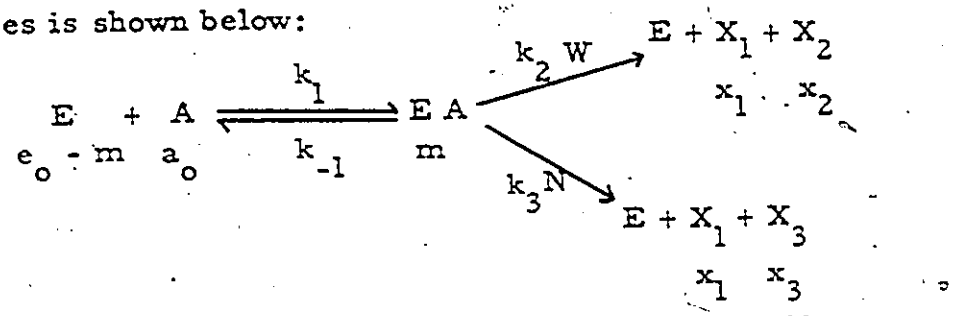
In this chapter, we develop the transient-phase kinetic equations for three basic mechanisms which involve addition of a nucleophile to intermediates in single-intermediate and in double-intermediate mechanisms. The analysis of results will be mainly to establish criteria for distinguishing the different mechanisms from transient-phase experiments.

THEORETICAL

Mechanism I

First, a single intermediate mechanism is considered, in which the enzyme-substrate complex may react with water, W, to form products X_1 and X_2; alteratively it may react with the nucleophile,

N, to form products x_1 and x_3. The mechanism and concentration of each species is shown below:



It is assumed, as is usually the case, that a_o, W, $N \gg e_o$.

The differential rate equations are

[1] $$\dot{m} = k_1 a_o (e_o - m) - (k_{-1} + k_2 W + k_3 N) m$$

[2] $$\dot{x}_1 = (k_2 W + k_3 N) m$$

[3] $$\dot{x}_2 = k_2 W m$$

[4] $$\dot{x}_3 = k_3 N m$$

Application of the Laplace-Carson transformation, replacing the differentials by operators, P, and algebraic solution of the resulting equations, leads to the following transforms for m, x_1, x_2 and x_3:

[5] $$m = \frac{k_1 a_o e_o}{(P + \lambda)}$$

[6] $$x_1 = \frac{k_1 (k_2 W + k_3 N) a_o e_o}{P (P + \lambda)}$$

[7] $$x_2 = \frac{k_1 k_2 W a_o e_o}{P (P + \lambda)}$$

[8] $$x_3 = \frac{k_1 k_3 N a_o e_o}{P (P + \lambda)}$$

where

[9] $$\lambda = k_1 a_o + k_{-1} + k_2 W + k_3 N$$

The transforms, eqs. [6] - [8] can be replaced by their originals, and the variations of x_1, x_2 and x_3 with time are found to be

$$[10] \qquad x_1 = \frac{k_1 (k_2 W + k_3 N) a_o e_o}{k_{-1} + k_2 W + k_3 N + k_1 a_o} t + \frac{k_1 (k_2 W + k_3 N) a_o e_o}{\lambda^2} (e^{-\lambda t} - 1)$$

$$[11] \qquad x_2 = \frac{k_1 (k_2 W + k_3 N) a_o e_o}{k_{-1} + k_2 W + k_3 N + k_1 a_o} t + \frac{k_1 k_2 W a_o e_o}{\lambda^2} (e^{-\lambda t} - 1)$$

$$[12] \qquad x_3 = \frac{k_1 k_3 N a_o e_o}{k_{-1} + k_2 W + k_3 N + k_1 a_o} t + \frac{k_1 k_3 N a_o e_o}{\lambda^2} (e^{-\lambda t} - 1)$$

Analysis

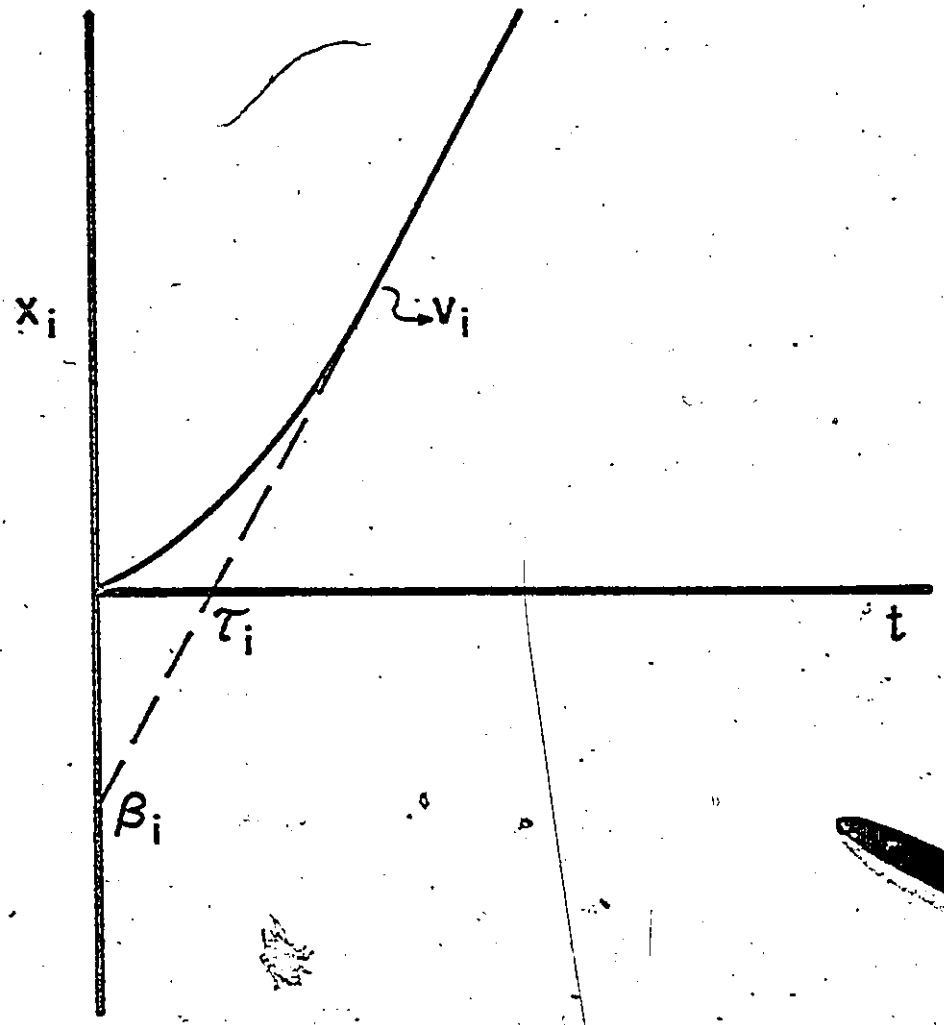
The variation of any product, X_i, with time t, eqs. [10] - [12] is a single exponential rise that leads to a linear increase corresponding to the initial steady state.

A general equation [13] can be written for the variation of any product X_i with time,

$$[13] \qquad x_i = v_i t + \beta_i (e^{-\lambda t} - 1)$$

where x_i corresponds to any product, x_1, x_2 or x_3, and v_i, β_i are the corresponding steady-state rate and pre-exponential term respectively. A schematic plot of eq. [13] is shown in Fig. 4. The slope of the linear part is v_i, and the linear part extrapolates to the time axis at a point τ, the induction period. The intercept on the x_i axis corresponds to the constant term β_i.

Fig. 4. Schematic plot of eq. 13 .


x_i
v_i
τ_i
t
β_i

The rate constants can be obtained from experiments without added nucleophiles as described in Chapter one. The rate constant for binding of nucleophile can be easily obtained by the following procedure.

A plot of $\ln (x_i - v_i \tau + \beta_i)$ against t leads to a linear monophasic variation. The slope of this linear plot is $-\lambda$. A plot of λ against N, the initial nucleophile concentration, is linear with a slope of k_3 (see eq. [9]).

Induction Period, τ

As discussed above, the three quantities τ_i, β_i, v_i can be obtained experimentally from a plot of x_i against t. It is easily shown that $\tau_i = \beta_i/v_i$; hence one can measure τ_i, β_i/v_i or both. Substitution of the appropriate values of β_i/v_i, or simple algebraic solution, leads to

$$[14] \qquad \tau_1 = \tau_2 = \tau_3 = \frac{1}{k_1 a_o + k_{-1} + k_2 W + k_3 N}$$

A plot of $\tau_i = \tau_1, \tau_2, \tau_3$ against a_o at different but fixed concentrations of nucleophile, N, is hyperbolic and shown schematically in Fig. 2.

It is easily shown from eq. [14] that

$$[15] \qquad \lim_{a_o \to 0} \tau_i = \tau_{io} = \frac{1}{k_{-1} + k_2 W + k_3 N}$$

$$[16] \qquad \lim_{a_o \to \infty} \tau_i = \tau_{i\infty} = 0$$

Also

$$[17] \qquad 1/\tau_{io} = k_{-1} + k_2 W + k_3 N$$

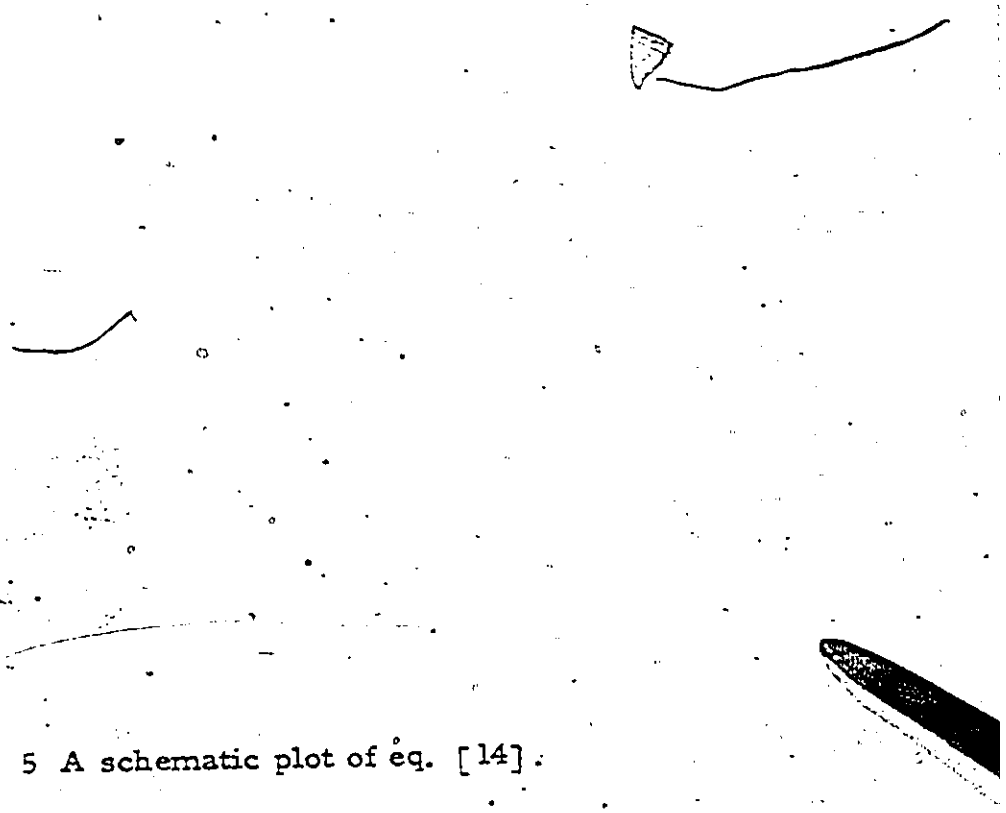
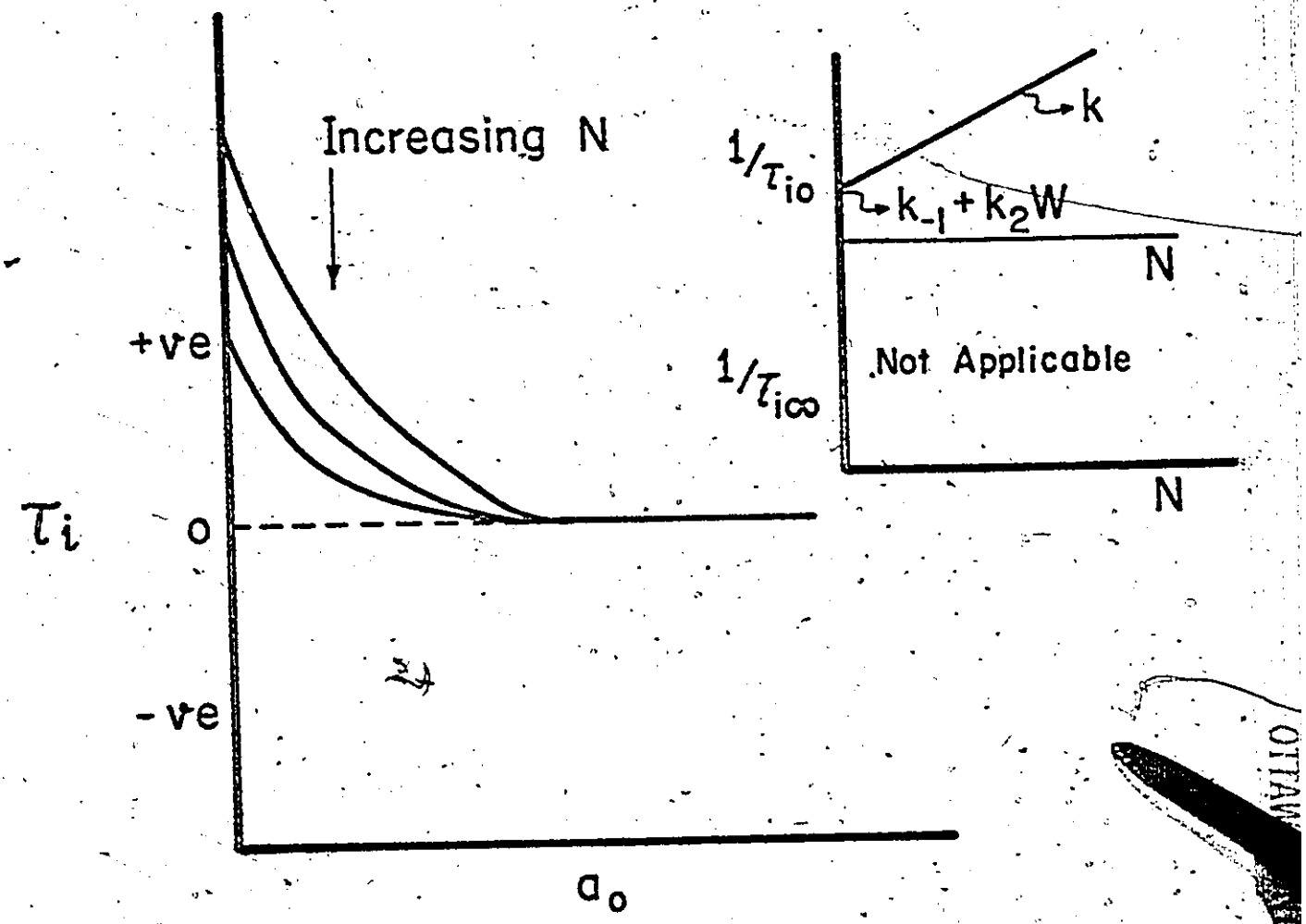


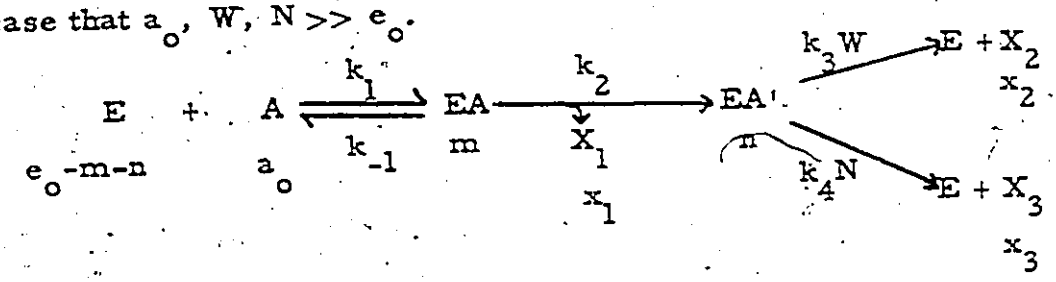
Fig. 5 A schematic plot of eq. [14].



and a plot of $1/\tau_{io}$ against N is shown schematically in the inner scale of Fig. 5.

Mechanism II

This is a double-intermediate mechanism, where the second-intermediate, e.g. an acyl enzyme, a phosphoryl enzyme, etc., reacts with water, W, to form product X_2, or it reacts with another nucleophile, N, to form product X_3. The mechanism, with the concentrations of each species, is shown below. It is assumed as usually is the case that a_o, W, $N >> e_o$.



The differential rate equations are

$$\dot{m} = k_1 a_o (e_o - m - n) - (k_{-1} + k_2) m \quad [18]$$

$$\dot{n} = k_2 m - (k_3 W + k_4 N) n \quad [19]$$

$$\dot{x}_1 = k_2 m \quad [20]$$

$$\dot{x}_2 = k_3 W n \quad [21]$$

$$\dot{x}_3 = k_4 N n \quad [22]$$

Applying the Laplace-Carson transformation, the following transforms are obtained for m and n:

$$m = \frac{k_1 a_o e_o [P + k_3 W + k_4 N]}{(P + \lambda_1)(P + \lambda_2)} \quad [23]$$

$$n = \frac{k_1 k_2 a_o e_o}{(P + \lambda_1)(P + \lambda_2)} \quad [24]$$

where

[25] $$\lambda_1 = \tfrac{1}{2}\left[-R - \sqrt{R^2 - 4\Omega}\right]$$

[26] $$\lambda_2 = \tfrac{1}{2}\left[-R + \sqrt{R^2 - 4\Omega}\right]$$

[27] $$R = k_1 a_o + k_3 W + k_4 N + k_{-1} + k_2$$

[28] $$\Omega = (k_{-1} + k_2)(k_3 W + k_4 N) + k_1 a_o (k_2 + k_3 W + k_4 N)$$

The originals for m and n are

[29] $$m = \frac{k_1 a_o e_o (k_3 W + k_4 N)}{\lambda_1 \lambda_2} - \frac{k_1 a_o e_o (k_3 W + k_4 N - \lambda_1)}{\lambda_1 (\lambda_2 - \lambda_1)} e^{-\lambda_1 t} - \frac{k_1 a_o e_o (k_3 W + k_4 N - \lambda_2)}{\lambda_2 (\lambda_1 - \lambda_2)} e^{-\lambda_2 t}$$

[30] $$n = \frac{k_1 k_2 a_o e_o}{\lambda_1 \lambda_2} - \frac{k_1 k_2 a_o e_o}{\lambda_1 (\lambda_2 - \lambda_1)} e^{-\lambda_1 t} - \frac{k_1 k_2 a_o e_o}{\lambda_2 (\lambda_1 - \lambda_2)} e^{-\lambda_2 t}$$

The variation of product x_1 with t is obtained by subsituting eq. [29] into [20] , and integration with the boundary conditions: $x_1 = 0$, $t = o$ ($\lambda_1 \lambda_2 = \Omega$); the result is

[31] $$x_1 = \frac{k_1 k_2 a_o e_o (k_3 W + k_4 N)}{(k_{-1} + k_2)(k_3 W + k_4 N) + k_1 a_o (k_2 + k_3 W + k_4 N)} t + \frac{k_1 k_2 a_o e_o (k_3 W + k_4 N - \lambda_1)}{\lambda_1^2 (\lambda_2 - \lambda_1)} (e^{-\lambda_1 t} - 1) + \frac{k_1 k_2 a_o e_o (k_3 W + k_4 N - \lambda_2)}{\lambda_2^2 (\lambda_1 - \lambda_2)} (e^{-\lambda_2 t} - 1)$$

The solutions for x_2 and x_3 are obtained by substituting eq. [30] into eqs [21] - [22] and integration with the boundary conditions: $x_2 = x_3 = 0$, $t = 0$:

$$[32] \qquad x_2 = \frac{k_1 k_2 k_3 W a_o e_o}{(k_{-1} + k_2)(k_3 W + k_4 N) + k_1 a_o (k_2 + k_3 W + k_4 N)} t$$

$$+ \frac{k_1 k_2 k_3 W a_o e_o}{\lambda_1^2 (\lambda_2 - \lambda_1)} (e^{-\lambda_1 t} - 1)$$

$$+ \frac{k_1 k_2 k_3 W a_o e_o}{\lambda_2^2 (\lambda_1 - \lambda_2)} (e^{-\lambda_2 t} - 1)$$

$$[33] \qquad x_3 = \frac{k_1 k_2 k_4 N a_o e_o}{(k_{-1} + k_2)(k_3 W + k_4 N) + k_1 a_o (k_2 + k_3 W + k_4 N)} t$$

$$+ \frac{k_1 k_2 k_4 N a_o e_o}{\lambda_1^2 (\lambda_2 - \lambda_1)} (e^{-\lambda_1 t} - 1)$$

$$+ \frac{k_1 k_2 k_4 N a_o e_o}{\lambda_2^2 (\lambda_1 - \lambda_2)} (e^{-\lambda_2 t} - 1)$$

Analysis

The solutions for x_1, x_2 and x_3 are all of the same general form. They consist of a biphasic exponential rise that leads to a linear variation. A generalized equation [34] can be written for x_i as a function of t:

$$[34] \qquad x_i = v_i t + \beta_i + \beta_j e^{-\lambda_1 t} + \beta_k e^{-\lambda_2 t}$$

where x_i is x_1, x_2 or x_3 and v_i and β_i are the initial steady-state rate and the constant term respectively for product X_i. The rate constants can be obtained as described in Chapter one for the mechanism without added nucleophiles. The binding rate constant for the nucleophile can be obtained by plotting $\ln (x_i - v_{it} - \beta_i)$ against t, which gives two linear regions, the slopes of which are $-\lambda_1$ and $-\lambda_2$. A plot of $\lambda_1 + \lambda_2 = R$, eq. [27], against N is linear with a slope if k_4.

Induction Period, τ_i

The linear part of an x_i vs t plot will extrapolate to the time axis at a point, τ, corresponding to the induction period. The extension will also intersect the x_i axis at a point corresponding to β_i, the constant term in the appropriate rate equation. It is easily shown that $\tau_i = \beta_i/v_i$. Substitution of the terms for β_i/v_i or simple algebraic solution leads to

$$[35] \qquad \tau_1 = \beta_1/v_1 = \frac{(k_3 W + k_4 N)^2 - k_1 k_2 a_o}{(k_3 W + k_4)\left[(k_{-1} + k_2)(k_3 W + k_4 N) + k_1 a_o (k_2 + k_3 W + k_4 N)\right]}$$

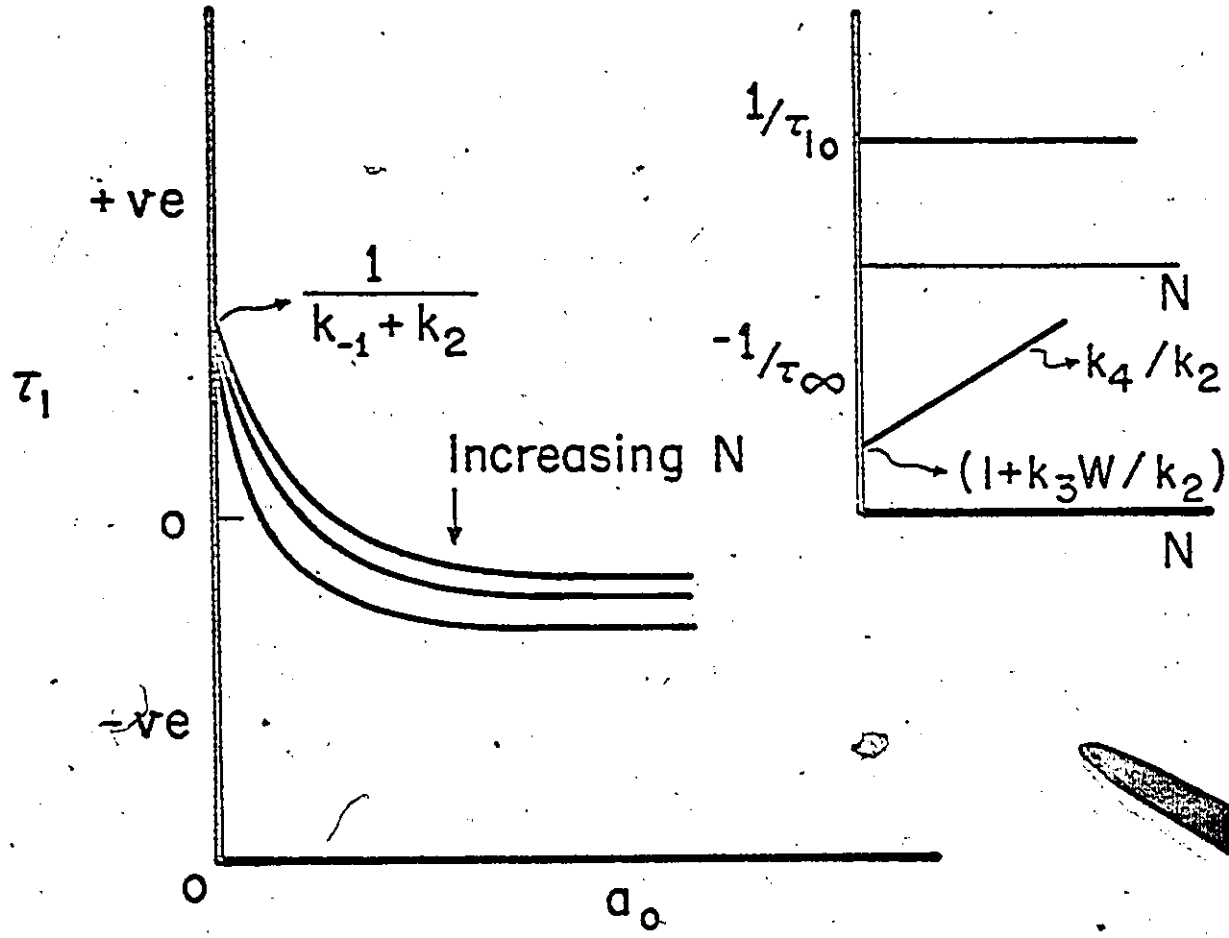
$$[36] \qquad \lim_{a_o = 0} \tau_1 = \tau_{10} = \frac{1}{k_{-1} + k_2}$$

$$[37] \qquad \lim_{a_o \to \infty} \tau_1 = \tau_{1\infty} = \frac{-k_2}{k_2 + k_3 W + k_4 N}$$

A plot of τ_1 against a_o with fixed levels of N is shown schematically in Fig. 6. The inner plots are for $1/\tau_{10} = k_{-1} + k_2$ and $1/\tau_{1\infty} = (k_2 + k_3 W + k_4 N)/k_2$ against N.

$$[38] \qquad \tau_2 = \tau_3 = \beta_2/v_2 = \beta_3/v_3 = \frac{k_1 a_o + k_3 W + k_4 N + k_{-1} + k_2}{(k_{-1} + k_2)(k_3 W + k_4 N) + k_1 a_o (k_2 + k_3 W + k_4 N)}$$

Fig. 6. Schematic plot of eq. [35].



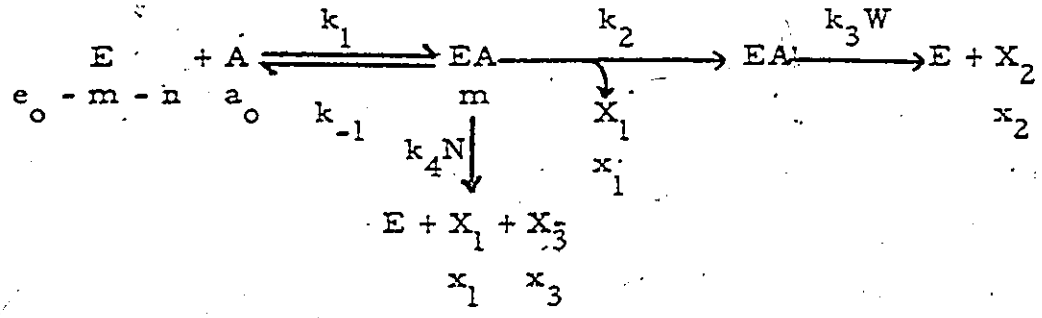
$$[39] \qquad \lim_{a_o = 0} \tau = \tau_{20} = \tau_{30} = \frac{k_3 W + k_4 N + k_{-1} + k_2}{(k_{-1} + k_2)(k_3 W + k_4 N)}$$

$$[40] \qquad \lim_{a_o \to \infty} \tau = \tau_{2\infty} = \tau_{3\infty} = \frac{1}{k_3 W + k_4 N + k_2}$$

A plot of $\tau_2 = \tau_3$ against a_o with fixed levels of N is shown schematically in Fig. 7. Plots of $1/\tau_{20} = 1/\tau_{30} = \frac{(k_{-1} + k_2)(k_3 W + k_4 N)}{k_3 W + k_4 N + k_{-1} + k_2}$ and $1/\tau_{2\infty} = 1/\tau_{3\infty} = k_3 W + k_4 N + k_2$ against N are also shown schematically in Fig. 7.

Mechanism III

This is a double-intermediate mechanism where the nucleophile, N, adds to the first intermediate to form the products X_1, X_3 and free enzyme, E. This intermediate also can give rise to product X_1 and EA', e.g. an acylenzyme or phosphorylenzyme, EA' then reacts with water, W, to give product X_2 and free enzyme. The mechanism and concentrations are shown under the condition that a_o, N, $W \gg e_o$:



The differential rate equations are

$$[41] \qquad \dot{m} = k_1 a_o (e_o - m - n) - (k_{-1} + k_2 + k_4 N) m$$

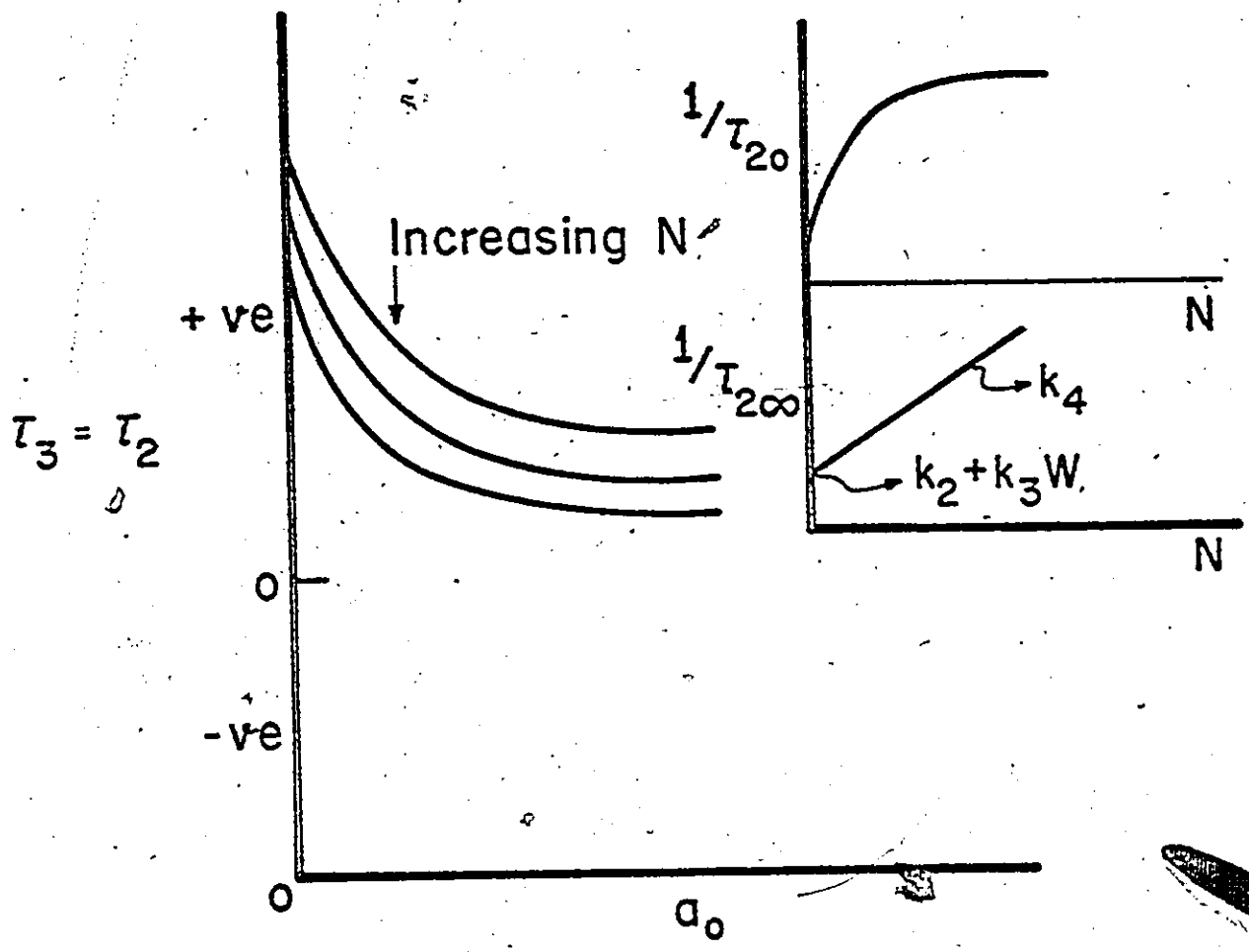
$$[42] \qquad \dot{n} = k_2 m - k_3 W n$$

$$[43] \qquad \dot{x}_1 = (k_2 + k_4 N) m$$

$$[44] \qquad \dot{x}_2 = k_3 W n$$

$$[45] \qquad \dot{x}_3 = k_4 N m$$

Fig. 7. Schematic plot of eq. [38].


Increasing N
+ ve
- ve
0
0
$\tau_3 = \tau_2$
$1/\tau_{20}$
N
$1/\tau_{2\infty}$
k_4
$k_2 + k_3 W_o$
N
a_o

Transformation of these equations according to the Laplace-Carson method leads to the following transforms for m and n:

$$[46] \qquad m = \frac{k_1 a_o e_o (P + k_3 W)}{(P + \lambda_1)(P + \lambda_2)}$$

$$[47] \qquad n = \frac{k_1 k_2 a_o e_o}{(P + \lambda_1)(P + \lambda_2)}$$

where

$$[48] \qquad \lambda_1 = \tfrac{1}{2} [- R - \sqrt{R^2 - 4 Q}]$$

$$[49] \qquad \lambda_2 = \tfrac{1}{2} [- R + \sqrt{R^2 - 4 Q}]$$

$$[50] \qquad R = k_1 a_o + k_{-1} + k_2 + k_4 N + k_3 W$$

$$[51] \qquad Q = k_1 a_o (k_2 + k_3 W) + k_3 W (k_{-1} + k_2 + k_4 N)$$

The originals for m and n are

$$[52] \qquad m = \frac{k_1 k_3 W a_o e_o}{\lambda_1 \lambda_2} - \frac{k_1 a_o e_o (k_3 W - \lambda_1}{\lambda_1 (\lambda_2 - \lambda_1)} e^{-\lambda_1 t} - \frac{k_1 a_o e_o (k_3 W - \lambda_2)}{\lambda_2 (\lambda_1 - \lambda_2)} e^{-\lambda_2 t}$$

$$[53] \qquad n = \frac{k_1 k_2 a_o e_o}{\lambda_1 \lambda_2} - \frac{k_1 k_2 a_o e_o}{\lambda_1 (\lambda_2 - \lambda_1)} e^{-\lambda_1 t} - \frac{k_1 k_2 a_o e_o}{\lambda_2 (\lambda_1 - \lambda_2)} e^{-\lambda_2 t}$$

The variation of products with time can be obtained by substituting for m and n in eqs [43] - [45] and integration with the boundary conditions: $x_1 = x_2 = x_3 = 0$, $t = 0$.

$$[54] \qquad x_1 = \frac{k_1 k_3 W (k_2 + k_4 N) a_o e_o}{k_1 a_o (k_2 + k_3 W) + k_3 W (k_{-1} + k_2 + k_4 N)} \; t$$

$$+ \frac{k_1 a_o e_o (k_2 + k_4 N) (k_3 W - \lambda_1)}{\lambda_1^2 (\lambda_2 - \lambda_1)} \; (e^{-\lambda_1 t} - 1)$$

$$+ \frac{k_1 a_o e_o (k_2 + k_4 N) (k_3 W - \lambda_2)}{\lambda_2^2 (\lambda_1 - \lambda_2)} \; (e^{-\lambda_2 t} - 1)$$

$$[55] \qquad x_2 = \frac{k_1 k_2 k_3 W a_o e_o}{k_1 a_o (k_2 + k_3 W) + k_3 W (k_{-1} + k_2 + k_3 N)} \; t$$

$$+ \frac{k_1 k_2 k_3 W a_o e_o}{\lambda_1^2 (\lambda_2 - \lambda_1)} \; (e^{-\lambda_1 t} - 1)$$

$$+ \frac{k_1 k_2 k_3 W a_o e_o}{\lambda_2^2 (\lambda_1 - \lambda_1)} \; (e^{-\lambda_2 t} - 1)$$

$$[56] \qquad x_3 = \frac{k_1 k_3 k_4 W N a_o e_o}{k_1 a_o (k_2 + k_3 W) + k_3 W (k_{-1} + k_2 + k_4 N)} \; t$$

$$+ \frac{k_1 k_4 N a_o e_o (k_3 W - \lambda_1)}{\lambda_1^2 (\lambda_2 - \lambda_1)} \; (e^{-\lambda_1 t} - 1)$$

$$+ \frac{k_1 k_4 N a_o e_o (k_3 W - \lambda_2)}{\lambda_2^2 (\lambda_1 - \lambda_2)} \; (e^{-\lambda_2 t} - 1)$$

Analysis

The analysis of eqs. [54] - [56] is similar to that of Mechanism II. The binding constant for the nucleophile can be obtained by the same plots as for Mechanism II.

Induction Period, τ.

The induction period for each product can be obtained as outlined for Mechanisms I and II. It is found that

[57] $$\tau_1 = \beta_1/v_1 = \tau_3 = \beta_3/v_3 = \frac{(k_3 W)^2 - k_1 k_2 a_o}{k_3 W [k_1 a_o (k_2 + k_3 W) + k_3 W (k_{-1} + k_2 + k_4 N)]}$$

[58] $$\lim_{a_o = 0} \tau_1 = \tau_{10} = \tau_{30} = \frac{1}{k_{-1} + k_2 + k_4 N}$$

[59] $$1/\tau_{10} = k_{-1} + k_2 + k_4 N$$

[60] $$\lim_{a_o \to \infty} \tau_1 = \tau_{1\infty} = \frac{-k_2}{k_3 W (k_2 + k_3 W)}$$

[61] $$1/\tau_{1\infty} = -\frac{k_3 W (k_2 + k_3 W)}{k_2}$$

Plots of eqs. [57], [59] and [61] are shown in Fig. [8].

[62] $$\tau_2 = \beta_2/v_2 = \frac{k_1 a_o + k_{-1} + k_2 + k_4 N + k_3 W}{k_1 a_o (k_2 + k_3 W) + k_3 W (k_{-1} + k_2 + k_4 N)}$$

[63] $$\lim_{a_o = 0} \tau_2 = \tau_{20} = \frac{k_{-1} + k_2 + k_4 N + k_3 W}{k_3 W (k_{-1} + k_2 + k_4 N)}$$

[64] $$1/\tau_{20} = \frac{k_3 W (k_{-1} + k_2 + k_4 N)}{k_{-1} + k_2 + k_4 N + k_3 W}$$

[65] $$\lim_{a_o \to \infty} \tau_2 = \tau_{2\infty} = \frac{1}{k_2 + k_3 W}$$

[66] $$1/\tau_{2\infty} = k_2 + k_3 W$$

Fig. 8. Schematic plots of eqs. [57], [59] and [61].

