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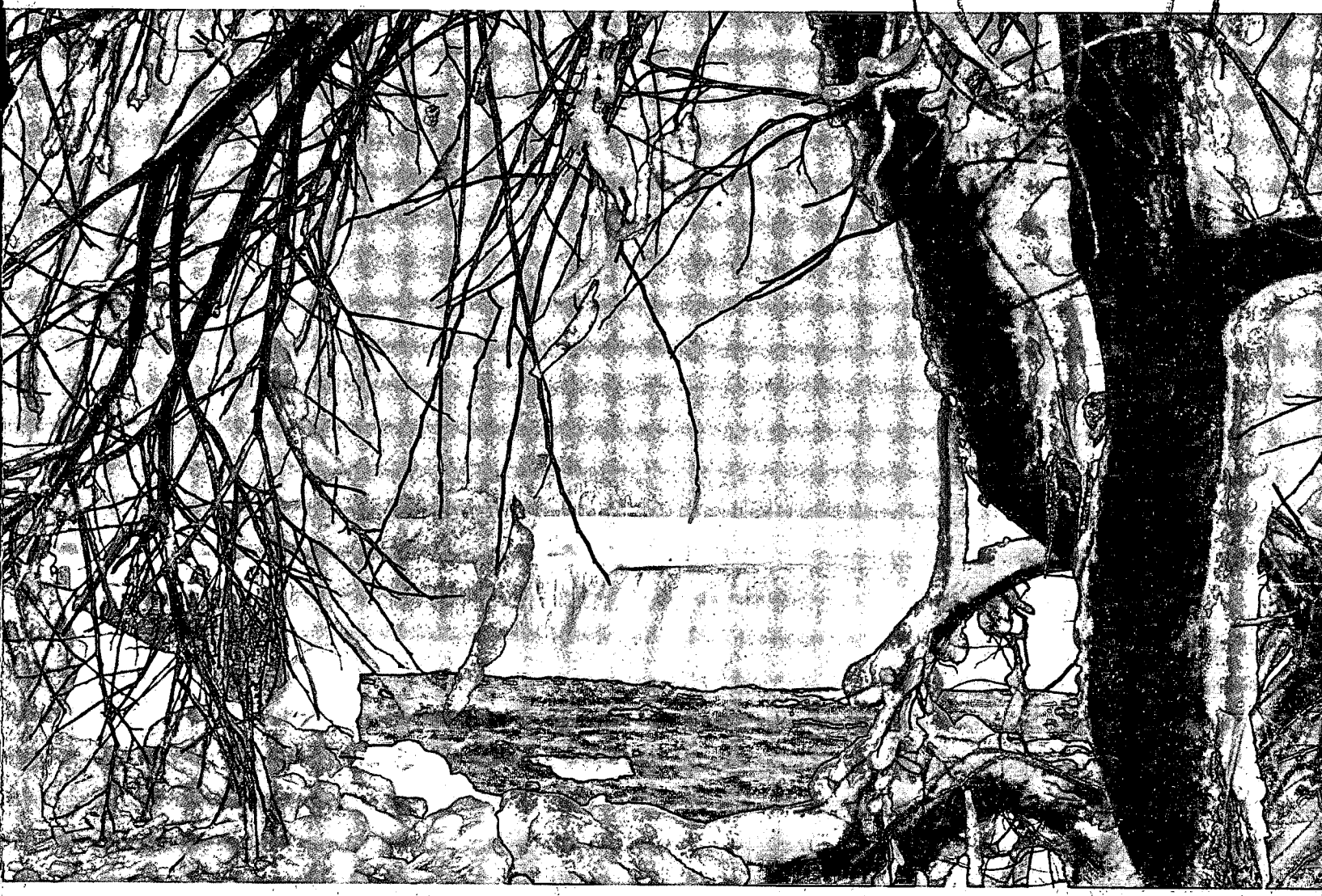
Enzyme Kinetics — A Review with Emphasis on Inhibition of Enzymes

James Maguire



SCIENTIFIC SERIES NO. 47
(Résumé en français)

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Cat. No.: En 36-502/47

Contract # KL 327-4-8069
Thorn Press Limited

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Abstract

Enzymes are found in the cells of all living things. They are responsible for the catalysis of the chemical reactions that occur in cells. The study of enzymology is fundamental to an understanding of the mechanisms of living processes. Enzymology plays an important part in environmental considerations because the effects of physical variables and added chemical compounds on chemical reactions can be assessed at the molecular or enzymatic level. This review demonstrates the potential value of the application of the techniques of enzyme kinetics to environmental problems.

Résumé

Les cellules de tout être vivant produisent des enzymes qui agissent comme catalyseur dans les réactions chimiques se déroulant dans ces cellules mêmes. Les études enzymologiques sont fondamentales pour la compréhension des mécanismes biologiques. L'enzymologie intervient, pour une grande part, dans les examens environnementaux car l'action des variables physiques et celle des réactifs chimiques sur les changements chimiques peuvent être évaluées à l'échelle moléculaire ou enzymatique. Cet examen démontre la valeur possible de l'application des techniques de la cinétique des enzymes au domaine touchant les problèmes environnementaux.

Enzyme Kinetics — A Review with Emphasis on Inhibition of Enzymes

James Maguire

INTRODUCTION

This review deals with the kinetics of enzyme action. It is devoted mainly to discussions of steady-state and transient-phase kinetics of enzyme-catalyzed reactions in the absence and presence of inhibitors that may be of environmental significance. Attention is also given to the effects of the pH of the medium. Finally, a section is presented on the relatively new field of insolubly supported enzyme systems. This review is not intended to be comprehensive, and the reader is referred to recent books on these subjects by Laidler and Bunting (1973), Jencks (1969), and Mahler and Cordes (1966), particularly for effects of temperature, ionic strength, and dielectric constant, kinetic isotope effects, the kinetics of two-substrate reactions, sigmoid kinetics, and allostery.