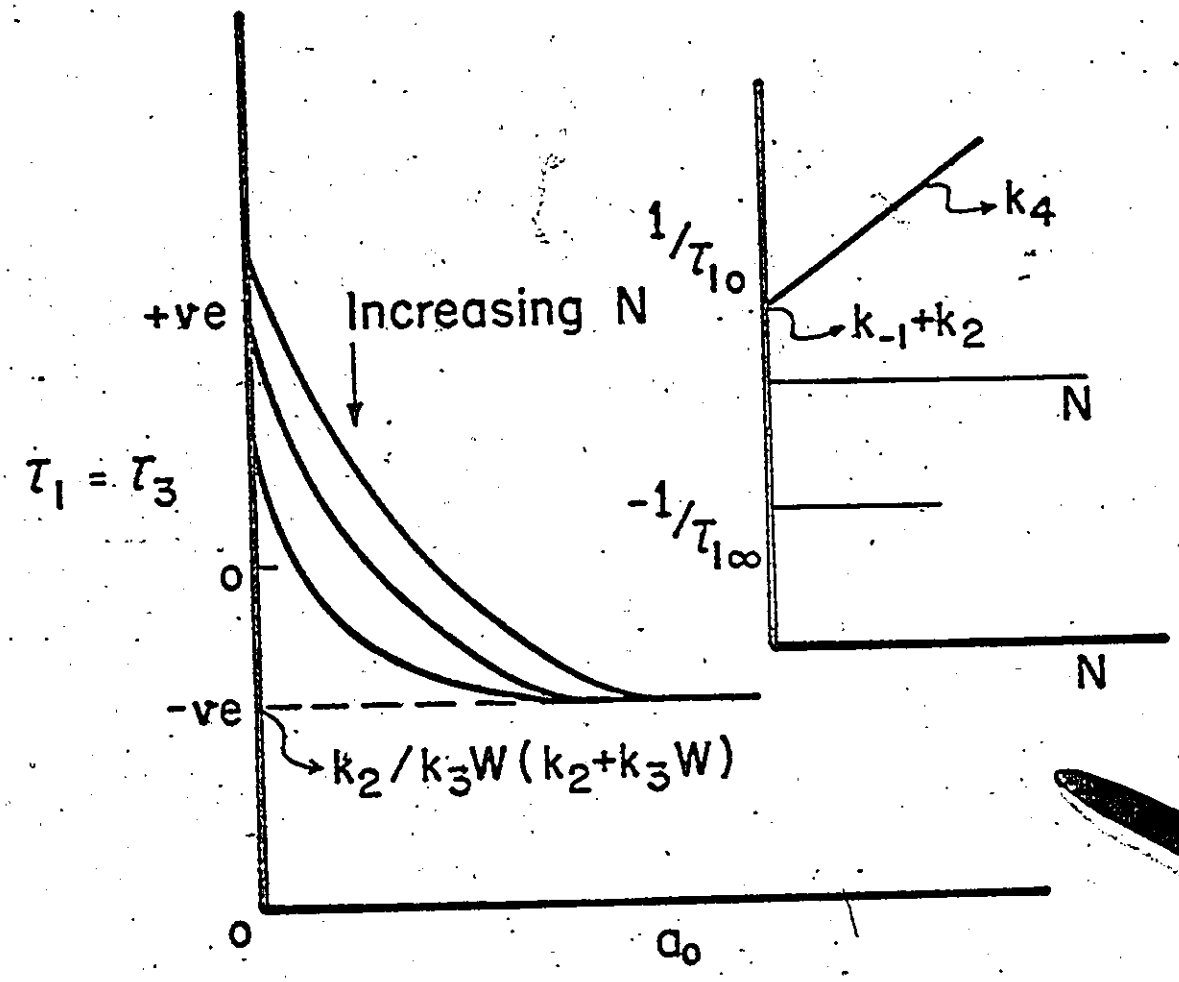
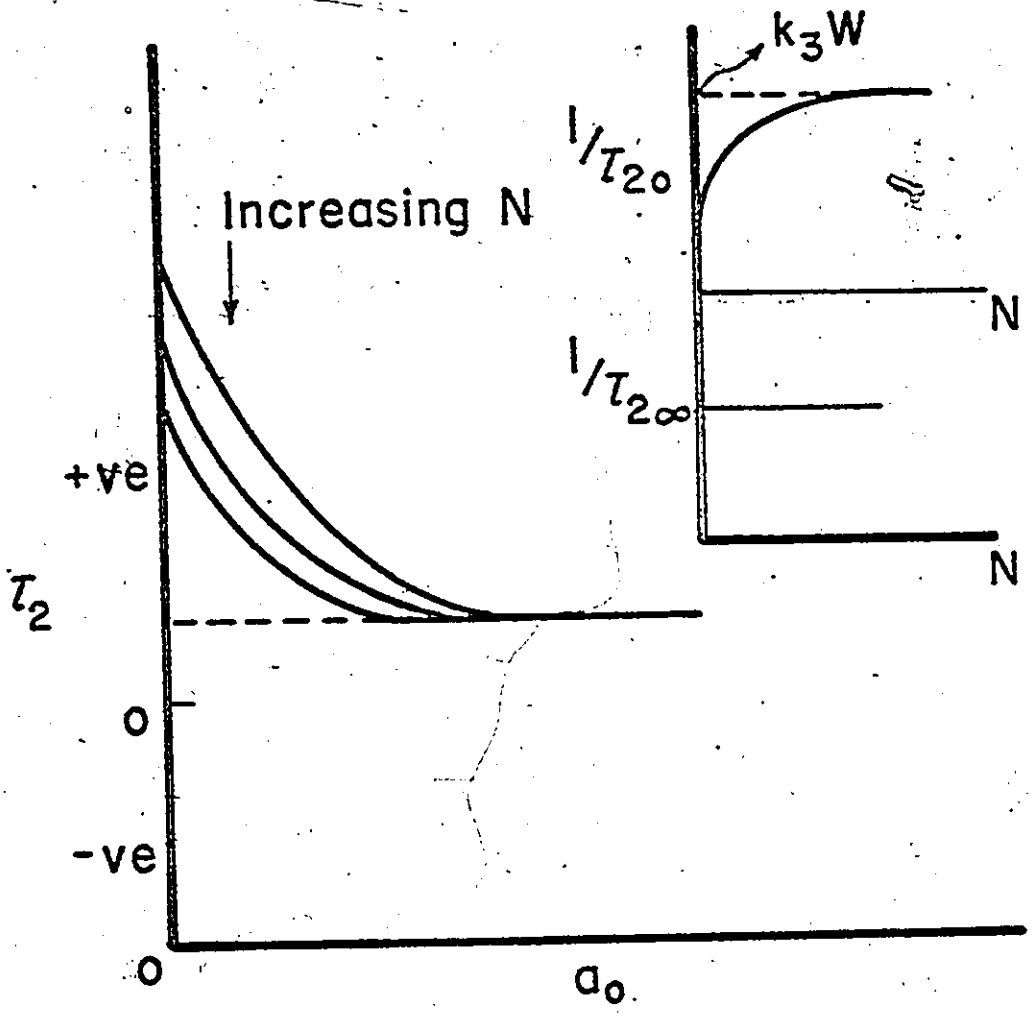


Fig. 9. Schematic plots of eqs [62] , [64] and [66].


k_3W
$1/\tau_{20}$
Increasing N
N
$1/\tau_{2\infty}$
N
+ve
τ_2
0
-ve
0
a_0

Discussion

As mentioned in the introduction, the purpose of this analysis is to establish criteria by which the three mechanisms can be distinguished.

If experimentally the formation of product X_1 was being monitored, then this would give rise to one of the induction periods denoted by τ_1. It is clear from Figs. [3] and [5] that the three mechanisms give a different pattern for the three parameters τ_1, $1/\tau_{10}$ and $1/\tau_{1\infty}$. While $\tau_{1\infty}$ for Mechanism I approaches zero, it approaches a constant negative value for Mechanism III. The same parameter, however, does not approach a constant value for Mechanism II.

Similarly if product X_2 or X_3 was being monitored, then this would give rise to induction periods τ_2 or τ_3 respectively. A simple inspection of the appropriate figures shows that there is a different pattern for each of the three mechanisms.

From the above discussion, it is obvious that the three mechanisms can be distinguished on the basis of their characteristic induction periods.

CHAPTER THREE

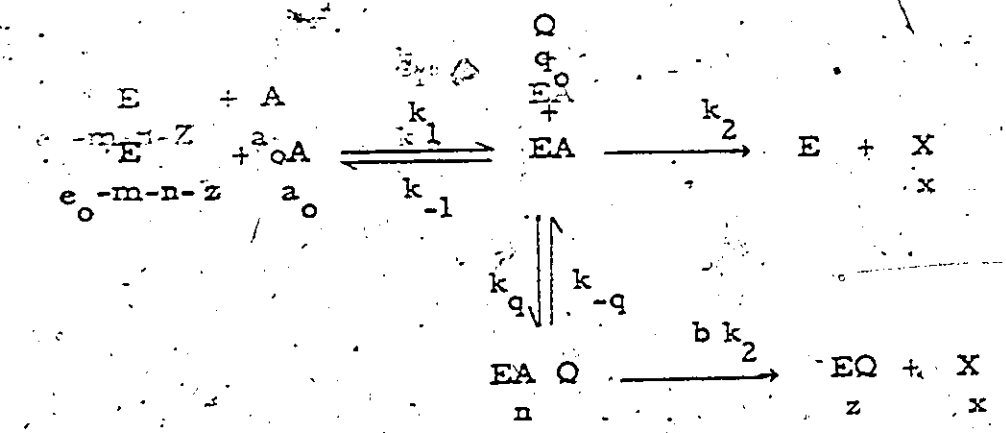
TRANSIENT-PHASE AND STEADY-STATE KINETICS FOR ENZYME ACTIVATION

Introduction

In some enzyme systems, a modifier may activate the enzyme by binding with the enzyme-substrate complex to form a ternary enzyme-substrate-modifier complex which breaks down at a comparable or faster rate than the binary enzyme-substrate complex. The theoretical steady-state kinetics of this system is well understood. This chapter deals with the transient-phase kinetics of the above system and includes the case in which the substrate molecule itself also acts as an activator. Finally, some data on carboxypeptidase A are analyzed with reference to derived equations.

No Steady-State

The reaction scheme first considered is



A is the substrate, which like the modifier is considered to be in great excess of the enzyme; Q is the modifier, in this case considered to be an activator (a special case, to be considered, is when Q = A). The concentrations of the various species are indicated in small letters.

The differential rate equations are

[1] $$\dot{m} = k_1 a_o (e_o - m - n - z) - \tilde{k} m - k_q q_o m + k_{-q} n$$

[2] $$\dot{n} = k_q q_o m - (k_{-q} + bk_2) n$$

[3] $$\dot{x} = \tilde{k}_2 m + bk_2 n$$

[4] $$\dot{z} = bk_2 n$$

where $\tilde{k} = k_{-1} + k_2$. If the differentials are replaced by operators, P, following the Laplace-Carson procedure, the result is

[5] $$(P + \tilde{k} + k_q q_o + k_1 a_o) m = k_1 a_o e_o - (k_1 a_o - k_{-q}) n - k_1 a_o z$$

[6] $$(P + k_{-q} + bk_2) n = k_q q_o n$$

[7] $$n = \frac{k_q q_o m}{P + k_{-q} + bk_2}$$

[8] $$z = \frac{bk_2 n}{P}$$

Substitution of the expressions for n and z into eq. [5] leads to

[9] $$m = \frac{k_1 a_o e_o P (P + k_{-q} + bk_2)}{(P + \lambda_1)(P + \lambda_2)(P + \lambda_3)}$$

where λ_i's are the negative roots of the equation

[10] $$P^3 + MP^2 + LP + C = 0$$

and

[11] $$M = k_1 a_o + k_q q_o + \tilde{k} + k_{-q} + bk_2$$

[12] $$L = k_{-q} (\tilde{k} + k_q q_o) + bk_2 (\tilde{k} + k_1 a_o + k_q q_o) + k_q k_1 a_o q_o$$

[13] $$Q = k_1 bk_2 k_q q_o a_o$$

From eqs. [3] and [7]

[14] $$\dot{x} = k_2 m + \frac{bk_2 k_q q_o m}{P + k_{-q} + bk_2}$$

hence

[15] $$x = \frac{k_2 (P + k_{-q} + bk_2 + bk_q q_o) m}{P (P + k_{-q} + bk_2)}$$

Substitution of eq. [9] for m into eq. [15] gives

[16] $$x = \frac{k_1 k_2 e_o a_o (P + k_{-q} + bk_2 + bk_q q_o)}{(P + \lambda_1)(P + \lambda_2)(P + \lambda_3)}$$

The original for x is

[17] $$x = \frac{k_1 k_2 a_o e_o (k_{-q} + bk_2 + bk_q q_o)}{\lambda_1 \lambda_2 \lambda_3}$$

$$- \sum_{i=1}^{3} \frac{k_1 k_2 a_o e_o (k_{-q} + bk_2 + bk_q q_o - \lambda_i)}{\lambda_i (P - \lambda_i)} e^{-\lambda_i t}$$

Since $\lambda_1 \lambda_2 \lambda_3 = k_1 bk_2 k_q q_o a_o$ this leads to

[18] $$x = e_o \left(\frac{k_{-q} + bk_2}{bk_q q_o} + 1 \right)$$

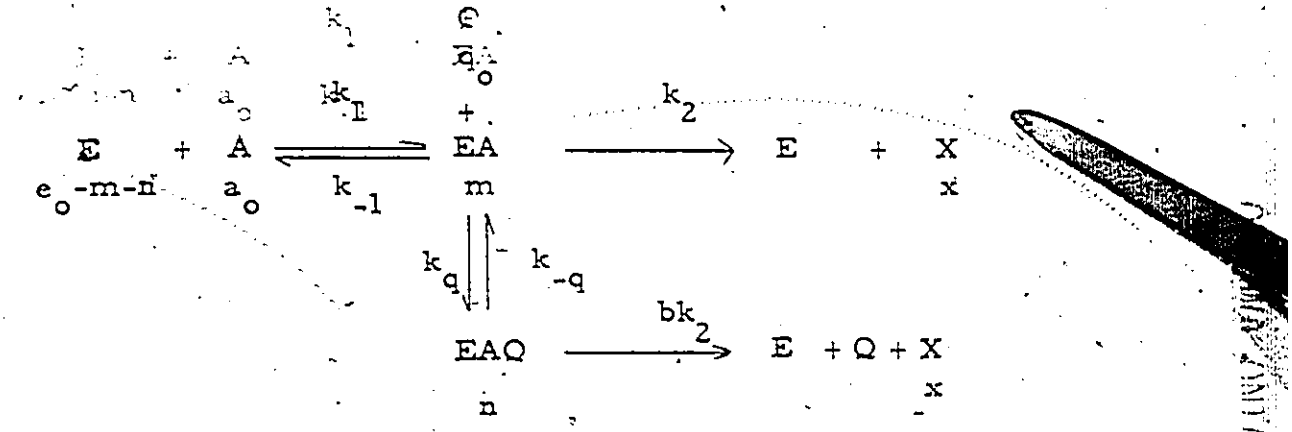
$$- \sum_{i=1}^{3} \frac{k_1 k_2 a_o e_o (k_{-q} + bk_2 + bk_q q_o - \lambda_i)}{\lambda_i (P - \lambda_i)} e^{-\lambda_i t}$$

Thus, the variation of product concentration x with t corresponds to a triphasic exponential rise, with a limiting value of x equal to the first term in eq. [18]. That is, no steady state is established.

Establishing a Steady-State

In those cases where Q is the substrate itself a steady state is generally found after an initial transient phase. A modification to the mechanism is therefore necessary. The absence of a steady state is attributable to the fact that EAQ decomposes into EQ + X, rather than into E + Q + X. The latter possibility is now considered.

The scheme is



The differential rate equations are now

[19] $$\dot{m} = k_1 a_o (e_o - m - n) - \tilde{k} m k_q q_o m + k_{-q} n$$

[20] $$\dot{n} = k_q q_o m - (k_{-q} + bk_2) n$$

[21] $$\dot{x} = k_2 m + bk_2 n$$

Replacing the differentials by operators P gives

[22] $$(P + \tilde{k} + k_q q_o + k_1 a_o) m = k_1 e_o a_o - (k_1 a_o - k_{-q}) n$$

[23] $$(P + k_{-q} + bk_2) n = k_q q_o m$$

[24] $$n = \frac{k_q q_o m}{(P + k_{-q} + bk_2)}$$

Substitution for n into eq. [22] leads to

$$[25] \qquad m = \frac{k_1 a_o e_o (P + k_{-q} + bk_2)}{(P + \lambda_1)(P + \lambda_2)}$$

where

$$[26] \qquad \lambda_1 = \tfrac{1}{2} (-R - \sqrt{R^2 - 4Q})$$

$$[27] \qquad \lambda_2 = \tfrac{1}{2} (-R + \sqrt{R^2 - 4Q})$$

and

$$[28] \qquad R = k_1 a_o + \tilde{k} + k_q q_o + k_{-q} + bk_2$$

$$[29] \qquad Q = k_{-q} (k_1 a_o + \tilde{k}) + bk_2 (k_1 a_o + \tilde{k} + k_q q_o) + k_1 k_q q_o a_o$$

It then follows from eq. [21] that

$$[30] \qquad x = \frac{k_1 k_2 a_o e_o (P + k_{-q} + bk_2 + bk_q q_o)}{P (P + \lambda_1)(P + \lambda_2)}$$

The original for x is

$$[31] \qquad x = \frac{k_1 k_2 a_o e_o (k_{-q} + bk_2 + bk_q q_o)}{\lambda_1 \lambda_2}$$

$$+ \frac{k_1 k_2 a_o e_o (k_{-q} + bk_2 + bk_q q_o - \lambda_1)}{\lambda_1^2 (\lambda_2 - \lambda_1)} (e^{-\lambda_1 t} - 1)$$

$$+ \frac{k_1 k_2 a_o e_o (k_{-q} + bk_2 + bk_q q_o - \lambda_2)}{\lambda_2^2 (\lambda_1 - \lambda_2)} (e^{-\lambda_2 t} - 1)$$

Introduction of expressions for λ_1 and λ_2 then gives

$$[32] \qquad x = \frac{k_2 a_o e_o (1 + \alpha q_o)}{K_m (1 + \frac{\alpha k_2}{k_1 K_m} q_o) + a_o (1 + \frac{\alpha}{b} q_o)} t$$

$$+ \beta_1 (e^{-\lambda_1 t} - 1) + \beta_2 (e^{-\lambda_2 t} - 1)$$

where

$$[33] \qquad \alpha = bk_q / (k_{-q} + bk_2)$$

$$[34] \qquad K_m = \frac{k_{-1} + k_2}{k_1} = \frac{\tilde{k}}{k_1}$$

β_1 and β_2 are the exponential coefficients as defined in the original for x, eq. [31]. Since eq. [32] contains a term linear in t (contrast eq. [18], the transient phase is followed by the establishment of a steady state. The mathematical condition for a steady state in the case where EQ is formed, is obtained by setting k_1 bk_2 k_q e_o a_o equal to zero in eq. [13].

Equation [32] may be written as

$$[35] \qquad x = vt + \beta_1 (e^{-\lambda_1 t} - 1) + \beta_2 (e^{-\lambda_2 t} - 1)$$

where

$$[36] \qquad v = \frac{k_2 a_o e_o (1 + \alpha q_o)}{K_m (1 + \frac{\alpha k_2 q_o}{k_1 K_m}) + a_o (1 + \frac{\alpha}{b} q_o)}$$

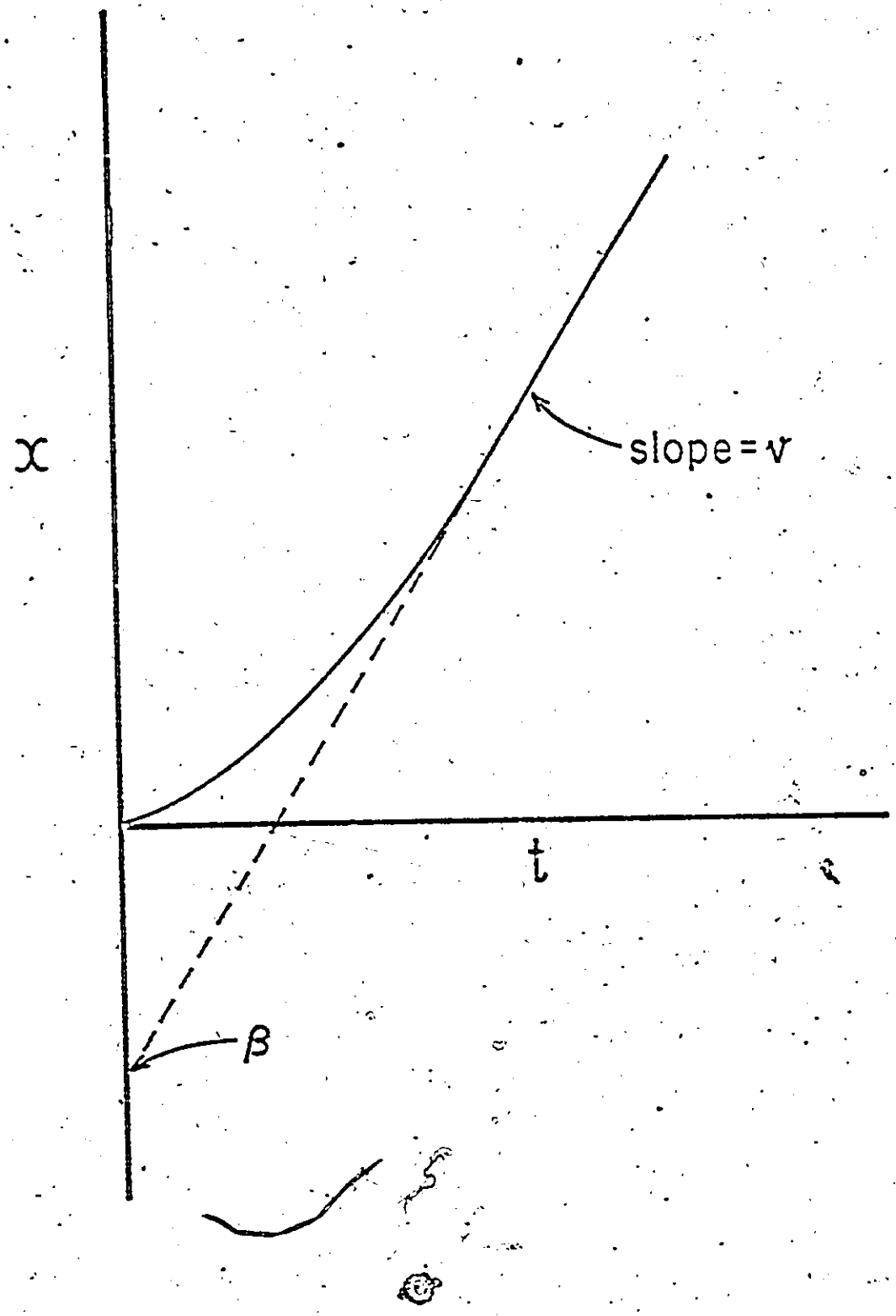
A schematic plot of x against t is shown in Fig. 10.

Analysis of Experimental Results

The Steady State

The steady-state phase of the reaction may be analyzed by means of a Lineweaver-Burk plot; according to eq. [36] a plot of $1/v$ against $1/a_o$

Fig. 10 Schematic plot of x against t (cf. eq. [35]).


x
slope = v
t
β

$1/a_o$ will have an intercept of

[37] $$\text{Intercept} = (1 + \frac{\alpha}{b} q_o)/k_2 e_o (1 + \alpha q_o)$$

and a plot of this intercept against q_o is of the form shown in Fig. 11; its intercept is $1/k_2 e_o$, from which k_2 can be determined. The initial slope of this plot is α/bk_2, so that α/b can be calculated. The limiting value of the curve is $1/bk_2 e_o$, hence b can be calculated, and hence α can also be obtained.

The slope of the Lineweaver-Burk plot is

[38] $$\text{Slope} = \frac{K_m \frac{\alpha k_2 q_o}{k_1}}{k_2 e_o (1 + \alpha q_o)}$$

and a plot of this against q_o is shown schematically in Fig. 12. The quantities k_1, k_2, α, K_m and b can be obtained from such plots.

The application of these methods to carboxypeptidase is considered later in this chapter.

The Pre-Steady-State

Analysis of the pre-steady state part of the reaction can be carried out as follows. Eq. [35] can be rearranged to give

[39] $$x - vt + \beta = \beta_1 e^{-\lambda_1 t} + \beta_2 e^{-\lambda_2 t}$$

A plot of $\ln (x - vt + \beta)$ against t gives two straight regions (biphasic) whose slopes are $-\lambda_1$ and $-\lambda_2$. From eqs. [28] and [29] the following relations hold between the λ_i's:

Fig. 11 A plot of Intercept of a Lineweaver-Burk plot against q_o, the activator concentration (cf. eq. [37]).

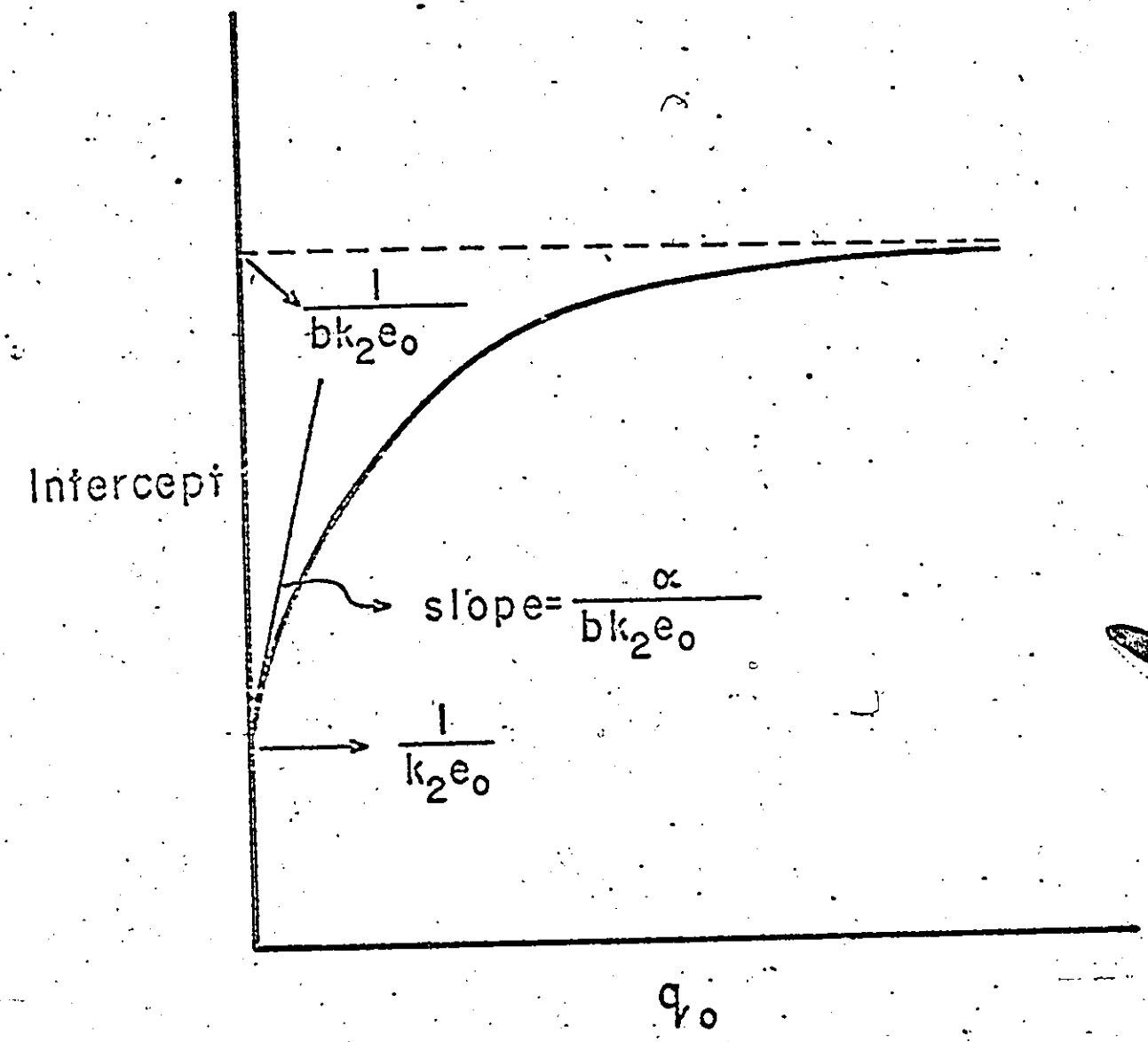
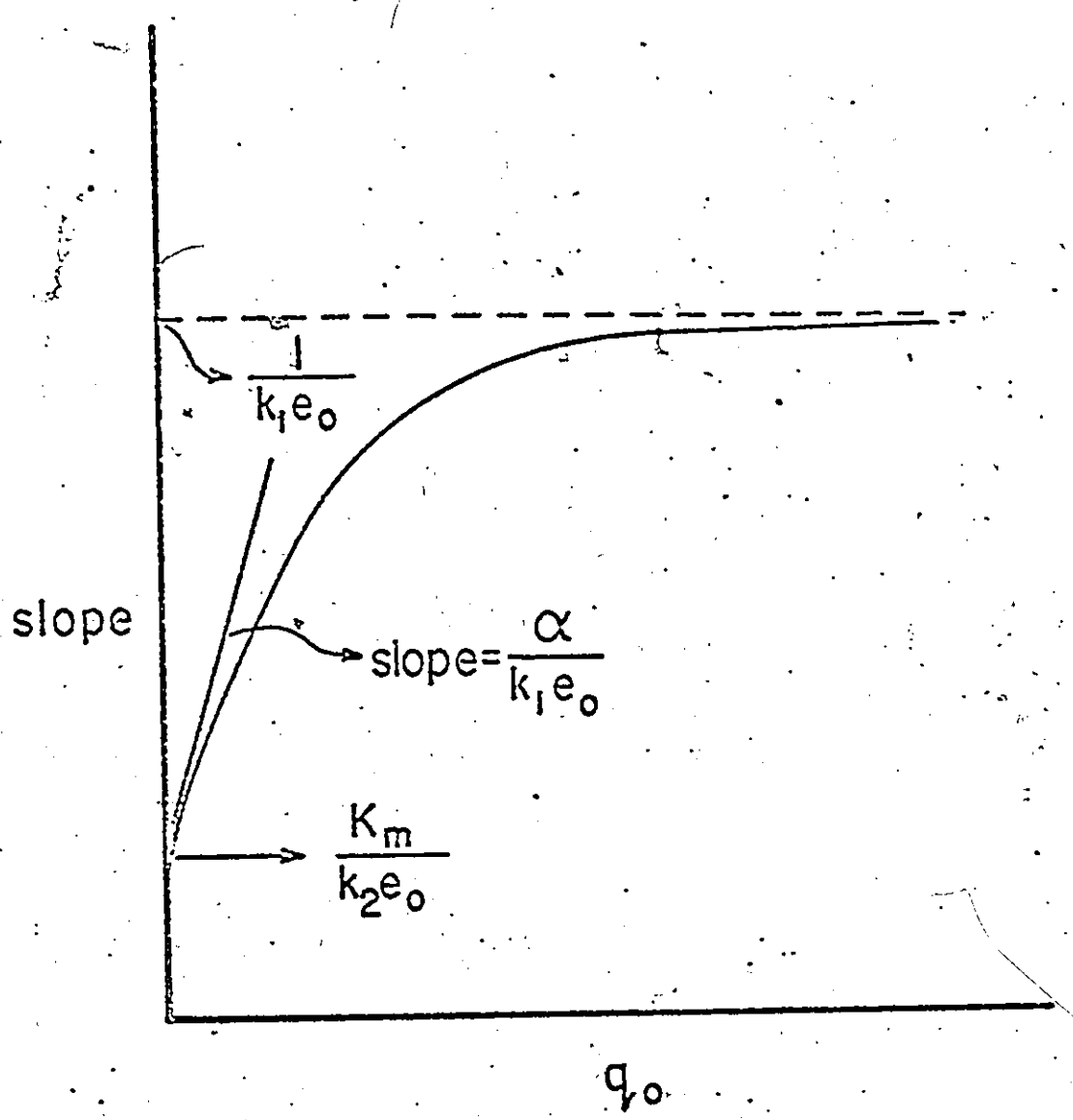


Fig. 12. A schematic plot of the slope of a Lineweaver-Burk plot against q_o, the activator concentration (cf. eq. [38]).


slope
$\frac{1}{k_1 e_o}$
slope $= \frac{\alpha}{k_1 e_o}$
$\frac{K_m}{k_2 e_o}$
q_o

$$[40] \qquad (\lambda_1 + \lambda_2) = R = k_1 a_o + k_q q_o + \tilde{k} + k_{-q} + bk_2$$

$$[41] \qquad \lambda_1 \lambda_2 = Q = k_1 a_o (k_{-q} + bk_2 + k_q q_o) + \tilde{k} (k_{-q} + bk_2) + bk_2 k_q q_o$$

$$[42] \qquad vQ = k_1 k_2 (k_{-q} + bk_2 + bk_q q_o) e_o a_o$$

Plots of R against a_o and q_o are both linear with slopes of k_1 and k_q respectively. From these plots $(\tilde{k} + k_{-q} + bk_2)$ can be calculated.

A plot of vQ against a_o is linear with

$$[43] \qquad \text{Slope} = k_1 k_2 (k_{-q} + bk_2 + bk_q q_o) e_o$$

and a plot of this slope against q_o gives $k_1 bk_2 k_q$ and $k_1 k_2 (k_{-q} + bk_2)$. Since k_1 and k_q are known, values of bk_2 and $k_2 (k_{-q} + bk_2)$ can be separated. Also $\tilde{k} + k_{-q}$ can be obtained from $(\tilde{k} + k_{-q} + bk_2)$

Since values of $\tilde{k}$ and k_2 can be calculated from systems in the absence of an activator, then k_{-q} and b can be calculated. Hence from transient-phase experiments in the absence and presence of an activator all rate constants can be calculated.

Application to Experimental Results

Introduction

Suitable data are available in the literature for some cases of substrate activation. For this situation $q_o = a_o$, and the initial steady-state rate becomes

$$[44] \qquad v = \frac{k_1 a_o e_o + \alpha k_2 e_o a_o^2}{K_m + a_o (1 + \frac{\alpha k_2}{k_1}) + \frac{\alpha}{b} a_o^2}$$

There are two kinds of substrate activation [32]:

(I) A non-sigmoidal v vs a_o dependence and

(II) A sigmoidal v vs a_o dependence.

Case I Non-Sigmoidal Activation

The carboxypeptidase-catalyzed hydrolysis of benzyloxy-carbonyl glycyl-L-phenyl-alanine shows non-sigmoidal behavior [33-38]. A plot of v_o/e_o against a_o is shown in Fig. 13, the data being those of Whitaker, Menger and Bender [33]. These workers observed that the activity at higher substrate concentrations is more than expected from the activity at lower substrate concentrations. This led the authors to the conclusion that there is a slight substrate activation at higher substrate concentrations. The authors corrected their data for this apparent substrate activation and calculated K_m(app) and k_{cat} as 5.53×10^3 M and 106 sec^{-1}, respectively. A plot of e_o/v against $1/a_o$ is shown in Fig. 14 for the data at the higher substrate concentrations. This figure shows non-sigmoidal activation according to the theoretical analysis of Botts [32], the criterion being that the extrapolated linear part of the curve intersects the negative part of the e_o/v_o axis.

Another plot which might prove to be useful for analysis of experimental results is based on a rearranged form of eq. [44],

$$[45] \qquad \frac{v}{e_o} = \frac{k_2}{1 + \frac{\alpha k_2}{k_1}} + \frac{\alpha k_2 a_o}{1 + \frac{\alpha k_2}{k_1}} + \frac{K_m}{1 + \frac{\alpha k_2}{k_1}} \frac{v}{e_o a_o}$$

when v/e_o is plotted against $v/e_o a_o$ a curve will be obtained. At low substrate concentrations, the term $\alpha k_2 a_o / (1 + \frac{\alpha k_2}{k_1})$ will become

Fig. 13 A plot of v_o/e_o against a_o for the data of ref. (33) for the carboxypeptidase A - catalyzed hydrolysis of benzylosycarbonylglycyl-L-phenyl alanine.

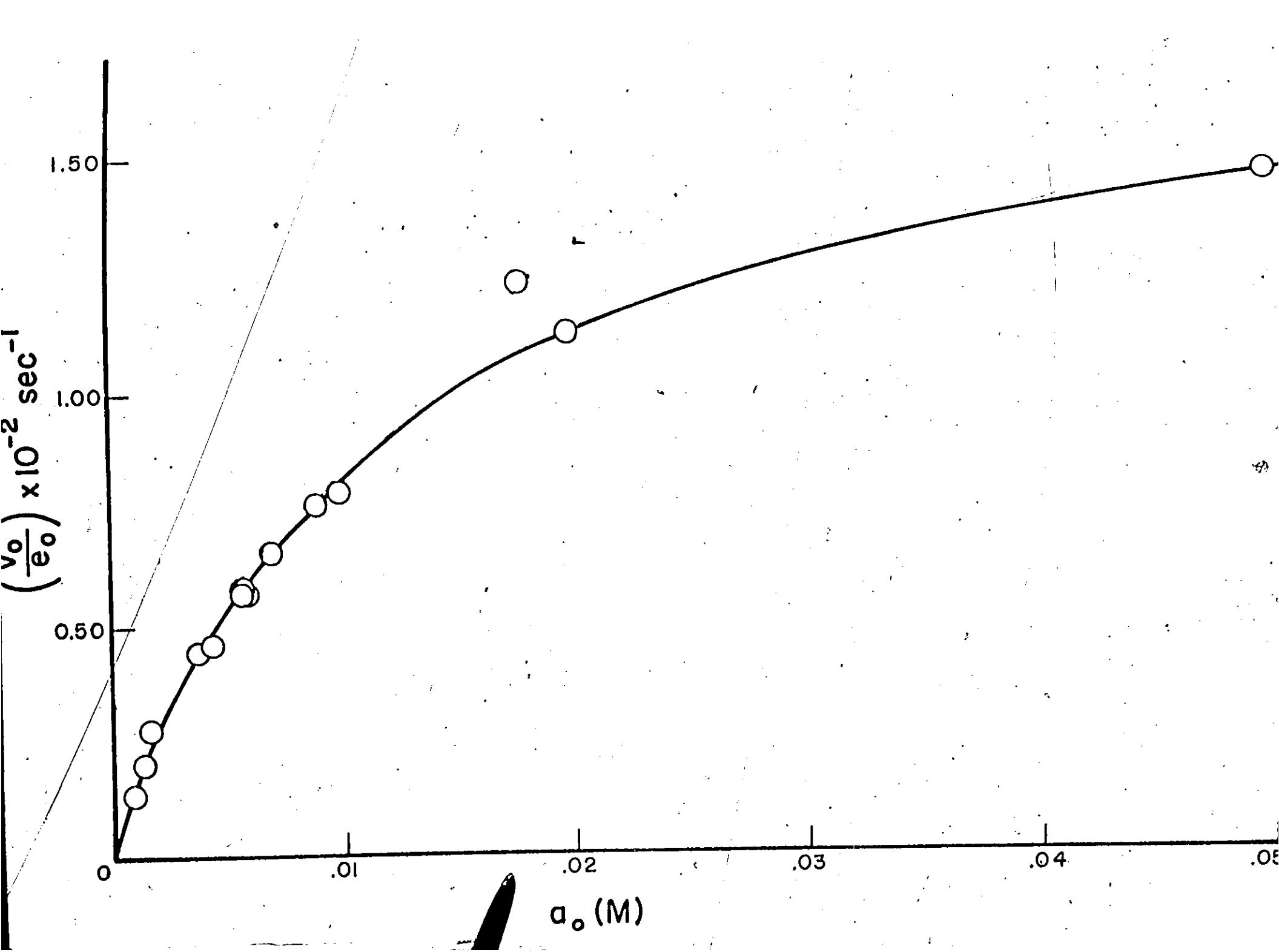

1.50
1.00
0.50
0
.01
.02
.03
.04
.05
$\left(\frac{v_o}{e_o}\right) \times 10^{-2}$ sec^{-1}
a_o (M)

Fig. 14 A plot of e_o/v against $1/a_o$ for the same data as in Fig. 13.

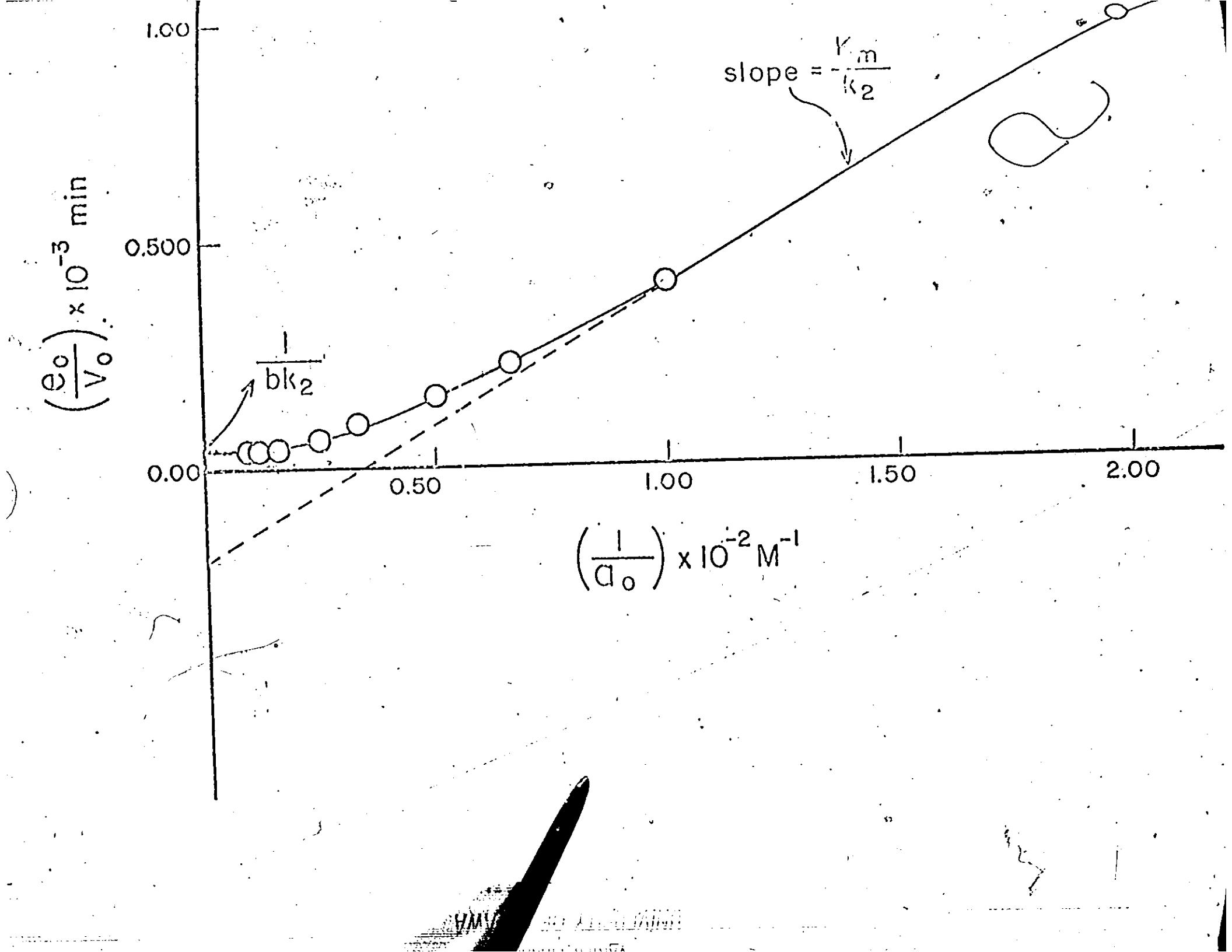
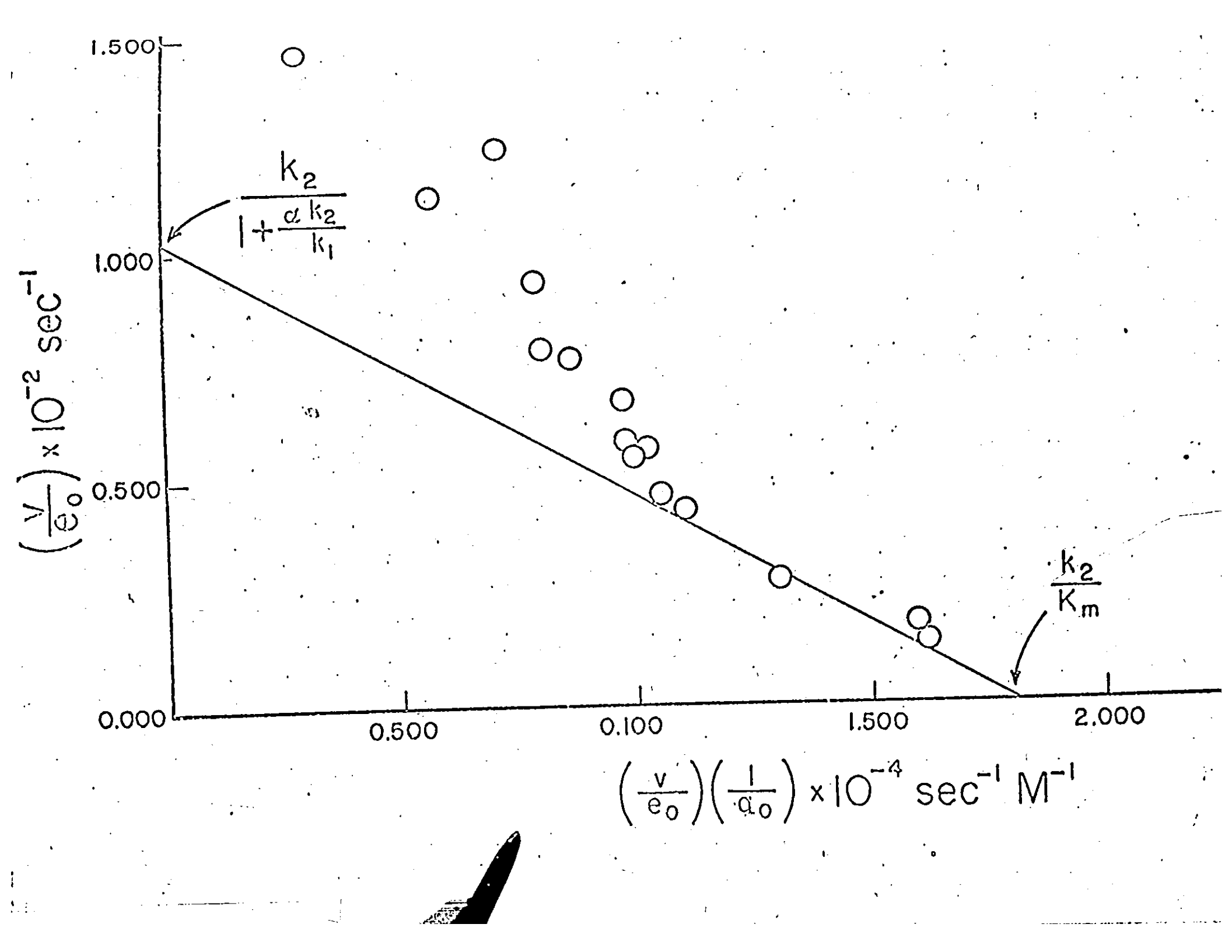

1.00
0.500
0.00
0.50
1.00
1.50
2.00
slope = $\frac{K_m}{k_2}$
$\frac{1}{bk_2}$
$\left(\frac{e_o}{V_o}\right) \times 10^{-3}$ min
$\left(\frac{1}{a_o}\right) \times 10^{-2} M^{-1}$

Fig. 15 Plot of v/e_o vs. $v/e_o\, a_o$ for the data of Fig. 13.


1.500
1.000
0.500
0.000
$\frac{k_2}{1+\frac{\alpha k_2}{k_1}}$
$\frac{k_2}{K_m}$
$\left(\frac{v}{e_o}\right) \times 10^{-2}\ sec^{-1}$
0.500
0.100
1.500
2.000
$\left(\frac{v}{e_o}\right)\left(\frac{1}{a_o}\right) \times 10^{-4}\ sec^{-1}\ M^{-1}$

negligible as compared to the term $K_m/(1 + \frac{a k_2}{k_1})$ at this portion the curve can be approximated by a line of slope $K_m/(1 + \frac{a k_2}{k_1})$. As K_m is known the term $(1 + a k_2/k_1)$ can be calculated. Such a plot is shown in Fig. 15 for the data of Whitaker et al.[33]. More experimental data will be required to calculate the numerical values of individual rate constants.

Case II Sigmoidal Activation

This is shown by the carboxypeptidase A catalyzed hydrolysis of O-hippuryl glycolate[39]. The v/e_o vs. a_o plot levels off at high a_o and eq. (44) must therefore be used as it stands. From the plot of v/e_o vs. a_o (ref. 39, Fig. 1).

$$\text{Initial Slope} = \frac{a k_2}{K_m} = 3.33 \times 10^{3}\ M^{-1}\ \text{sec}^{-1}$$

$$V_{max} = bk_2 = k_{cat} = 416\ \text{sec}^{-1}$$

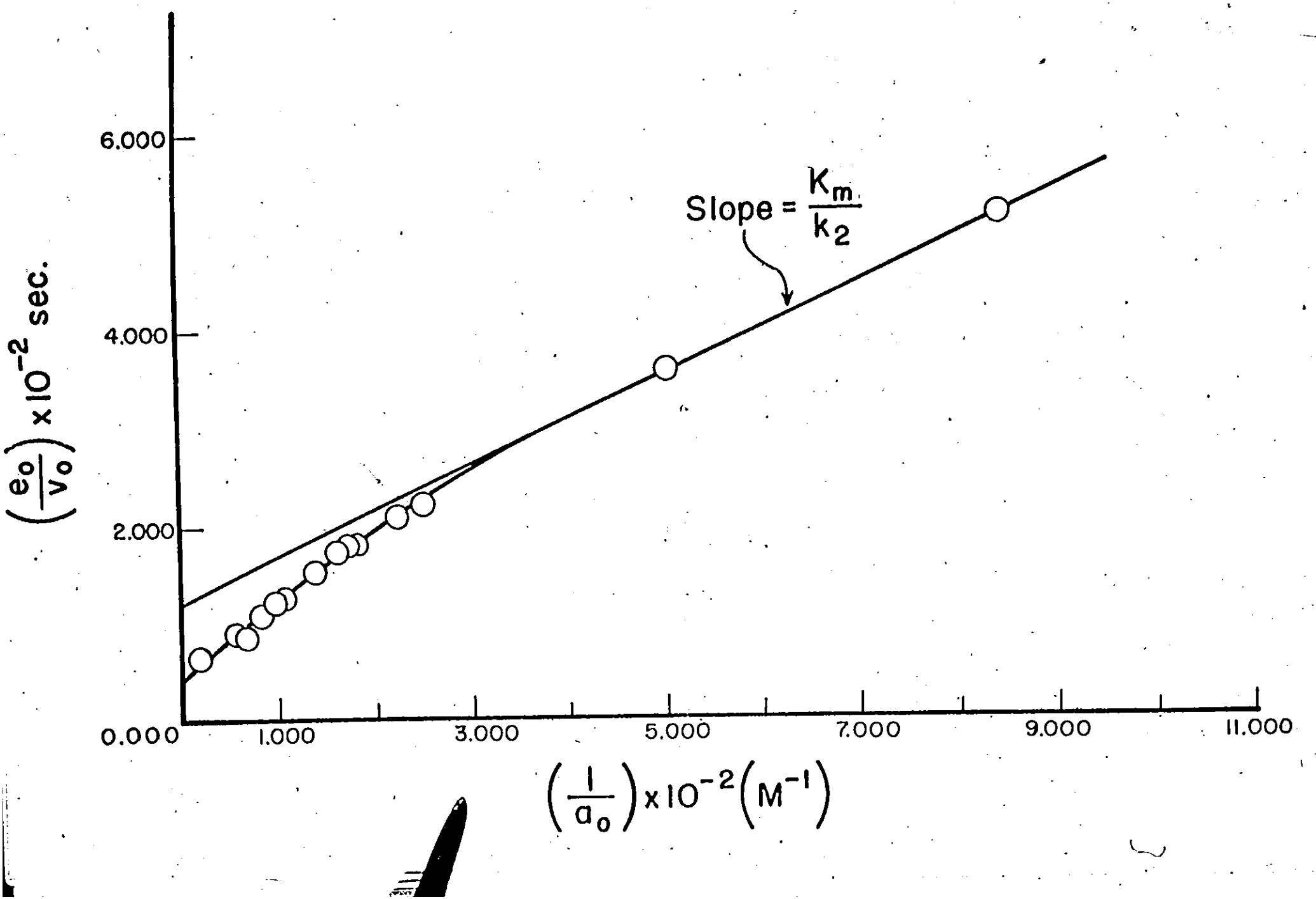
From the eq. (44) the following rearrangement is made

$$[46] \qquad \frac{e_o}{v} = \frac{\frac{a}{b} + (1 + \frac{a k_2}{k_1})(\frac{1}{a_o}) \qquad K_m (\frac{1}{a_o})^2}{a k_2 + k_2 (\frac{1}{a_o})}$$

Fig. 16 shows a plot of e_o/v against $1/a_o$. Then the slope of the linear part is $K_m/k_2 = 2.75 \times 10^{-3}$ M. sec. The intercept is $1/bk_2$ and from $a k_2/K_m$ and K_m/k_2, a can be obtained, hence $a = 15.8$. From this study k_2 and K_m cannot be separated. It would be possible to obtain the k_2 and K_m values from a study with an external activator Q,

by use of the equations previously obtained. It is to be noted that the numerical values calculated for α, K_m/k_2, and k_{cat} are approximate values.

Fig. 16 Plot of e_o/v_o against $1/a_o$ for the data of ref. 39 for the carboxypeptidase A catalyzed hydrolysis of O-hippuryl-glycolate. The data has been read off Fig. 1, ref. 39.


6.000
4.000
2.000
0.000
$\left(\frac{e_o}{v_o}\right) \times 10^{-2}$ sec.
Slope = $\frac{K_m}{k_2}$
1.000
3.000
5.000
7.000
9.000
11.000
$\left(\frac{1}{a_o}\right) \times 10^{-2} (M^{-1})$

CHAPTER FOUR

TRANSIENT-PHASE OF TWO SYSTEMS EXHIBITING SIGMOID KINETICS IN THE STEADY-STATE

Introduction

Under steady-state conditions, a hyperbolic relationship between reaction velocity and initial substrate concentration provided a unifying basis for the early development of enzyme kinetics, deviations from this relationship often being treated as minor perturbations. However beginning with the discovery of feedback regulations, non-hyperbolic, especially sigmoidal, relationships have emerged as extensively observed features in enzymic activities.

In a sigmoidal relationship, the slope of the plot of reaction velocity against substrate concentration first increases and then decreases, as shown schematically in Fig. 17. Rate equations, in the steady-state, have been worked for various models (See ref. 10, chapter 11 references therein).

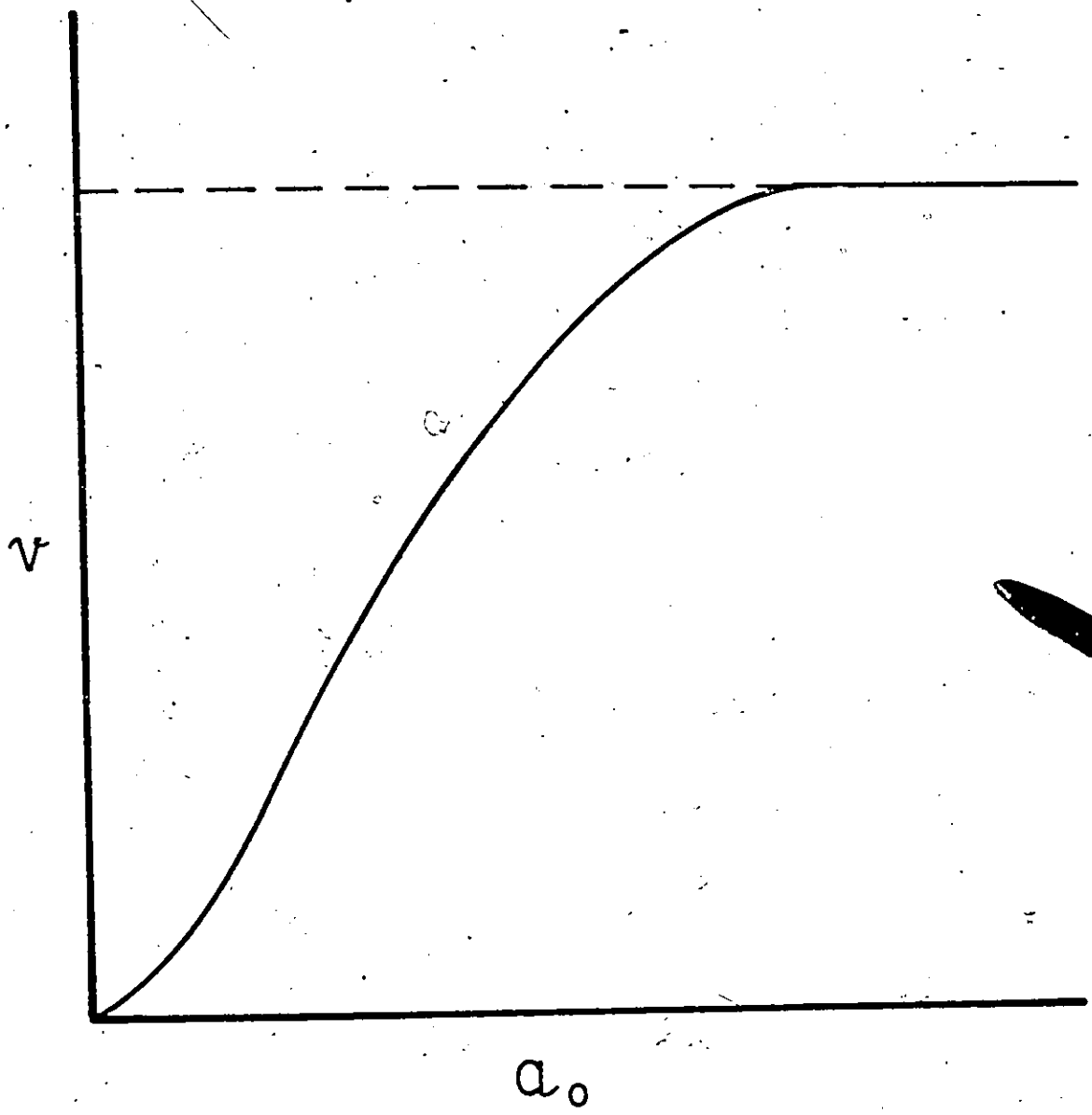
The development of fast reaction techniques permits observation of the kinetics in the transient phase of the reaction. So far there has been no theoretical treatment of the transient-phase kinetics of systems that exhibit sigmoidal behavior.

The present chapter deals with two simple models that lead to sigmoidal behavior:

(I) Two forms of the monomeric enzyme.

(II) Binding of two molecules of substrate to one molecule of a monomeric enzyme.

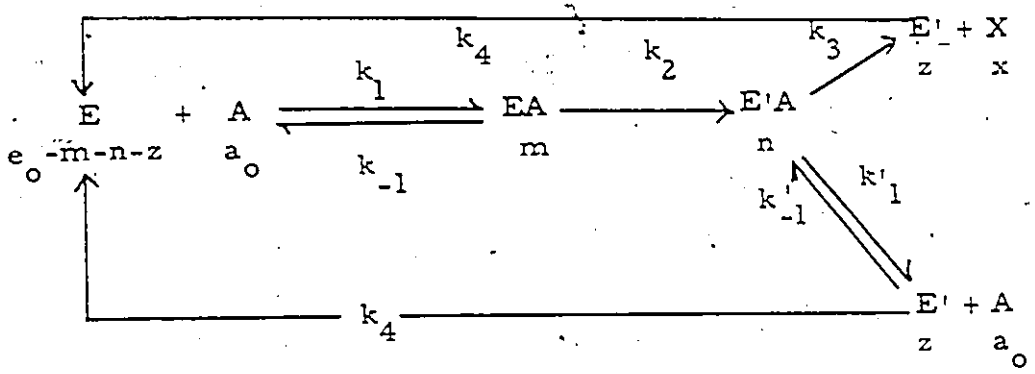
Fig. 17 A schematic plot of reaction velocity v against a_o, the initial substrate concentration showing a sigmoidal dependence of v on a_o.


v
a_o

Two Forms of the Enzyme

Introduction

It has been suggested [40-43] that a monomeric enzyme which exists in two forms of different activities shows sigmoidal behavior. A specific mechanism of this type has been suggested by Rabin [40].



The concentrations of the species are shown in small letters. It is assumed in the above scheme that the substrate concentration, a_o, is in large excess of the enzyme concentration, e_o. It is also assumed that the substrate concentration does not change effectively during the short transient phase and the initial steady-state cycle.

In Rabin's mechanism, the enzyme form E' is more reactive than form E; E'A can give rise to E' and product x, but EA cannot. Thus the form E'A is activated by the presence of substrate; this is usually referred to as a substrate-induced activation. At high substrate concentrations, there is an abnormal increase in rate because A forces the conversion of E into E'; at low substrate concentrations the rate varies with a higher power than the first so that sigmoidal kinetics results.

Solution

The differential rate equations are

[1] $$\dot{m} = k_1 a_o (e_o - m - n - z) - (k_{-1} + k_2) m$$

[2] $$\dot{n} = k_2 m - (k_3 + k'_1)n + k_{-1}' a_o z$$

[3] $$\dot{z} = (k'_1 + k_3) n - (k'_{-1} a_o + k_4) z$$

[4] $$\dot{x} = k_3 n$$

Replacing the differentials by operator P and algebraic solution of the resulting equations leads to the following transforms for m, n, z and x.

[5] $$m = \frac{k_1 a_o e_o [P^2 + P (k'_1 + k_3 + k_4 + k'_{-1} a_o) + k_4 (k'_{-1} + k_3)]}{(P + \lambda_1) (P + \lambda_2) (P + \lambda_3)}$$

[6] $$n = \frac{k_1 k_2 a_o e_o (P + k_4 + k'_{-1} a_o)}{(P + \lambda_1) (P + \lambda_2) (P + \lambda_3)}$$

[7] $$z = \frac{k_1 k_2 a_o e_o (k_3 + k'_1)}{(P + \lambda_1) (P + \lambda_2) (P + \lambda_3)}$$

[8] $$x = \frac{k_1 k_2 k_3 a_o e_o (P + k_4 + k'_{-1} a_o)}{P (P + \lambda_1) (P + \lambda_2) (P + \lambda_3)}$$

where $-\lambda_1$, $-\lambda_2$ and $-\lambda_3$ are the roots of the equation

[9] $$P^3 + MP^2 + NP + Q = 0$$

and

[10] $$M = (k_1 + k'_{-1}) a_o + k_{-1} + k_2 + k_3 + k'_1 + k_4$$

[11] $$N = (k_1 a_o + k_{-1} + k_2) (k_3 + k'_1 + k_4 + k'_{-1} a_o) + k_4 (k'_1 + k_3) + k_1 k_2 a_o$$

[12] $$Q = k_4 (k_{-1} + k_2) (k'_1 + k_3) + k_1 k'_{-1} k_2 a^2_o + a_o [k_1 k_2 (k_3 + k_4 + k'_1) + k_1 k_4 (k'_1 + k_3)]$$

The transforms, eqs. [5] - [8], can be easily replaced by the respective originals. Of special interest is the variation of x with time, t. Hence

$$[13] \qquad x = v_{st} + \sum_{i=1}^{3} \frac{k_1 k_2 [k_4 + k'_{-1} a_o - \lambda_i] e_o a_o}{\lambda_i^2 (P - \lambda_i)} (e^{-\lambda_i t} - 1)$$

where v_s, the steady-state rate, is

$$[14] \qquad v_s = \frac{k_1 k_2 k_3 [k_4 a_o + k'_{-1} a_o^2] e_o}{k_4 (k_{-1} + k_2)(k'_1 + k_3) + a_o [k_1 k_2 (k_4 + k_3 + k'_1) + k_1 k_4 (k'_1 + k_3)] + k_1 k'_{-1} k_2 a_o^2}$$

Analysis of Results

The steady-state rate, eq. [14], is the same as obtained from a strictly steady-state treatment[10, 40].

The variation of x with t, eq. [13], is a three exponential approach to the steady-state (linear in t). A plot of x against t will consist of a transient (exponential) rise that leads to a linear variation at later times. The slope of the linear part is v_s, and extension of this linear part intercepts the time axis at a point corresponding to the induction period τ. the intercept on the x-axis corresponding to β:

$$[15] \qquad \beta = - \sum_{i=1}^{3} \frac{k_1 k_2 [k_4 + k'_{-1} a_o - \lambda_i] e_o a_o}{\lambda_i^2 (P - \lambda_i)}$$

Eq. [13] can be written as

$$[16] \qquad x - v_s t + \beta = \sum_{i=1}^{3} \frac{k_1 k_2 [k_4 + k'_{-1} a_o - \lambda_i] e_o a_o}{\lambda_i^2 (P - \lambda_i)} e^{-\lambda_i t}$$

A plot of $\ln (x - v_s t + \beta)$ against t is triphasic and the slopes of the three straight regions correspond to $-\lambda_1$, $-\lambda_2$ and $-\lambda_3$ which obey the following relations :

[17] $$\lambda_1 \lambda_2 \lambda_3 = Q$$

[18] $$\sum_{i=1}^{3} \lambda_i = M$$

[19] $$vQ = k_1 k_2 k_3 [k_4 + k'_{-1} a_o] a_o e_o$$

Also, eq. [14] can be written as

[20] $$\frac{e_o}{v_s} = \frac{k_1 k'_{-1} k_2 + [k_1 k_2 (k_4 + k_3 + k'_1) + k_1 k_4 (k'_1 + k_3)] (1/a_o)}{k_1 k'_{-1} k_2 k_3 + k_1 k_2 k_3 k_4 (1/a_o)} + \frac{k_4 (k_{-1} + k_2) (k'_1 + k_3)(1/a_o)^2}{k_1 k'_{-1} k_2 k_3 + k_1 k_2 k_3 k_4 (1/a_o)}$$

A plot of e_o/v_s against $(1/a_o)$ is linear at high values of $(1/a_o)$ and then curves down. The intercept on the e_o/v_s axis is $(1/k_3)$. The slope of the linear part is

$$\text{slope} = \frac{(k_{-1} + k_2) (k'_1 + k_3)}{k_1 k_2 k_3}$$

From eq. [10] , a plot of M against a_o is linear with slope = $(k_1 + k'_{-1})$ and intercept = $k_{-1} + k_2 + k_3 + k'_1 + k_4$.

A plot of vQ/a_o against a_o, eq. [19] is linear with slope = $k_1 k_2 k'_{-1} k_3 e_o$ and intercept = $k_1 k_2 k_3 k_4 e_o$.

A value of $\gamma = k_4 (k_{-1} + k_2) (k'_1 + k_3)$ can be calculated from the above combinations of rate constants. Hence, from eq. [12],

[21] $$Q = \gamma + a_o [k_1 k_2 (k_4 + k_3 + k'_1) + k_1 k_4 (k'_1 + k_3)] + k_1 k'_{-1} k_2 a_o^2$$

and

[22] $$\frac{Q - \gamma}{a_o} = k_1 k_2 (k_4 + k_3 + k'_1) + k_1 k_4 (k'_1 + k_3) + k_1 k'_{-1} k_2 a_o$$

A plot of $\frac{Q - \gamma}{a_o}$ against a_o is linear with slope = $k_1 k'_{-1} k_2$

and

$$\text{intercept} = k_1 k_2 (k_4 + k_3 + k'_1) + k_1 k_4 (k'_1 + k_3)$$

By algebraic manipulation, various other combinations of rate constants can be calculated. Unfortunately, the individual rate constants cannot be separated, except for k_3. However all the phenomenological terms in the steady-state rate equation can be calculated by combination of steady-state and transient-phase experiments.

Binding of Two Substrate Molecules

Introduction

Worcel et al.[44] showed that sigmoidal kinetics can arise if the enzyme binds two molecules of substrate to form a ternary complex which undergoes reaction to give the enzyme, substrate and product. A more general mechanism[45, 46] has been considered where n molecules of substrate can bind to the enzyme. In this section only the attachment of two substrate molecules is considered.

Mechanism

The mechanism of the reaction can be represented as

$$\underset{e_o-m-n}{E} + \underset{a_o}{A} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{m}{EA} \xrightarrow{k_2 \, A \, a_o} \underset{n}{EA_2} \xrightarrow{k_3} E + \underset{a_o}{A} + \underset{x}{X}$$

The concentration of each species is shown below it. It is assumed, as usual, that $a_o >> e_o$.

The mechanism as shown above involves site interaction. If the binding of substrate at each of two or more identical sites involves no interaction between the sites, then normal hyperbolic behavior is obtained.

Worcel et al.[44] obtained evidence that this mechanism applies to the enzyme nicotinamide adenine dinucleotide oxidase.

Solution

The differential rate equations are

$$\dot{m} = k_1 a_o (e_o - m - n) - (k_{-1} + k_2 a_o) m \quad [23]$$

$$\dot{n} = k_2 a_o m - k_3 n \quad [24]$$

$$\dot{x} = k_3 n \quad [25]$$

Replacing the differentials by operators P and algebraic solution leads to the following transforms for m, n and x:

$$m = \frac{k_1 a_o e_o (P + k_3)}{(P + \lambda_1)(P + \lambda_2)} \quad [26]$$

$$n = \frac{k_1 k_2 a_o^2 e_o}{(P + \lambda_1)(P + \lambda_2)} \quad [27]$$

$$x = \frac{k_1 k_2 k_3 a_o^2 e_o}{P(P + \lambda_1)(P + \lambda_2)} \quad [28]$$

where

$$\lambda_1 = \tfrac{1}{2}(-R - \sqrt{R^2 - 4Q}) \quad [29]$$

$$\lambda_2 = \tfrac{1}{2}(-R + \sqrt{R^2 - 4Q}) \quad [30]$$

[31] $$R = (k_1 + k_2)\, a_o + k_{-1} + k_3 = \lambda_1 + \lambda_2$$

[32] $$C = k_{-1} k_3 + k_3 (k_1 + k_2)\, a_o + k_1 k_2 a_o^2 = \lambda_1 \lambda_2$$

The transforms for m, n, and x can easily be replaced by their originals. Only the variation of product x with time is considered below.

The original for x is

[33] $$x = \frac{k_1 k_2 k_3 a_o^2 e_o}{\lambda_1 \lambda_2}\, t + \sum_{i=1}^{2} \frac{k_1 k_2 k_3 a_o^2 e_o}{\lambda_i^2 (P - \lambda_i)} \left(e^{-\lambda_i t} - 1\right)$$

The first term in equation [33] corresponds to the initial steady state. Substituting the value of $\lambda_1 \lambda_2 = C$, leads to v_s, the steady-state rate:

[34] $$v_s = \frac{k_1 k_2 k_3 a_o^2 e_o}{k_{-1} k_3 + k_3 (k_1 + k_2)\, a_o + k_1 k_2 a_o^2}$$

Analysis of Results

The variation of x, eq. [33], with time is biphasic, leading to a linear part. The slope of the linear part is v_s. Extension of this linear part intercepts the time axis at a point corresponding to the induction period, τ, and the intercept on the x axis corresponds to $\beta = -(\beta_1 + \beta_2)$. Hence eq. [33] can be written as

[35] $$x - v_{st} + \beta = \beta_1 e^{-\lambda_1 t} + \beta_2 e^{-\lambda_2 t}$$

where

[36] $$\beta / v_s = \tau = \frac{(k_1 + k_2)\, a_o + k_{-1} + k_3}{k_{-1} k_3 + k_3 (k_1 + k_2)\, a_o + k_1 k_2 a_o^2}$$

or

$$[37] \qquad v_s/\beta = 1/\tau = \frac{k_{-1} k_3 (k_1 + k_2) a_o + k_1 k_2 a_o^2}{k_{-1} + k_3 + (k_1 + k_2) a_o}$$

A plot of $\ln (x - v_{st} + \beta)$ against t will consist of two straight regions whose slopes correspond to $-\lambda_1$ and $-\lambda_2$. Hence R and Q can be determined. A plot of R against a_o is linear, eq. [31], with slope = $(k_1 + k_2)$ and intercept = $k_{-1} + k_3$.

A plot of $1/\tau$ against a_o, eq. 37, is linear at high values of a_o, and then curves and intercepts the $1/\tau$ axis:

Slope of linear part = $k_1 k_2/(k_1 + k_2)$

Intercept on $1/\tau$ axis = $k_{-1} k_3/(k_{-1} + k_3)$

A plot of $v_s Q$ against a_o^2 is linear:

$$[38] \qquad v_s Q = k_1 k_2 k_3 a_o^2 e_o$$

and the slope is $k_1 k_2 k_3 e_o$.

From manipulation of the above values, one can separate k_3, k_{-1}, $k_1 k_2$ and $k_1 + k_2$. It is unfortunate that k_1 and k_2 cannot be separated.

CHAPTER FIVE

TRANSIENT-PHASE AND STEADY-STATE KINETICS FOR INHIBITED ENZYME SYSTEMS: SINGLE INTERMEDIATE MECHANISMS

Introduction

Up to now there has been no theoretical attack on the problem of pre-steady-state kinetics for enzyme systems in which inhibitors are present. Steady-state investigations on such systems have revealed that there are various patterns of behavior. From the phenomenological point of view we recognize the following extreme cases [10]:

(1) Pure non-competitive inhibition, in which the degree of inhibition is independent of substrate concentration.

(2) Competitive inhibition, in which the degree of inhibition is reduced on adding substrate.

(3) Anticompetitive (also known as coupling and uncompetitive) inhibition, where the degree of inhibition is increased as the substrate concentration is increased.

Various other types of behavior have been observed.

As far as mechanisms are concerned, it has been common in the past to interpret the various types of inhibition with respect to a simple Michaelis-Menten mechanism:

$$E + A \rightleftharpoons EA \longrightarrow E + X$$

where E is the enzyme, A is the substrate, X is the product and EA is the addition complex. It can be shown that if an inhibitor Q combines only with E (and not with EA) there will be competitive inhibition; if it combines only with EA (and not with E) there will be anticompetitive inhibition; while if it combines equally strongly with E and EA there

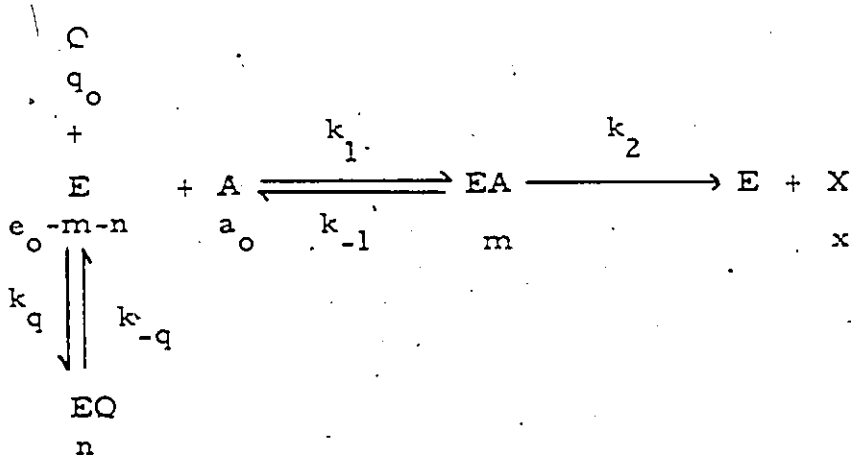
will be pure non-competitive behavior. Intermediate cases are dealt with in terms of varying strengths of combination of Q with E and EA.

The present chapter develops the transient-phase equations for systems in which E, A and Q are present, on the basis of the single-intermediate (EA) mechanism above. The next chapter will treat the problem on the basis of a two-intermediate mechanism, which is known to apply in a number of cases.

Competitive Inhibition

Reversible Case

The mechanism is



It is assumed, as in most experimental studies, that the substrate and inhibitor are in excess of the enzyme; the concentrations can then be approximated as shown above in small letters. The differential equations are:

[1] $$\dot{m} = k_1 a_o (e_o - m - n) - (k_{-1} + k_2) m$$

[2] $$\dot{n} = k_q q_o (e_o - m - n) - k_{-q} n$$

[3] $$\dot{x} = k_2 m$$

The differentials are replaced by operators P, and we obtain the transform equation for m:

$$[4] \qquad m = \frac{k_1 a_o e_o (P + k_{-q})}{(P + \lambda_1)(P + \lambda_2)}$$

where

$$[5] \qquad \lambda_1 = \tfrac{1}{2} (-R - \sqrt{R^2 - 4Q})$$

$$[6] \qquad \lambda_2 = \tfrac{1}{2} (-R + \sqrt{R^2 - 4Q})$$

$$[7] \qquad R = k_1 a_o + \tilde{k} + k_q q_o + k_{-q}$$

$$[8] \qquad Q = k_1 k_{-q} a_o + \tilde{k} (k_q q_o + k_{-q})$$

where $\tilde{k} = k_{-1} + k_2$. The original for m is

$$[9] \qquad m = \frac{k_1 k_{-q} a_o e_o}{\lambda_1 \lambda_2} - \frac{k_1 a_o e_o (k_{-q} - \lambda_1)}{\lambda_1 (\lambda_1 - \lambda_2)} e^{-\lambda_1 t} - \frac{k_1 a_o e_o (k_{-q} - \lambda_2)}{\lambda_2 (\lambda_2 - \lambda_1)} e^{-\lambda_2 t}$$

Introduction of this into eq. [3] and integration with t = 0, n = 0, and use of the relation $\lambda_1 \lambda_2 = Q$, then leads to

$$[10] \qquad x = \frac{k_2 a_o e_o}{a_o + K_m (1 + \frac{q}{K_q})} t + \frac{k_1 k_2 a_o e_o (k_{-q} - \lambda_1)}{\lambda_1^2 (\lambda_2 - \lambda_1)} (e^{-\lambda_1 t} - 1) + \frac{k_1 k_2 a_o e_o (k_{-q} - \lambda_2)}{\lambda_2^2 (\lambda_1 - \lambda_2)} (e^{-\lambda_2 t} - 1)$$

where

$$[11] \qquad K_m = \tilde{k}/k_1 \text{ and } K_q = k_{-q}/k_q$$

The first term in eq. [10] corresponds to the steady state, the behavior being competitive; the other two terms account for the transient behavior. Fig. 18 shows the behavior in a schematic fashion.

If v is the steady-state rate, i. e.

$$[12] \qquad v = \frac{k_2 e_o a_o}{a_o + K_m (1 + \frac{q_o}{K_q})}$$

and β (the sum of the pre-exponential terms) is the intercept on the x-axis (cf. Fig. 18) then

$$[13] \qquad \beta/v = \tau = -\frac{1}{k_{-q}} + \frac{k_1 a_o + \tilde{k} + k_q q_o + k_{-q}}{k_1 k_{-q} a_o + \tilde{k}(k_q q_o + k_{-q})}$$

Eq. [10] can be written as

$$[14] \qquad x - vt + \beta = \beta_1 e^{-\lambda_1 t} + \beta_2 e^{-\lambda_2 t}$$

and plots of ln $(x - vt + \beta)$ against t will consist of two straight regions with slopes of $-\lambda_1$ and $-\lambda_2$. The λ_i's obey the relations

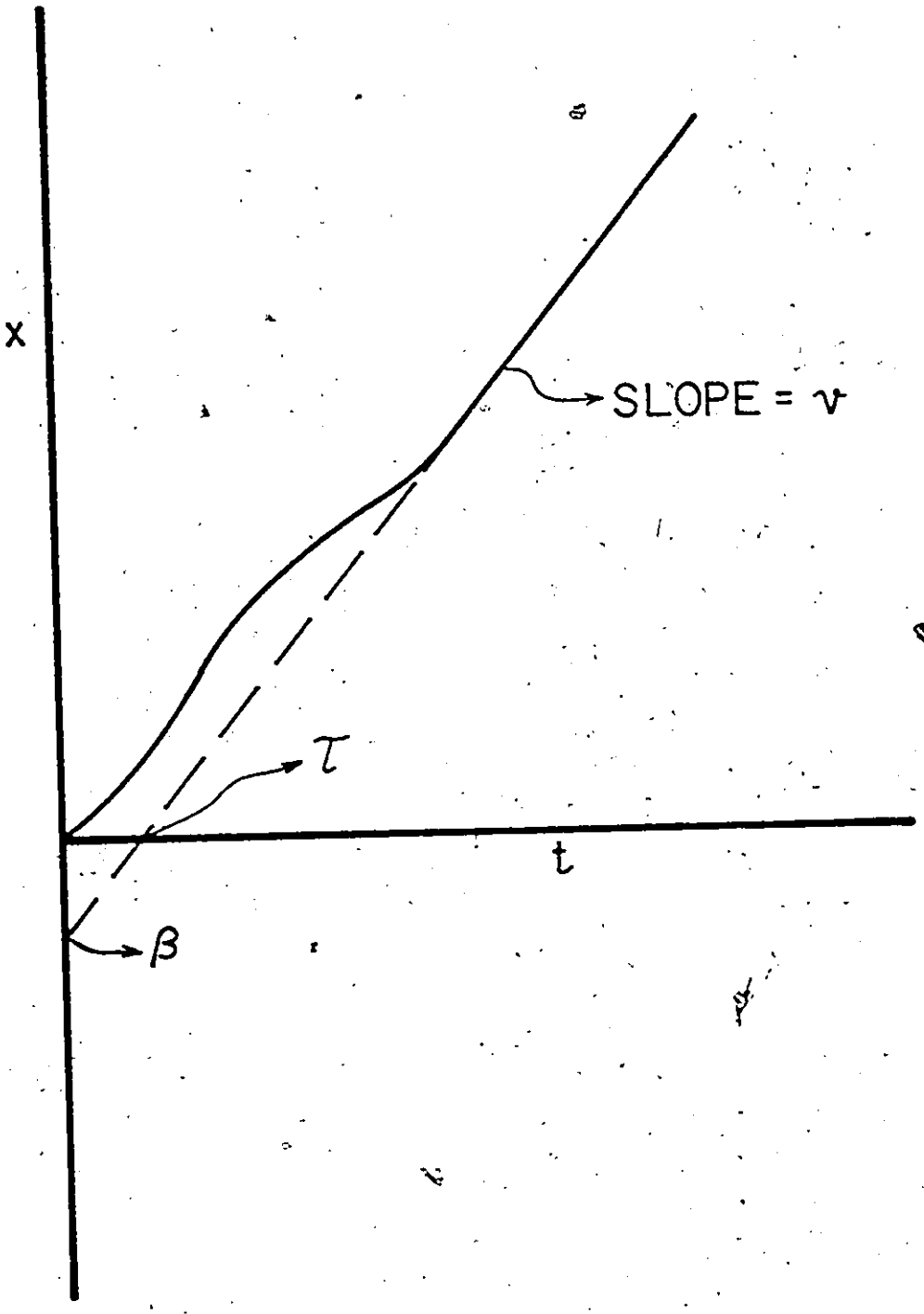
$$[15] \qquad (\lambda_1 + \lambda_2) = k_1 a_o + \tilde{k} + k_q q_o + k_{-q}$$

$$[16] \qquad \lambda_1 \lambda_2 = k_1 k_{-q} a_o + \tilde{k}(k_q q_o + k_{-q})$$

$$[17] \qquad v \lambda_1 \lambda_2 = k_1 k_{-q} a_o e_o$$

Plots of $(\lambda_1 + \lambda_2)$ against a_o and q_o are linear with slopes of k_1 and k_q, respectively. Also, plots of $\lambda_1 \lambda_2$ against a_o and q_o are linear with slopes of $k_1 k_{-q}$ and $k_q \tilde{k}$, respectively. Therefore, k_1, k_q, k_{-q}

Fig. 18 A schematic plot of x vs. t (cf. eq. [10].


x
SLOPE = v
τ
t
β

and $\tilde{k} = k_{-1} + k_2$ can be calculated. The rate constants k_{-1} and k_2 can be calculated from the uninhibited case (cf. chapter one).