The outstanding characteristic of nearly all biochemical reactions is the rapidity with which they occur through the mediation of natural catalysts called enzymes, whose high degree of specificity and efficiency direct biochemical reactions quickly through defined pathways. Enzymic reactions were used by man long before written history; the process of fermentation is perhaps the best-known example. The recognition that living cells are responsible for alcoholic and other types of fermentation was one of the great achievements of the nineteenth century. Enzymology received a powerful impetus when the Buchner brothers showed that yeast cells, ground with sand and squeezed under high pressure, gave a cell-free juice capable of fermenting sugar and at the same time producing alcohol and carbon dioxide. It is now apparent that yeast juice contains a complex mixture of enzymes which can catalyze these transformations, and that enzymes can function outside the cell as well as inside.

A substantial part of the study of the chemistry of living cells is today devoted to the enzymes, for it is now understood that all physiological functions—muscular contraction, nerve conduction, excretion by the kidney, and so forth—are inextricably linked to the activity of enzymes. A complex process such as muscular contraction may be dissected into a series of enzyme-catalyzed reactions. Many of these reactions have now been studied *in vitro* as isolated systems with pure, crystalline enzymes.

The properties of enzymes are compounded of catalyst properties and protein properties. Like other catalysts, enzymes lower the free energies of activation of the reactions they catalyze. Like at least some other catalysts, they show a "saturation effect," i.e., the catalyzed reactions have rates independent of reactant concentrations when those concentrations are sufficiently high. Unlike most other catalysts, however, enzymes have pH and temperature dependence properties that are typical of proteins. Plots of enzyme activity against pH show, in part, inflections at the pK (the negative logarithm of the dissociation constant) values of the ionizable groups of proteins, and the thermal stability of enzyme activity is characteristically that of protein tertiary structure.

The characteristic property and function of enzymes is the catalysis of chemical reactions. Any fundamental study of this catalytic function must be based on quantitative measurements of the rate of the catalyzed reaction. From the effect of varying the conditions on the rate, certain inferences may be made about the mechanism of enzyme action. Ideally, such studies should be brought into relation with chemical and structural studies on the enzyme in order to obtain a clearer picture of the process, but this is only possible if the enzyme has been isolated in a high degree of purity. Many enzymes have not been so purified, and kinetic studies are the only approach possible at present.

The mechanism of an enzyme reaction has, for the most part, been investigated from a somewhat different point of view than the study of an ordinary chemical reaction. With most chemical reactions the structures of the reacting molecules are known, and information about their structures is not required from the kinetic investigations. With enzyme reactions, however, little was known about the detailed structure of the enzyme, and all information possible was obtained from the kinetic study. For example, studies of the pH dependence of the rate have given the values of the pK's of those groups in the enzyme that are concerned in the catalytic action. From a study of the way in which these pK's vary with solvent composition, it is possible in some cases to make a firm identification of the groups.

Until recently, such kinetic results have been the main source of information about the nature of the active centres of enzymes. X-ray investigations of protein structures have now, however, reached the point at which we are learning the exact structures of some enzymes, so it will be possible to correlate structural and kinetic information. Already there have been X-ray studies in which not only has the structure of the enzyme been determined, but also that of the enzyme-substrate complex. No doubt the next few years will produce many more structural results on enzymes, enzyme-substrate complexes, and enzyme-inhibitor complexes, and it will be possible to relate these structures to the kinetics. However, structural studies will never entirely supersede kinetic studies. Kinetic investigations will always be needed to provide information about the detailed course of enzyme reactions and about the nature of the very short-lived activated complexes; purely structural studies can never provide such information.

The question of enzyme inhibition is important in the consideration of environmental problems because many chemical compounds that find their way into the environment are capable of reversible or irreversible inhibition of enzymes, resulting in disease or death to man, other animals, and plants; it is desirable to know the toxic levels of such substances and their mechanisms of action. In general, organophosphates used as pesticides attack the central nervous system by attaching themselves to the enzyme acetylcholinesterase; heavy metal ions may form disulfide derivatives with sulfhydryl groups of enzymes, causing them to lose activity; and cyanide, sulfide, and azide ions, and carbon monoxide, can form inactive complexes with metallo-enzymes and proteins, such as the cytochromes, causing respiratory failure. Chelating agents such as nitrilotriacetic acid (N, N-bis (carboxymethyl)-glycine, NTA) may remove metal ions that are needed for the activity of particular enzymes; and various pesticide formulations may denature enzymes and proteins by precipitating them.

Mercury, cadmium, and lead all show a strong affinity for ligands such as phosphates, cysteinyl and histidyl side chains of proteins, purines, pteridines, and porphyrins. Hence, all three elements can act at a large number of biochemical sites; all inhibit a large number of enzymes having functional sulfhydryl groups; all bind to and affect the conformation of nucleic acids; and all disrupt pathways of oxidative phosphorylation, although in each instance the precise response depends upon the individual properties of the metal.

On the other hand, Hg, Cd, and Pb can all induce catalytic activity in certain enzymes, e.g., by substituting for the zinc atoms at the active centre of bovine pancreatic carboxypeptidase A (Coleman and Vallee, 1961). The Hg, Cd, and Pb carboxypeptidases all actively hydrolyze ester substrates, the Cd enzyme, in fact, being significantly more active than the native Zn enzyme. However, in contrast to Zn-carboxypeptidase, the Hg, Cd, and Pb enzymes do not hydrolyze peptide substrates. Hence, these metals alter both catalytic efficiency and specificity. It is evident that Cd could be said both to "activate" and to "inhibit" carboxypeptidase, depending on whether ester or peptide substrates are used. This circumstance should be recalled when evaluating the large number of reports of enzyme activation or inhibition by mercury, cadmium, and lead.

Mercury, as well as cadmium and lead, can interfere with cerebral pyruvate metabolism and simulate the symptoms and signs of thiamine deficiency (Thompson and Whittaker, 1947; Peters and Wakelin, 1946), probably by interaction with the -SH groups of lipoic acid, pantethine, and coenzyme A. The Hg^{++} ion and organic mercurials interact with -SH and S-S groups of proteins in many systems, and the consequences for structure and function of proteins have been well reviewed (e.g., Riordan and Vallee, 1967). Hence, the biochemical basis of toxicological effects of Hg and its derivatives is generally sought through mercury-sulfur interactions. It is difficult, however, to localize the reaction in organized systems; in a mixture of proteins, such as exists in cells, the identification of that protein most sensitive to mercurials presents problems because of the ubiquity of functional -SH groups. The existence of the S-Hg-S bond in proteins has been demonstrated through simultaneous elimination of Hg by ethylenediamine tetraacetic acid (N, N′-1, 2-ethandiylbis [N-(carboxymethyl)]-glycine, EDTA) and carboxymethylation of the -SH groups thus released (Burstein and Sperling, 1970). Mercury also interacts with tryptophan and characteristically alters its absorption spectrum (Ramachandran and Witkop, 1964) while quenching fluorescence and phosphorescence. Analogous fluorescence quenching of phenylalanine and tyrosine has been reported (Chen, 1971). Hg also interacts with phosphoryl groups of cell membranes (Passow *et al.*, 1971) and with amino and carboxyl groups of enzymes (Cook and Perisutti, 1947).