Irreversible Case

If the inhibition is irreversible (i.e. $k_{-q} = 0$) the resulting equation is

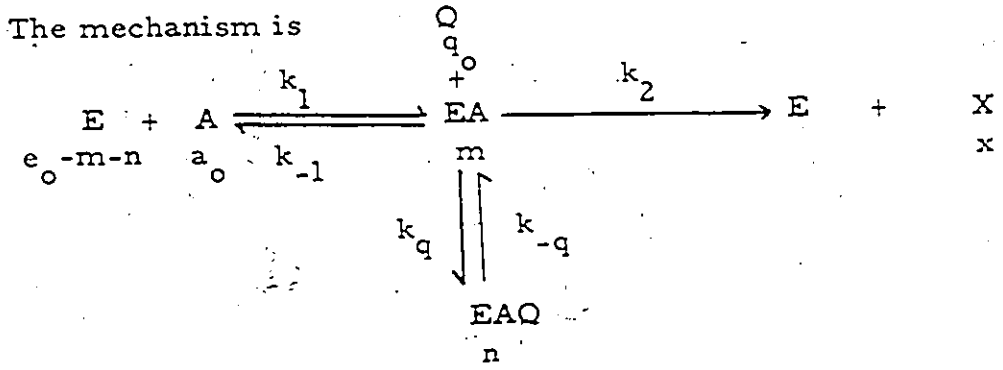
$$[18] \qquad x = \frac{k_2 e_o a_o}{K_m k_q q_o} - \frac{k_1 k_2 e_o a_o}{\lambda_1 (\lambda_2 - \lambda_1)} e^{-\lambda_1 t} - \frac{k_1 k_2 e_o a_o}{\lambda_2 (\lambda_1 - \lambda_2)} e^{-\lambda_2 t}$$

Since there is no linear term in t, there is no steady state. This criterion will be useful in distinguishing between reversible and irreversible inhibition. From the limiting value of x, equal to the first term in eq. [18], k_q can be calculated.

Anticompetitive Inhibition

Reversible Case

The mechanism is



The concentrations, shown above, correspond to the usual case of the substrate and inhibitor being in great excess of the enzyme.

The differential equations are

$$[19] \qquad \dot{m} = k_1 a_o (e_o - m - n) - \tilde{k} m - k_q q_o m + k_{-q} n$$

[20] $$\dot{n} = k_q q_o m - k_{-q} n$$

[21] $$\dot{x} = k_2 m$$

The transform for m is

[22] $$m = \frac{k_1 a_o e_o (P + k_{-q})}{(P + \lambda_1)(P + \lambda_2)}$$

where P is the operator and

[23] $$\lambda_1 = \tfrac{1}{2}(-R - \sqrt{R^2 - 4C})$$

[24] $$\lambda_2 = \tfrac{1}{2}(-R + \sqrt{R^2 - 4C})$$

[25] $$R = \lambda_1 + \lambda_2 = k_1 a_o + \tilde{k} + k_q q_o + k_{-q}$$

[26] $$C = \lambda_1 \lambda_2 = k_1 k_{-q} a_o + k_{-q} \tilde{k} + k_1 k_q a_o q_o$$

The original for m is

[27] $$m = \frac{k_1 k_{-q} a_o e_o}{\lambda_1 \lambda_2} - \frac{k_1 a_o e_o (k_{-q} - \lambda_1)}{\lambda_1 (\lambda_2 - \lambda_1)} e^{-\lambda_1 t} - \frac{k_1 a_o e_o (k_{-q} - \lambda_2)}{\lambda_2 (\lambda_1 - \lambda_2)} e^{-\lambda_2 t}$$

Substitution of eq. [27] into [21] and integration with the boundary conditions, t = 0, x = o, leads to (after substituting $\lambda_1 \lambda_2 = C$)

[28] $$x = \frac{k_2 e_o a_o}{K_m + a_o (1 + \frac{q_o}{K_q})} t + \frac{k_1 k_2 a_o e_o (k_{-q} - \lambda_1)}{\lambda_1^2 (\lambda_2 - \lambda_1)} (e^{-\lambda_1 t} - 1) + \frac{k_1 k_2 a_o e_o (k_{-q} - \lambda_2)}{\lambda_2^2 (\lambda_1 - \lambda_2)} (e^{-\lambda_2 t} - 1)$$

where $K_q = k_{-q}/k_q$. The first term represents the steady state, the behavior being anticompetitive; the other two terms correspond to a biphasic exponential rise leading to the steady state.

Eq. [28] can be written as

$$[29] \qquad x = vt + \beta + \beta_1 e^{-\lambda_1 t} + \beta_2 e^{-\lambda_2 t}$$

where v is the steady state rate and β is the intercept on the x-axis of a plot of x vs. t after extension of the linear part.

$$[30] \qquad \beta/v = \tau = -\frac{1}{k_{-q}} + \frac{k_1 a_o + \tilde{k} + k_q q_o + k_{-q}}{k_1 a_o (k_{-q} + k_q q_o) + k_q \tilde{k}}$$

A plot of $\ln (x - vt - \beta)$ vs. t is biphasic (two straight regions) with slopes of $-\lambda_1$ and $-\lambda_2$. From eq. [25], plots of $(\lambda_1 + \lambda_2)$ against a_o and q_o are linear with slopes of k_1 and k_q, respectively. From these plots $\tilde{k} + k_{-q}$ can be obtained. Since $\tilde{k} = k_{-1} + k_2$ can be obtained from the uninhibited reaction, k_{-q} can be calculated.

Irreversible Case

A similar treatment of the irreversible case ($k_{-q} = 0$) leads to

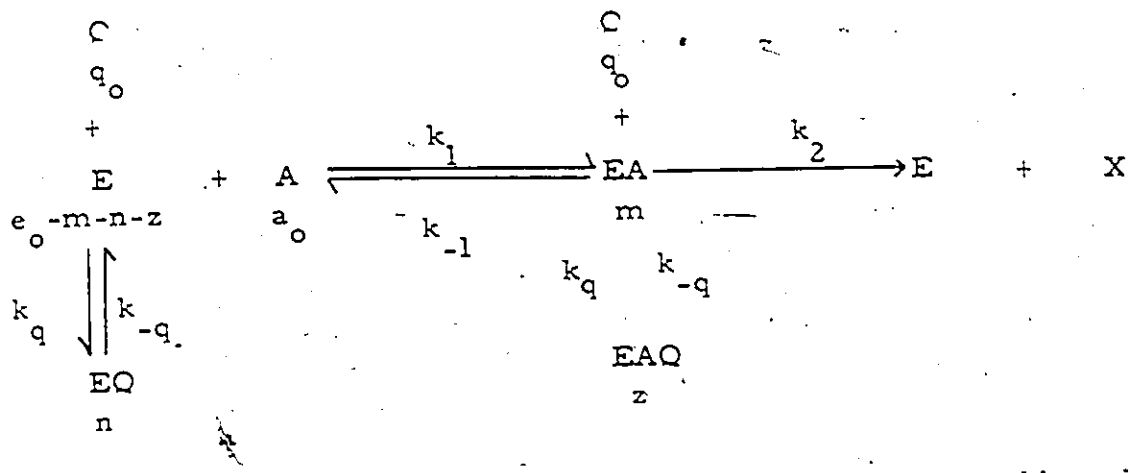
$$[31] \qquad x = \frac{k_2 e_o}{k_q q_o} - \frac{k_1 k_2 a_o e_o}{\lambda_1 (\lambda_2 - \lambda_1)} e^{-\lambda_1 t} - \frac{k_1 k_2 a_o e_o}{\lambda_2 (\lambda_1 - \lambda_2)} e^{-\lambda_2 t}$$

There is now no steady state, which distinguishes this case from the reversible case. The values of k_q can readily be obtained from the analysis of experimental data, k_2, e_o, q_o being known.

Pure Non-Competitive Inhibition

Reversible Case

The mechanism is now



For the pure non-competitive inhibition the inhibitor must combine with EA in the same manner as with E. The concentrations shown above correspond to the substrate and the inhibitor in excess of the enzyme concentration. The differential equations are:

[32] $$\dot{m} = k_1 a_o (e_o - m - n - z) - \tilde{k} m - k_q q_o m + k_{-q} z$$

[33] $$\dot{n} = k_q q_o (e_o - m - n - z) - k_{-q} n$$

[34] $$\dot{z} = k_q q_o m - k_{-q} z$$

[35] $$\dot{x} = k_2 m$$

The transform of m is

[36] $$m = \frac{k_1 a_o e_o (P + k_{-q})^2}{(P + \lambda_1)(P + \lambda_2)(P + \lambda_3)}$$

where P is the differential operator and

[37] $$\lambda_1 = k_q q_o + k_{-q}$$

[38] $\lambda_2 = \frac{1}{2}(-R + \sqrt{R^2 - 4\Omega})$

[39] $\lambda_3 = \frac{1}{2}(-R + \sqrt{R^2 - 4\Omega})$

[40] $R = k_1 a_o + \tilde{k} + k_q q_o + k_{-q}$

[41] $\Omega = k_{-q}(k_1 a_o + \tilde{k})$

The original for m is

[42] $$m = \frac{k_1 k_{-q}^2 a_o e_o}{\lambda_1 \lambda_2 \lambda_3} - \sum_{i=1}^{3} \frac{k_1 a_o e_o (\lambda_i - k_{-q})^2}{\lambda_i (P - \lambda_i)} e^{-\lambda_i t}$$

Substituting eq. [42] into [35] and integration with the boundary conditions, t = 0, x = 0 , leads to (since $\lambda_1 \lambda_2 \lambda_3 = \lambda_1 \Omega$):

[43] $$x = \frac{k_2 a_o e_o}{(K_m + a_o)(1 + \frac{q_o}{K_q})} t + \sum_{i=1}^{3} \frac{k_1 k_2 a_o e_o (\lambda_i - k_{-q})^2}{\lambda_i^2 (P - \lambda_i)} (e^{-\lambda_i t} - 1)$$

There is thus a triphasic exponential rise of x with t, until a steady state is reached.

Eq. [43] can be written as

[44] $$x - vt + \beta = \sum_{i=1}^{3} \beta_i e^{-\lambda_i t}$$

where

[45] $$v = \frac{k_2 a_o e_o}{(K_m + a_o)(1 + \frac{q_o}{K_q})}$$

[46] $$\beta = -\sum_{i=1}^{3} \beta_i = -\sum_{i=1}^{3} \frac{k_1 k_2 a_o e_o (\lambda_i - k_{-q})^2}{\lambda_i^2 (P - \lambda_i)}$$

$$[47] \qquad \beta/v = \tau = -\frac{1}{k_q q_o + k_{-q}} - \frac{2}{k_{-q}} + \frac{k_1 a_o + \tilde{k} + k_q q_o + k_{-q}}{k_{-q}(k_1 a_o + \tilde{k})}$$

A plot of ln $(x - vt + \beta)$ against t consists of three linear regions whose slopes are $-\lambda_1$, $-\lambda_2$, $-\lambda_3$. However

$$[48] \qquad (\lambda_1 + \lambda_2 + \lambda_3) = k_1 a_o + 2 k_q q_o + 2 k_{-q} + \tilde{k}$$

$$[49] \qquad \lambda_1 \lambda_2 \lambda_3 = k_1 k_{-q}(k_{-q} + k_q q_o) a_o + k_{-q} \tilde{k} (k_{-q} + k_q q_o)$$

Plots of $(\lambda_1 + \lambda_2 + \lambda_3)$, eq. [48], against a_o and q_o give k_1 and $2 k_q$ as slopes, respectively. Also, $2 k_{-q} + \tilde{k}$ can be calculated from these plots. Since $\tilde{k} = k_{-1} + k_2$ can be calculated from the uninhibited case, k_{-q} can be calculated from k_q and $K_q = k_{-q}/k_q$, the latter being known from steady-state analysis. It is to be noted that several other plots can be devised.

Irreversible Case

For the case of irreversible inhibition $(k_{-q} = 0)$

$$[50] \qquad x = \frac{k_1 k_2 a_o e_o}{k_q q_o (k_1 a_o + \tilde{k} + k_q q_o)} - \frac{k_1 k_2 a_o e_o}{\lambda_1 (\lambda_2 - \lambda_1)} e^{-\lambda_1 t} - \frac{k_1 k_2 a_o e_o}{\lambda_2 (\lambda_1 - \lambda_2)} e^{-\lambda_2 t}$$

There is now no steady state, which distinguishes this case experimentally from the reversible case. If x_f is the final value of x (limit x as $t = \infty$), then

$$\frac{1}{x_f} = \frac{k_q q_o}{k_2 e_o} + \frac{\tilde{k} + k_q q_o}{k_1 k_2 e_o} \cdot \frac{1}{a_o}$$

From a plot of $1/x_f$ against $1/a_o$, k_1 and k_q can be determined since k_2 is known.

CHAPTER SIX

TRANSIENT-PHASE AND STEADY-STATE KINETICS FOR INHIBITED ENZYME SYSTEMS: DOUBLE-INTERMEDIATE MECHANISM

Introduction

A number of reactions catalyzed by enzymes are known to occur by a mechanism involving a second intermediate, the general scheme being

$$E + A \rightleftharpoons EA \underset{X}{\longrightarrow} EA' \longrightarrow E + Y$$

Here E is the enzyme and A the substrate; EA is an addition complex which splits off one product X to form a second intermediate EA'. This finally forms the free enzyme and the second product Y, usually by hydrolysis. The reactions of proteolytic and other hydrolytic enzymes have frequently been found to occur with two identifiable intermediates, EA' being an acylated enzyme.

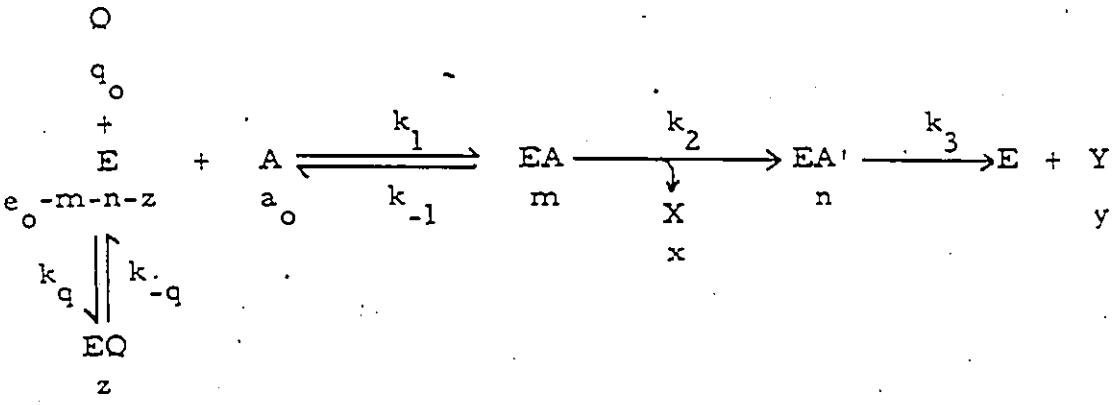
The inhibition behavior is complicated by the occurrence of two intermediates, since either or both may combine with an inhibitor molecule. The relevant steady-state equations were worked out by Krupka and Laidler [47], who discussed the conditions under which one can obtain non-competitive, anticompetitive and competitive behavior.

The present chapter is concerned with the corresponding treatment of the transient phase. It is confined to the usual case in which the substrate and inhibitor concentrations are greatly in excess of the concentration of the enzyme.

Mechanism I

Reversible Case

The inhibitor Q becomes attached to the free enzyme, and not to EA or EA':



The differential rate equations are

$$\dot{m} = k_1 a_o (e_o - m - n - z) - \tilde{k} m \quad [1]$$

$$\dot{n} = k_2 m - k_3 n \quad [2]$$

$$\dot{z} = k_q q_o (e_o - m - n - z) - k_{-q} z \quad [3]$$

$$\dot{x} = k_2 m \quad [4]$$

$$\dot{y} = k_3 n \quad [5]$$

where $\tilde{k} = k_{-1} + k_2$. Replacement of the differentials by operators P and algebraic solution leads to

$$m = \frac{k_1 a_o e_o (P + k_{-q})(P + k_3)}{P^3 + MP^2 + LP + C} \quad [6]$$

where

$$M = k_1 a_o + k_q q_o + k_{-q} + \tilde{k} + k_3 \quad [7]$$

$$L = (\tilde{k} + k_3)(k_q q_o + k_{-q}) + k_1 k_{-q} a_o + \tilde{k} k_3 + k_1 a_o (k_2 + k_3) \quad [8]$$

$$Q = \tilde{k} k_3 (k_q q_o + k_{-q}) + k_1 k_3 k_{-q} a_o + k_1 k_2 k_{-q} a_o \quad [9]$$

To obtain the original for m we require the roots of the polynomial

[10] $$P^3 + MP^2 + LP + C = 0$$

These roots cannot be obtained explicitly; we denote them by $-\lambda_1$, and $-\lambda_2$ and $-\lambda_3$; eq. [6] then can be written as

[11] $$m = \frac{k_1 a_o e_o (P + k_{-q})(P + k_3)}{(P + \lambda_1)(P + \lambda_2)(P + \lambda_3)}$$

The original for m is

[12] $$m = \frac{k_1 k_{-q} k_3 a_o e_o}{\lambda_1 \lambda_2 \lambda_3} - \frac{k_1 a_o e_o (\lambda_1 - k_{-q})(\lambda_1 - k_3)}{\lambda_1 (\lambda_2 - \lambda_1)(\lambda_3 - \lambda_1)} e^{-\lambda_1 t}$$

$$- \frac{k_1 a_o e_o (\lambda_2 - k_{-q})(\lambda_2 - k_3)}{\lambda_2 (\lambda_1 - \lambda_2)(\lambda_3 - \lambda_2)} e^{-\lambda_2 t}$$

$$- \frac{k_1 a_o e_o (\lambda_3 - k_{-q})(\lambda_3 - k_3)}{\lambda_3 (\lambda_1 - \lambda_3)(\lambda_2 - \lambda_3)} e^{-\lambda_3 t}$$

Use of eq. [4] and integration subject to the boundary condition x = 0, t = 0, and with the substitution $\lambda_1 \lambda_2 \lambda_3 = Q$ leads to

[13] $$x = \frac{k_2 a_o e_o}{K_m (1 + \frac{q_o}{K_q}) + a_o (1 + \frac{k_2}{k_3})} t$$

$$+ \sum_{i=1}^{3} \frac{k_1 k_2 a_o e_o (\lambda_i - k_{-q})(\lambda_i - k_3)}{\lambda_1^2 (P - \lambda_i)} (e^{-\lambda_i t} - 1)$$

where $K_m = \tilde{k}/k_1$ and $K_q = k_{-q}/k_q$. The first term, linear in t, corresponds to the steady state. Eq. [13] is of the form

[14] $$x - vt + \beta = \beta_1 e^{-\lambda_1 t} + \beta_2 e^{-\lambda_2 t} + \beta_3 e^{-\lambda_3 t}$$

where

$$\beta = -\sum_{i-1}^{3} \beta_i$$

$$v = \frac{k_2 a_o e_o}{K_m \left(1 + \frac{q_o}{K_q}\right) + a_o \left(1 + \frac{k_2}{k_3}\right)}$$

Fig. 19 shows schematically the dependence of x on t (cf. eq. [13]); there is a triphasic exponential approach to the steady state.

In principle, data can be analyzed with respect to these equations, but in practice the analysis will generally be rather difficult. The quantities β and v can be determined from a plot of x against t (cf. Fig. 19), and then ln $(x - vt + \beta)$ can be plotted against t. In general this plot will have three straight regions, the slopes of which give the values of λ_1, λ_2 and λ_3. However $(\lambda_1 + \lambda_2 + \lambda_3) = M$ (cf. eq. [7]) and plots of M against a_o and q_o are linear with slopes of k_1 and k_q respectively. From these plots $\tilde{k} + k_q + k_3$ can be obtained from the intercepts. Since $\tilde{k} + k_3$ can be obtained from the uninhibited case then k_{-q} can be calculated. Alternatively k_{-q} can be calculated from k_q and $K_q = k_{-q}/k_q$ where the latter can be obtained from the steady-state analysis. Maguire, Hijazi and Laidler [27] applied this to the α-chymotrypsin-catalyzed hydrolysis of p-nitrophenyl acetate in the presence of indole which was shown to competitively inhibit the reaction. A plot of their data for the function ln $(x - vt + \beta)$ against t is shown in Fig. 20. This figure should be contrasted with Fig. 3 where the same plot is shown for the uninhibited case. In agreement with theoretical prediction the uninhibited case shows a biphasic behavior while the competitively inhibited case shows a triphasic behavior.

It is to be noted that various other plots can be used to separate the values k_q and k_{-q}.

Fig. 19 A schematic plot for the dependence of x on t (cf. eq. [13]).

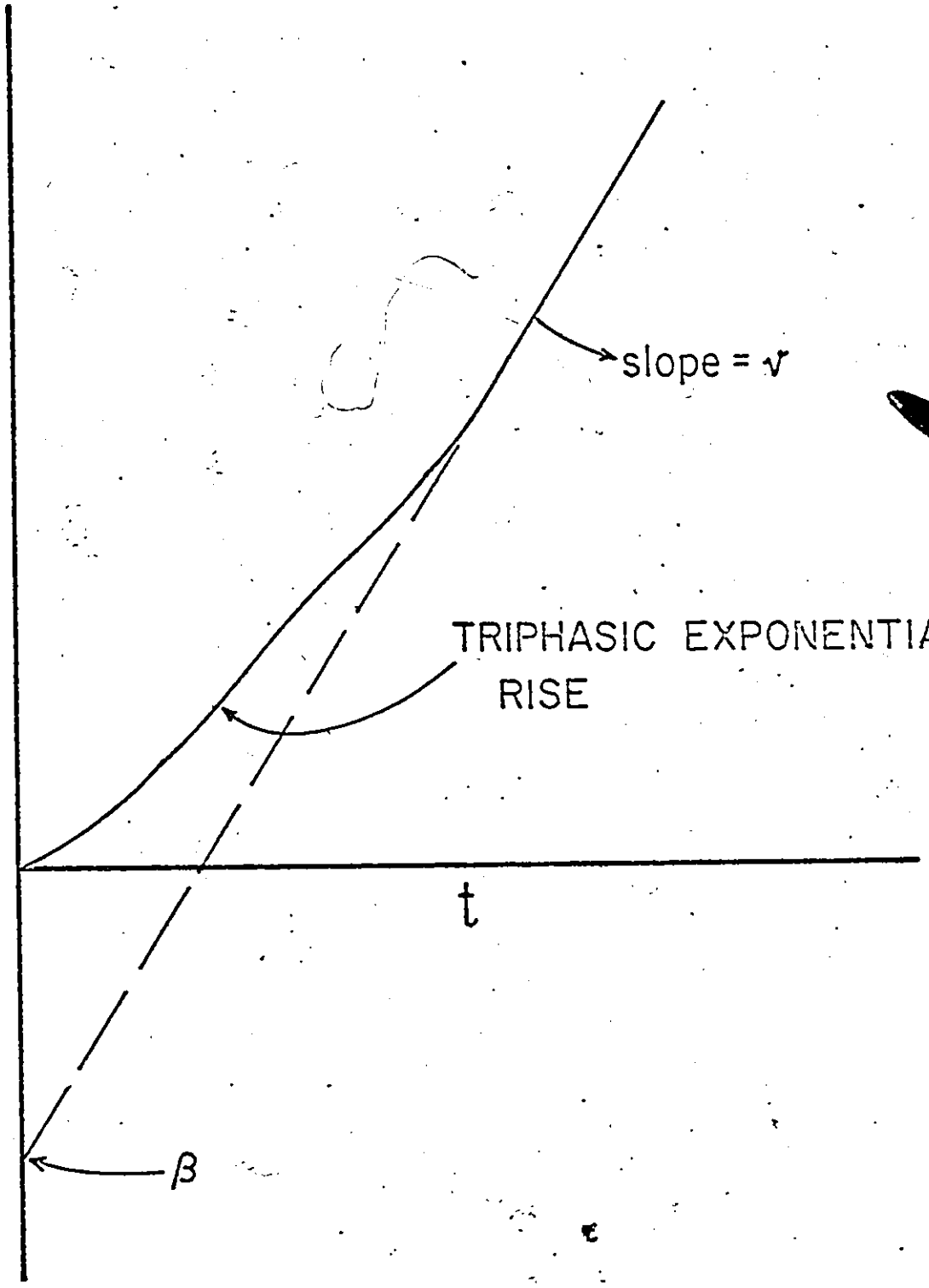
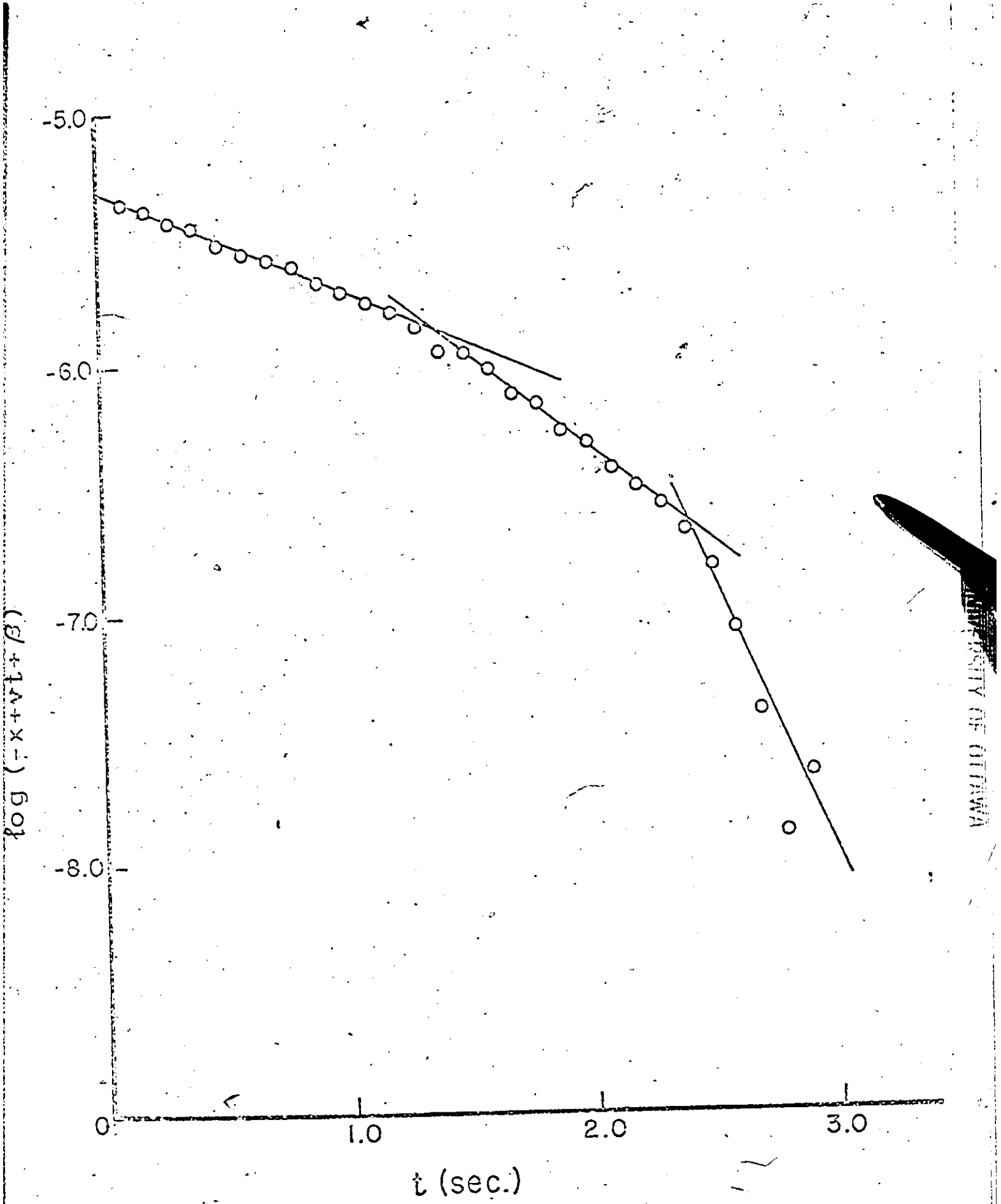

x
slope = v
TRIPHASIC EXPONENTIAL
RISE
t
β

Fig. 20 A plot of ln (x - vt + β) against t for the Indole-inhibited α-chymotrypsin-catalyzed reaction of p-nitrophenyl acetate.


-5.0
-6.0
-7.0
-8.0
0
1.0
2.0
3.0
log (-x+vt+β)
t (sec.)

In some cases plots of ln $(x - vt + \beta)$ will have only two or one straight portions; this can arise if one or two of the λ_1, λ_2 and λ_3 values is much smaller than the remainder. This obviously makes the analysis impossible, since some of the values are not revealed by the data.

Irreversible Case

An interesting special case is when the inhibition is irreversible ($k_{-q} = 0$). The solution is now

$$[15] \qquad x = \frac{k_2 a_o e_o}{K_m k_q q_o} - \frac{k_1 k_2 a_o e_o (k_3 - \lambda_1)}{\lambda_1 (\lambda_2 - \lambda_1)(\lambda_3 - \lambda_1)} e^{-\lambda_1 t}$$

$$- \frac{k_1 k_2 a_o e_o (k_3 - \lambda_2)}{\lambda_2 (\lambda_1 - \lambda_2)(\lambda_3 - \lambda_1)} e^{-\lambda_2 t} - \frac{k_1 k_2 a_o e_o (k_3 - \lambda_3)}{\lambda_3 (\lambda_1 - \lambda_3)(\lambda_2 - \lambda_3)} e^{-\lambda_3 t}$$

where $-\lambda_1$, $-\lambda_2$ and $-\lambda_3$ are the roots of eq. [10] with

$$[16] \qquad M = k_q q_o + \tilde{k} + k_1 a_o + k_3$$

$$[17] \qquad L = k_3 (k_1 a_o + k_q q_o + \tilde{k}) + k_1 k_2 a_o + \tilde{k} k_q q_o$$

$$[18] \qquad Q = \tilde{k} k_3 k_q q_o$$

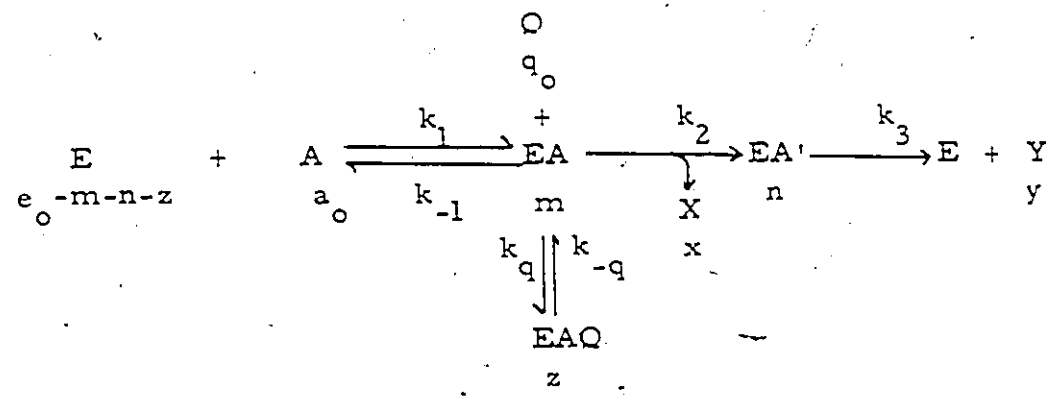
Since eq. [15] has no term linear in t, there is no steady state; x levels off at a limiting value of $k_2 a_o e_o / K_m k_q q_o$. Irreversible inhibition can thus be distinguished from reversible inhibition by this lack of a steady state. In this irreversible case the value of k_q can be determined from the limiting value of x, since the other quantities are known. It is essential in applying this method to ensure that the substrate is well in

excess of the inhibitor, so that there shall be little depletion of substrate by the time the reaction ceases.

Mechanism II

Reversible Case

The inhibitor becomes attached only to EA:



The differential rate eequations are

[19] $$\dot{m} = k_1 a_o (e_o - m - n - z) - (\tilde{k} + k_q q_o) m + k_{-q} z$$

[20] $$\dot{n} = k_2 m - k_3 n$$

[21] $$\dot{z} = k_q q_o m = k_{-q} z$$

[22] $$\dot{x} = k_2 m$$

[23] $$\dot{y} = k_3 n$$

The transform for m is

[24] $$m = \frac{k_1 a_o e_o [P^2 + P (k_{-q} + k_3) + k_{-q} k_3]}{P^3 + MP^2 + LP + Q}$$

where P is the differential operator, and

[25] $$M = k_1 a_o + \tilde{k} + k_q q_o + k_{-q} + k_3$$

[26] $$L = k_{-q}(k_1 a_o + \tilde{k}) + k_1 a_o k_q q_o + k_3 (k_1 a_o + \tilde{k} + k_q q_o + k_{-q}) + k_1 k_2 a_o$$

[27] $$C = k_3 [k_{-q}(k_1 a_o + \tilde{k}) + k_1 k_q a_o q_o] + k_1 k_2 k_{-q} a_o$$

Eq. [24] can be written as

[28] $$m = \frac{k_1 a_o e_o [P^2 + P(k_{-q} + k_3) + k_{-q} k_3]}{(P + \lambda_1)(P + \lambda_2)(P + \lambda_3)}$$

where $-\lambda_1$, $-\lambda_2$ and $-\lambda_3$ are the three roots of the cubic. The original is then

[29] $$m = \frac{k_1 k_{-q} k_3 a_o e_o}{\lambda_1 \lambda_2 \lambda_3} - \sum_{i=1}^{3} \frac{k_1 a_o e_o (\lambda_i - k_{-q})(\lambda_i - k_3)}{\lambda_i (P - \lambda_i)} e^{-\lambda_i t}$$

Introduction of this into eq. [22], and integration with the initial conditions, x = 0, t = 0, leads to

[30] $$x = \frac{k_2 a_o e_o}{K_m + a_o (1 + \frac{k_2}{k_3} + \frac{q_o}{K_q})} t + \sum_{i=1}^{3} \frac{k_1 k_2 a_o e_o (\lambda_i - k_{-q})(\lambda_i - k_3)}{\lambda_i^2 (P - \lambda_i)} (e^{-\lambda_i t} - 1)$$

The coefficient of t in this expression is the steady-state rate for this case of anticompetitive inhibition. The concentration of x approahces the steady-state in a triphasic exponential manner. Analysis of results proceeds as in the case of Mechanism I.

Irreversible Case

For irreversible inhibition by this mechanism ($k_{-q} = 0$) the solution is

$$[31] \qquad x = \frac{k_2 e_o}{k_q q_o} - \sum_{i=1}^{3} \frac{k_1 k_2 a_o e_o (k_3 - \lambda_i)}{\lambda_i (P - \lambda_i)} e^{-\lambda_i t}$$

where $-\lambda_1$, $-\lambda_2$ and $-\lambda_3$ are the roots of eq. [10] with

$$[32] \qquad M = k_1 a_o + \tilde{k} + k_q q_o + k_3$$

$$[33] \qquad L = k_1 k_q a_o q_o + k_3 (k_1 a_o + \tilde{k} + k_q q_o) + k_1 k_2 a_o$$

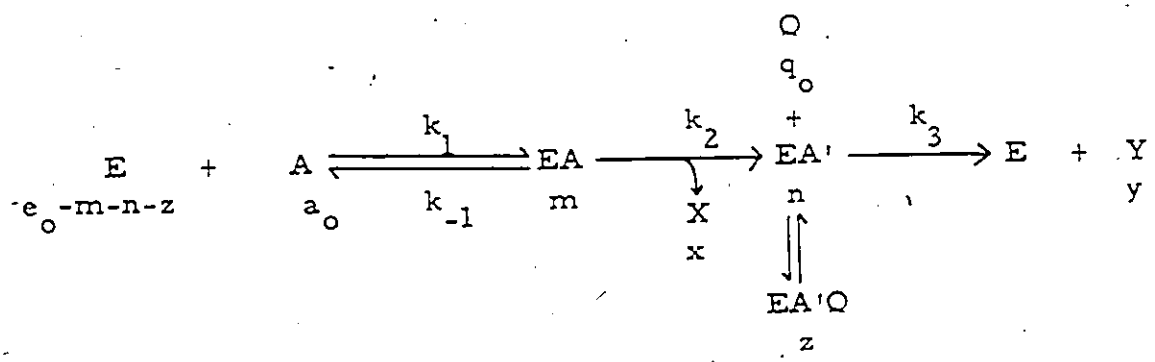
$$[34] \qquad Q = k_1 k_3 a_o k_q q_o$$

There is now no steady state. Experimentally, k_q can be obtained from the limiting value of x, equal to the first term in eq. [31]. Again, the substrate must be well in excess of inhibitor.

Mechanism III

Reversible Case

The inhibitor becomes attached only to EA':



The differential rate equations are

$$[35] \qquad \dot{m} = k_1 a_o (e_o - m - n - z) - \tilde{k} m$$

$$[36] \qquad \dot{n} = k_2 m - k_3 n - k_q q_o n + k_{-q} z$$

$$[37] \qquad \dot{z} = k_q q_o n - k_{-q} z$$

$$[38] \qquad \dot{x} = k_2 \dot{m}$$

$$[39] \qquad \dot{y} = k_3 n$$

The transform of m is

$$[40] \qquad m = \frac{k_1 a_o e_o [P^2 + P(k_3 + k_q q_o + k_{-q}) + k_{-q} k_3)}{P^3 + MP^2 + LP + Q}$$

where

$$[41] \qquad M = k_1 a_o + \tilde{k} + k_3 + k_q q_o + k_{-q}$$

$$[42] \qquad L = (k_3 + k_q q_o + k_{-q})(k_1 a_o + \tilde{k}) + k_1 k_q a_o + k_{-q} k_3$$

$$[43] \qquad Q = k_1 a_o k_{-q} k_3 + \tilde{k} k_{-q} k_3 + k_1 k_2 a_o (k_{-q} + k_q q_o)$$

Eq. [40] can be written as

$$[44] \qquad m = \frac{k_1 a_o e_o [P^2 + P(k_3 + k_q q_o + k_{-q}) + k_{-q} k_3]}{(P + \lambda_1)(P + \lambda_2)(P + \lambda_3)}$$

where $-\lambda_1$, $-\lambda_2$ and $-\lambda_3$ are the roots of the cubic.

The original for m is then

$$[45] \qquad m = \frac{k_1 k_{-q} k_3 a_o e_o}{\lambda_1 \lambda_2 \lambda_3} - \sum_{i=1}^{3} \frac{k_1 a_o e_o [\lambda_i^2 - \lambda_i (k_3 + k_q q_o + k_{-q})}{\lambda_i (P - \lambda_i)} + \frac{k_{-q} k_3]}{\lambda_i (P - \lambda_i)} e^{-\lambda_i t}$$

Substitution of eq. [45] into [38], followed by integration with the initial conditions, x = 0, t = 0, leads to

$$[46] \qquad x = \frac{k_2 a_o e_o}{K_m + a_o + \frac{k_2}{k_3} a_o (1 + \frac{q_o}{K_q})} t$$

$$+ \sum_{i=1}^{3} \frac{[\lambda_i^2 - \lambda_i (k_3 + k_q q_o + k_{-q}) + k_{-q} k_3]}{\lambda_i^2 (P - \lambda_i)} (e^{-\lambda_i t} - 1)$$

The first term corresponds to the steady-state. As expected, there is no inhibition if $k_2 << k_3$; otherwise there is anticompetitive inhibition. As before, the steady state is approached in an exponential manner. Analysis of experimental results can be carried out as explained for Mechanism I, and leads to values of k_q and k_{-q}.

Irreversible Case

The result for irreversible inhibition by this mechanism is

$$[47] \qquad x = e_o (1 + \frac{k_3}{k_q q_o}) - \sum_{i=1}^{3} \frac{k_1 k_2 a_o e_o (k_3 + k_q q_o - \lambda_i)}{\lambda_i (P - \lambda_i)} e^{-\lambda_i t}$$

where $-\lambda_1$, $-\lambda_2$, $-\lambda_3$ are the roots of eq. [10] with

[48] $$M = k_3 + k_q q_o + k_1 a_o + \tilde{k}$$

[49] $$L = k_1 a_o (k_3 + k_q q_o) + \tilde{k} (k_3 + k_q q_o) + k_1 k_2 a_o$$

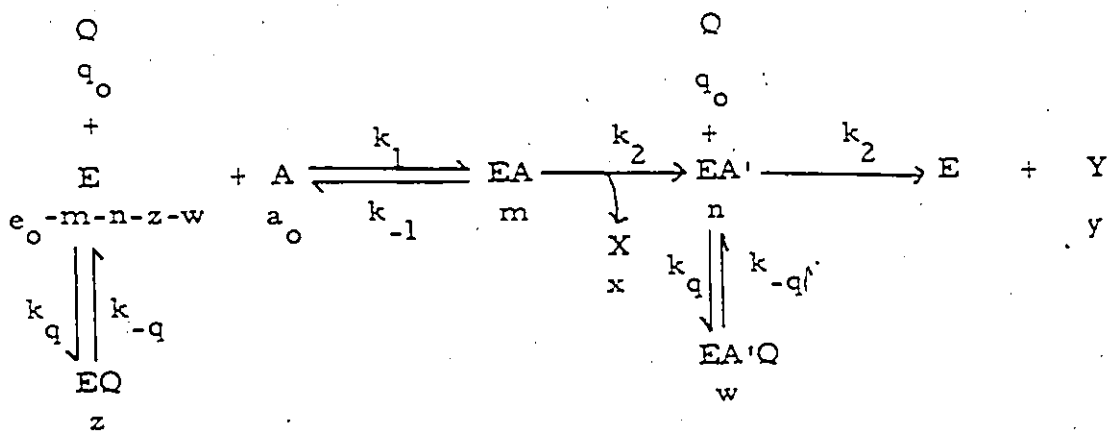
[50] $$Q = k_1 k_2 k_q a_o q_o$$

Again there is no steady state and the k_q value can be calculated from the experimental limiting value of x.

Mechanism IV

Reversible Case

The inhibitor becomes attached to both E and EA! the rate and equilibrium constants being the same



The rate equations are

[51] $$\dot{m} = k_1 a_o (e_o - m - n - z - w) - \tilde{k} m$$

[52] $$\dot{n} = k_2 m - (k_2 + k_q q_o) n + k_{-q} w$$

[53] $$\dot{z} = k_q q_o (e_o - m - n - z - w) - k_{-q} z$$

[54] $$\dot{w} = k_q q_o n - k_{-q} w$$

[55] $\dot{x} = k_2 m$

[56] $\dot{y} = k_3 n$

The transform for m is

$$[57] \qquad m = \frac{k_1 a_o e_o (P + k_{-q})[P^2 + P(k_3 + k_q q_o + k_{-q}) + k_{-q} k_3]}{P^4 + LP^3 + MP^2 + NP + Q}$$

where

[58] $L = 2 k_q q_o + 2 k_{-q} + k_1 a_o + \tilde{k} + k_3$

[59] $M = (k_1 a_o + \tilde{k}) k_{-q} + \tilde{k} k_q q_o + k_{-q} k_3 + k_1 k_2 a_o$

[60] $N = (k_3 + k_q q_o + k_{-q})[(k_1 a_o + \tilde{k}) k_{-q} + \tilde{k} k_q q_o]$

$\quad + k_{-q} k_3 [k_q q_o + k_{-q} + k_1 a_o + \tilde{k}] + k_1 k_2 a_o (k_q q_o + 2 k_{-q})$

[61] $Q = k_{-q} k_3 [(k_1 a_o + \tilde{k}) k_{-q} + \tilde{k} k_q q_o] + k_1 k_2 k_{-q} a_o (k_q q_o + k_{-q})$

Eq. [57] may be written as

$$[62] \qquad m = \frac{k_1 a_o e_o (P + k_{-q})[P^2 + P(k_3 + k_q q_o + k_{-q}) + k_{-q} k_3]}{(P + \lambda_1)(P + \lambda_2)(P + \lambda_3)(P + \lambda_4)}$$

where $-\lambda_1$, $-\lambda_2$, $-\lambda_3$ and $-\lambda_4$ are the four roots of the quartic

[63] $P^4 + LP^3 + MP^2 + NP + Q = 0$

The original is

$$[64] \qquad m = \frac{k_1 k_{-q}^2 k_3 a_o e_o}{\lambda_1 \lambda_2 \lambda_3 \lambda_4}$$

$$- \sum_{i=1}^{4} \frac{k_1 a_o e_o (k_{-q} \lambda_i)[\lambda_i^2 - \lambda_i (k_3 + k_q q_o + k_{-q}) + k_{-q} k_3]}{\lambda_i (P - \lambda_i)} e^{-\lambda_i t}$$

Substitution of this into eq. [55] and integration with the boundary conditions, $t = 0$, $x = 0$, leads to

$$[65] \qquad x = \frac{k_2 a_o e_o}{(K_m + \frac{k_2 + k_3}{k_3} a_o)(1 + \frac{q_o}{K_q})} t$$

$$+ \sum_{i=1}^{4} \frac{k_1 k_2 a_o e_o (k_{-q} - \lambda_i)[\lambda_i^2 - \lambda_i (k_3 + k_q q_o + k_{-q}) + k_{-q} k_3]}{\lambda_i^2 (P - \lambda_i)}$$

$$-(e^{-\lambda_i t} - 1)$$

where $K_m = \tilde{k}/k_1$ and $K_q = k_{-q}/k_q$.

The first term, linear in t, corresponds to the steady state, the behavior being non-competitive. A plot of x against t will be of the same form as Fig. 19, except that now the steady state is approached in a quadriphasic manner. The analysis of the pre-steady-state will probably always be prohibitively difficult in practice; in principle it can be done by the same methods as for the preceeding mechanisms.

Irreversible Case

The variation of x with t when inhibition is irreversible ($k_{-q} = 0$) is

$$[66] \qquad x = \frac{k_1 k_2 a_o e_o (k_3 + k_q q_o)}{\tilde{k} k_q q_o (k_3 + k_q q_o) + k_1 k_2 k_q q_o a_o}$$

$$- \sum_{i=1}^{3} \frac{k_1 k_2 a_o e_o (k_3 + k_q q_o - \lambda_i)}{\lambda_i (P - \lambda_i)} e^{-\lambda_i t}$$

where $-\lambda_1$, $-\lambda_2$ and $-\lambda_3$ are the roots of the equation

$$[67] \qquad P^3 + LP^2 + MP + N = 0$$

and

$$[68] \qquad L = 2 k_q q_o + k_1 a_o + \tilde{k} + k_3$$

$$[69] \qquad M = k_1 k_2 a_o + \tilde{k} k_q q_o$$

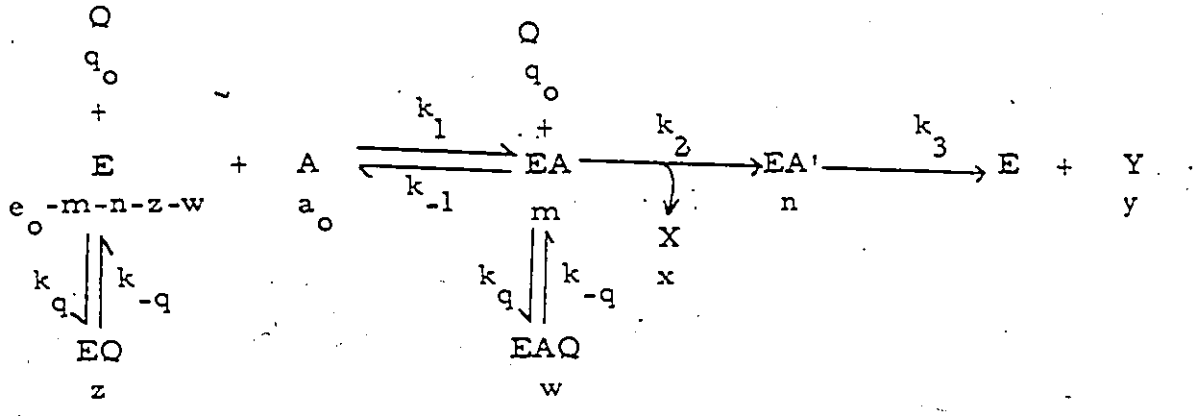
$$[70] \qquad N = \tilde{k} k_q q_o (k_3 + k_q q_o) + k_1 k_2 k_q q_o a_o$$

From eq. [66], there is no steady state. Analysis of results can be carried out as for other mechanisms.

Mechanism V

Reversible Case

The inhibitor becomes attached to both E and EA, the rate and equilibrium constants being the same for both:



The rate equations are

[71] $$\dot{m} = k_1 a_o (e_o - m - n - z - w) - \tilde{k} m - k_q q_o m + k_{-q} w$$

[72] $$\dot{n} = k_2 m - k_3 n$$

[73] $$\dot{z} = k_q q_o (e_o - m - n - z - w) - k_{-q} z$$

[74] $$\dot{w} = k_q q_o n - k_{-q} w$$

[75] $$\dot{x} = k_2 m$$

[76] $$\dot{y} = k_3 n$$

The transform for m is

[77] $$m = \frac{k_1 a_o e_o (P + \tilde{k})(P + k_{-q})(P + k_3)}{P^4 + MP^3 + NP^2 + LP + Q}$$

where

[78] $$M = k_1 a_o + \tilde{k} + 2 k_q q_o + 2 k_{-q} + k_3$$

[79] $$N = (k_q q_o + k_{-q})(k_1 a_o + \tilde{k} + k_q q_o) + (k_{-q} + k_3)(k_1 a_o + \tilde{k} + 2 k_q q_o + k_{-q}) + k_{-q} k_3 + k_1 k_2 a_o - k_{-q} k_q q_o$$

[80] $$L = (k_{-q} + k_3)\,[k_1 a_o (k_{-q} + k_q q_o) + (\tilde{k} + k_q q_o)(k_{-q} + k_q q_o)]$$

$$+ k_{-q} k_3 [k_1 a_o + \tilde{k} + 2 k_q q_o + k_{-q}] + 2 k_1 k_2 k_{-q} a_o$$

$$- k_q k_{-q} q_o (k_3 + k_q q_o + k_{-q})$$

[81] $$Q = k_1 k_{-q} k_3 a_o (k_{-q} + k_q q_o) + k_{-q} k_3 \tilde{k} (k_{-q} + k_q q_o)$$

$$+ k_1 k_2 k_{-q}^2 a_o$$

Eq. [77] may be written as

[82] $$m = \frac{k_1 a_o e_o (P + k_{-q})(P + k_{-q})(P + k_3)}{(P + \lambda_1)(P + \lambda_2)(P + \lambda_3)(P + \lambda_4)}$$

where $-\lambda_1$, $-\lambda_2$, $-\lambda_3$ and $-\lambda_4$ are the four roots of the polynomial

[83] $$P^4 + MP^3 + NP^2 + LP + Q = 0$$

The original for m is

[84] $$m = \frac{k_1 k_{-q}^2 k_3 a_o e_o}{\lambda_1 \lambda_2 \lambda_3 \lambda_4} - \sum_{i=1}^{4} \frac{k_1 a_o e_o (k_{-q} - \lambda_i)^2 (k_3 - \lambda_i)}{\lambda_i (P - \lambda_i)} e^{-\lambda_i t}$$

Substitutions of eq. [84] into [75] and integration subject to the boundary conditions t = 0, x = 0, leads to

[85] $$x = \frac{k_2 a_o e_o}{K_m (1 + \frac{q_o}{K_q}) + a_o (1 + \frac{q_o}{K_q}) + \frac{k_2 a_o}{k_3}}\, t$$

$$+ \sum_{i=1}^{4} \frac{k_1 k_2 a_o e_o (k_{-q} - \lambda_i)^2 (k_{-3} - \lambda_i)}{\lambda_i^2 (P - \lambda_i)} (e^{-\lambda_i t} - 1)$$

where $K_m = \tilde{k}/k_1$ and $k_q = k_{-q}/k_q$

Irreversible Case

For the irreversible inhibition

$$[86] \qquad x = \frac{k_1 k_2 a_o e_o}{k_1 k_q q_o a_o + k_q q_o (\tilde{k} + k_q q_o)}$$

$$- \sum_{i=1}^{3} \frac{k_1 k_2 a_o e_o (k_3 - \lambda_i)}{\lambda_i (P - \lambda_i)} e^{-\lambda_i t}$$

where $-\lambda_1$, $-\lambda_2$ and $-\lambda_3$ are the roots of the equation

$$[87] \qquad P^3 + MP^2 + NP + L = 0$$

and

$$[88] \qquad M = k_1 a_o + \tilde{k} + 2 k_q q_o + k_3$$

$$[89] \qquad N = k_q q_o (k_1 a_o + \tilde{k} + k_q q_o) + k_3 (k_1 a_o + \tilde{k} + 2 k_q q_o) + k_1 k_2 a_o$$

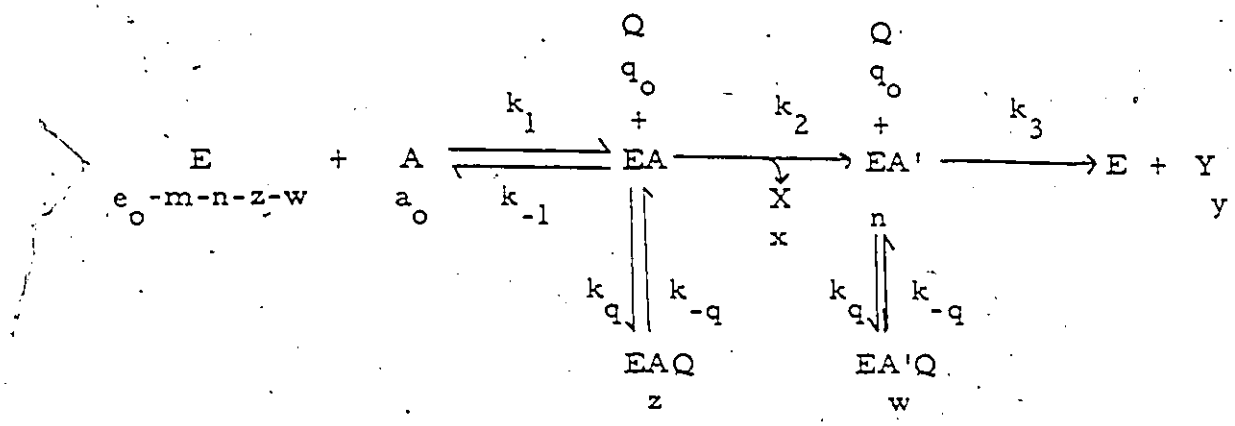
$$[90] \qquad L = k_3 [k_1 k_q q_o a_o + k_q q_o (\tilde{k} + k_q q_o)]$$

Again, no steady state is established and analysis can be carried out as before.

Mechanism VI

Reversible Case

The inhibitor becomes attached to EA and EA' only, the rate and equilibrium constants being the same:



The rate equations are

[91] $$\dot{m} = k_1 a_o (e_o - m - n - z - w) - \tilde{k} m - k_q q_o m + k_{-q} z$$

[92] $$\dot{n} = k_2 m + k_{-q} w - k_q q_o n - k_3 n$$

[93] $$\dot{z} = k_q q_o m - k_{-q} z$$

[94] $$\dot{w} = k_q q_o n - k_{-q} w$$

[95] $$\dot{x} = k_2 m$$

[96] $$\dot{y} = k_3 n$$

The transform for m is

[97] $$m = \frac{k_1 a_o e_o (P + k_{-q}) [P^2 + P (k_q q_o + k_{-q} + k_3) + k_{-q} k_3]}{P^4 + MP^3 + NP^2 + LP + Q}$$

where

[98] $$M = k_1 a_o + \tilde{k} + 2k_q q_o + 2k_{-q} + k_3$$

[99] $$N = k_{-q} \tilde{k} + k_1 a_o (k_{-q} + k_q q_o) + k_{-q} k_3 + k_1 k_2 a_o + (k_q q_o + k_3 + k_{-q})(k_1 a_o + \tilde{k} + k_q q_o + k_{-q})$$

[100] $$Q = k_{-q}^2 k_3 \tilde{k} + k_{-q} k_1 k_3 a_o (k_{-q} + k_q q_o) + k_1 k_2 k_{-q} a_o (k_{-q} + k_q q_o)$$

Eq. [97] can be written as

[101] $$m = \frac{k_1 a_o e_o (P + k_{-q})[P^2 + P(k_q q_o + k_{-q} + k_3) + k_{-q} k_3]}{(P + \lambda_1)(P + \lambda_2)(P + \lambda_3)(P + \lambda_4)}$$

where $-\lambda_1$, $-\lambda_2$, $-\lambda_3$ and $-\lambda_4$ are the roots of the polynomial

[102] $$P^4 + MP^3 + NP^2 + LP + Q = 0$$

The original for m is

[103] $$m = \frac{k_{-q}^2 k_1 k_3 a_o e_o}{\lambda_1 \lambda_2 \lambda_3 \lambda_4} \sum_{i=1}^{4} \frac{k_1 a_o e_o (k_{-q} - \lambda_i)[\lambda_i^2 - \lambda_i(k_q q_o + k_{-q} + k_3) + k_{-q} k_3]}{\lambda_i (P - \lambda_i)} e^{-\lambda_i t}$$

Substitution of eq. [103] into [95] and integration subject to the boundary conditions $t = 0$, $x = 0$ leads to

[104] $$x = \frac{k_2 a_o e_o}{K_m + a_o\left(1 + \frac{q}{K_q}\right) + \frac{k_2}{k_3} a_o \left(1 + \frac{q_o}{K_q}\right)} \cdot t + \sum_{i=1}^{4} \frac{k_1 k_2 a_o e_o (k_{-q} - \lambda_i)[\lambda_i^2 - \lambda_i(k_q q_o + k_{-q} + k_3) + k_{-q} k_3}{\lambda_i^2 (P - \lambda_i)} (e^{-\lambda_i t} - 1)$$

hence $K_m = \tilde{k}/k_1$ and $K_q = k_{-q}/k_q$

Irreversible Case

The solution now is

$$[105] \qquad x = \frac{e_o (k_q q_o + k_3)}{k_q q_o} - \sum_{i=1}^{3} \frac{k_1 k_2 a_o e_o (k_q q_o + k_3 - \lambda_i)}{\lambda_i (P - \lambda_i)} e^{-\lambda_i t}$$

where $-\lambda_1$, $-\lambda_2$, $-\lambda_3$ are the roots of the equation

$$[106] \qquad P^3 + MP^2 + NP + L = 0$$

and

$$[107] \qquad M = k_1 a_o + \tilde{k} + 2 k_q q_o + k_3$$

$$[108] \qquad N = k_1 a_o k_q q_o + (k_q q_o + k_3)(k_1 a_o + \tilde{k} + k_q q_o) + k_1 k_2 a_o$$

$$[109] \qquad L = k_1 k_2 a_o k_q q_o$$

As with other irreversible inhibitions, no steady state is established and analysis in principle can be carried out as before.

CHAPTER SEVEN

TRANSIENT-PHASE AND STEADY-STATE KINETICS FOR TWO SUBSTRATE SYSTEMS

Introduction

Some enzymes are known to catalyze reactions between two substrates, or between one substrate and a co-enzyme. Cleland [9] classified the kinetic mechanisms for these two systems into two major categories: (a) ping pong bi bi mechanism in which products can be released before all substrates have added to the enzyme * (b) sequential mechanisms in which the products cannot be released before all substrates have added to the enzyme. Under the latter category falls the Theorell-Chance, the ordered ternary-complex and the random ternary-complex mechanisms. On the basis of inhibition studies, it is possible to distinguish between these mechanisms [10]. In the present chapter, transient-phase and steady-state solutions are given for all four mechanisms under the usual assumptions that the two substrates or the substrate and co-enzyme are present at a larger concentration than the enzyme. This can be easily realized in practice. The theoretical significance of this assumption is that the substrate concentrations can be treated as constants during the transient phase and the initial steady-state phase of the reaction.

As in the previous chapters, the equations have been integrated using the Laplace-Carson transformation technique.

Theorell-Chance Mechanism

Mechanism and Solution

The mechanism is

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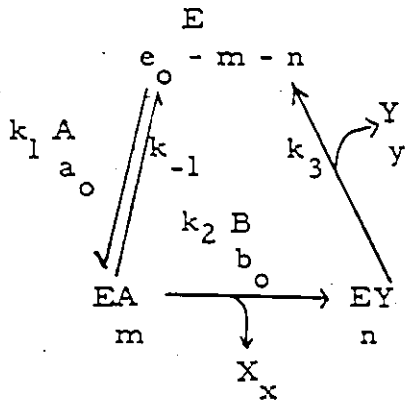
* Double-intermediate single-substrate mechanisms are frequently of this type, the water molecule acting as second substrate.



The reactants are A and B, and the products are X and Y. It is assumed, as is usually the case, that the enzyme concentration is much less than that of A and B. The concentrations, at any time t, are shown in small letters in the scheme above.

The differential rate equations are

[1] $$\dot{m} = k_1 a_o (e_o - m - n) - (k_{-1} + k_2 b_o) m$$

[2] $$\dot{n} = k_2 B_o m - k_3 n$$

[3] $$\dot{x} = k_2 b_o m$$

[4] $$\dot{y} = k_3 n$$

The transforms of m and n are found to be

[5] $$n = \frac{k_2 b_o m}{P + k_3} = \frac{k_1 k_2 a_o b_o}{(P + \lambda_1)(P + \lambda_2)}$$

[6] $$m = \frac{k_1 a_o e_o (P + k_3)}{(P + \lambda_1)(P + \lambda_2)}$$

where

[7] $$\lambda_1 = \tfrac{1}{2}(- R - \sqrt{R^2 - 4 Q})$$

[8] $$\lambda_2 = \tfrac{1}{2}(- R + \sqrt{R^2 - 4 Q})$$

[9] $$R = k_1 a_o + k_{-1} + k_2 b_o + k_3$$

[10] $$Q = k_3 (k_1 a_o + k_{-1} + k_2 b_o) + k_1 k_2 a_o b_o$$

The originals for m and n are

[11] $$m = \frac{k_1 k_2 a_o e_o}{\lambda_1 \lambda_2} - \frac{k_1 a_o e_o (k_3 - \lambda_1)}{\lambda_1 (\lambda_2 - \lambda_1)} e^{-\lambda_1 t} - \frac{k_1 a_o e_o (k_3 - \lambda_2)}{\lambda_2 (\lambda_1 - \lambda_2)} e^{-\lambda_2 t}$$

[12] $$n = \frac{k_1 k_2 a_o b_o e_o}{\lambda_1 \lambda_2} - \frac{k_1 k_2 a_o b_o e_o}{\lambda_1 (\lambda_2 - \lambda_1)} e^{-\lambda_1 t}$$

$$- \frac{k_1 k_2 a_o b_o e_o}{\lambda_2 (\lambda_1 - \lambda_2)} e^{-\lambda_2 t}$$

The rate of production of the first product X is

[13] $$\dot{x} = k_2 b_o m$$

Substituting the solution from m and putting $\lambda_1 \lambda_2 = Q$, and integration with the boundary condition that $t = 0$, $x = 0$, leads to

[14] $$x = \frac{k_1 k_2 k_3 a_o b_o e_o}{k_3 (k_1 a_o + k_{-1} + k_2 b_o) + k_1 k_2 a_o b_o} t$$

$$+ \frac{k_1 k_2 a_o b_o e_o (k_3 - \lambda_1)}{\lambda_1^2 (\lambda_2 - \lambda_1)} (e^{-\lambda_1 t} - 1)$$

$$+ \frac{k_1 k_2 a_o b_o e_o (k_3 - \lambda_2)}{\lambda_2^2 (\lambda_1 - \lambda_2)} (e^{-\lambda_2 t} - 1)$$

Similarly, the solution for the second product Y is

$$[15] \qquad Y = \frac{k_1 k_2 k_3 a_o b_o e_o}{k_3 (k_1 a_o + k_{-1} + k_2 b_o) + k_1 k_2 a_o b_o} \; t$$

$$+ \frac{k_1 k_2 k_3 a_o b_o e_o}{\lambda_1^2 (\lambda_2 - \lambda_1)} (e^{-\lambda_1 t} - 1) + \frac{k_1 k_2 k_3 a_o b_o e_o}{\lambda_2^2 (\lambda_1 - \lambda_2)} (e^{-\lambda_2 t} - 1)$$

The first term in each of eqs. [14] and [15] corresponds to the steady state. The variation of x with t is shown schematically in Fig. 21; the linear steady-state behavior is approached in a biphasic exponential fashion. The variation of y with t is of the same form.

Analysis of Results

If the formation of X has been studied experimentally, in the transient-phase and steady-state regions, the results can be analyzed as follows. Equation [14] can be written as

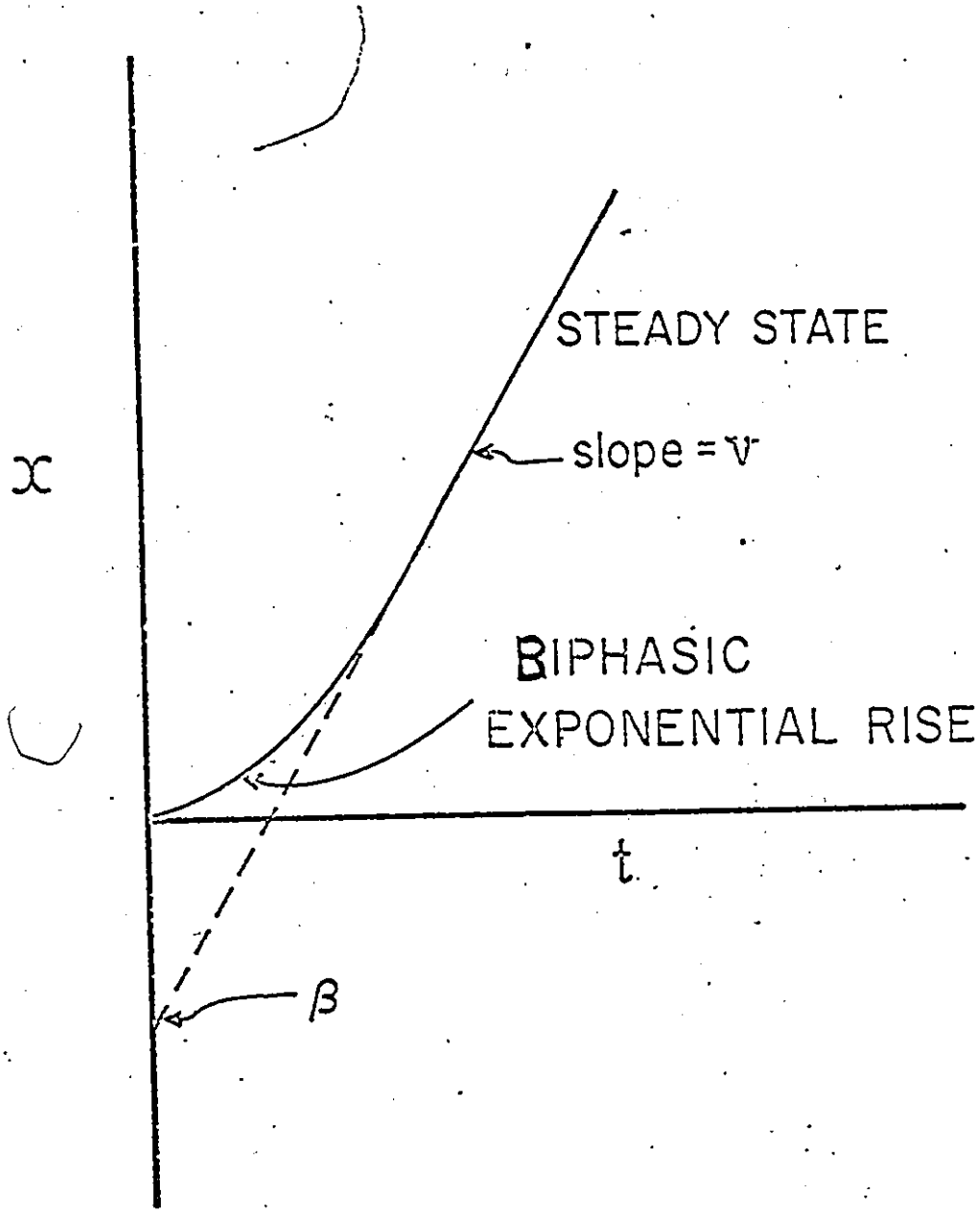
$$[16] \qquad x = vt + \beta + \beta_1 e^{-\lambda_1 t} + \beta_2 e^{-\lambda_2 t}$$

where $\beta = -(\beta_1 + \beta_2)$. Extrapolation of the linear part of the curve (cf. Fig. 21) will has an intercept on the x axis equal to β, and from the expressions for β_1 and β_2 we find that

$$[17] \qquad \beta/v = \tau = -\frac{1}{k_3} + \frac{k_1 a_o + k_{-1} + k_2 b_o + k_3}{k_3 (k_1 a_o + k_{-1} + k_2 b_o) + k_1 k_2 a_o b_o}$$

Also, a plot of $\ln (x - vt - \beta)$ against t will consist of two straight regions, of slopes $-\lambda_1$ and $-\lambda_2$. λ_1 and λ_2 obey the following relations

Fig. 21. A schematic plot of x vs t (cf. eq. [14]).


STEADY STATE
slope = v
x
BIPHASIC
EXPONENTIAL RISE
t
β

[18] $$\lambda_1 \lambda_2 = k_1 (k_3 + k_2 b_o) a_o + k_{-1} k_3 + k_2 k_3 b_o$$

[19] $$(\lambda_1 + \lambda_2) = k_1 a_o + k_{-1} + k_2 b_o + k_3$$

Thus a plot of $(\lambda_1 + \lambda_2)$ against a_o at constant b_o is linear with a slope of k_1. The intercept of this plot is $k_{-1} + k_2 b_o + k_3$; a plot of this intercept against b_o is linear with slope of k_2 and intercept of $k_{-1} + k_3$.

Similarly $\lambda_1 \lambda_2$ can be plotted against b_o, a schematic plot of which is shown in Fig. 22. The slope is $k_1 k_2 a_o + k_2 k_3$; k_1, k_2, and a_o in this slope are known so that k_3 can be calculated, and consequently k_{-1}. The procedure thus allows k_1, k_2, k_3 and k_{-1} to be calculated.

It is noted that various other methods of analyzing the data can also be devised.

Similar procedures apply to the second product Y. Eq. [15] can be written as

[20] $$y = vt + \beta + \beta_1 e^{-\lambda_1 t} + \beta_2 e^{-\lambda_2 t}$$

where v is again the steady-state rate (the same as for X), and

[21] $$\beta/V = \tau = \frac{k_1 a_o + k_{-1} + k_2 b_o + k_3}{k_3 (k_1 a_o + k_{-1} + k_2 b_o) + k_1 k_2 a_o b_o}$$

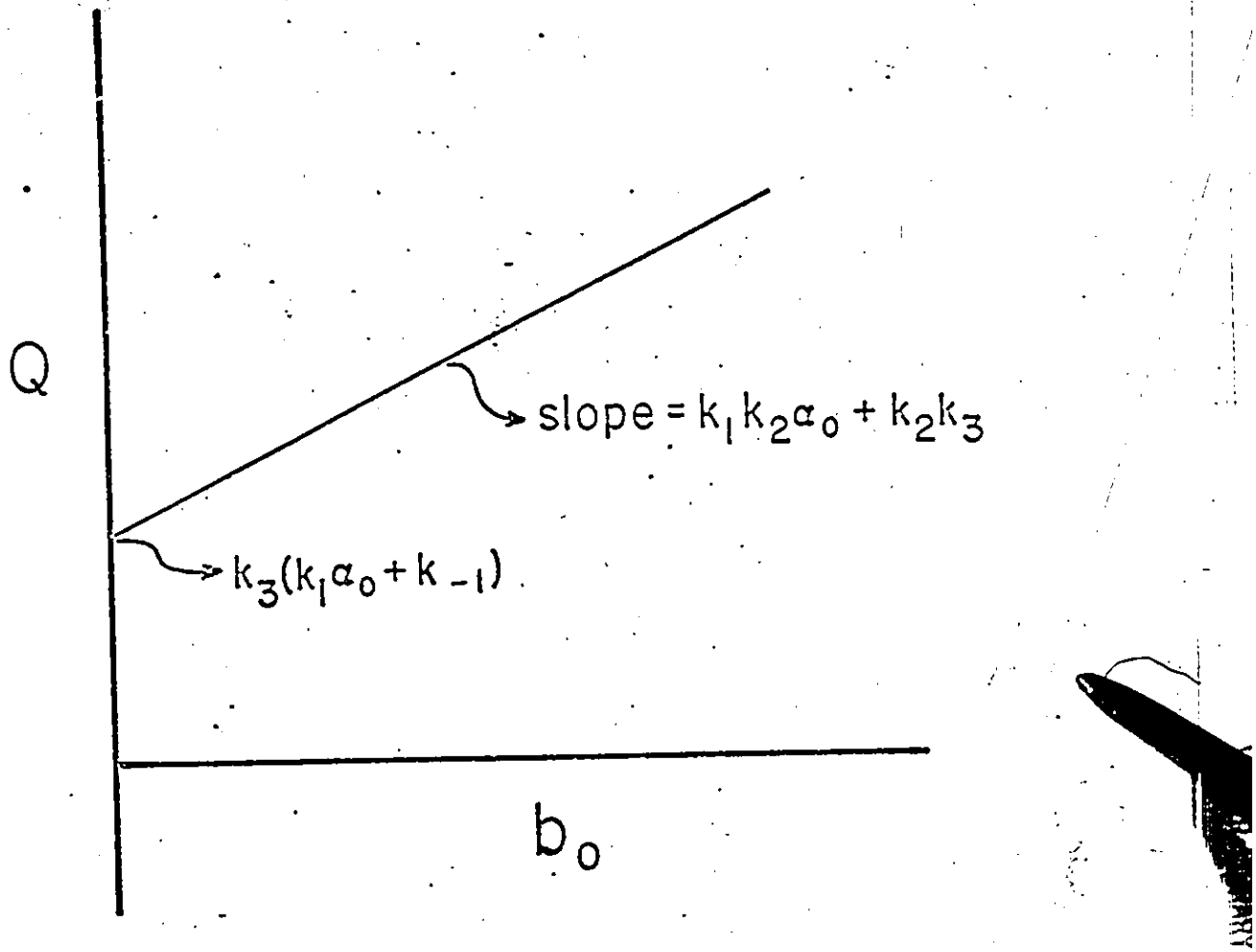
As before, v and β can be obtained from a plot of y against t (compare Fig. 21). A plot of ln $(y - vt - \beta)$ against t can then be used to get λ_1 and λ_2 and various plots can be used to calculate the rate constants.

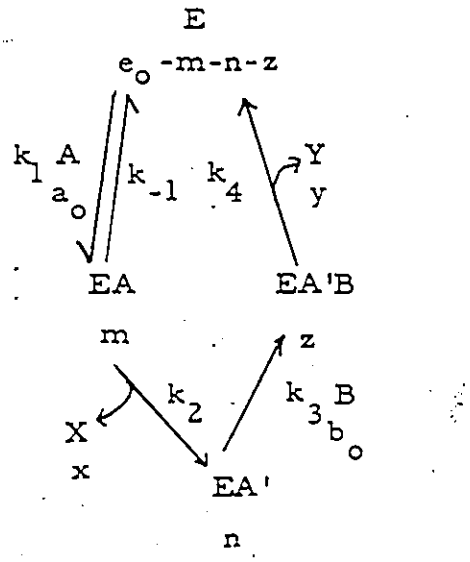
Ping Pong Bi Bi Mechanism

Mechanism and Solution

The mechanism is

Fig. 22 A schematic plot of $\lambda_1 \lambda_2$ against b_o (cf. eq. [18])


Q
slope = $k_1 k_2 \alpha_0 + k_2 k_3$
$k_3(k_1 \alpha_0 + k_{-1})$
b_0



The concentrations of the various species, on the assumption of excess A and B as compared with E, are given in small letters. The differential rate equations are

[22] $$\dot{m} = k_1 a_o (e_o - m - n - z) - (k_{-1} + k_2) m$$

[23] $$\dot{n} = k_2 m - k_3 b_o n$$

[24] $$\dot{z} = k_3 b_o n - k_4 z$$

[25] $$\dot{x} = k_2 m$$

[26] $$\dot{y} = k_4 z$$

The transform of m is

[27] $$m = \frac{k_1 a_o e_o (P + k_3 b_o)(P + k_4)}{(P + \lambda_1)(P + \lambda_2)(P + \lambda_3)}$$

where the λ_i's are the negative roots of the polynomial

[28] $$P^3 + MP^2 + LP + Q = 0$$

where

[29] $$M = k_1 a_o + k_{-1} + k_2 + k_3 b_o + k_4$$

[30] $$L = k_3 k_4 b_o + (k_3 b_o + k_4)(k_1 a_o + k_{-1} + k_2) + k_1 k_2 a_o$$

[31] $$Q = k_3 k_4 b_o (k_1 a_o + k_{-1} + k_2) + k_1 k_2 k_4 a_o + k_1 k_2 k_3 a_o b_o$$

The transform of z is

[32] $$z = \frac{k_1 k_2 k_3 a_o b_o e_o}{(P + \lambda_1)(P + \lambda_2)(P + \lambda_3)}$$

and the originals are

[33] $$m = \frac{k_1 k_3 k_4 a_o b_o e_o}{\lambda_1 \lambda_2 \lambda_3} - \sum_{i=1}^{3} \frac{k_1 a_o e_o (\lambda_i - k_3 b_o)(\lambda_i - k_4)}{\lambda_i (P - \lambda_i)} e^{-\lambda_i t}$$

[34] $$z = \frac{k_1 k_2 k_3 a_o b_o e_o}{\lambda_1 \lambda_2 \lambda_3} - \sum_{i=1}^{3} \frac{k_1 k_2 k_3 a_o b_o e_o}{\lambda_i (P - \lambda_i)} e^{-\lambda_i t}$$

Substitution of these expressions into eqs. [25] and [26], integration subject to the boundary conditions t = 0, x = 0, y = 0, and substituting $\lambda_1 \lambda_2 \lambda_3 = Q$, leads to

[35] $$x = \frac{k_1 k_2 k_3 k_4 a_o b_o e_o}{k_1 k_2 k_4 a_o + k_3 k_4 (k_{-1} + k_2) b_o + k_1 k_3 (k_2 + k_4) a_o b_o} t$$

$$+ \sum_{i=1}^{3} \frac{k_1 k_2 a_o e_o (\lambda_i - k_3 b_o)(\lambda_i - k_4)}{\lambda_i^2 (P - \lambda_i)} (e^{-\lambda_i t} - 1)$$

$$[36] \qquad y = \frac{k_1 k_2 k_3 k_4 a_o b_o e_o}{k_1 k_2 k_4 a_o + k_3 k_4 (k_{-1} + k_2) b_o + k_1 k_3 (k_2 + k_4) a_o b_o} t$$

$$+ \sum_{i=1}^{3} \frac{k_1 k_2 k_3 k_4 a_o b_o e_o}{\lambda_i^2 (P - \lambda_i)} (e^{-\lambda_i t} - 1)$$

Analysis of Results

The first term in both of these expressions correspond to the steady state; the remaining terms correspond to a triphasic exponential approach to the steady state. The general form of the x vs t and y vs t curves will be similar to that in Fig. 21. Equations [35] and [36] can be written as

$$[37] \qquad \left.\begin{matrix} x \\ y \end{matrix}\right\} = vt + \beta + \beta_1 e^{-\lambda_1 t} + \beta_2 e^{-\lambda_2 t} + \beta_3 e^{-\lambda_3 t}$$

where $\beta = -(\beta_1 + \beta_2 + \beta_3)$, v is the steady-state rate and β the intercept on the x(y) axis. A plot of $\ln (x - vt - \beta)$ or $\ln (y - vt - \beta)$ against t will in general have three straight portions, the slopes of which are $-\lambda_1$, $-\lambda_2$ and $-\lambda_3$. The product $\lambda_1 \lambda_2 \lambda_3 = Q$ which is given by eq. [31]. The sum $(\lambda_1 + \lambda_2 + \lambda_3)$ is M which is given by eq. [29]. Plots of M against a_o at constant b_o, and against b_o at constant a_o, will then give k_1, k_3, and $k_{-1} + k_2 + k_4$. A plot of Q against b_o at constant a_o will be linear with an intercept of $k_1 k_2 k_4 a_o$; hence $k_2 k_4$ is known. We also have that

$$[38] \qquad v = \frac{k_1 k_2 k_3 k_4 a_o b_o e_o}{Q}$$

and a plot of vQ against $a_o b_o$ will give $k_1 k_2 k_3 k_4$; this also gives k_3. The slope of the plot of Q against b_o is

$$k_1 k_3 (k_2 + k_4) a_o + k_3 k_4 (k_{-1} + k_2)$$

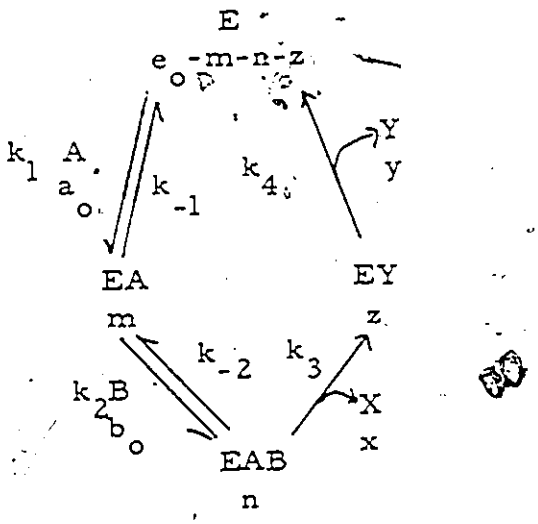
and a plot of this against a_o gives $k_1 k_3 (k_2 + k_4)$; hence $k_2 + k_4$ can be determined, and k_{-1} can also be obtained. This plot also gives $k_3 k_4 (k_{-1} + k_2)$ as intercept, since k_3, $k_2 k_4$ and k_{-1} are known, k_2 and k_4 can be calculated. Therefore, using this procedure all the rate constants can be calculated.