The complexes of Cd and Zn with amino acids and peptides have been compared (Sillén and Martell, 1964). The stability constants of the Zn complexes are greater than those of Cd when nitrogen and oxygen serve as ligands, but Cd binds more firmly to free sulfur groups. Interaction of Cd with polythiols is particularly strong and probably of considerable biological significance, so much so that preferential binding of Cd vs Zn has been suggested as being indicative of a dithiol configuration in the active site of an enzyme (Gaber and Fluharty, 1968). Cd either

activates or inhibits a large number of enzyme systems *in vitro*, and administration of Cd to animals alters the activity of many such enzymes *in vivo* (Vallee and Ulmer, 1972).

Lead, like mercury and cadmium, forms mercaptides with the -SH group of cysteine, and less stable complexes with other amino acid side chains (Sillén and Martell, 1964). The stability constants of complexes of Pb^{++} with serine and threonine are equivalent to those with Zn^{++}. Proteins with large numbers of free -SH groups, e.g. thionein, bind Pb^{++} firmly both *in vitro* and *in vivo* (Ulmer, 1966). The formation of Pb-albumin and Pb-γ-globulin complexes has served as a means of fractionating serum proteins (Aoki and Hori, 1964). Pb inhibits most enzymes bearing a single functional -SH group less readily than either Hg^{++} or Cd^{++}. The concentrations of Pb^{++} generally required to inhibit -SH and other enzymes *in vitro*, i.e. 10^{-3} to 10^{-4} *M*, far exceed those observed in tissues and fluids from individuals with clinically evident Pb intoxication. However, much lower concentrations of Pb^{++} inhibit a small group of enzymes, e.g. lipoamide dehydrogenase, certain ATPases, and δ-aminolevulinic acid (ALA) dehydrase.

In addition to these studies of direct environmental relevance, many studies exist about interactions of metal ions with small molecules, which serve as model compounds for enzymes and proteins (e.g., Fuhr and Rabenstein, 1973).

A number of recent studies deal with inhibition of enzymes by chlorinated hydrocarbons. Davis *et al.* (1972) have reported that the common insecticides, polychlorinated biphenyls, and herbicides inhibit Na^+, K^+-activated adenosine triphosphatase (Na, K-ATPase) of rainbow trout to decreasing extents. Desaiah *et al.* (1972) have reported that polychlorinated biphenyls show prominent inhibitory effects on oligomycin-insensitive Mg^{++}-ATPase. Pardini (1971) has shown that polychlorinated biphenyls inhibit various beef heart mitochondrial systems; similar results were obtained by Moffett and Yarbrough (1972) for DDT, toxaphene, and dieldrin, and by Christensen (1971/72) with metal cations. Pocker *et al.* (1971) claim that inhibition of carbonic anhydrase with DDT, DDE, and dieldrin is more physical than chemical in that impairment of catalytic efficiency can only be documented in opalescent test solutions in which the insecticides are present in excess of their solubility limit; slowly-forming precipitates exclude enzyme from solution and furnish a physical explanation of the supposed inhibition. This finding is of interest because substantial evidence has accumulated indicating that high concentrations of DDT, DDE, and dieldrin in raptor birds may be causally related to abnormally thin eggshells and nesting failures (Risebrough *et al.*, 1968). Carbonic anhydrase is present in the oviduct of birds and is believed to be important in eggshell formation (Bitman *et al.*, 1970; Heald *et al.*, 1968).

The preceding paragraphs support the assertion that the study of enzyme kinetics is a valuable diagnostic aid in dealing with inhibition from an environmental and medical point of view. Too often these studies are incomplete. When such problems are approached in a rational, quantitative fashion, the way may be clear to an understanding of the mechanism of enzyme action and inhibition, and the design of agents which will alleviate, or prevent, undesirable enzyme inhibition. The rest of this review describes various approaches that may be used in the elucidation of the mechanism of enzyme action.

THE STEADY STATE IN ENZYME KINETICS

During the latter part of the nineteenth century a number of investigations were made of the kinetics of enzyme reactions, and it was found that the behaviour observed frequently differed from that found in the simple homogeneous chemical reactions studied up to that time. In particular, it was found that often the rate of the reaction was not simply proportional to the concentrations of the reacting substances. Brown (1902) showed that a given amount of invertase brings about the breakdown of a constant amount of sucrose in unit time in solutions of varying concentration. He also discovered that in a given run the amount transformed is not proportional to time, but shows a falling-off from linearity; the apparent order with respect to time is therefore greater than zero. To explain these observations, Brown proposed the hypothesis that a definite complex forms between enzyme and substrate. This concept of the enzyme-substrate complex has been of enormous importance in enzyme kinetics, and has been amply supported by more recent evidence.

In most instances, enzymes are such efficient catalysts that concentrations must be very low to make the reaction occur during a convenient time interval. Typical enzyme concentrations range from approximately 10^{-8} to 10^{-10} *M*, while substrate concentrations are usually greater than 10^{-6} *M*. Under these conditions, all reaction intermediates are present at much smaller concentrations than the substrates, so that they can be considered, after a short induction period, to be in a steady state. A majority of enzyme reactions have been investigated under these conditions, and such experiments are appropriately called steady-state kinetic studies.

The classical one-intermediate reaction scheme that may apply to some one-substrate systems, uncomplicated

by the reverse reactions or the effects of modifiers (activators or inhibitors), is:

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA \xrightarrow{k_2} E + X \qquad (1)$$

where E is enzyme, A is substrate, EA is an enzyme-substrate complex, and X is the product. We can derive an expression for the rate of this reaction by using the steady-state approximation and the enzyme conservation relation. The rate of appearance of product is written as:

$$v = \frac{d[X]}{dt} = k_2[EA] \qquad (2)$$

By the steady-state approximation, we assume that since the enzyme and its intermediates are in concentrations very much less than that of the substrate, the rate of change of the concentrations of these species is zero (i.e., they are consumed as fast as they are produced), and:

$$\frac{d[E]}{dt} = -k_1[E][A] + (k_{-1} + k_2)[EA] = 0 \qquad (3)$$

$$\frac{d[EA]}{dt} = k_1[E][A] - (k_{-1} + k_2)[EA] = 0 \qquad (4)$$

and the enzyme conservation relation is:

$$[E]_0 = [E] + [EA] \qquad (5)$$

Combining eq. 5 with either eq. 3 or 4,

$$[EA] = \frac{[E]_0[A]}{\frac{k_{-1} + k_2}{k_1} + [A]} \qquad (6)$$

and, from eq. 2,

$$v = k_2[EA] = \frac{k_2[E]_0[A]}{\frac{k_{-1} + k_2}{k_1} + [A]} \qquad (7)$$

which may be written as:

$$v = \frac{V[A]}{K_A + [A]} \qquad (8)$$

where V, the maximum velocity, is equal to $k_2[E]_0$, and K_A, the Michaelis constant, is equal to $(k_{-1} + k_2)/k_1$. If we plot v vs [A] (Fig. 1), we have a rectangular hyperbola from which we may derive values of V and K_A. A more manageable plotting form, the Lineweaver-Burk plot, results from taking the reciprocal of eq. 8:

$$\frac{1}{v} = \frac{1}{V} + \frac{K_A}{V}\frac{1}{[A]} \qquad (9)$$

and a plot of 1/v vs 1/[A] results in a straight line of slope K_A/V and intercept 1/V (Fig. 2).

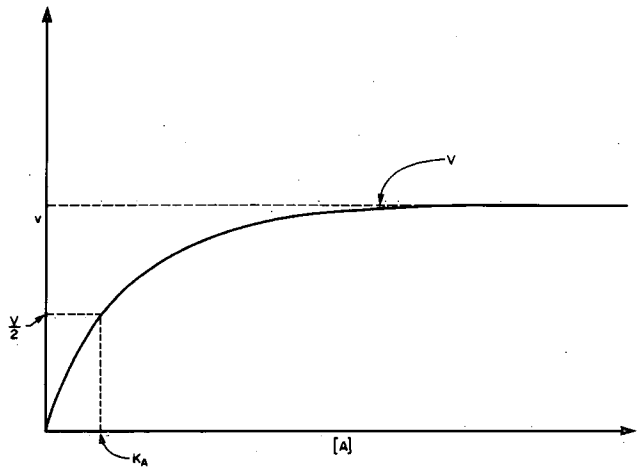


Figure 1. A plot of initial velocity, v, vs the concentration of substrate, A (eq. 8). The maximum velocity, V, is given by the horizontal portion of the curve, and the Michaelis constant, K_A, corresponds to that concentration of substrate which yields half-maximal velocity.

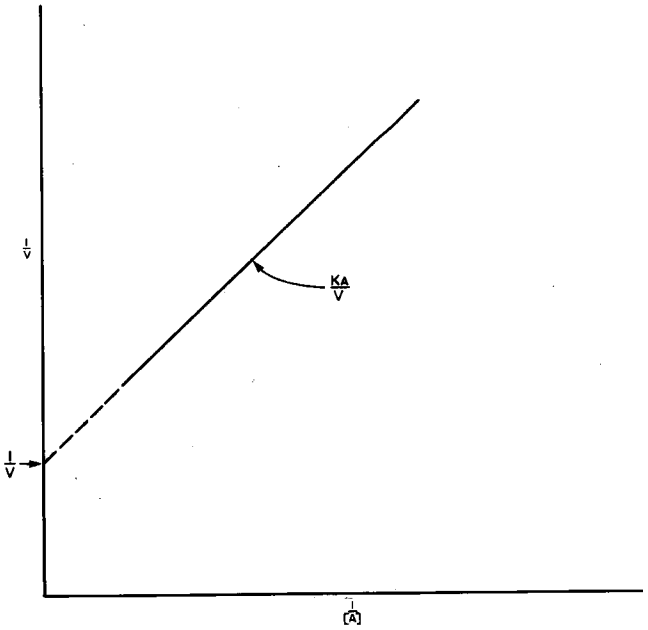


Figure 2. A Lineweaver-Burk plot of 1/v vs 1/[A] for a typical enzyme-catalyzed reaction (eq. 9). The slope is K_A/V and the intercept is 1/V.

A rather more realistic enzyme reaction scheme, e.g. for ester hydrolysis, in which an acyl enzyme is formed with simultaneous production of alcohol prior to the deacylation step producing acid, is the following:

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA \xrightarrow{k_2} EA' \xrightarrow{k_3} E + Y \qquad (10)$$
$$\qquad\qquad\qquad\qquad\quad \searrow X$$

The steady-state derivation for this reaction scheme yields

$$v = \frac{V[A]}{K_A + [A]} \qquad (11)$$

where, now,

$$V = \frac{k_2 k_3}{k_2 + k_3} [E]_0 \qquad (12)$$

and

$$K_A = \frac{k_3 (k_{-1} + k_2)}{k_1 (k_2 + k_3)} \qquad (13)$$

In these two treatments we have neglected certain reverse rate constants because many enzyme-catalyzed reactions are irreversible with regard to these steps. It is important to realize that all types of mechanisms of enzyme reactions (involving any number of intermediates, and with or without reverse rate constants in steps subsequent to initial complex formation) yield Michaelis-Menten behaviour, i.e., they obey an equation of the form of eqs. 8 and 11, but the maximum velocity and Michaelis constant are made up of different collections of rate constants, depending upon the chosen reaction scheme. The corollary is that observance of Michaelis-Menten behaviour does not indicate the number of intermediates involved.

The validity of the steady-state hypothesis in enzyme kinetics has often been discussed (Hommes, 1962; Walter and Morales, 1964; Walter, 1966, Wong, 1965), and the matter is complicated, but it seems generally true that the steady-state hypothesis is satisfactory provided that the ratio of the substrate concentration to the enzyme concentration is one thousand or greater.