Various other methods of analysis can also be used.

Ordered Ternary-Complex Mechanism

Mechanism and Solution

The mechanism is



The differential rate equations are

[39] $$\dot{m} = k_1 a_o (e_o - m - n - z) - (k_{-1} + k_2 b_o) m + k_{-2} n$$

[40] $$\dot{n} = k_2 b_o m - (k_{-2} + k_3) n$$

[41] $$\dot{z} = k_3 n - k_4 z$$

[42] $$\dot{x} = k_3 n$$

[43] $$\dot{y} = k_4 z$$

The transforms for n and z are

[44] $$n = \frac{k_1 k_2 a_o b_o e_o (P + k_4)}{(P+\lambda_1)(P + \lambda_2)(P + \lambda_3)}$$

[45] $$z = \frac{k_1 k_2 k_3 a_o b_o e_o}{(P + \lambda_1)(P + \lambda_2)(P + \lambda_3)}$$

where $-\lambda_1$, $-\lambda_2$, and $-\lambda_3$ are roots of the polynomial

[46] $$P^3 + MP^2 + LP + Q = 0$$

with

[47] $$M = k_1 a_o + k_4 + k_{-1} + k_{-2} + k_3 + k_2 b_o$$

[48] $$L = k_4 (k_1 a_o + k_{-1} + k_2 b_o + k_{-2} + k_3) + k_{-2} (k_1 a_o + k_{-1}) + k_3 (k_1 a_o + k_{-1} + k_2 b_o) + k_1 k_2 a_o b_o$$

[49] $$Q = k_{-2} k_4 (k_1 a_o + k_{-1}) + k_3 k_4 (k_1 a_o + k_{-1} + k_2 b_o) + k_1 k_2 k_4 a_o b_o + k_1 k_2 k_3 a_o b_o$$

The originals for n and z are

$$[50] \qquad n = \frac{k_1 k_2 k_4 a_o b_o e_o}{\lambda_1 \lambda_2 \lambda_3} - \sum_{i=1}^{3} \frac{k_1 k_2 a_o b_o e_o (k_4 - \lambda_i)}{\lambda_i (P - \lambda_i)} e^{-\lambda_i t}$$

$$[51] \qquad z = \frac{k_1 k_2 k_3 a_o b_o e_o}{\lambda_1 \lambda_2 \lambda_3} - \sum_{i=1}^{3} \frac{k_1 k_2 k_3 a_o b_o e_o}{\lambda_i (P - \lambda_i)} e^{-\lambda_i t}$$

Substitution of these equations for n and z into eqs. [42] and [43], and integration with the initial conditions t = 0, x = 0, y = 0, leads to

$$[52] \qquad x = \frac{k_1 k_2 k_3 k_4 a_o b_o e_o}{k_{-2} k_4 (k_1 a_o + k_{-1}) + k_3 k_4 (k_1 a_o + k_{-1} + k_2 b_o) + k_1 k_2 (k_3 + k_4) a_o b_o} t$$

$$+ \sum_{i=1}^{3} \frac{k_1 k_2 k_3 a_o b_o e_o (k_4 - \lambda_i)}{\lambda_i^2 (P - \lambda_i)} (e^{-\lambda_i t} - 1)$$

$$[53] \qquad y = \frac{k_1 k_2 k_3 k_4 a_o b_o e_o}{k_{-2} k_4 (k_1 a_o + k_{-1}) + k_3 k_4 (k_1 a_o + k_{-1} + k_2 b_o) + k_1 k_2 (k_3 + k_4) a_o b_o} t$$

$$+ \sum_{i=1}^{3} \frac{k_1 k_2 k_3 k_4 a_o b_o e_o}{\lambda_i^2 (P - \lambda_i)} (e^{-\lambda_i t} - 1)$$

Analysis of Results

Equations for x and y are both of the form

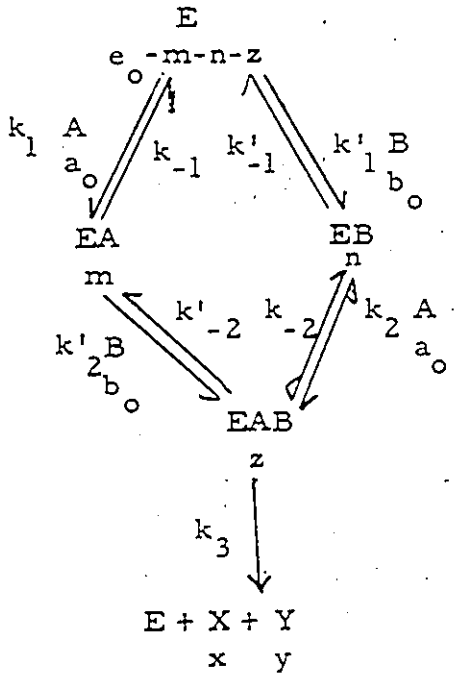
$$[54] \qquad \left.\begin{matrix} x \\ y \end{matrix}\right\} = vt + \beta + \beta_1 e^{-\lambda_1 t} + \beta_2 e^{-\lambda_2 t} + \beta_3 e^{-\lambda_3 t}$$

where v is the steady-state rate and β is equal to $-(\beta_1 + \beta_2 + \beta_3)$. The x vs t and y vs t curves thus show a linear steady-state portion, which is approached in a triphasic exponential manner (compare Fig. 21). The quantities β and v can be determined from the intercept and slope of such a plot, and λ_1, λ_2, and λ_3 from plots of ln (x-- vt - β) or ln (y - vt - β) against t. We also have that $\lambda_1 \lambda_2 \lambda_3 = Q$, $\lambda_1 + \lambda_2 + \lambda_3 = M$ and $vQ = k_1 k_2 k_3 k_4 a_o b_o e_o$.

Further analysis to obtain the rate constants is carried out by plotting Q, vQ against a_o at constant b_o, and against b_o at constant a_o, as previously described. Plots of M against a_o at constant b_o and b_o at constant a_o lead at once to values of k_1 and k_2, respectively.

Random Ternary-Complex Mechanism

The mechanism is



Againt it is assumed that a_o, $b_o >> e_o$, and the concentrations are given above in small letters. Even the steady-state analysis of this mechanism is very complicated, and leads to clumsy results which do not permit an analysis of experimental data [48, 49]. The complete transient-phase equations have been developed but only an outline of the conclusions is presented since it is not likely that they can be utilized.

The form of the equations for the products x and y is

$$[55] \qquad \left.\begin{matrix} x \\ y \end{matrix}\right\} = vt + \sum_{i=1}^{4} \frac{f_1 (\lambda_i^2 - f_2 \lambda_i + f_3)}{\lambda_i^2 (P - \lambda_i)} (e^{-\lambda_i t} - 1)$$

where f_1, f_2 and f_3 are complicated expressions involving the rate constants. The λ_i's are the roots of a quartic equation in P, the differential operator. The steady-state rate v has the same complex form obtained by Botts and Morales [48] and by Laidler [49].

Eq. [55] is of the form

$$[56] \qquad \left.\begin{matrix} x \\ y \end{matrix}\right\} = vt + \beta + \beta_1 e^{-\lambda_1 t} + \beta_2 e^{-\lambda_2 t} + \beta_3 e^{-\lambda_3 t} + \beta_4 e^{-\lambda_4 t}$$

where $\beta = -(\beta_1 + \beta_2 + \beta_3 + \beta_4)$. A plot of x or y against t will yield β as an intercept, by extrapolating the linear part, on the x(y) axis. v is the slope of the linear part. A plot could then be made of ln $(x - vt - \beta)$ against t, which in principle would yield λ_1, λ_2, λ_3, λ_4. However, only in very favourable cases would it be possible to separate these values. In any case, further analysis to yield the rate constants appears to be out of the question.

CHAPTER EIGHT

INFLUENCE OF PREMIXING ON TRANSIENT-PHASE KINETICS FOR TWO-SUBSTRATE ENZYME SYSTEMS

Introduction

In the previous chapter we have presented equations for the pre-steady-state and steady-state kinetics of two-substrate systems occurring by the following mechanisms:

(1) Theorell-Chance
(2) Ping Pong Bi Bi
(3) Ordered ternary complex
(4) Random ternary complex

It was assumed that the concentrations of the substrates A and B were in great excess of that of the enzyme E, and that the three ingredients, E, A and B were mixed together at zero time.

In the present chapter we derive the equations that apply to certain pre-mixing procedures. In the case of the first three mechanisms, one substrate, which we denote as A, interacts first with the enzyme, the other, B, being involved later in the reaction. The pre-steady-state kinetics will therefore be modified if we premix E and A, and then add B at zero time. On the other hand, for these three mechanisms, premixing E and B will lead to the same pre-steady-state kinetics as bringing E, A and B together at zero time.

Equations for the various cases are derived in the present paper and attention is also given to the situation in which the concentration of A is much less than that of E and B. It will be seen that the resulting equations lead to criteria for distinguishing between the various mechanisms on the basis of transient-phase studies under different conditions of premixing. The equations for the random ternary-complex mechanism

have been omitted from the present chapter, since they are too complicated to be useful in interpreting experimental data; however, certain qualitative conclusions are drawn about the influence of different premixing conditions.

Theoretical

Figures 23, 24 and 25 show the Theorell-Chance, Ping Pong Bi Bi and Ordered Ternary-Complex Mechanisms, and give the concentrations of the various species for the case a_o, $b_o \gg e_o$. Two special cases are considered:

(i) Both substrates in excess of enzyme; a_o, $b_o \gg e_o$;

(ii) Enzyme and substrate B in excess of substrate A

$$e_o, b_o \gg a_o$$

The concentrations of the various species for these special cases are shown, for the three mechanisms, in Table 1.

Two different premixing conditions are considered:

(i) E|A|B, E|A + B or E + B|A. The vertical stroke indicates mixing at t = 0, the positive sign premixing; thus E|A + B means that A and B are premixed, their mixed solutions being added to E at t = 0. By definition B is the second substrate involved in the reaction; premixing of B with A, or of B with E, therefore, does not affect the kinetics. The equations for this case were given in the previous chapter.

(ii) E + A|B. This type of premixing will affect the kinetics, in that there will be more rapid reaction in the transient phase.

TABLE 1

Concentrations at Time t

Theorell-Chance	E	A	B	EA	EA'	EB	EAB	EA'B	EY	X	Y
1. $a_o, b_o >> e_o$	e_o-m-n	a_o	b_o	m	-	-	-	-	n	x	y
2. $b_o, e_o >> a_o$	e_o	a_o-m-n-y	b_o	m	-	-	-	-	n	x	y
Ping-Ping-Bi-Bi											
3. $a_o, b_o >> e_o$	e_o-m-n-z	a_o	b_o	m	n	-	z	-	-	x	y
4. $e_o, b_o >> a_o$	e	a_o-m-n-y-z	b_o	m	e_o-z-m-e	-	-	z	-	x	y
Ordered Ternary Complex											
5. $a_o b_o >> e_o$	e_o-m-n-z	a_o	b_o	m	-	-	n	-	z	x	y
6. $e_o, b_o >> a_o$	e_o	a_o-m-n-y-z	b_o	m	-	-	n	-	z	x	y

Fig. 23 The Theorell-Chance mechanism, showing the concentrations of the various species for the case a_o, $b_o \gg e_o$.

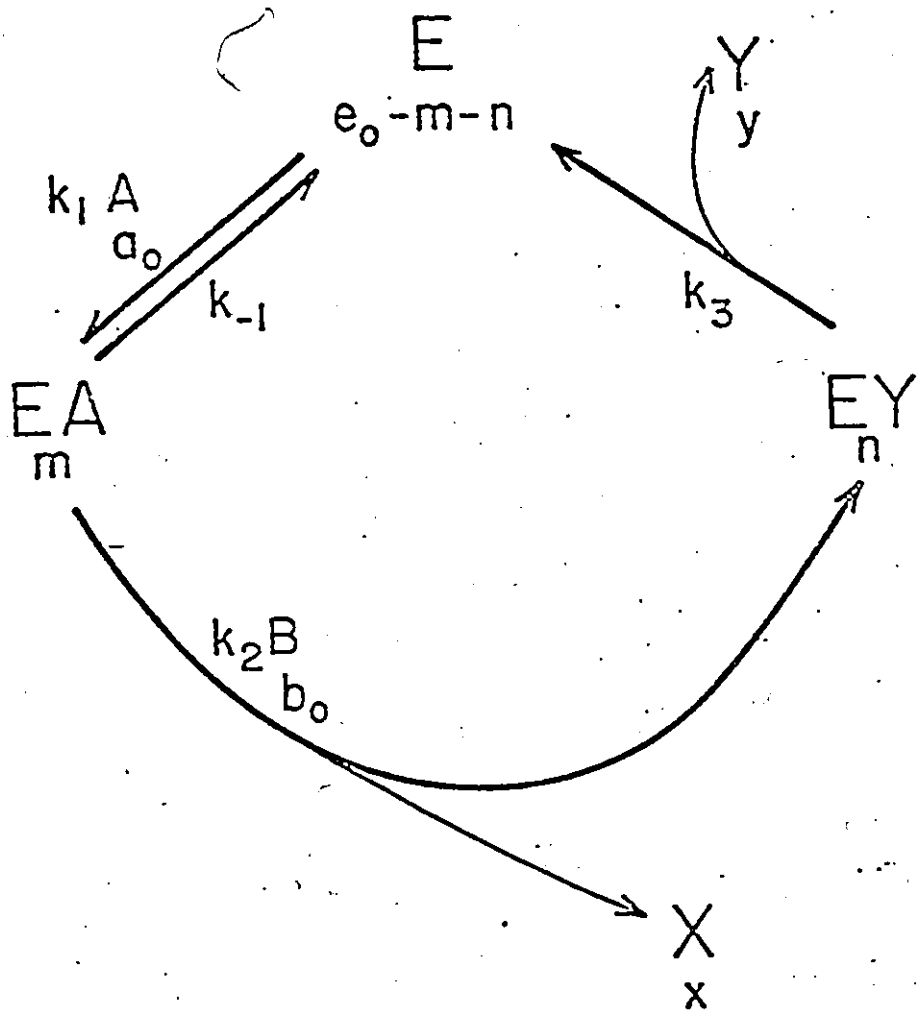

E
e_o-m-n
Y
y
k_1 A
a_o
k_{-1}
k_3
EA
m
EY
n
k_2B
b_o
X
x

Fig. 24 The Ping Pong Bi Bi mechanism, showing the concentrations of the various species for the case $a_o, b_o \gg e_o$.

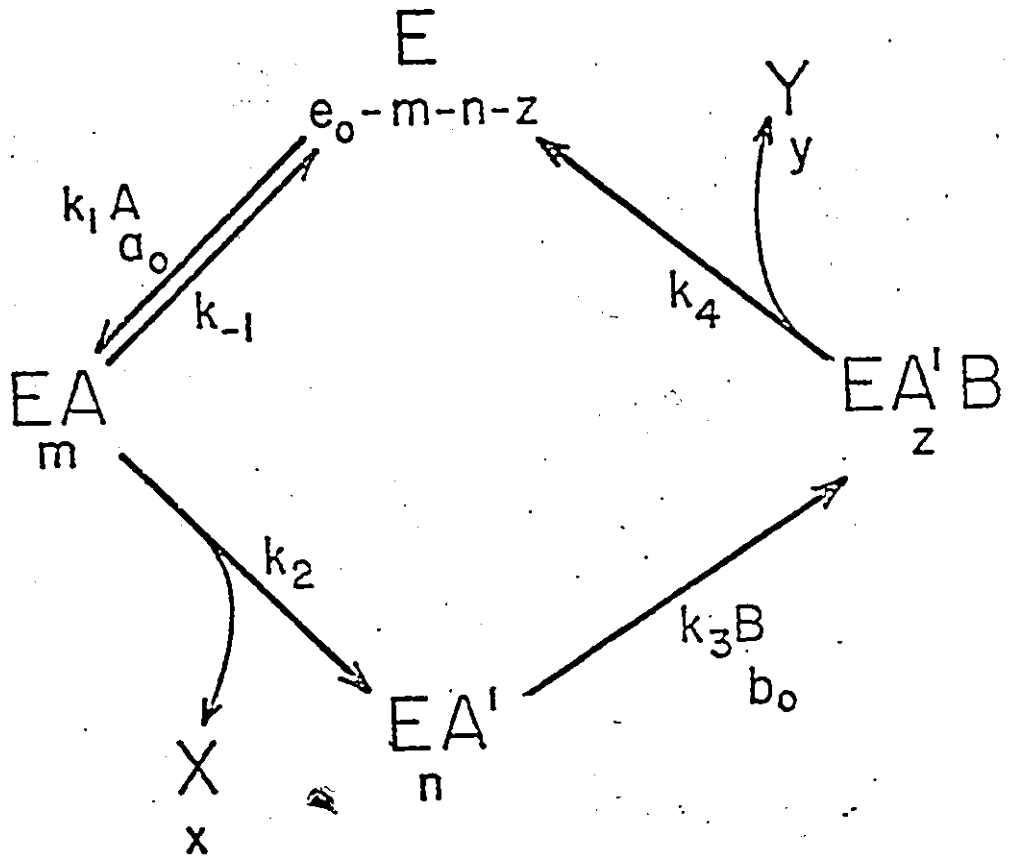
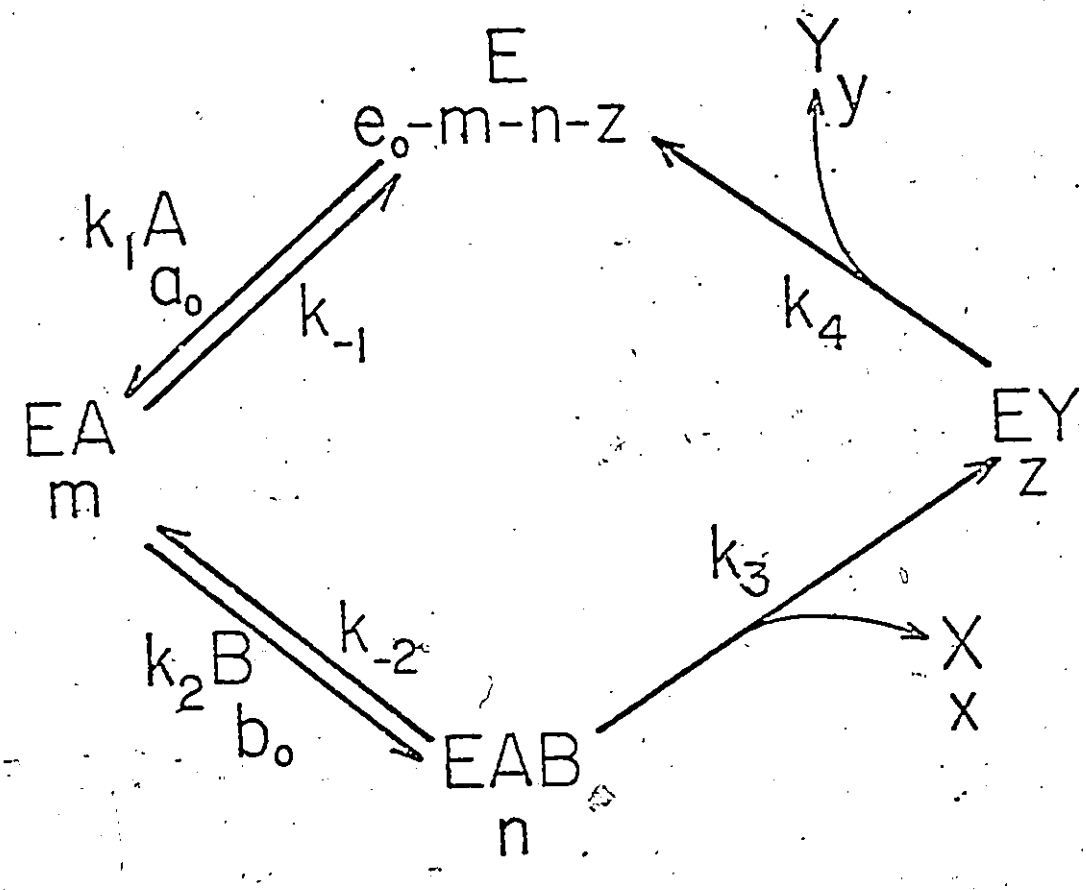

E
$e_o - m - n - z$
Y
y
k_1 A
a_o
k_{-1}
k_4
EA
m
EA'B
z
k_2
k_3B
b_o
X
x
EA'
n

Fig. 25 The Ordered Ternary-Complex mechanism, showing the concentrations of the various species for the case a_o, $b_o \gg e_o$.


E
e_o-m-n-z
Y
y
k_1A
a_o
k_{-1}
k_4
EA
m
EY
z
k_3
k_2B
b_o
k_{-2}
X
x
EAB
n

Theorell-Chance Mechanism

The Theorell-Chance mechanism is shown in Fig. 23, and the equations for the rates of formation of X (the first product produced) and Y (the product later produced) are given in Table 2, for a_o, $b_o \gg e_o$ and the two premixing conditions stated above. The equations were obtained by the use of the Laplace-Carson transform method. The constants λ_1 and λ_2 are always positive quantities, and we arbitrarily define λ_1 as the one having the larger value. It is easily shown that λ_1 must be greater than k_3; λ_2 can be either greater of less than k_3.

Analysis of the experimental data with reference to the equations for x and y was discussed in the previous chapter.

If, on the other hand, E and A are premixed, the equilibrium $E + A \rightleftharpoons EA$ is established; if m_o is the value of m at t = 0 we have the equilibrium equation

$$[1] \qquad k_1 a_o e_o = k_{-1} m_o$$

hence

$$[2] \qquad m_o = \frac{k_1 a_o e_o}{k_{-1}}$$

The procedure for solving the differential equations for this case is similar to that for the previous case, the only modification being that in replacing the differentials by operators P, the transformation is $Pm - Pm_o$. The solutions for this case are shown in the lower part of Table 2.

It is to be noted that for both sets of premixing conditions there is a biphasic exponential rise of x or y to the steady state, the steady state being identical in the two cases. However, in the second case the

Rate equations for the theoretical mechanism with $a_o \neq b_o$

Premixing	Formation of X	Formation of Y
E|A|B	$x = \dfrac{k_1k_2k_3e_oa_ob_o}{k_3(k_1a_o+k_{-1}+k_2b_o)+k_1k_2a_ob_o}\,t$	$y = \dfrac{k_1k_2k_3e_oa_ob_o}{k_3(k_1a_o+k_{-1}+k_2b_o)+k_1k_2a_ob_o}\,t$
E|A + B	$-\dfrac{k_1k_2e_oa_ob_o(\lambda_1-k_3)}{\lambda_1^2(\lambda_1-\lambda_2)}\,(1-e^{-\lambda_1 t})$	$+\dfrac{k_1k_2k_3e_oa_ob_o}{\lambda_1^2(\lambda_1-\lambda_2)}\,(1-e^{-\lambda_1 t})$
or E + B|A	$+\dfrac{k_1k_2e_oa_ob_o(\lambda_2-k_3)}{\lambda_1^2(\lambda_1-\lambda_2)}\,(1-e^{-\lambda_2 t})$	$-\dfrac{k_1k_2k_3e_oa_ob_o}{\lambda_2^2(\lambda_1-\lambda_2)}\,(1-e^{-\lambda_2 t})$
E + A|B	$x = \dfrac{k_1k_2k_3e_oa_ob_o}{k_3(k_1a_o+k_{-1}+k_2b_o)+k_1k_2a_ob_o}\,t$ $-\dfrac{k_1k_2e_oa_ob_o(1-\lambda_1/k_{-1})(\lambda_1-k_3)}{\lambda_1^2(\lambda_1-\lambda_2)}\,(1-e^{-\lambda_1 t})$ $+\dfrac{k_1k_2e_oa_ob_o(1-\lambda_2/k_{-1})(\lambda_2-k_3)}{\lambda_2^2(\lambda_1-\lambda_2)}\,(1-e^{-\lambda_2 t})$	$y = \dfrac{k_1k_2k_3e_oa_ob_o}{k_3(k_1a_o+k_{-1}+k_2b_o)+k_1k_2a_ob_o}\,t$ $+\dfrac{k_1k_2k_3e_oa_ob_o(1-\lambda_1/k_{-1})}{\lambda_1^2(\lambda_1-\lambda_2)}\,(1-e^{-\lambda_1 t})$ $-\dfrac{k_1k_2k_3e_oa_ob_o(1-\lambda_2/k_{-1})}{\lambda_2^2(\lambda_1-\lambda_2)}\,(1-e^{-\lambda_2 t})$

λ_1 and λ_2 are the negatives of the roots of the quadratic equation

$$p^2 + (k_1a_o + k_{-1} + k_2b_o + k_3)p + k_3(k_1a_o + k_{-1} + k_2b_o) + k_1k_2a_ob_o = 0$$

exponential rise is more rapid; this is because of the equilibrium concentration of EA that is established during the premixing. A study of the pre-steady state thus permits one to distinguish A (the first substrate) from B; premixing with A will enhance the pre-steady-state kinetics, while premixing with B will either leave it unaffected or (if an abortive complex is formed with B) will actually reduce the pre-steady-state rate.

The equations for the situation in which the concentration of A is limiting (e_o, $b_o >> a_o$) are shown in Table 3, for the two sets of premixing conditions. λ_1 and λ_2 are positive, with $\lambda_1 > \lambda_2$. It is to be noted that there is now no steady state, x and y rising to the value a_o. The exponential rise is biphasic for x, triphasic for y. Analysis of experimental results for this situation could be carried out as follows. A plot of ln x against t is biphasic, the slopes of the two lines being $-\lambda_1$ and $-\lambda_2$. From the equation at the foot of Table 3

$$[3] \qquad \lambda_1 + \lambda_2 = k_1 e_o + k_{-1} + k_2 b_o$$

$$[4] \qquad \lambda_1 \lambda_2 = k_1 k_2 e_o b_o$$

so that a plot of $\lambda_1 + \lambda_2$ against e_o gives k_1, and against b_o gives k_2; k_{-1} can then be obtained. Alternatively, $\lambda_1 \lambda_2$ could be plotted against $e_o b_o$. A similar procedure could be applied to y.

It is to be seen that the different premixing procedures give similar behavior (x biphasic, y triphasic, no steady state). However, with a_o limiting, premixing E and A gives a faster exponential rise than E|A|B, E|A + B, etc.

TABLE 3

Rate equations for the Theorell-Chance mechanism, with e_o, $b_o \gg a_o$

Premixing	Formation of X	Formation of Y
E|A|B,	$x = a_o + \frac{k_1k_2e_oa_ob_o}{\lambda_1(\lambda_1-\lambda_2)} e^{-\lambda_1 t}$	$y = a_o - \frac{k_1k_2k_3e_oa_ob_o}{\lambda_1(\lambda_1-\lambda_2)(\lambda_1-k_3)} e^{-\lambda_1 t}$
E|A + B	$- \frac{k_1k_2e_oa_ob_o}{\lambda_2(\lambda_1-\lambda_2)} e^{-\lambda_2 t}$	$+ \frac{k_1k_2k_3e_oa_ob_o}{\lambda_2(\lambda_1-\lambda_2)(\lambda_2-k_3)} e^{-\lambda_2 t}$
or E + B|A		$- \frac{k_1k_2k_3e_oa_ob_o}{k_3(\lambda_1-k_3)(\lambda_2-k_3)} e^{-k_3 t}$
E + A|B	$x = a_o + \frac{k_1k_2e_oa_ob_o(1-\lambda_1/k_{-1})}{\lambda_1(\lambda_1-\lambda_2)} e^{-\lambda_1 t}$	$y = a_o - \frac{k_1k_2k_3e_oa_ob_o(1-\lambda_1/k_{-1})}{\lambda_1(\lambda_1-\lambda_2)(\lambda_1-k_3)} e^{-\lambda_1 t}$
	$- \frac{k_1k_2e_oa_ob_o(1-\lambda_2/k_{-1})}{\lambda_2(\lambda_1-\lambda_2)} e^{-\lambda_2 t}$	$+ \frac{k_1k_2k_3e_oa_ob_o(1-\lambda_2/k_{-1})}{\lambda_2(\lambda_1-\lambda_2)(\lambda_1-k_3)} e^{-\lambda_2 t}$
		$- \frac{k_1k_2k_3e_oa_ob_o(1-k_3/k_{-1})}{k_3(\lambda_1-k_3)(\lambda_2-k_3)} e^{-k_3 t}$

λ_1 and λ_2 are the negatives of the roots of the quadratic equation

$$p^2 + (k_1e_o + k_{-1} + k_2b_o)p + k_1k_2e_ob_o = 0$$

Ping Pong Bi Bi

The mechanism is shown in Fig. 24, which gives the concentrations corresponding to the condition a_o, $b_o >> e_o$ (cf. also Table 1). The resulting expressions for this condition are given in the upper part of Table 4; they were worked out in detail in the previous chapter. The λ's are the negative roots of the equation

$$[5] \qquad P^3 + MP^2 + LP + Q = 0$$

with

$$[6] \qquad M = k_1 a_o + k_{-1} + k_2 + k_3 b_o + k_4$$

$$[7] \qquad L = k_3 k_4 b_o + (k_3 b_o + k_4)(k_1 a_o + k_{-1} + k_2) + k_1 k_2 a_o$$

$$[8] \qquad Q = k_3 k_4 b_o (k_1 a_o + k_{-1} + k_2) + k_1 k_2 k_4 a_o + k_1 k_2 k_3 a_o b_o$$

The premixing of E and A gives rise to the product X; a study of its formation in the absence of B is a good way of arriving at the constants k_1, k_{-1} and k_2. The kinetic equation during this premixing process is

$$[9] \qquad x = e_o + \frac{k_1 k_2 e_o a_o}{\lambda_1 (\lambda_1 - \lambda_2)} e^{-\lambda_1 t} - \frac{k_1 k_2 e_o a_o}{\lambda_2 (\lambda_1 - \lambda_2)} e^{-\lambda_2 t}$$

where $-\lambda_1$ and $-\lambda_2$ are the roots of

$$[10] \qquad P^2 + (k_1 a_o + k_{-1} + k_2) P + k_1 k_2 a_o = 0$$

The constants k_1, k_{-1} and k_2 can readily be obtained, by analogous methods to those described above and in the previous chapters.

At the end of the E + A premixing, E has all been converted into EA'; the initial concentration of this is therefore e_o. The kinetic equations for the rates of formation of X and Y for this case are given

Rate Constants for the Ping Pong Bi Bi mechanism, with a_o, b_o $\ll$ e_o

Premixing	Formation of X	Formation of Y
E|A|B,	$x = \frac{k_1k_2k_3k_4e_oa_ob_o}{k_1k_2k_4a_o+k_3k_4(k_{-1}+k_2)b_o+k_1k_3(k_2+k_4)a_ob_o}\,t$	$y = \frac{k_1k_2k_3k_4e_oa_ob_o}{k_1k_2k_4a_o+k_3k_4(k_{-1}+k_3)b_o+k_1k_3(k_2+k_4)a_ob_o}\,t$
E|A + B or E + B|A	$-\sum_{i=1}^{3}\frac{k_1k_2e_oa_o(\lambda_1-k_3b_o)(\lambda_1-k_4)}{\lambda_1^2(P-\lambda_1)}(1-e^{-\lambda_1 t})$	$-\sum_{i=1}^{3}\frac{k_1k_2k_3k_4e_oa_ob_o}{\lambda_1^2(P-\lambda_1)}(1-e^{-\lambda_1 t})$
E + A|B	$x = x_o + \frac{k_1k_2k_3k_4e_oa_ob_o}{k_3k_4b_o+k_1k_2k_4a_o+k_1k_2k_3a_ob_o}\,t$ $-\sum_{i=1}^{3}\frac{k_1k_2k_3k_4e_oa_ob_o}{\lambda_1^2(P-\lambda_1)}(1-e^{-\lambda_1 t})$	$y = \frac{k_1k_2k_3k_4e_oa_ob_o}{k_3k_4b_o+k_1k_2k_4a_o+k_1k_2k_3a_ob_o}\,t$ $-\sum_{i=1}^{3}\frac{k_3k_4e_ob_o[\lambda_1^2-\lambda_1(k_1a_o+k_{-1}+k_2)+k_1k_2a_o]}{\lambda_1^2(P-\lambda_1)}(1-e^{-\lambda}$

in the lower part of Table 4; the λ's are the negatives of the roots of the equation

$$P^3 + MP^2 + NP + C = 0 \quad [11]$$

with

$$M = k_1 a_o + k_{-1} + k_2 + k_3 b_o + k_4 \quad [12]$$

$$N = k_1 k_2 a_o + (k_3 b_o + k_4)(k_1 a_o + k_{-1} + k_2) \quad [13]$$

$$C = k_3 k_4 b_o + k_1 k_2 k_3 a_o b_o + k_1 k_2 k_4 a_o \quad [14]$$

The determination of rate constants can be carried out as previously. It will be noted that for this case of E + A| B the steady-state rates are the same for X as for Y but that they are different from those for E| A| B.

The equations for the case in which the concentration of A is limiting (e_o, $b_o >> a_o$) are given in Table 5. The λ_1 and λ_2 values for the first premixing case are the negatives of the roots of

$$P^2 + P(k_1 e_o + k_{-1} + k_2) + k_1 k_2 e_o = 0 \quad [15]$$

and $\lambda_3 = k_3 b_o$. In the case of E + A premixing, A is all converted into EA. during the premixing, and there is no further production of X after B is added; the equation for the formation of Y is given in Table 5.

Again we may wish to measure X formation during the premixing of E with A, in the absence of B. The equation for this, with $e_o \gg a_o$, is

$$x = a_o + \frac{k_1 k_2 e_o a_o}{\lambda_1 (\lambda_1 - \lambda_2)} - \frac{k_1 k_2 e_o a_o}{\lambda_2 (\lambda_1 - \lambda_2)} e^{-\lambda_2 t} \quad [16]$$

where λ_1 and λ_2 are the negatives of the roots of the equation

TABLE 5

Rate Constants for the Ping Pong Bi Bi mechanism, with e_o, $b_o >> a_o$

Premixing	Formation of X	Formation of Y
E|A|B, E|A + B or E + B|A	$x = a_o + \frac{k_1 k_2 e_o a_o}{\lambda_1(\lambda_1-\lambda_2)} e^{-\lambda_1 t} - \frac{k_1 k_2 e_o a_o}{\lambda_2(\lambda_1-\lambda_2)} e^{-\lambda_2 t}$	$y = a_o + \frac{k_1 k_2 k_3 k_4 e_o a_o b_o}{\lambda_1(\lambda_1-\lambda_2)(\lambda_1-\lambda_3)(\lambda_1-k_4)} e^{-\lambda_1 t} - \frac{k_1 k_2 k_3 k_4 e_o a_o b_o}{\lambda_2(\lambda_1-\lambda_2)(\lambda_2-\lambda_3)(\lambda_2-k_4)} e^{-\lambda_2 t} + \frac{k_1 k_2 k_3 k_4 e_o a_o b_o}{\lambda_3(\lambda_1-\lambda_3)(\lambda_2-\lambda_3)(\lambda_3-k_4)} e^{-\lambda_3 t} - \frac{k_1 k_2 k_3 k_4 e_o a_o b_o}{k_4(\lambda_1-k_4)(\lambda_2-k_4)(\lambda_3-k_4)} e^{-k_4 t}$
E + A|B	NO FURTHER FORMATION OF X	$y = a_o - \frac{k_4 a_o}{k_4-k_3 b_o} e^{-k_3 b_o t} + \frac{k_3 b_o a_o}{k_4-k_3 b_o} e^{-k_4 t}$

[17] $$P^2 + (k_1 e_o + k_{-1} + k_2) P + k_1 k_2 e_o = 0$$

The results for this case are easily analyzed to give k_1, k_{-1} and k_2.

This Ping Pong Bi Bi mechanism is easily distinguished from the others on the basis of transient-phase studies, since it is the only one in which product X is produced in the absence of one reactant B. The reactants A and B are readily distinguished in this way, and rate constants are easily determined.

Ordered Ternary-Complex Mechanism

The various solutions for the ordered ternary-complex mechanism (Fig. 25) are given in Tables 6 and 7. Analysis of results can be carried out by methods similar to those considered previously. Again, premixing of E with A gives more rapid pre-steady state kinetics; premixing of E with B has no effect.

Random Ternary-Complex Mechanism

Even the steady-state equation [48, 49] for the random ternary-complex mechanism (Fig. 26) is extremely complicated, too much so to be applied to experimental results. The pre-steady-state equations are even more complicated; they can be worked out by the Laplace-Carson transform method, but would be useless for interpreting experimental results.

On the qualitative side it can be noted that for this mechanism premixing of E with either A or B will lead to no change in steady-state kinetics, but to an acceleration of the pre-steady-state kinetics relative to E| A| B, E|A + B. This allows a clear distinction of this mechanism

Rate Constants for the Ordered Ternary Complex mechanism, with $a_o, b_o >> e_o$

Premixing	Formation of X	Formation of Y
E|A|B, E|A + B or E + B|A	$x = \frac{k_1k_2k_3k_4e_oa_ob_o}{k_{-2}k_4(k_1a_o+k_{-1})+k_3k_4(k_1a_o+k_{-1}+k_2b_o)+k_1k_2(k_3+k_4)a_ob_o}\,t - \sum_{i=1}^{3}\frac{k_1k_2k_3k_4e_oa_ob_o(k_4-\lambda_i)}{\lambda_i^2(P-\lambda_i)}(1-e^{-\lambda_i t})$	$y = \frac{k_1k_2k_3k_4e_oa_ob_o}{k_{-2}k_4(k_1a_o+k_{-1})+k_3k_4(k_1a_o+k_{-1}+k_2b_o)+k_1k_2(k_3+k_4)a_ob_o} - \sum_{i=1}^{3}\frac{k_1k_2k_3k_4e_oa_ob_o}{\lambda_i^2(P-\lambda_i)}(1-e^{-\lambda_i t})$
E + A|B	$x = \frac{k_1k_2k_3k_4e_oa_ob_o}{k_{-2}k_4(k_1a_o+k_{-1})+k_3k_4(k_1a_o+k_{-1}+k_2b_o)+k_1k_2(k_3+k_4)a_ob_o}\,t - \sum_{i=1}^{3}\frac{k_1k_2k_3e_oa_ob_o(1-\lambda_i/k_{-1})(k_4-\lambda_i)}{\lambda_i^2(P-\lambda_i)}(1-e^{-\lambda_i t})$	$y = \frac{k_1k_2k_3k_4e_oa_ob_o}{k_{-2}k_4(k_1a_o+k_{-1})+k_3k_4(k_1a_o+k_{-1}+k_2b_o)+k_1k_2(k_3+k_4)a_ob_o}\,t - \sum_{i=1}^{3}\frac{k_1k_2k_3k_4e_oa_ob_o(1-\lambda_i/k_{-1})}{\lambda_i^2(P-\lambda_i)}(1-e^{-\lambda_i t})$

The λ's are the negatives of the roots of the polynominal

$$p^3 + Mp^2 + Lp + Q = 0$$

with

$$M = k_1a_o + k_4 + k_{-1} + k_{-2} + k_3 + k_2b_o$$

$$L = k_4(k_1a_o + k_{-1} + k_2b_o + k_{-2} + k_3) + k_{-2}(k_1a_o + k_{-1}) + k_3(k_1a_o + k_{-1} + k_2b_o) + k_1k_2a_ob_o$$

$$Q = k_{-2}k_4(k_1a_o + k_{-1}) + k_3k_4(k_1a_o + k_{-1} + k_2b_o) + k_1k_2k_4a_ob_o + k_1k_2k_3a_ob_o$$

Rate Constants for the Ordered Ternary Complex Mechanism, with e_o, $b_o >> a_o$

Premixing	Formation of X	Formation of Y
E⁄A⁄B, E⁄A + B or E + B⁄A	$x = a_o - \sum_{i=1}^{3} \frac{k_1 k_2 k_3 e_o a_o b_o}{\lambda_i (P-\lambda_i)} e^{-\lambda_i t}$	$y = a_o - \sum_{i=1}^{4} \frac{k_1 k_2 k_3 k_4 e_o a_o b_o}{\lambda_i (P-\lambda_i)} e^{-\lambda_i t}$
E + A⁄B	$x = a_o - \sum_{i=1}^{3} \frac{k_1 k_2 k_3 e_o a_o b_o (1-\lambda_i/k_{-1})}{\lambda_i (P-\lambda_i)} e^{-\lambda_i t}$	$y = a_o - \sum_{i=1}^{4} \frac{k_1 k_2 k_3 k_4 e_o a_o b_o (1-\lambda_i/k_{-1})}{\lambda_i (P-\lambda_i)} e^{-\lambda_i t}$

The λ's are the negatives of the roots of the polynominal

$$P^3 + MP^2 + LP + Q = 0$$

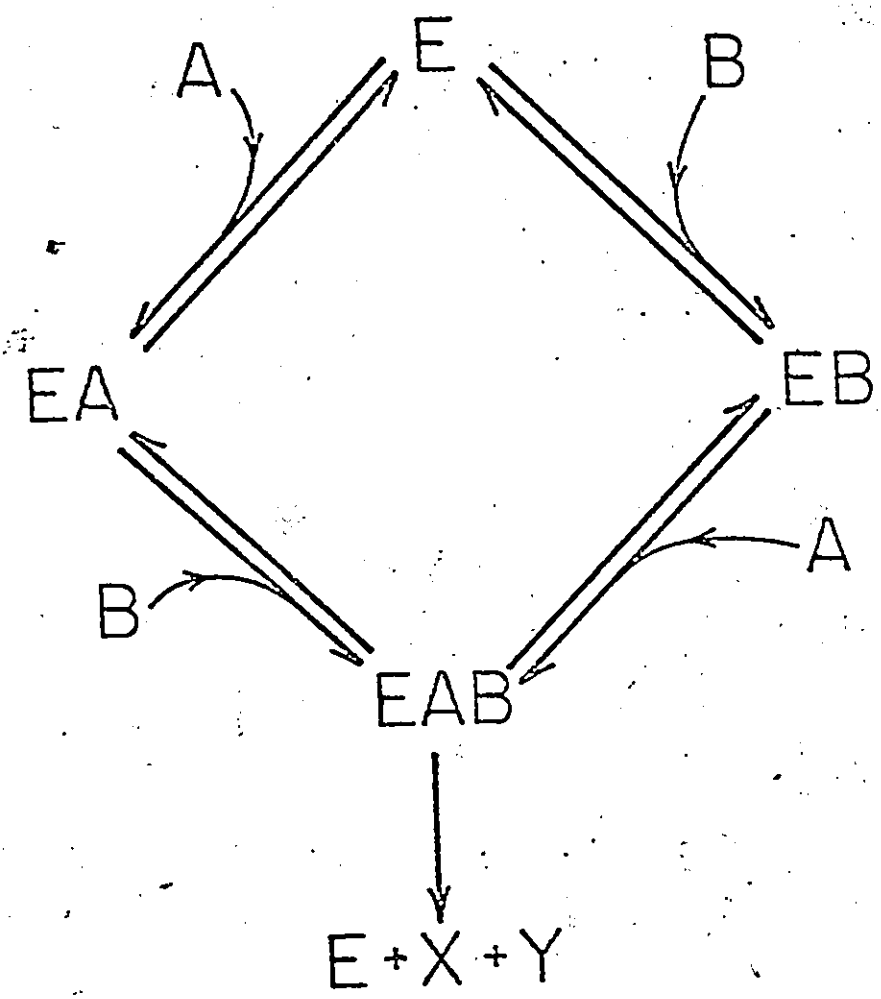
with

$$M = k_1 e_o + k_{-1} + k_2 b_o + k_{-2} + k_3$$

$$L = (k_{-2} + k_3)(k_1 e_o + k_{-2}) + k_2 k_3 b_o + k_1 k_2 e_o b_o$$

$$Q = k_1 k_2 k_3 b_o e_o$$

Fig. 26 The Random Ternary-Complex Mechanism.