THE TRANSIENT PHASE OF ENZYME-CATALYZED REACTIONS

Besides the steady-state methods treated in the previous section, other procedures yield quantitative data of value in characterizing the reactions that comprise an enzyme mechanism. These methods deal with the direct measurement of rates in enzyme-catalyzed reactions under circumstances in which the property observed does not reflect the overall steady state. This category includes the study of the transient phase (pre-steady-state) of reactions, using equipment that resolves events on a scale of milli- or microseconds.

We have already developed suitable differential equations describing the steady-state situation for the one-substrate mechanism. Integrated forms of these equations yield time course curves for the steady state. For initial velocities in the absence of product and where $[A]_0 >> [E]_0$ we have, as in eqs. 2 and 4,

$$\frac{d[X]}{dt} = k_2[EA] \qquad (14)$$

and

$$\frac{d[EA]}{dt} = k_1[A]_0([E]_0 - [EA]) - (k_{-1} + k_2)[EA] \qquad (15)$$

Application of the steady-state approximation to eq. 15 gives:

$$[EA]_0 = \frac{k_1[A]_0[E]_0}{k_{-1} + k_2 + k_1[A]_0} = \frac{[A]_0[E]_0}{K_A + [A]_0} \qquad (16)$$

This is the hypothetical concentration of EA at zero time under the steady-state assumption. It is the steady-state level assumed for the initial velocity in conventional steady-state analysis (Fig. 3). During the time after the steady state has been reached but before much product has accumulated, $[EA]_0$ equals [EA] to a very good approximation. After that time, dropping the subscript zero from $[EA]_0$ and replacing $[A]_0$ by $[A]_0 - x$ provides a general equation for [EA] during the whole steady state if $[A]_0 >> [E]_0$. For the time before the steady state is achieved, eq. 15 can be integrated to yield a non-steady-state expression for [EA] in terms of $[EA]_0$ and time:

$$[EA] = [EA]_0 \left(1 - \exp \frac{-k_1[E]_0[A]_0 t}{[EA]_0}\right) \qquad (17)$$

If we substitute eq. 16 into 17,

$$[EA] = \frac{[E]_0[A]_0}{K_A + [A]_0} \left(1 - \exp -k_1 t (K_A + [A]_0)\right) \qquad (18)$$

It can be seen that this function approaches the value of the first term (i.e., of the hypothetical steady state $[EA]_0$) when t is large. The exponential term is of importance only at low values of t, where it causes [EA] to diminish to zero at zero time. Thus eq. 18 describes the time course of [EA] during the transient pre-steady-state phase of the reaction. The solid curve in Figure 3 describes the actual time course of [EA]. Also shown in Figure 3 are lines illustrating how the foregoing equations describe various portions of the curve at different times.

These relations provide a basis for analysis of data obtained by direct observation of an enzymic intermediate. It will ordinarily be more useful to have a time course for product formation, as it can usually be followed experimentally. The differential expression for the transient phase is obtained by combining eqs. 14 and 18:

$$\frac{d[X]}{dt} = \frac{k_2[E]_0[A]_0}{K_A + [A]} \left(1 - \exp -k_1 t(K_A + [A]_0)\right) \quad (19)$$

This equation can also be integrated with $t = 0$, $[X] = 0$ describing the initial condition in the reaction mixture, to yield the time course of the product X;

$$[X] = \frac{k_2[E]_0[A]_0 t}{K_A + [A]_0} - \frac{k_2[E]_0[A]_0}{(K_A + [A]_0)(k_{-1} + k_2 + k_1[A]_0)} \times \left(1 - \exp -k_1 t(K_A + [A]_0)\right) \quad (20)$$

Note that when t is large, a plot of [X] vs t has a slope of $k_2[E]_0[A]_0/(K_A + [A]_0)$, i.e., this is the steady-state rate, as expected. Extrapolation of this steady-state rate to $[X] = 0$ gives the relation:

$$\frac{k_2[E]_0[A]_0 t}{K_A + [A]_0} = \frac{k_2[E]_0[A]_0}{(K_A + [A]_0)(k_{-1} + k_2 + k_1[A]_0)} \quad (21)$$

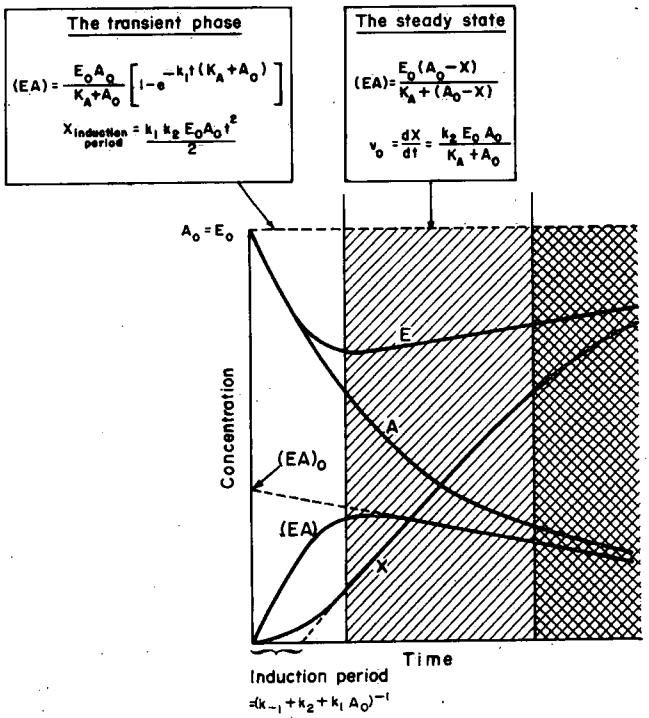


Figure 3. The time course of events during the transient phase for a one-intermediate mechanism (eqs. 16-22). From *Enzymic Catalysis* by John Westley, reproduced by permission of Harper and Row (1969, p. 131).

from which:

$$t_{[X]=0} = k_{-1} + k_2 + k_1[A]_0 \quad (22)$$

Figure 3 shows this result graphically. In practical use, this extrapolated time, called the induction period, is determined at several values of $[A]_0$, and eq. 22 is then used to obtain separate values for k_1 and the sum $k_{-1} + k_2$. Since the value of k_2 is generally known from steady-state studies ($V = k_2[E]_0$), this procedure permits evaluation of all the rate constants in the mechanism.

Laidler (1955) and Gutfreund (1955) have applied similar treatments to the study of the transient phase of reaction schemes more complicated than that of a single-intermediate reaction.

As mentioned before, special equipment is needed to monitor the kinetics of such fast reactions. One such piece of equipment is the stopped-flow apparatus. In this apparatus, two reactants are driven by compressed air-driven syringes into a mixing chamber, whence they flow to an observation chamber, and the flow is stopped by mechanical means to allow kinetic observation. The reaction is followed spectrophotometrically, with monochromatic light entering the reaction cuvette from one end and encountering a photomultiplier tube at the other end. The output is displayed on an oscilloscope. The reaction can be observed from about two milliseconds after mixing, and first-order reactions with rate constants up to about 300 s^{-1} may be followed. Figure 4 is a schematic diagram of the commercially available Durrum stopped-flow apparatus, and Figure 5 is a photograph of the apparatus. Flow methods have been reviewed by Chance (1963), Roughton (1963), Caldin (1964), and Gibson (1969).

Although the flow methods have permitted measurements on a milliseconds time scale, rather than the seconds scale accessible with conventional methods, many important reactions still cannot be approached directly without yet faster techniques. Eigen and his associates developed the relaxation methods, which are capable of dealing with kinetic observations on a microseconds time scale. Bimolecular reactions with rate constants as high as $10^9 M^{-1} s^{-1}$ can be measured by these powerful techniques. A number of studies have made important applications of relaxation kinetics to enzyme-catalyzed reactions (e.g., Erman and Hammes, 1966).

The general principles on which relaxation analysis is based are straightforward. A reaction at equilibrium is subjected to a rapid change in an intensive variable (e.g., temperature or pressure) that affects the equilibrium. Adjustment of the reaction to the new equilibrium is then observed by spectrophotometric or other means. In a typical

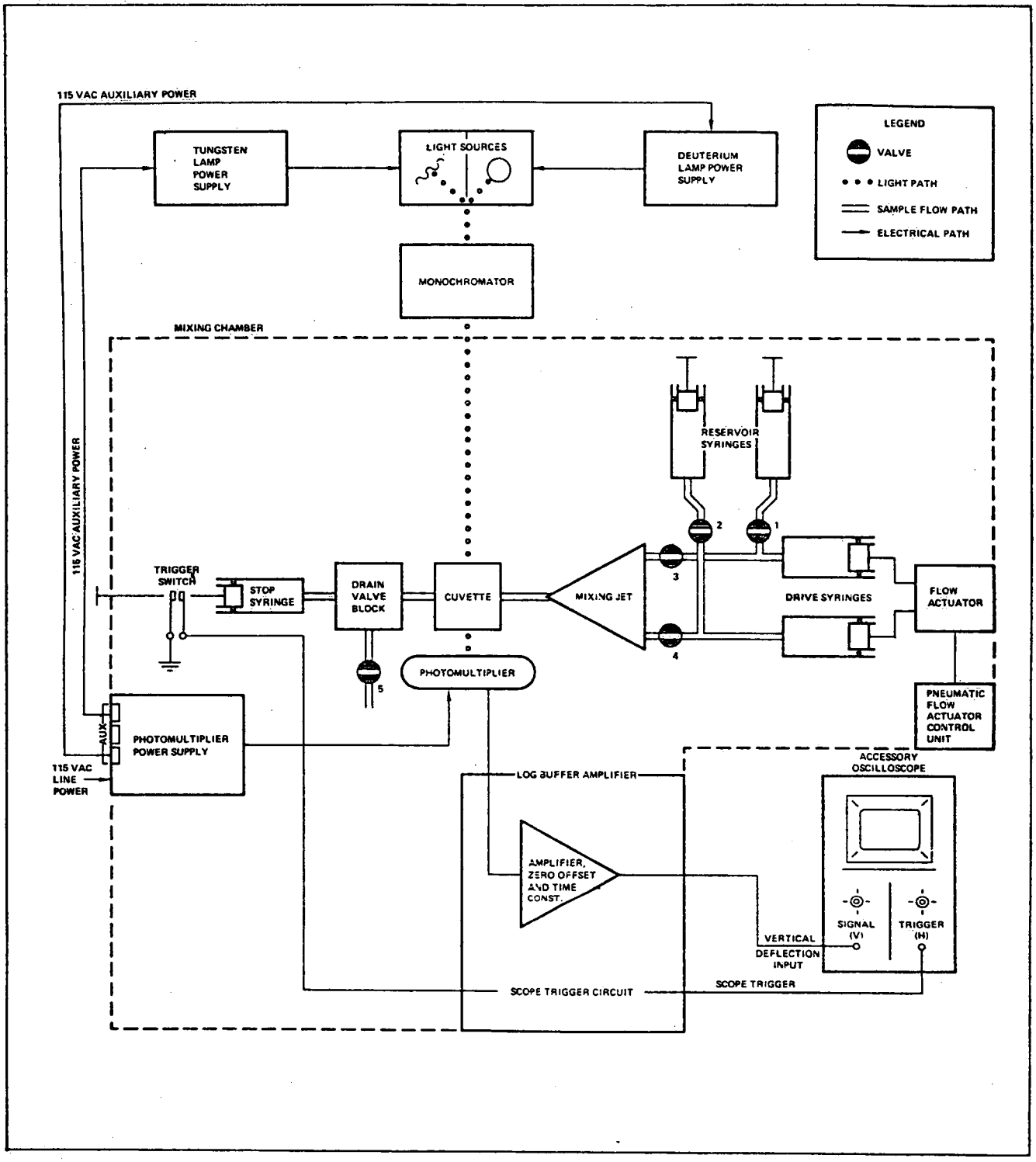


Figure 4. A schematic diagram of a stopped-flow apparatus. Courtesy of Durrum Instrument Corporation.