E
A
B
EA
EB
B
A
EAB
E + X + Y

from the other three, for which E + B premixing cannot increase rates (it may decrease them, if an abortive-EB complex is formed).

Discussion

The main conclusions of the present work are summarized in Table 8. They can be further summerized as follows:

(1). When the concentration of E is limiting, there is always a steady state preceded by a phase consisting of the sum of exponentials.

(2). When the concentration of the first substrate A is limiting there is no steady-state; the concentrations of X and Y rise to a limiting value equal to the initial concentration of A, the rise being represented by the sum of exponentials. (The case of B being limited was also investigated, but no analytical solution appears to be possible: presumably, there would again be no steady state, the concentrations of X rising to the initial concentration of B).

(3) Premixing of E and B affeets the kinetics only in the case of the random ternary-complex mechanism; it leads to more rapid initial reaction as compared with E|A|B, etc.

(4) Premixing E and A followed by addition of B leads to more rapid initial reaction, but does not affect the number of phases, except for the Ping Pong Bi Bi mechanism.

(5) Premixing does not affect the steady-state kinetics except for the Ping Pong Bi Bi mechanism; this is a special case, because the starting material is now altered.

SUMMARY OF KINETIC EQUATIONS

Initial Mixing Conditions	Formation of X	Formation of Y	Remarks
E|A|B; E|A + B; E + B|A; a_o, b_o >> e_o	Biphasic	Biphasic	Steady state established
E + A|B; a_o, b_o >> e_o	Biphasic	Biphasic	Faster rise to steady state
E|A|B; E|A + B; E + B|A; e_o, b_o >> a_o	Biphasic	Triphasic	No steady state
E + A|B; b_o e_o >> a_o	Biphasic	Triphasic	No steady state; faster rise
Ping Pong Bi Bi			
E|A|B; E|A + B; E + B|A a_o, b_o >> e_o	Triphasic	Triphasic	Steady state
E + A|B; a_o, b_o >> e_o	Triphasic	Triphasic	Faster rise to steady state
E|A|B; E|A + B; E + B|A e_o, b_o >> a_o	Biphasic	Quadriphasic	No steady state
E + A|B; e_o, b_o >> a_o	No further formation of X	Biphasic	No steady state; faster production of Y
Ordered Ternary Complex			
E|A|B; E|A + B; E + B|A a_o, b_o >> e_o	Triphasic	Triphasic	Steady state
E + A|B; a_o, b_o >> e_o	Triphasic	Triphasic	Faster rise to steady state
E|A|B; E|A + B; A + B|A; e_o, b_o >> a_o	Triphasic	Quadriphasic	No steady state
E + A|B; e_o, b_o >> a_o	Triphasic	Quadriphasic	No steady state, faster rise

Results for Horse Liver Alcohol Dehydrogenase

Recently some transient-phase studies [50-54] have been carried out with horse liver alcohol dehydrogenase (LADH), and, in particular, Luisi and Favilla [53] have studied the effect of premixing the enzyme and co-enzyme. The results have been interpreted as implying that there are two catalytic sites on the enzyme which differ in their activity. However, the theoretical equations derived in the present chapter show that no such conclusion is necessary; the experimental results are consistent with a Theorell-Chance mechanism occurring at a single catalytic site.

The main experimental results of the studies, with premixing of E and A (co-enzyme), can be summarized as follows, with reference to the theoretical treatment of the present paper:

(1) With $e_o \gg a_o$, or b_o there is a biphasic exponential formation of product X, completion of the reaction corresponding to depletion of the limiting reagent. This is consistent with the equation given in Table 3 for the Theorell-Chance mechanism. Two catalytic sites are not required to explain the biphasic character.

(2) During the biphasic formation of product X there is an initial exponential formation of approximately half of the product, followed by a slower exponential formation of the other half. This is explained by the equation in Table 3 if λ_1 and λ_2 are fairly close to one another. The exponential terms (amplitude factors) are then approximately equal, leading to the result that $a_o/2$ will be produced initially followed by the remaining $a_o/2$.

(3) A Similar result was found with a_o, $b_o \gg e_o$ and E + A | B; in this case a steady-state is attained. There was an initial rapid product of X, followed by slower exponential processes; this is predicted by the equations in Table 2.

(4) The amplitudes of the pre-exponential terms under the above conditions were proportional to e_o, a_o and b_o. This is predicted by the equations in Table 2.

We conclude that the results are fully explained by the equations in Tables 2 and 3 of the present chapter, and that the transient-phase studies provide no evidence for two non-equivalent catalytic sites.

CHAPTER NINE

GENERAL DISCUSSION OF THE TRANSIENT-PHASE KINETIC APPROACH

Introduction

In the theoretical treatment presented throughout this thesis, and under the general condition that the enzyme concentration is the limiting reagent, the variation of a product x with time has the general form:

$$x = vt + \beta + \sum_{i=1}^{n} \beta_i e^{-\lambda_i t}$$

where v is the initial steady-state rate of the reaction and $\beta = -\sum_{i=1}^{n} \beta_i$ is the intercept of the extrapolated steady-state line on the product axis of an x vs. t plot. In general, plots of x vs. t will consist of a linear steady-state portion which is approached asymptotically by an exponential rise. The number of exponentials might not be revealed by a simple visual inspection. Two general methods can be used to detect the number of exponentials: (1) Numerical fitting by computer analysis, which will not be discussed further. (2) Plots of ln $(x - vt - \beta)$ against t, which will consist of a number of linear regions. The number of these linear regions, which determines the phasicity, gives the number of experimentally detectable exponential functions. The numerical values of the slopes of these linear regions correspond to the values of the λ_i's. Hence all the effective λ_i's can be calculated. The number and numerical values of λ_i's are very important in determining the number of experimentally detectable intermediates and in determining the numerical values of the rate constants. These two aspects will be discussed in some detail.

Number of Intermediates

In the theoretical solutions presented above for the condition of enzyme concentration as limiting reagent, a generalization can be

made: the number of kinetically important intermediates, (that is, the number of forms in which the enzyme occurs minus one, the free enzyme not being counted) is the same as the number of exponential terms. This rule has evolved from a simple inspection of the mechanisms and the number of exponential terms obtained in the theoretical solution. The intermediates can be simple binary or ternary complexes as well as chemically-attached intermediates like an acyl enzyme or phosphoryl enzyme, etc. Table 9 lists all the mechanisms treated in this thesis under the conditions of limiting enzyme concentration. It shows that the above rule applies in general for the treated mechanisms. The rule, therefore can be used to predict the number of expected exponential terms in any proposed mechanism. On the other hand, if the number of exponential terms have been observed experimentally, it is necessary to propose a mechanism which has a minimum number of intermediates as the number of experimentally observed exponentials. It is apparent that the proposed mechanism is phenomenological and is only true under the specified experimental conditions. That is, if any other intermediates are present with shorter life times than the experimental detection time, they will not be detected. As mentioned above the proposed mechanism is only apparent and its validity should be treated by comparing it with the theoretical solution of that mechanism. It is necessary however to have more quantitative tests for the validity of this mechanism. A general and quantitative test which can be applied will be discussed in the following section and some other tests applicable to specific mechanisms will also be discussed; other tests can be derived on the basis of the theoretical solutions.

Before discussing the available tests for mechanisms, we will show that the prediction technique can be applied satisfactorily to other mechanisms than the ones listed in Table 9.

Table 9

Summary of Transient-Phase Equations

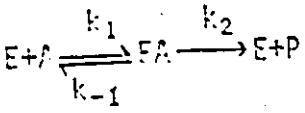
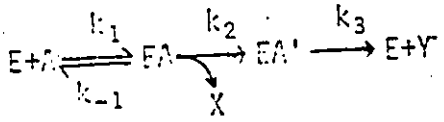
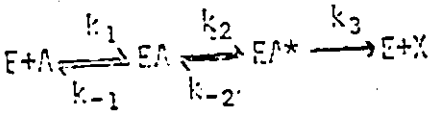
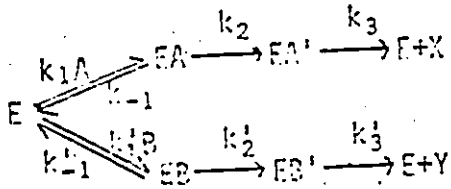
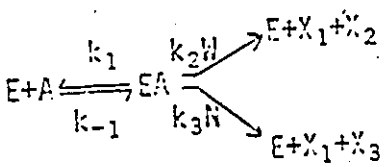
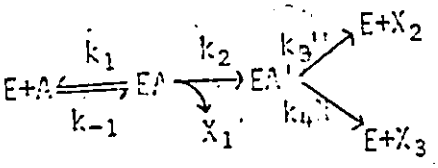
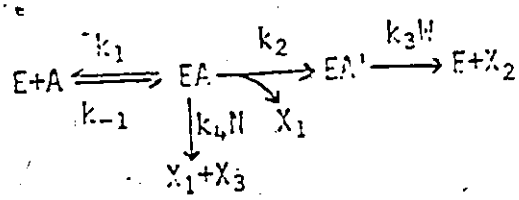
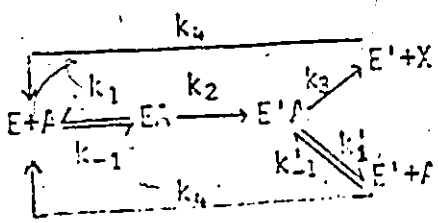
Type of Mechanism	Mechanism	No. of Exponentials, n	$\sum_{i=1}^{n} \lambda_i$
Simple Michaelis-Menten		1	$k_1 a_0 + k_{-1} + k_2$
Michaelis-Menten with second intermediate		2	$k_1 a_0 + k_{-1} + k_2 + k_3$
Reversible conformation change in EA		2	$k_1 a_0 + k_{-1} + k_2 + k_{-2} + k_3$
Two competing substrates		4	$k_1 a_0 + k_1' b_0 + k_{-1} + k_{-1}' + k_2 + k_2' + k_3 + k_3'$
Simple Michaelis-Menten with competing nucleophile N		1	$k_1 a_0 + k_{-1} + k_2'' N + k_3'' N$
Second intermediate with competing nucleophile		2	$k_1 a_0 + k_3'' N + k_4'' N + k_{-1} + k_2$
Second intermediate with competing nucleophile		2	$k_1 a_0 + k_3 N + k_4 N + k_{-1} + k_2$
A mechanism for sigmoid kinetics		3	$(k_1 + k_{-1}') a_0 + k_{-1} + k_2 + k_3 + k_1' + k_4$

Table 9 (cont.)

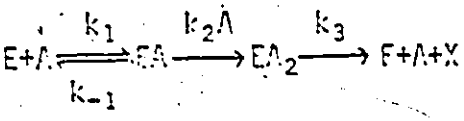
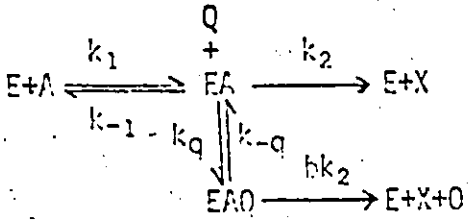
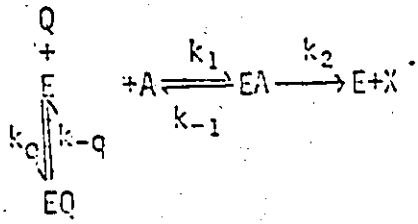
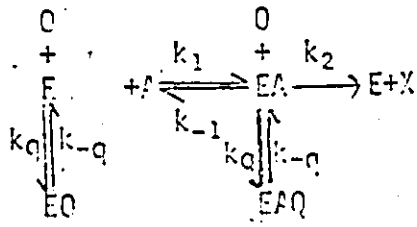
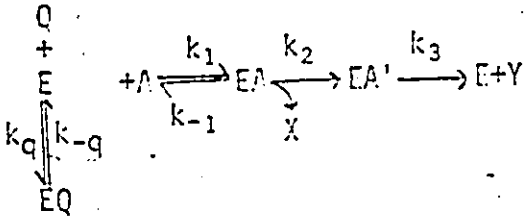
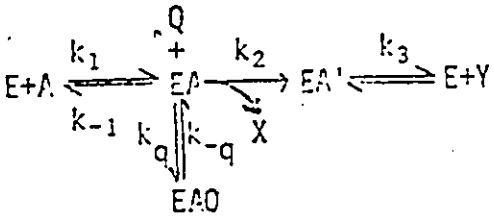
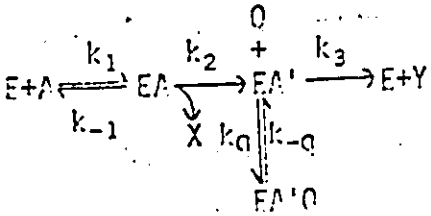
A mechanism for substrate activation		2	$(k_{-1}+k_2)a_0+k_{-1}+k_3$
Classical anticompetitive inhibition		2	$k_1a_0+k_qq_0+k_{-1}+k_2+k_{-q}+bk_2$
Classical competitive inhibition		2	$k_1a_0+k_{-1}+k_2+k_qq_0+k_{-q}$
Classical non-competitive inhibition		3	$k_1a_0+2k_qq_0+2k_{-q}+k_{-1}+k_2$
Competitive inhibition; two intermediates		3	$k_1a_0+k_qq_0+k_{-q}+k_{-1}+k_2+k_3$
Inhibition; two intermediates		3	$k_1a_0+k_qq_0+k_{-q}+k_{-1}+k_2+k_3$
Inhibition; two intermediates		3	$k_1a_0+k_qq_0+k_{-q}+k_{-1}+k_2+k_3$

Table 9 (cont.)

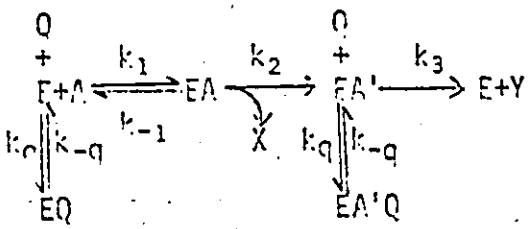
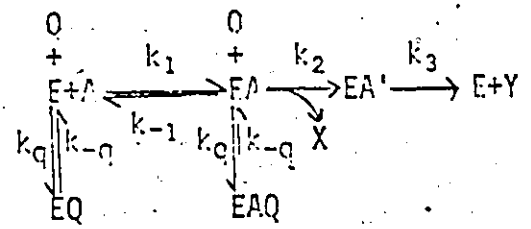
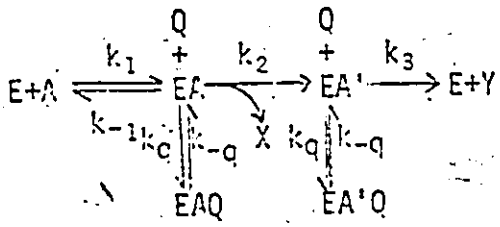
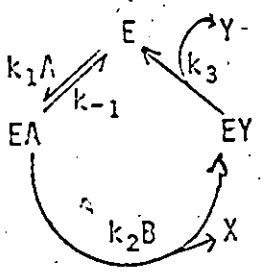
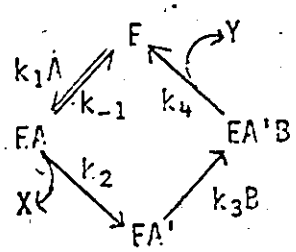
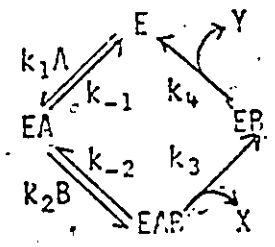
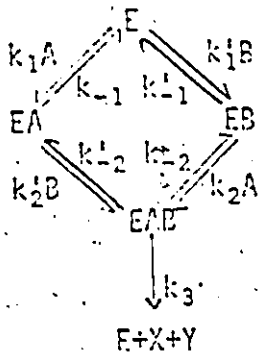
Inhibition; two intermediates		4	$k_1a_0+2k_qq_0+k_{-1}+k_2+k_3+2k_{-q}$
Inhibition; two intermediates		4	$k_1a_0+2k_qq_0+k_{-1}+k_2+k_3+2k_{-q}$
Inhibition; two intermediates		4	$k_1a_0+2k_qq_0+k_{-1}+k_2+k_3+2k_{-q}$
Theorell-Chance		2	$k_1a_0+k_{-1}+k_2b_0+k_3$
Ping Pong bi bi		3	$k_1a_0+k_3b_0+k_{-1}+k_2+k_4$
Ordered ternary complex		3	$k_1a_0+k_2b_0+k_{-1}+k_{-2}+k_3+k_4$

Table 9 (cont.)

Random ternary complex		4 $(k_1+k_2)a_o+(k'_1+k'_2)b_o+k_{-1}+k'_{-1}+k_{-2}+k'_{-2}+k_3$

a_o = initial concentration of substrate A

b_o = initial concentration of substrate B

W = concentration of water

N = concentration of alternative nucleophile

q_o = initial concentration of modifier Q

In a simple Michaelis-Menten scheme, if the enzyme-substrate complex undergoes a rearrangement (chemical shift or conformational change) before releasing the products, the reaction mechanism can be represented as

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EA^* \xrightarrow{k_3} E + X$$

Two intermediates, EA and EA^*, are present in this mechanism. According to our method of prediction, if the product X is being measured as a function of time under the conditions of $a_o >> e_o$, then the steady state will be approached asymptotically by a biphasic (two-exponential) rise. A solution of this mechanism has been given by Ouellet and Laidler [12] and we have resolved the kinetics by use of the Laplace-Carson technique. The result is

$$x = vt + \beta + \sum_{i=1}^{2} \beta_i e^{-\lambda_i t}$$

where

$$v = \frac{k_1 k_2 k_3 a_o e_o}{k_1 a_o (k_2 + k_{-2} + k_3) + k_{-1}(k_{-2} + k_3) + k_2 k_3}$$

$$\beta = -\sum_{i=1}^{2} \beta_i$$

$$\beta_i = \frac{k_1 k_2 k_3 a_o e_o}{\lambda_i^2 (P - \lambda_i)}$$

P is the differential operator and λ_i's are the negative roots of the quadratic

$$P^2 + P(k_1 a_o + k_{-1} + k_2 + k_{-2} + k_3) + k_1 a_o (k_2 + k_{-2} + k_3) + k_{-1}(k_{-2} + k_3) + k_2 k_3 = 0$$

Quantitative Test

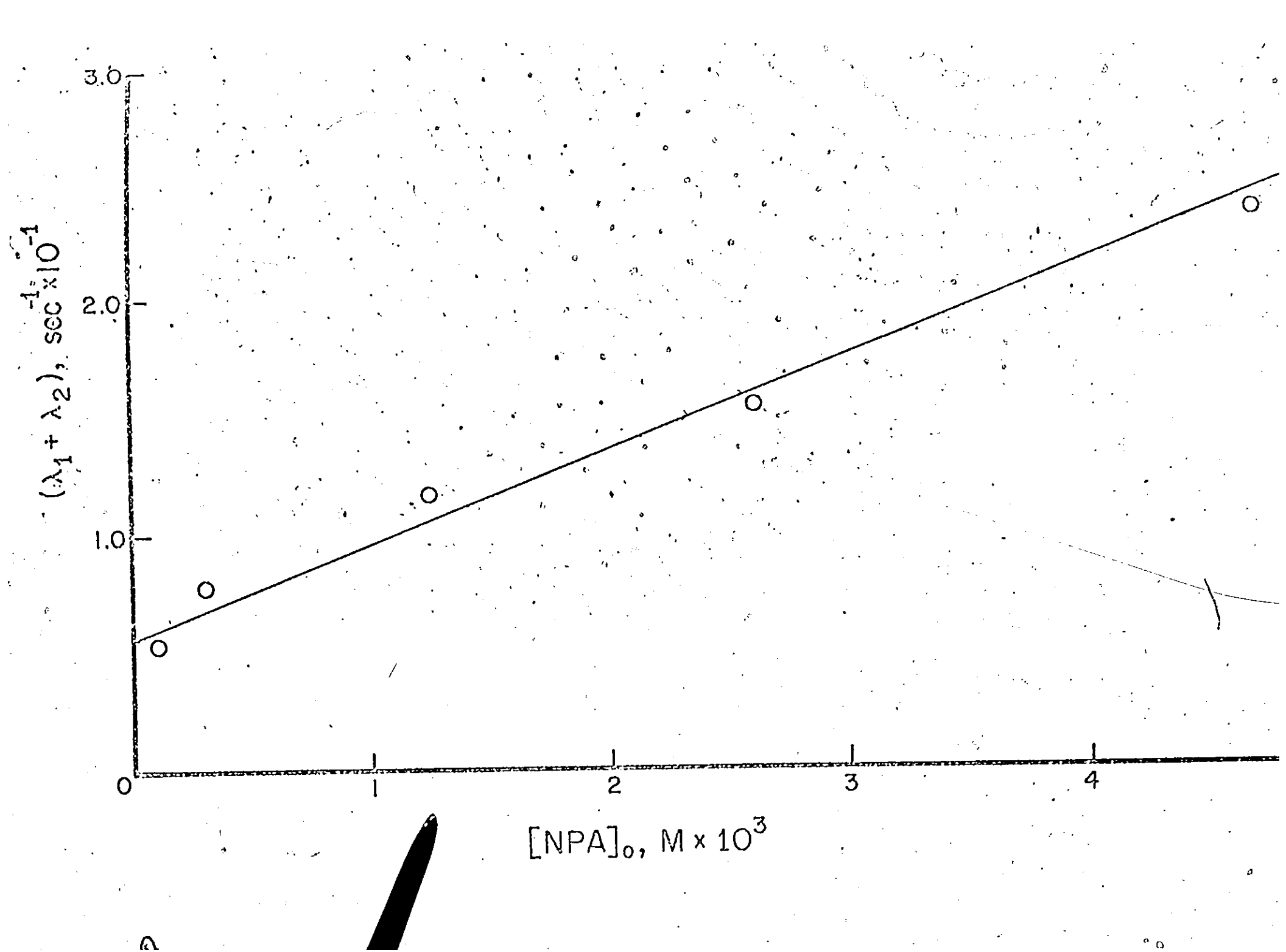
Table 9 shows that the theoretical values of λ_i's obey the relation where $\sum_{i=1}^{n} \lambda_i$ is equal to the sum of all the kinetic steps in any reaction mechanism, both the bimolecular and unimolecular steps. It can in general be written as

$$\sum_{i=1}^{n} \lambda_i = \sum k_i C_i + \sum k_j$$

where $k_i C_i$ represents the bimolecular steps and the summation is over all the bimolecular steps in the reaction sequence; k_j represents a unimolecular step and the summation is over all unimolecular steps. C_i is the initial concentration of a reagent, other than free enzyme, that is involved in a bimolecular step in the sequence.

The procedure to be followed is to vary the initial concentration of one reagent, a substrate, modifier, etc., and keep the concentrations of other reagents constant. This step is followed by an experimental determination of λ_i's from plots of $\ln (x - vt - \beta)_n$ against t. The determined values of λ_i's can then be used to plot $\sum_{i=1}^{n} \lambda_i$ against the initial concentration of the varied reagent. According to the theoretical result this plot must be linear. If the experiments bear out this result then a bimolecular step involving this reagent is kinetically effective and experimentally detectable. This procedure can be repeated for other reagents and hence bimolecular steps will be either confirmed or excluded. The only experimental application for this type of test has been done by Maguire, Hijazi and Laidler [27]. A plot of $\sum_{i-1}^{2} \lambda_i$ against a_o is shown in Fig. 27 for the α-chymotrypsin-catalyzed hydrolysis of p-nitrophenylacetate. After the confirmation or exclusion of all the bimolecular steps, the unimolecular steps can be fixed depending on the number of exponentials that are experimentally observable. Hence

Fig. 27 A plot of $\lambda_1 + \lambda_2$ against as for the α-chymotrypsin catalyzed hydrolysis of p-nitrophenylacetate (Maguire et al.[27]).


3.0
2.0
1.0
0
$(\lambda_1 + \lambda_2)$, $sec^{-1} \times 10^{-1}$
1
2
3
4
$[NPA]_o$, $M \times 10^3$

the reaction mechanism can be constructed from experiments in a piece-wise fashion. It is obvious that this test does not by itself lead to a unique mechanism, but it does establish whether a bimolecular step is being resolved experimentally or not. To establish a unique mechanism, if ever possible, one resorts to various other tests that can be specifically devised for the possible mechanisms. An example of such tests is the effect of pre-mixing on two substrate reactions. Some other tests which can be used are (a) the plots of $\prod_{i=1}^{n} \lambda_i$ against a varied reagent concentration (b) the dependence, in some cases, of the induction period on the varied reagent concentration (c) plots of v / $\prod_{i=1}^{n} \lambda_i$ against the varied substrate concentration. It is to be noted that various other tests can be specifically designed for a mechanism such as an inhibition mechanism. In the following sections, some of the published results on transient-phase kinetics will be discussed. The choice of myosin, alkaline phosphatase and α-chymotrypsin has been dictated by the availability of the experimental data.

Myosin

The hydrolysis of nucleoside triphosphates by myosin and actomyosin has been the subject of a large number of studies in both the steady state and the pre-steady state. In this brief section we will discuss the basic features of the pre-steady state studies. Tonomura et al.[55-62] have studied the pre-steady state by monitoring the phosphate production and the proton production. Taylor et al.[63-65] repeated some of the studies using a chemical-quench flow apparatus. The observed features are:

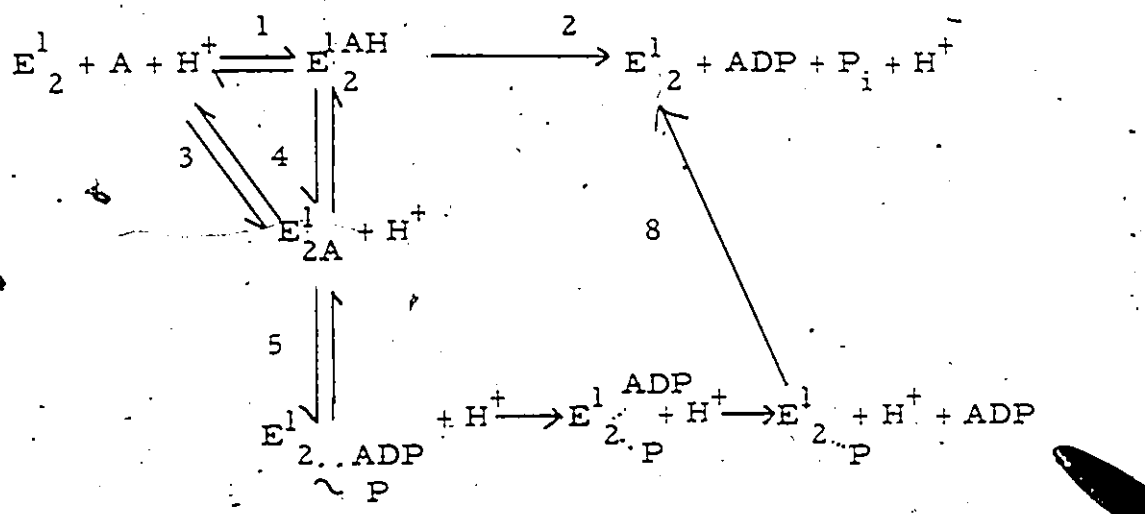
(1) Myosin at pH 8.0 and 20° yielded early bursts of phosphate liberation for the substrates MgATP, MgITP and CaATP[63]. The early bursts are single exponential approaches to the steady-state line. Extrapolation of the steady-state line leads to negative values for the

induction period, τ, on the time axis. Tonomura and his collaborators obtained similar results with phosphate liberation. The latter failed to distinguish between the observed rate constants, λ, and the specific rate constants for bimolecular and unimolecular steps in the reaction scheme, which can be measured only over a range of substrate concentrations. Since phosphate measurements are constructed point by point [63] it would be difficult to observe a second exponential, if it existed. In some cases [63] a second exponential has been observed but was discarded as due to experimental error. However, as will be shown later, the presence of the "burst" instead of a "lag" proves the existence of a second exponential.

(2) Finlayson and Taylor [64] observed a two-exponential rise to the steady state by monitoring the proton liberation in a stopped-flow apparatus in the presence of an indicator. This was also observed by Tokiwa and Tonomura [61]; later Tonomura et al. [58, 60] discarded the first exponential (absorption step) as being too fast to measure but retained it on the basis of a stoichiometric argument, since one mole of ATP produces one mole of H^+. However, the liberation step was confirmed [58, 60]; there is an exponential rise in all these observations and an extension of the linear steady-state line to the time axis gives a negative induction period which will be shown as a qualitative proof of the existence of a second exponential as mentioned above.

(3) In the presence of EDTA with ATP as substrate, it was observed that there is no burst in the production of both phosphates and protons [55, 56, 58-65]. The product formation (phosphates or protons) followed a distinct "lag" (single exponential) before the steady state. The extension of the steady state line gives a positive induction period, τ, which is consistent with a single-intermediate Michaelis-Menten scheme.

Both groups proposed similar mechanisms to explain the experimental results. Tonomura proposed that since the enzyme has two active sites (denoted by 1 and 2) then the mechanism in the presence of divalent metals is



where E^1_2 is myosin with its two active sites. A is the substrate, ATP. E^{1A}_2 and E^1_{2A} are enzyme-substrate complexes with the substrate bound to the first and second site respectively. $E^1_{2\cdots ADP}^{\sim P}$ is a complex where there has been a chemical shift of the phosphate from the substrate to the enzyme. This complex can exchange phosphate with terminal phosphate of ATP. $E^1_{2\cdots ADP}^{\sim P}$ and $E^1_{2\cdots P}$ cannot exchange their phosphates. This scheme involves the clear assumption that there are two kinetically and chemically different sites on the enzyme. While site 1 catalyzes the reaction without a phosphoryl complex or phosphoryl intermediate, the second site does (site 2). Step 3 is sometimes included and sometimes not; its significance is not clear from Tonomura's discussions. There are five intermediates and one of them is being formed by two routes. By our simple correlation rule we expect six exponentials. Experimentally only a maximum of two exponentials have been observed or can be inferred. Tonomura et al.[55-62] proposed that in the presence

of EDTA, the latter blocks step 4 in the reaction mechanism and thus a simple Michaelis-Menten scheme is observed. However, step 3 should also be blocked; otherwise the observed experimental results cannot be explained. This is a very plausible explanation since it is not clear why EDTA should block the hydrolysis by site 2 and not by site 1. After the discussion of Taylor's mechanisms, we will propose an explanation for the observed phenomenon for both cases of hydrolysis in the presence of EDTA as well as in its absence.

Taylor et al.[63-65] essentially proposed similar but less involved mechanisms than Tonomura's. In the presence of Me^{++}, Taylor proposed the following mechanism

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA \underset{k_2}{\overset{k_2}{\rightleftharpoons}} EA^* \xrightarrow{k_3} E + \text{Products}$$

A solution for this mechanism gives the following result for the variation of any product x (H^+, P_i or ADP) with time

$$x = \frac{k_1 k_2 k_3 a_o e_o}{\lambda_1 \lambda_2} + \sum_{i=1}^{2} \frac{k_1 k_2 k_3 a_o e_o}{\lambda_i^2 (P - \lambda_i)} (e^{-\lambda_i t} - 1)$$

where the λ_i's are the negative roots of the quadratic equation with

$$(\lambda_1 + \lambda_2) = k_1 a_o + k_{-1} + k_2 + k_{-2} + k_3$$

$$\lambda_1 \lambda_2 = k_1 a_o (k_2 + k_{-1} + k_3) + k_{-1} (k_{-2} + k_3) + k_2 k_3$$

This mechanism can explain the observed biphasic nature of the P_i and H^+ liberation. However a simple algebraic manipulation leads to a value for the induction period

$$\tau = \beta/v = \frac{\lambda_1 + \lambda_2}{\lambda_1 \lambda_2}$$

which is positive under all conditions. This is contrary to the experimental evidence where the presence of Me^{++} the induction period is always negative. This simple test rules out the applicability of this mechanism.

Lymn and Taylor [63] proposed that in the presence of EDTA, the reaction mechanism is a simple Michaelis-Menten scheme (single-intermediate). This is true. The authors did not commit themselves to explain this change in mechanism due to EDTA.

A simple explanation is proposed here to explain the experimental behavior. Since EDTA is known to chelate the divalent metals Mg^{++} and Ca^{++}, then the change in the mechanism due to the presence of EDTA should be correlated to its ability to remove from solution the free metal ions through chelation. Hence the metal ions should be directly involved in the reaction in the absence of EDTA. A theoretical analysis of the scheme

$$\begin{array}{ccccc} & & Q & & \\ & & + & & \\ E + A & \rightleftharpoons & EA & \longrightarrow & E + \text{Products} \\ & & \Downarrow\Uparrow & & \\ & & EAQ & \longrightarrow & E + \text{Products} \end{array}$$

where Q can be the metal ion or the substrate molecule proved to be incompatible with experimental results.

Formally the inhibition by metal ions according to the following scheme:

$$\begin{array}{ccccccc} & & & & Q & & \\ & & & & + & & \\ E + A & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & EA & & \xrightarrow{\quad k_2 \quad} & & E + \text{Products} \\ & & & k_q \Downarrow & \Uparrow k_{-q} & & \\ & & & EAQ & & & \end{array}$$

can explain qualitatively the observed behavior. The variation of products with time is found to be biphasic; the induction period, τ is found to be

$$\tau = \frac{-k_1 k_q q_o a_o + k_{-q}(k_{-q} + k_q q_o)}{k_1 k_{-q}(k_{-q} + k_q q_o) a_o + k_{-q} k_q (k_{-1} + k_2) q_o}$$

which gives negative values of τ under the condition

$$k_1 k_q q_o a_o \gg k_{-q}(k_{-q} + k_q q_o)$$

Also the $k_{obs} = \lambda_2$ under an approximate evaluation of λ_1 and λ_2 would be

$$\lambda_2 = k_{obs} = \frac{k_1 a_o (k_{-q} + k_q q_o) + (k_{-1} + k_2)}{k_1 a_o + \tilde{k} + k_q q_o + k_{-q}}$$

which has a hyperbolic dependence on substrate concentration as is observed experimentally [63].

Another plausible mechanism which can intuitively explain the observed experimental behavior, though an analytical treatment is not possible, is

$$A + Me^{++} \rightleftharpoons A \cdot Me$$

$$E + A\,Me \rightleftharpoons EAMe \longrightarrow E + \text{Products}$$

$$E + A \rightleftharpoons EA \longrightarrow E + \text{Products}$$

Here the metal ion binds to the substrate and the binary complex, AMe, is the one that undergoes binding to the enzyme and consequently hydrolyzes. The substrate molecules can also hydrolyze through direct binding of A with E in a Michaelis-Menten scheme. This type of mechanism has been proposed to explain some of the steady-state results [62, 66-68]. More recently [69] it has been proposed that the inhibition mechanism is the one that takes place and the effect of EDTA is due to its ability to

chelate trace amounts of Me^{++} which could otherwise inhibit the reaction.

The activation of the substrate by metal ions cannot be solved, as mentioned above. However if we consider intuitively that the two reaction paths are due to different substrates, A and AMe, but lead to the same products, then we would expect a biphasic approach to the steady state with each path contributing a single exponential to the total variation of any measured product with time. If we adopt this scheme we are obliged to conclude that the mechanism through AMe is faster than that through A.

It is not known at this time [70] whether the divalent metal ions bind to a distinct site on myosin (inhibition by binding to EA) or whether they exert their influence by forming a stereochemically specific complex, AMe, with the polyphosphate portion of the nucleoside substrate. However both mechanisms can explain the fact that in the presence of EDTA only a single exponential rise to the steady state is observed, with a positive induction period. The variation of products with time is of the form

$$x = \frac{k_1 k_2 a_o e_o}{k_1 a_o + k_{-1} + k_2} t + \frac{k_1 k_2 a_o e_o}{(k_1 a_o + k_{-1} + k_2)^2} (e^{-\lambda t} - 1)$$

and

$$\tau = 1/(k_1 a_o + k_{-1} + k_2)$$

EDTA removes all the metal ions such that all steps involving these ions are now nonexistent.

The Michaelis-Menten scheme is the simplest possible mechanism for an enzyme-catalyzed reaction, and its solution consists of a single exponential rise to the steady state. Under all conditions the induction

period for this mechanism is positive. An observation of a negative value for the induction period, therefore, forces the conclusion that there exists more than one exponential function and consequently more than a single intermediate.

Alkaline Phosphatase

In recent years, there has been a wide interest in the transient-phase kinetics of alkaline-phosphatase-catalyzed reactions [71-77]. In spite of the impressive number of articles in this area, the interpretation of the results varied from one group to the other. However, in most cases the interpretation of the pre-steady-state data has been in terms of the transient product formation (the intercept of the extrapolated steady state line on the product concentration axis of an x vs. t plot). This parameter is very complicated even for the simplest enzyme mechanism and consequently this parameter cannot be used as a sensitive test of any mechanism. Another complication is the use of comparable concentrations of enzyme and substrate, in which case theoretical treatment presented above will fail. On the other extreme some workers have used too high a substrate concentration such that even the steady-state rate will simplify to

$$v = K_{cat} E_o$$

and consequently the pre-steady state part of the reaction is too fast to be resolved by the experimental techniques in use, especially the stopped-flow apparatus.

A maximum of two exponential terms in the approach to the steady state has been resolved by Fernley and Walker [83] by monitoring the first

product released (4-methylumbelliferone) from the substrate 4-methylumbelliferyl phosphate. The authors have been able to observe that a low substrate concentrations (5μM) in the pH range of 3.8 - 6.3 there was an initial rapid liberation of 4-methylumbelliferone with a negative induction period. On the other hand at very low substrate concentrations (0.1μM) in the pH range 4.9-5.9 an initial lag with a positive induction period has been observed from which the values of k_1 and k_{-1} were calculated. This is a clear indication that Fernley and Walker [83] have been able to resolve the two-exponential approach to the steady state which is predicted for a double-intermediate mechanism, where theoretically the variation of product concentration with time is

$$x = vt + \beta + \sum_{i=1}^{2} \beta_i e^{-\lambda_i t}$$

where

$$\tau = v/\beta = \frac{k_3^2 - k_1 k_2 a_o}{k_3^2 (k_{-1} + k_2) + k_1 k_3 a_o (k_2 + k_3)}$$

and other terms are as given in Chapter one. It is seen that at very low substrate concentration $\tau = \frac{1}{k_{-1} + k_2}$ which is a positive quantity. At higher, but still low substrate concentrations, where $k_1 k_2 a_o \gg k_3^2$,

$$\tau = \frac{- k_1 k_2 a_o}{k_3^2 (k_{-1} + k_2) + k_1 k_3 (k_2 + k_3) a_o}$$

which is a negative quantity as observed experimentally. At still higher substrate concentrations

$$\tau = \beta/v = - \frac{k_2}{k_3 (k_2 + k_3)}$$

and the induction period is independent of substrate concentration. Hence the experimental observations can be qualitatively reconciled with a double-intermediate mechanism. Only Fernley et al, [74, 80, 83, 85, 86] have tried to explain their transient-phase experiments without going into farfetched conclusions.