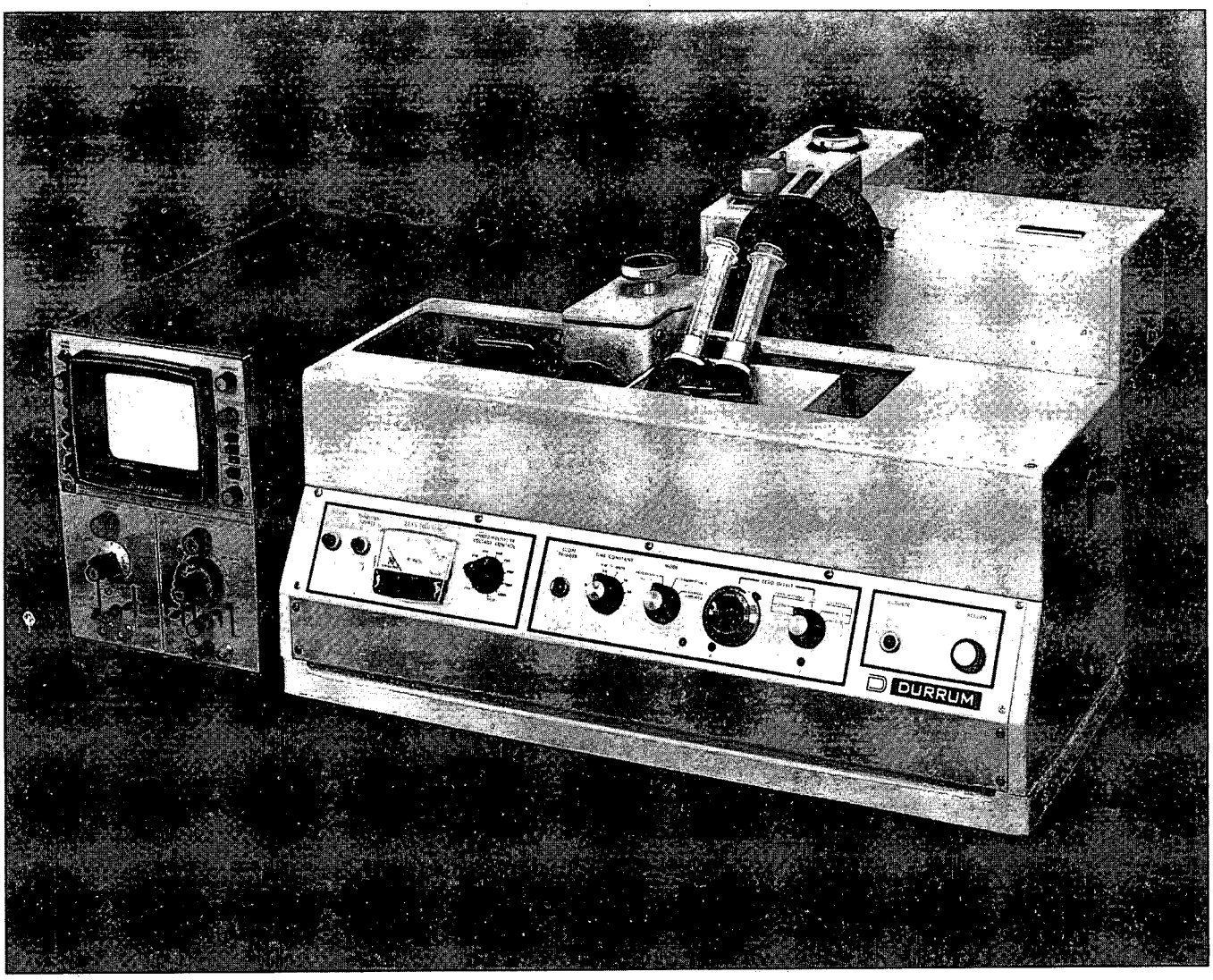


Figure 5. A photograph of a stopped-flow apparatus. Courtesy of Durrum Instrument Corporation.

experiment, an enzyme-catalyzed reaction is at equilibrium in a specially equipped spectrophotometer cell. A large electrical capacitor is discharged through the solution, raising the temperature 10 degrees within one millisecond (a "temperature jump") and observation begins. Clearly, oscilloscope recording of the event during the "relaxation" to the new equilibrium position can start very soon after those events begin. The advantage of the method is that neither mixing nor transportation of the reaction system are necessary to initiate the observation of changes. Thus the time-limiting features of the flow methods are avoided.

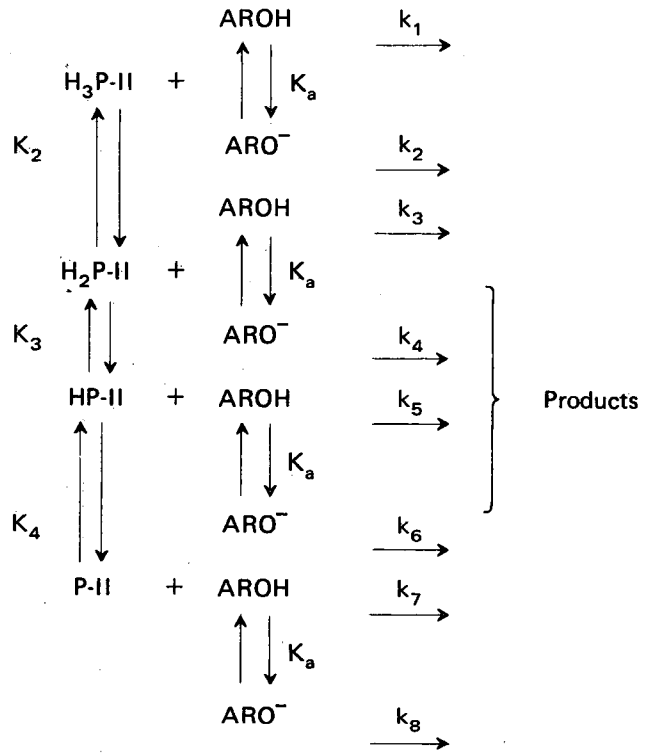
THE EFFECT OF pH ON THE KINETICS OF ENZYME-CATALYZED REACTIONS

Biological systems, particularly living cells, are more sensitive to changes of temperature and pH than most non-biological chemical reactions, and this is due largely to the properties of the enzymes upon which these systems depend. A particularly important tool in the investigation of the kinetics of enzyme-catalyzed reactions is the variation of the pH of the reaction medium; this section gives an account of a new approach to the problem.