Gutfreund et al.[71-73, 79, 81, 82] observed only one exponential approach to the steady state. They have proposed a conformational change of the enzyme-substrate complex before any product is released. The mechanism is

$$E + A \underset{}{\overset{1}{\rightleftharpoons}} EA \overset{2}{\rightleftharpoons} EA^* \xrightarrow{3} E^* \xrightarrow{k_4} E + P_2$$
$$\downarrow P_1$$

Where the rate-determining step is the conversion of EA to E*A. Clearly, for this mechanism, one would expect to observe a three-exponential approach to the steady state. This mechanism has actually been proposed to explain the fact that different substrates are hydrolyzed at the same rate regardless of the leaving product P_1 even when production of P_1 is rate limiting. Further evidence for this mechanism proposed by Gutfreund and his collaborators is from an inhibition study using 2-hydroxy-5-nitrobensylphosphonate[82]. Halford et al.[82] proposed that the only mechanism which can explain the experimental results (dependence of the amplitude, β, and observed rate constant, λ, on inhibitor concentration) is the following scheme

$$E + I \rightleftharpoons EI \rightleftharpoons EI^*$$

In connection with this work, it should be noted that

(1) Halford et al.[82] obtained evidence suggesting that one molecule of I is attached to one of enzyme, but they did not establish this conclusively.

(2) The conditions used in the experiments ranged from enzyme in excess to inhibitor in excess. If the concentrations are comparable, the pre-steady-state equations cannot be solved analytically. It is to be noted that Gurfreund and his collaborators did not offer a theoretical treatment of the kinetic system they proposed in the transient phase.

Further evidence for the conclusions of Halford et al.[82] was obtained from relaxation kinetics.

A different conformational change has been proposed by Reid and Wilson [87]. They proposed that the enzyme is found in two conformational states and the rate of interconversion of these two enzyme forms is the rate-determining step. A similar argument to the one presented above for Gutfreund's mechanism can be raised against Wilson's mechanism. The transient-phase experiments do not justify the proposed mechanism.

Chymotrypsin

Chymotrypsin is probably one of the most extensively studied enzymes from the kinetic point of view. It has been used to test theories as well as to lead to models for enzyme-catalyzed reactions. A comprehensive review of this enzyme is beyond the scope of this work. The transient-phase studies on this enzyme has been adequately reviewed [88-91]. In this section we will try to correlate the theoretical treatment presented here and the experimental observations. Two methods have been used to follow the transient kinetics of α-chymotrypsin:

(1) The direct measurement of the reaction products as a function of time.

(2) The displacement of proflavin by the substrate from a proflavin-enzyme complex where the change of proflavin-enzyme complex is being monitored as a function of time.

Direct Observation of Products. The most convenient and first substrate for α-chymotrypsin that has been studied in the transient phase is p-nitrophenylacetate [92]. Hartley and Kilby [92] proposed that the reaction mechanism is a double-intermediate mechanism

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA \xrightarrow[P_1]{k_2} EA' \xrightarrow{k_3} E + P_2$$

where A is p-nitrophenyl acetate, EA is the enzyme-substrate complex, P_1 is p-nitrophenol, and EA' is an acylenzyme and P_2 is the acetate. Later workers[27,93-99] studied the same reaction and obtained similar results indicating that the double-intermediate mechanism is probably an adequate description for the observed kinetics. Maguire et al.[27] were able to calculate the rate constants k_1 and k_{-1} as well as to prove that there is a biphasic approach to the steady state. This observation is in agreement with the theoretical predictions presented in Chapter one.

Proflavin-Displacement Method. The α-chymotrypsin-catalyzed hydrolysis of its specific substrates is generally too fast to be monitored by direct observation of one of the products. Another problem with some of the specific substrates is that the products cannot be monitored by spectroscopic methods. A new method has been developed experimentally to study these reactions. Proflavin is known to be a competitive inhibitor with the specific substrates of α-chymotrypsin. Since also proflavin changes its absorption spectrum upon binding to the enzyme, its enzyme complex can be used for monitoring the kinetics of the reaction[100-106]. The appropriate reaction mechanism is

(F = proflavin)

$$\begin{array}{l} F \\ + \\ E \\ k_q \big\downarrow \big\uparrow k_{-q} \\ EF \end{array} \quad + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA \xrightarrow[P_1]{k_2} EA' \xrightarrow{k_3} E + P_2$$

This mechanism has been solved in Chapter six where analysis of data have been presented in terms of P_1. However, experimentally the enzyme and proflavin are pre-mixed in one syringe and then their mixture is mixed with a substrate solution in a stopped-flow apparatus where the change in absorbance of EF complex is followed at 465 mu, where the absorbance due to EF is maximum. Hess et al.[100-106] envisaged the reaction to be composed of the following steps. " (a) An initial rapid decrease in concentration of the enzyme-proflavin complex, EF, considered to reflect the formation of an enzyme-substrate complex (EA). (b) A second decrease in the concentration of EF, considered to occur as a result of the formation of another intermediate, EA. This step is resolvable by the instrument. (c) A period during which essentially no change in EF occurs - a period considered to reflect the time during which there is maintained a steady-state concentration of the intermediate EA. The length of this period depends on substrate concentration. (d) Finally, an increase in the concentration of EF, considered to be a result of the decomposition of EA and regeneration of enzyme." [106] Hess et al.[102, 103, 106] showed that step (c) has the following dependence on the concentration

$$k_{obs} = \frac{k_2 a_o}{a_o + \frac{k_1}{k_{-1}}\left(1 + \frac{F_o}{K_q}\right)} + k_3$$

under the conditions $a_o >> e_o$ and $F_o >> e_o$ where all the terms are as defined in the reaction scheme.

On the basis of the above rationale, one would expect to find a four-exponential variation of EF with t. In fact this can be seen visually in the oscilloscope traces reported by Hess et al.[103, 106]

The above observation can be explained by the theoretical treatment where, from Chapter six, EF can be calculated. It is found to be

$$[EF] = \frac{k_q F_o (Q)}{\lambda_1 \lambda_2 \lambda_3 \lambda_4} - \sum_{i=1}^{4} \frac{k_q q_o e_o (-\lambda_i^3 + m \lambda_i^2 - L \lambda_i + Q)}{\lambda_i (P - \lambda_i)} e^{-\lambda_i t}$$

where λ_i's are the roots of a very complicated polynomial of the fourth degree, P is the differential operator, and F_o is the initial proflavin concentration. Two important properties, however, are less complicated than the rest:

$$\sum_{i=1}^{4} \lambda_i = k_1 a_o + 2 k_q F_o + 2 k_{-q} + k_2 + k_{-1} + k_3$$

$$\prod_{i=1}^{4} \lambda_i = (k_q F_o + k_{-q}) Q + k_1 k_{-q} a_o e_o (k_2 + k_3)$$

$$Q = (k_{-1} + k_2)(k_q F_o + k_{-q}) + k_1 k_{-q} a_o (k_2 + k_3)$$

There is yet another problem: the theoretical solution is for the case where E and F are not pre-mixed, which is contrary to the experimental conditions. For pre-mixing E and F we do not expect an effect on the λ_i's. The only effect will be on the pre-exponential terms. Therefore the equations for $\Sigma \lambda_i$ and $\pi \lambda_i$ are still valid and can be used to calculate some of the rate constants.

CHAPTER TEN

KINETIC EQUATIONS FOR CONSECUTIVE REVERSIBLE REACTIONS WITH SPECIAL REFERENCE TO PROTEIN DENATURATIONS

Introduction

Protein denaturations are usually studied kinetically by following the course of change of some property y, such as optical rotation. Sometimes the time-course of this property exhibits behaviour consistent with a single-stage mechanism

$$N \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} D$$

It is convenient to define a function

$$\frac{y - y_{eq}}{y_o - y_{eq}}$$

where y is the property at time t, y_o that at t = 0 and y_{eq} that at t = ∞ . It is easy to show that a plot of $\ln [(y - Y_{eq})/(y_o - y_{eq})]$ against t will be linear for this simple mechanism.

It has sometimes been found, however, that plots of this kind give not a single straight line, but two straight regions; they are then said to be biphasic. This means that at least two exponential terms are involved in the variation of y with t, and requires that an additional species X is involved in the mechanism.

It has frequently been assumed that X is a species which has properties intermediate between N and D, and which is formed as a kinetic intermediate on the pathway between N and D:

I $N \rightleftharpoons X \rightleftharpoons D$

Such a mechanism has been postulated by Simpson and Kauzmann [107] for the urea denaturation of ovalbumin, followed through its change in optical rotation. It is equally possible, however, to explain biphasic behaviour in terms of the mechanism

II $N \rightleftharpoons D \rightleftharpoons X$

and

III $X \rightleftharpoons N \rightleftharpoons D$

A mechanism similar to II was assumed by Chase [108], and has been more recently considered by Tanford [109, 110], who regards X as an incorrectly folded species which cannot be converted into the correctly folded form N without first being unfolded into D. A mechanism similar to II was postulated by Wright and Schomaker [111], but was not considered by Tanford. In mechanism III X can be regarded as an incorrectly folded species which cannot be unfolded to give D without first being correctly folded into state N.

In a recent paper Ikai and Tanford [114] have considered some experimental results in terms of mechanisms I and II. For the guanidine hydrochloride denaturation of cytochrome C, for example, they show that the type of variation of y with t is inconsistent with mechanism I, and they conclude that mechanism II applies. However, they do not establish that the behaviour is consistent with mechanism II, and they do not consider the possibility of mechanism III.

In the present work we have made a complete analysis of the kinetic implications of the three mechanisms, and have classified the different patterns of behaviour.

Theoretical

The states N and D are well-defined states of the protein. One can start with the pure native protein N, and carry out a study of the denaturation process, an equilibrium being eventually established. One can also start with the pure denatured protein D and study the kinetics of renaturation. Since N and D can be isolated, the values of the property y related to the pure species, viz. y_N and y_D, can be determined. The species X is not, however, in general isolatable, so that its contribution y_X to the property cannot be obtained. A convenient way [110] of dealing with this difficulty is to define a quantity α by

$$\alpha = \frac{y_X - y_N}{y_D - y_N} \qquad [1]$$

The property y is related to y_N, y_D and y_X by

$$y = (1 - f_X - f_D)\, y_N + f_X y_X + f_D\, y_D \qquad [2]$$

$$y = y_N + f_X (y_X - y_N) + f_D (y_D - y_N) \qquad [3]$$

where f_X and f_D are the fractions of the protein molecules in the states X and D at time t. It is also convenient [110] to define an apparent equilibrium constant by

$$K_{app} = \frac{y_{eq} - y_N}{y_D - y_{eq}} \qquad [4]$$

Experimentally, K_{app} has been found [110] to be a positive quantity for all systems investigated.

All three mechanisms lead to solutions of the form

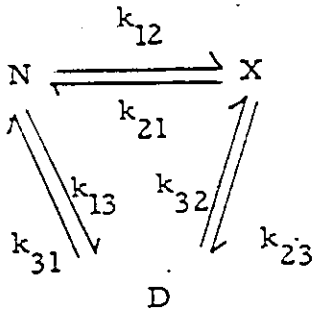
$$\frac{y - y_{eq}}{y_o - y_{eq}} = P_1 e^{-\theta_1 t} + P_2 e^{-\theta_2 t} \quad [5]$$

where

$$P_1 + P_2 = 1 \quad [6]$$

This explains why the plots of log $[(y - y_{eq})/(y_o - y_{eq})]$ are of a biphasic character.

The three mechanisms can be considered as special cases of the general mechanism



A solution for this case has been given by Szabó [112]. The initial conditions for the present problem are

$$t = 0,\ N = N_o,\ X = X_o,\ D = D_o \quad [7]$$

and the solutions are then

$$N_o - N = \frac{1}{\lambda_1 - \lambda_2}\left[\frac{\lambda_2 T'}{\theta_1}(e^{-\theta_1 t} - 1) - \frac{\lambda_1 T''}{\theta_2}(e^{-\theta_2 t} - 1)\right] \quad [8]$$

$$X_o - X = \frac{1}{\lambda_1 - \lambda_2}\left[-\frac{T'}{\theta_1}(e^{-\theta_1 t} - 1) + \frac{T''}{\theta_2}(e^{-\theta_2 t} - 1)\right] \quad [9]$$

$$D_o - D = \frac{1}{\lambda_1 - \lambda_2}\left[\frac{(\lambda_2 - 1)T'}{\theta_1}(e^{-\theta_1 t} - 1) + \frac{(\lambda_1 - 1)T''}{\theta_2}(e^{-\theta_2 t} - 1)\right] \quad [10]$$

$$[11] \qquad \theta_{1(2)} = \frac{S_1 + S_2}{2} \pm \sqrt{\frac{(S_1 - S_2)^2}{4} + (k_{12} - k_{32})(k_{21} - k_{31})}$$

$$[12] \qquad S_1 = k_{12} + k_{31} + k_{13}$$

$$[13] \qquad S_2 = k_{21} + k_{23} + k_{32}$$

$$[14] \qquad \lambda_1 = \frac{k_{21} - k_{31}}{S_2 - \theta_1}$$

$$[15] \qquad \lambda_2 = \frac{k_{21} - k_{31}}{S_2 - \theta_2}$$

$$[16] \qquad T' = T_1 + \lambda_1 T_2$$

$$[17] \qquad T'' = T_1 + \lambda_2 T_2$$

$$[18] \qquad T_1 = (k_{12} + k_{13}) N_o - k_{21} X_o - k_{31} D_o$$

$$[19] \qquad T_2 = -k_{12} N_o + (k_{21} + k_{23}) X_o - k_{32} D_o$$

The solutions for the three special cases were obtained by taking particular values for the rate constants and initial conditions. Solutions were obtained for the three mechanisms for the reactions occurring in both directions - i. e., starting with pure N and pure D. The intermediate state X is one which by hypothesis cannot be isolated, but which contributes to the property y.

Mechanism I

$$N \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} X \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} D$$

The solutions are obtained by setting $k_{13} = k_{31} = 0$. For denaturation the initial conditions are

[20] $t = 0,\ N = N_o,\ X = D = 0,\ y_o = y_N$

The resulting time-dependence expressions for N, X and D are given in the Appendix. The final solution is

[21] $$\frac{y - y_{eq}}{y_o - y_{eq}} = P_{1F}\, e^{-\theta_1 t} + P_{2F}\, e^{-\theta_2 t}$$

where

[22] $$P_{1F} = -\left(\frac{1 + K_{app}}{K_{app}}\right) \frac{k_{12}}{\theta_1(\theta_1 - \theta_2)} \left[a\,(k_{32} - \theta_1) + k_{23}\right]$$

[23] $$P_{2F} = \left(\frac{1 + K_{app}}{K_{app}}\right) \frac{k_{12}}{\theta_2(\theta_1 - \theta_2)} \left[a\,(k_{32} - \theta_2) + k_{23}\right]$$

For renaturation the initial conditions are

[24] $t = 0,\ D = D_o,\ N = X = 0,\ y_o = y_D$

and the solution is

[25] $$\frac{y - y_{eq}}{y_o - y_{eq}} = P_{1R}\, e^{-\theta_1 t} + P_{2R}\, e^{-\theta_2 t}$$

where

[26] $$P_{1R} = (1 + K_{app}) \frac{k_{32}}{\theta_1(\theta_1 - \theta_2)} \left[a\,(k_{12} - \theta_1) + \theta_1 - k_{12} - k_{21}\right]$$

[27] $$P_{2R} = (1 + K_{app}) \frac{k_{32}}{\theta_2(\theta_1 - \theta_2)} \left[a\,(\theta_2 - k_{12}) + k_{12} + k_{21} - \theta_2\right]$$

It follows from the above equations (cf. Ikai and Tanford [4]) that

$$[28] \qquad \frac{P_{1F}}{P_{1R}} = -\frac{1}{K_{app}}\left[\frac{k_{12}\,(k_{32} - \theta_1)}{k_{32}\,(k_{12} - \theta_1)}\right]$$

Experimentally it is found that K_{app} is always positive; this implies that y_{eq} always lies between y_N and y_D. Since, moreover, θ_1 must, from eqn (11), be greater than any of the individual rate constants, it follows from eqn (28) that

$$[29] \qquad \frac{P_{1F}}{P_{1R}} < 0$$

There are thus two possibilities:

$$[30] \quad \text{(a)} \quad P_{1F} > 0,\ P_{1R} < 0; \quad \text{(b)} \quad P_{1F} < 0,\ P_{1R} > 0$$

Similarly it is found that

$$[31] \qquad \frac{P_{2F}}{P_{2R}} = -\frac{1}{K_{app}}\left[\frac{k_{12}\,(k_{32} - \theta_2)}{k_{32}\,(k_{12} - \theta_2)}\right]$$

This ratio can be positive or negative. In the former case P_{2F} and P_{2R} must both be positive; both can not be negative in view of (6) and (29). In the latter case either can be positive and the other negative. In the light of the above the following are the only combinations of coefficients that are compatible with Mechanism I:

$P_{1F} < 0$	$P_{1F} > 0$	$P_{1F} < 0$	$P_{1F} > 1$
$P_{2F} > 1$	$P_{2F} > 0$	$P_{2F} > 1$	$P_{2F} < 0$
$P_{1R} > 0$	$P_{1R} < 0$	$P_{1R} > 1$	$P_{1R} < 0$
$P_{2R} > 0$	$P_{2R} > 1$	$P_{2R} < 0$	$P_{2R} > 1$

The patterns of kinetic behaviour related to these four possibilities are shown schematically in Figure 28.

Mechanism II

$$N \underset{k_{31}}{\overset{k_{13}}{\rightleftharpoons}} D \underset{k_{23}}{\overset{k_{32}}{\rightleftharpoons}} X$$

The conditions are now $k_{12} = k_{21} = 0$ and, for denaturation,

[32] $t = 0,\ N = N_o,\ X = D = 0,\ y_o = y_N$

The solution is eqn. (21) where now

[33] $$P_{1F} = -\left(\frac{1 + K_{app}}{K_{app}}\right)\left[\frac{k_{13}\,(\alpha\, k_{32} + k_{23} - \theta_1)}{\theta_1\,(\theta_1 - \theta_2)}\right]$$

[34] $$P_{2F} = \left(\frac{1 + K_{app}}{K_{app}}\right)\left[\frac{-k_{13}\,(\alpha\, k_{32} + k_{23} - \theta_2)}{\theta_2\,(\theta_1 - \theta_2)}\right]$$

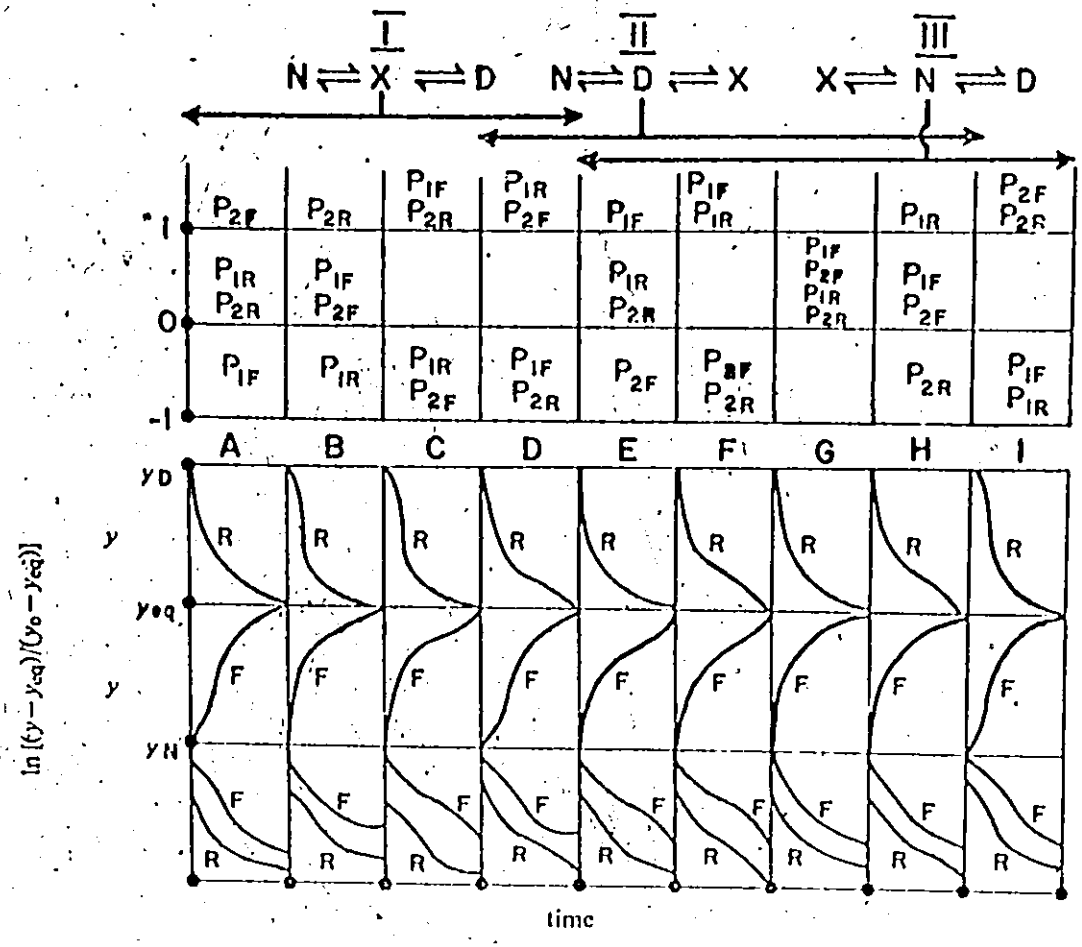
For renaturation the solution is eqn. (25) with

[35] $$P_{1R} = (1 + K_{app})\left[\frac{k_{32}\,(1 - \alpha)\,(k_{23} + k_{32} + k_{31} - \theta_2) + k_{31}\,(\theta_1 - k_{23})}{\theta_1\,(\theta_1 - \theta_2)}\right]$$

[36] $$P_{2R} = -(1 + K_{app})\left[\frac{k_{32}\,(1 - \alpha)(k_{23} + k_{32} + k_{31} - \theta_1) + k_{31}\,(\theta_2 - k_{23})}{\theta_2\,(\theta_1 - \theta_2)}\right]$$

Analysis of this case reveals that P_{1R} must be positive. This conclusion together with the normalization condition (6) leads to the following possible combination for Mechanism II:

Fig. 28 The different patterns for Mechanisms I, II and III



$\ln[(y-y_{eq})/(y_0-y_{eq})]$

$P_{1F} > 0$	$P_{1F} < 0$	$P_{1F} > 1$	$P_{1F} > 1$	$P_{1F} > 0$
$P_{2F} > 0$	$P_{2F} > 1$	$P_{2F} < 0$	$P_{2F} < 0$	$P_{2F} > 0$
$P_{1R} > 0$	$P_{1R} > 1$	$P_{1R} > 0$	$P_{1R} > 1$	$P_{1R} > 1$
$P_{2R} > 0$	$P_{2R} < 0$	$P_{2R} > 0$	$P_{2R} < 0$	$P_{2R} < 0$

The plots are shown in Figure 28.

Mechanism III

$$X \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} N \underset{k_{31}}{\overset{k_{13}}{\rightleftharpoons}} D$$

The solution for denaturation is eqn. (21) with

$$[37] \qquad P_{1F} = -\left(\frac{1 + K_{app}}{K_{app}}\right)\left[\frac{(k_{12} + k_{21} + k_{13} - \theta_2)(k_{12} + k_{21} - k_{12}\,a - \theta_1)}{\theta_1(\theta_1 - \theta_2)}\right]$$

$$[38] \qquad P_{2F} = \left(\frac{1 + K_{app}}{K_{app}}\right)\left[\frac{(k_{12} + k_{21} + k_{13} - \theta_1)(k_{12} + k_{21} - k_{12}\,a - \theta_2)}{\theta_2(\theta_1 - \theta_2)}\right]$$

The solution for renaturation is eqn. (25) with

$$[39] \qquad P_{1R} = (1 + K_{app})\left[\frac{k_{31}(a\,k_{12} + \theta_1 - k_{12} - k_{21})}{\theta_1(\theta_1 - \theta_2)}\right]$$

$$[40] \qquad P_{2R} = -(1 + K_{app})\left[\frac{k_{31}(a\,k_{12} + \theta_2 - k_{12} - k_{21})}{\theta_2(\theta_1 - \theta_2)}\right]$$

It is now found that

$$[41] \qquad \frac{P_{1F}}{P_{1R}} = \frac{1}{K_{app}}\left(\frac{k_{12} + k_{21} + k_{13} - \theta_2}{k_{31}}\right)$$

Since K_{app} is positive and θ_2 is less than $k_{12} + k_{21} + k_{13}$ it follows that P_{1F}/P_{1R} is positive. Similarly

$$\frac{P_{2F}}{P_{2R}} = \frac{1}{K_{app}} \left(\frac{k_{12} + k_{21} + k_{13} - \theta_1}{k_{31}} \right) \quad [42]$$

which can be positive or negative. In the former case the normalization condition (6) allows the first three possibilities shown below. If the ratio is negative the last two possibilities are allowed.

$P_{1F} > 0$	$P_{1F} < 0$	$P_{1F} > 1$	$P_{1F} > 0$	$P_{1F} > 1$
$P_{2F} > 0$	$P_{2F} > 1$	$P_{2F} < 0$	$P_{2F} > 0$	$P_{2F} < 0$
$P_{1R} > 0$	$P_{1R} < 0$	$P_{1R} > 1$	$P_{1R} > 1$	$P_{1R} > 0$
$P_{2R} > 0$	$P_{2R} > 1$	$P_{2R} < 2$	$P_{2R} < 0$	$P_{2R} > 0$

The plots are shown in Figure 28.

Discussion

It follows from the preceding treatment that in some cases the y vs. t curves permit a decision to be made as to which of the three mechanisms applies, but that in some cases there is ambiguity. Thus patterns A, B, and C belong exclusively to Mechanism I, and pattern I is exclusive to Mechanism III. Pattern D, however, is possible with either Mechanism I or II, and E, F, G and H belong to either II or III. It is to be noted that it is never possible, from the y vs. t curves, to be sure that Mechanism II applies.

As an example of the use of the above type of analysis may be mentioned work of Tanford et al.[109, 110, 113] on the denaturation of β-lactoglobulin by guanidine hydrochloride. These workers conclude that Mechanism II applies, partly on the basis of studies of the degree of exposure of certain peptide groups. It would appear, however, from

the kinetic studies that P_{1F}, P_{2F}, P_{1R}° and P_{2R} are all positive, so that either Mechanism II or III might apply.

Appendix

The expressions for N, X and D as functions of time are as follows:

Mechanism I

[43] $$N = N_o\left[\frac{k_{21}k_{32}}{\theta_1\theta_2} + \frac{k_{12}(\theta_1 - k_{23} - k_{32})}{\theta_1(\theta_1 - \theta_2)}\, e^{-\theta_1 t} + \frac{k_{12}(k_{23} + k_{32} - \theta_2)}{\theta_1(\theta_1 - \theta_2)}\, e^{-\theta_2 t}\right]$$

[44] $$X = N_o\left[\frac{k_{12}k_{32}}{\theta_1\theta_2} + \frac{k_{12}(k_{32} - \theta_1)}{\theta_1(\theta_1 - \theta_2)}\, e^{-\theta_1 t} + \frac{k_{12}(\theta_2 - k_{32})}{\theta_2(\theta_1 - \theta_2)}\, e^{-\theta_2 t}\right]$$

[45] $$D = N_o\left[\frac{k_{12}k_{23}}{\theta_1\theta_2} + \frac{k_{12}k_{23}}{\theta_1(\theta_1 - \theta_2)}\, e^{-\theta_1 t} - \frac{k_{12}k_{23}}{\theta_2(\theta_1 - \theta_2)}\, e^{-\theta_2 t}\right]$$

Mechanism II

[46] $$N = N_o\left[\frac{k_{31}k_{23}}{\theta_1\theta_2} + \frac{k_{13}(\theta_1 - k_{23} - k_{32})}{\theta_1(\theta_1 - \theta_2)}\, e^{-\theta_1 t} + \frac{k_{13}(k_{23} + k_{32} - \theta_2)}{\theta_2(\theta_1 - \theta_2)}\, e^{-\theta_2 t}\right]$$

[47] $$X = N_o \left[\frac{k_{13}\, k_{32}}{\theta_1 \theta_2} + \frac{k_{13}\, k_{32}}{\theta_1 (\theta_1 - \theta_2)} e^{-\theta_1 t} - \frac{k_{13}\, k_{32}}{\theta_2 (\theta_1 - \theta_2)} e^{-\theta_2 t} \right]$$

[48] $$D = N_o \left[\frac{k_{13}\, k_{23}}{\theta_1 \theta_2} + \frac{k_{13} (k_{23} - \theta_1)}{\theta_1 (\theta_1 - \theta_2)} e^{-\theta_1 t} + \frac{k_{13} (\theta_2 - k_{23})}{\theta_2 (\theta_1 - \theta_2)} e^{-\theta_2 t} \right]$$

Mechanism III

[49] $$N = N_o \left[\frac{k_{21}\, k_{31}}{\theta_1 \theta_2} - \frac{(k_{21} - \theta_1)(k_{12} + k_{21} + k_{13} - \theta_2)}{\theta_1 (\theta_1 - \theta_2)} e^{-\theta_1 t} + \frac{(k_{21} - \theta_2)(k_{12} + k_{21} + k_{13} - \theta_1)}{\theta_2 (\theta_1 - \theta_2)} e^{-\theta_2 t} \right]$$

[50] $$X = N_o \left[\frac{k_{12}\, k_{31}}{\theta_1 \theta_2} - \frac{k_{12} (k_{12} + k_{21} + k_{13} - \theta_2)}{\theta_1 (\theta_1 - \theta_2)} e^{-\theta_1 t} + \frac{k_{12} (k_{12} + k_{21} + k_{13} - \theta_1)}{\theta_2 (\theta_1 - \theta_2)} e^{-\theta_2 t} \right]$$

[51] $$D = N_o \left[\frac{k_{21}\, k_{13}}{\theta_1 \theta_2} + \frac{(k_{12} + k_{21} - \theta_1)(k_{12} + k_{21} + k_{13} - \theta_2)}{\theta_1 (\theta_1 - \theta_2)} e^{-\theta_1 t} - \frac{(k_{12} + k_{21} - \theta_2)(k_{12} + k_{21} + k_{13} - \theta_1)}{\theta_2 (\theta_1 - \theta_2)} e^{-\theta_2 t} \right]$$

The following is an example of the procedure for obtaining the P values; it refers to Mechanism III and the denaturation process. The initial conditions are

[52] $t = 0,\ N = N_o,\ X = D = 0,\ y_o = y_N$

K_{app} and α are given by eqn (4) and (1); y_{eq} (cf. eqn. (4) can be written as

[53] $$y_{eq} = \frac{K_{app}\, y_D + y_N}{1 + K_{app}}$$

The property y is given by eqn. (3), and

[54] $$f_X = \frac{x}{N_o} \qquad f_D = \frac{D}{N_o}$$

Substitution of the values for y, y_{eq} and y_o leads, after simplification, to

[55] $$\frac{y - y_{eq}}{y_o - y_{eq}} = 1 - \left(\frac{1 + K_{app}}{K_{app}}\right)(\alpha f_X + f_D)$$

Substitution for f_X and f_D form the concentration equation, followed by simplification and normalization ($P_{1F} + P_{2F} = 1$) then leads to

[56] $$\frac{y - y_{eq}}{y_o - y_{eq}} = P_{1F}\, e^{-\theta_1 t} + P_{2F}\, e^{-\theta_1 t}$$

where P_{1F} and P_{2F} are given by eqn. (37) and (38)

Since the completion of this study, Tanford et al.[114-116] have obtained similar theoretical results and applied their results to experimental data on lysozyme and cytochrome C.

APPENDIX A

The Laplace-Carson Transformation

The operations of setting up the transformed functions (transforms) and replacing the latter by the originals are based on the so-called Laplace transform.

The transform F(P) of a function f (t) subject to the Laplace transformation is defined as

$$F(P) = \int_0^\infty e^{-Pt} f(t)\, dt$$

The integration is over two functions f(t) and e^{-Pt}; both functions are dependent on t. After the integration has been performed and the limits t = 0 and t = ∞ substituted, the integral found will be independent of the variable t; hence, the function F(P) will be only a function of P.

By use of the definition of F(P), it is possible to determine an F(P) dependent only on P for every function f(t) which depends on t only. The integral we are discussing has been found for many functions f(t). We now have tables that contain the transforms with their corresponding originals. Hence, given a function F(P) (transform), the function f(t) (original) can be found. This is the operator method for solving differential equations.

Besides the Laplace transform, there is a similar Laplace-Carson transformation. This is defined by the integral

$$F(P) = P\int_0^\infty e^{-Pt} f(t)\, dt$$

This expression was used in this thesis to integrate the differential rate equations. The Laplace-Carson transformation gives the transform of a constant as the constant itself. This will be shown below.

Consider a function $f(t) = A$; its transform is

$$F(P) = P\int_0^\infty A\, e^{-Pt}\, dt = -A\left| e^{-Pt}\right|_0^\infty = A$$

Consider a function $f(t) = t$; its transform is defined by

$$F(P) = P\int_0^\infty t\, e^{-Pt}\, dt$$

Integration by parts.

$$P\int_0^\infty t\, e^{-Pt}\, dt = -\int_0^\infty t\, d\, e^{-Pt} = -\left| t\, e^{-Pt}\right|_0^\infty + \int_0^\infty e^{-Pt}\, dt$$

$$= 0 - \left| \frac{1}{P}\, e^{-Pt}\right|_0^\infty = \frac{1}{P}$$

Therefore

$$F(P) = P\int_0^\infty t\, e^{-Pt}\, dt = \frac{1}{P}$$

An actual solution of a differential equation will now be presented. It is required to solve the differential equation

$$\frac{dx}{dt} + \lambda x = 1$$

where x and t are variables and λ is a constant. Replace $\frac{d}{dt}$ by P:

$$P x + \lambda x = 1$$

Consider P as a constant and solve algebraically

$$x = \frac{1}{P + \lambda}$$

This is the transform of x. The solution of x as a function of t, the original, is obtained from the table as

$$x = \frac{1}{\lambda} (1 - e^{-\lambda t})$$

This procedure can be applied to any linear differential equation where the coefficients of variable x are constants. A table of transforms and originals, covering the cases dealt with in the present work, is given on the next page.

A Table of Transforms and Originals

Transforms	Originals
$\frac{1}{P}$	t
$\frac{1}{P+\lambda}$	$\frac{1}{\lambda} - \frac{1}{\lambda} e^{-\lambda t}$
$\frac{P}{P+\lambda}$	$e^{-\lambda t}$
$\frac{1}{(P+\lambda_1)(P+\lambda_2)}$	$\frac{1}{\lambda_1\lambda_2} - \frac{1}{\lambda_1(\lambda_2-\lambda_1)} e^{-\lambda_1 t} - \frac{1}{\lambda_2(\lambda_1-\lambda_2)} e^{-\lambda_2 t}$
$\frac{P}{(P+\lambda_1)(P+\lambda_2)}$	$\frac{1}{\lambda_2-\lambda_1} e^{-\lambda_1 t} + \frac{1}{\lambda_1-\lambda_2} e^{-\lambda_2 t}$
$\frac{P^2+b_1P+b_2}{(P+\lambda_1)(P+\lambda_2)}$	$\frac{b_2}{\lambda_1\lambda_2} - \frac{\lambda_1^2 - b_1\lambda_1 + b_2}{\lambda_1(\lambda_2-\lambda_1)} e^{-\lambda_1 t} - \frac{\lambda_2^2 - b_1\lambda_2 + b_2}{\lambda_2(\lambda_1-\lambda_2)} e^{-\lambda_2 t}$
$\frac{1}{(P+\lambda_1)(P+\lambda_2)(P+\lambda_3)}$	$\frac{1}{\lambda_1\lambda_2\lambda_3} - \frac{1}{\lambda_1(\lambda_2-\lambda_1)(\lambda_3-\lambda_1)} e^{-\lambda_1 t} - \frac{1}{\lambda_2(\lambda_1-\lambda_2)(\lambda_3-\lambda_1)} e^{-\lambda_2 t} - \frac{1}{\lambda_3(\lambda_1-\lambda_3)(\lambda_2-\lambda_3)} e^{-\lambda_3 t}$
$\frac{P+b}{(P+\lambda_1)(P+\lambda_2)(P+\lambda_3)}$	$\frac{b}{\lambda_1\lambda_2\lambda_3} - \frac{b-\lambda_1}{\lambda_1(\lambda_2-\lambda_1)(\lambda_3-\lambda_1)} e^{-\lambda_1 t} - \frac{b-\lambda_2}{\lambda_2(\lambda_1-\lambda_2)(\lambda_3-\lambda_2)} e^{-\lambda_2 t} - \frac{b-\lambda_3}{\lambda_3(\lambda_1-\lambda_3)(\lambda_2-\lambda_3)} e^{-\lambda_3 t}$

CLAIMS TO ORIGINAL RESEARCH

1. The Laplce-Carson transformation technique is applied to the single-substrate mechanisms. The single-intermediate and the double-intermediate cases were solved under two limiting cases": (a) enzyme concentration limiting (b) substrate concentration limiting. This gave the same results as had previously been obtained by other mathematical techniques. The solution of the double-intermediate mechanism at limiting substrate concentration, in terms of the second product, is reported for the first time. A new method of analyzing the experimental data is discussed.

2. The transient-phase kinetics applicable to a scheme involving two competing substrates is solved and analysis of experimental results is discussed.

3. The transient-phase kinetic equations for three mechanisms in the presence of an added nucleophile are presented, and criteria for distinguishing the three mechanisms are discussed. Also, analysis of results to calculate the rate constants from experimental data is discussed.

4. The transient-phase kinetic equations are developed for a single-intermediate mechanism in the presence of an activator. It was found that if the enzyme-substrate-activator complex breaks down into enzyme-activator complex and products a steady state can not be established. However, if the enzyme-substrate-activator complex breaks down into enzyme, activator and products, the steady state can be established. In both cases analysis of results is carried out to yield the specific rate constants.

5. The transient-phase kinetic equations applicable to two systems exhibiting sigmoidal behavior in the steady state are obtained, and a discussion of the methods of analyzing experimental data is presented.

6. The transient-phase kinetic equations are reported for a single intermediate mechanism in the presence of inhibitor. The cases considered are competitive, non-competitive and anti-competitive inhibitions. For each mechanism two types of inhibition are considered, reversible and irreversible. Analysis of results to calculate the specific rate constants is also presented.

7. A variety of inhibition mechanisms applicable in double-intermediate mechanisms are considered, and the transient-phase kinetic equations are reported. For each mechanism two types of inhibition are considered, reversible and irreversible. Analysis of results to yield the specific rate constants is also discussed.

8. Transient-phase kinetic equations applicable to two-substrate enzyme reactions are obtained. The mechanisms considered are (a) Theorell-Chance mechanism, (b) Ping Pong Bi Bi mechanism, (c) Ordered ternary-complex mechanism, (d) Random ternary-complex mechanism. Methods of analysis to yield the specific rate constants are also suggested.

9. The effect on the kinetics of pre-mixing two of the three reagents in two-substrate reactions has been thoroughly investigated. The mechanisms considered are (a) Theorell-Chance mechanism (b) Ping Pong Bi Bi mechanism (c) Ordered ternary-complex mechanism. All possible combinations are considered. The relative concentrations of the three reagents have been found to affect the kinetics. Two cases of relative concentrations are considered a_o, $b_o >> e_o$, and b_o, $e_o >> a_o$.

The case of $e_o, a_o \gg b_o$ was also considered, but analytical solutions were not possible. However, the behavior is predicted to be similar to that with a_o limiting. The effect of pre-mixing on the mechanisms has also been discussed. Criteria are established to distinguish among the four mechanisms. Methods of calculating the specific rate constants are also discussed.

10. A correlation rule between the number of exponentials and the number of intermediates is suggested, with enzyme concentration limiting and one of the products being measured as a function of time. From this rule has evolved a technique for predicting the expected number of exponentials. A test is discussed to confirm or reject bimolecular steps in the reaction mechanism, as to whether or not these steps are resolved by the experimental technique.

It was also found that the sum of all the exponents, $\sum_{i=1}^{n} \lambda_i$, is equal to the sum of all the kinetic steps in the reaction scheme.

11. The kinetic patterns applicable to three-state denaturation mechanisms

I $N \rightleftharpoons X \rightleftharpoons D$

II $N \rightleftharpoons D \rightleftharpoons X$

III $X \rightleftharpoons N \rightleftharpoons D$

in both directions are classified. The possibility of distinguishing the three mechanism on the basis of kinetic studies is discussed.

12. A unified mathematical method, the Laplace-Carson transformation technique, is applied for all the transient-phase kinetic study. It is found that this method is versatile and simple for integrating the rate equations under the conditions, $a_o \gg e_o$ and $e_o \gg a_o$.

13. On the basis of the theoretical treatment, some experimental data are discussed for carboxypeptidase A, liver alcohol dehydrogenase, myosin, alkaline phosphatase and α-chymotrypsin.

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