In general the shape of log k vs pH plots is informative with regard to the identity of ionizable groups in the enzyme that participate in the reaction in a catalytically important manner. If, for example, a group of pK 7 participates in a reaction, then the log k - pH plot will show an inflection at pH 7. Dixon's rules (Dixon and Webb, 1964) state, in particular, that a decrease in slope with increasing pH in a rate-pH plot is indicative of the pK of a catalytically important group. Important details are pointed out in the following example which describes two methods of treating an experimental log k-pH plot.

The reaction to be considered is the oxidation of p-cresol by lactoperoxidase compound II (Maguire and Dunford, 1973). The plot of log k vs pH is shown in Figure 6. The plot is well defined, and can be seen with the aid of Dixon's rules to depend on the acid dissociation of the substrate at pH 10 and the ionization of three kinetically important groups having pK values of about 2, 6, and 9.5. The minimum reaction scheme suggested by the data is given by Scheme I, in which P-II, HP-II, H_2P-II, and H_3P-II are the four kinetically distinct states of protonation of compound II, the K's are acid dissociation constants, the k's are bimolecular rate constants, and AROH and ARO^- refer to the protonated and dissociated forms of p-cresol, respectively:

Scheme I



The H's have been omitted for convenience. The vertical arrows denote fast non-rate-determining proton transfers. The corresponding kinetic expression is:

$$k = \left[\frac{k_1[H^+]^4}{K_aK_2K_3K_4} + \left(k_3 + k_2\frac{K_a}{K_2}\right)\frac{[H^+]^3}{K_aK_3K_4} + \left(k_5 + \frac{k_4K_a}{K_3}\right)[H^+]^2 + \left(k_7 + \frac{k_6k_a}{K_4}\right)\frac{[H^+]}{K_a} + k_8\right] \Bigg/ \left[\left(1 + \frac{[H^+]}{K_4} + \frac{[H^+]^2}{K_3K_4} + \frac{[H^+]^3}{K_2K_3K_4}\right)\left(1 + \frac{[H^+]}{K_a}\right)\right] \quad (23)$$

The smooth curve in Figure 6 shows the best fit to eq. 23.

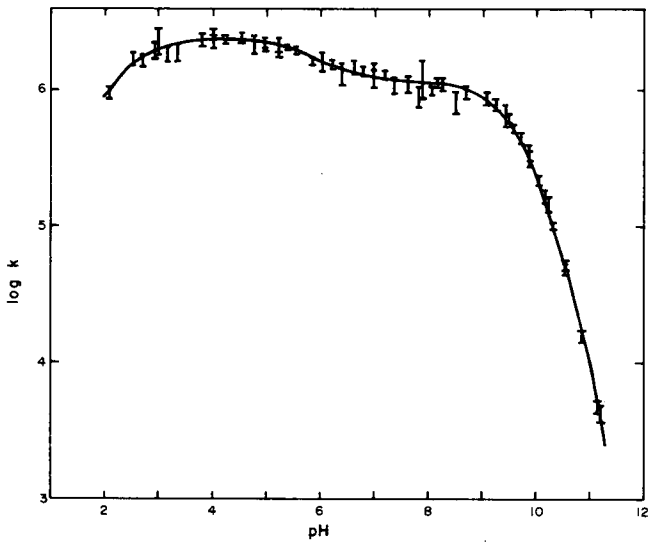
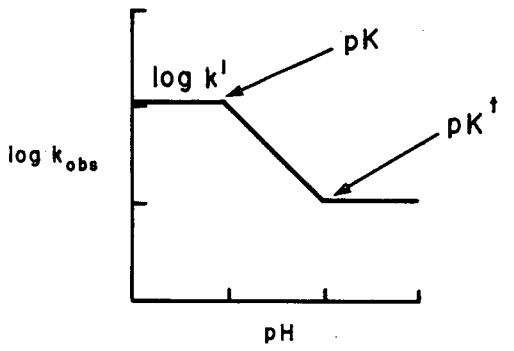


Figure 6. A plot of log k vs pH for the reaction between lactoperoxidase compound II and p-cresol (Maguire and Dunford, 1973). The solid line is a curve calculated by computer for the fitting of the data to eq. 23, and the error bars represent standard deviations in the rate constant, k. Reproduced by permission of the National Research Council of Canada from the Canadian Journal of Chemistry, 51, 1721-23 (1973).

An alternative and algebraically more simple method of analyzing the log k vs pH plot, which avoids the need to consider kinetically indistinguishable or insignificant rate processes, is provided by the use of transition-state acid-dissociation constants. Critchlow and Dunford (1972) have shown that an acid dissociation in the transition state gives rise to an increase in slope in the plot of log k vs pH, whereas an acid dissociation in the ground state causes a decrease in slope. The number and approximate pK_a values of such ionizations may therefore be read off the plot by an extension of Dixon's rules in the same way as the pK values were read before. The essential significance of this treatment may be arrived at more simply by considering the experimental log k-pH profile in a less complicated plot, e.g.,



The change of slope on passing from the horizontal portion of the plot at high pH to the portion of unit negative slope must be caused by a change in the reaction route from one involving a given set of reactants to one involving a different set containing a total of one more proton. The transition state at medium (and low) pH therefore contains one more proton than that for reaction at high pH, and the point at which the curve begins to rise in going to lower pH may be associated formally with a transition-state acid dissociation. The subsequent return to a horizontal relationship at lower pH is of course due to the protonation of the ground state and is covered by Dixon's rules.

To use this treatment in describing the mechanism of a reaction each transition-state acid dissociation constant ($pK^{\dagger}$) must be paired with that for the ground-state dissociation constant (pK) considered to involve the same ionizing group, although both members of each pair may not be found in the pH range of the study. The pK difference between ground- and transition-states, which may be regarded as expressing the sensitivity of the rate to the ionization in question, provides a qualitative measure of the change in environment accompanying the activation process. For example, if protonation of a particular protein residue facilitates a reaction, then $pK^{\dagger} > pK$, as in the simple reaction considered above. Conversely, if $pK^{\dagger} < pK$, then protonation of the residue will retard the rate of the reaction.

In the actual derivation of an equation describing the observed rate constant as a function of pH, dimensionless Michaelis functions (Dixon and Webb, 1964) are used, and the final result is (Critchlow and Dunford, 1972):

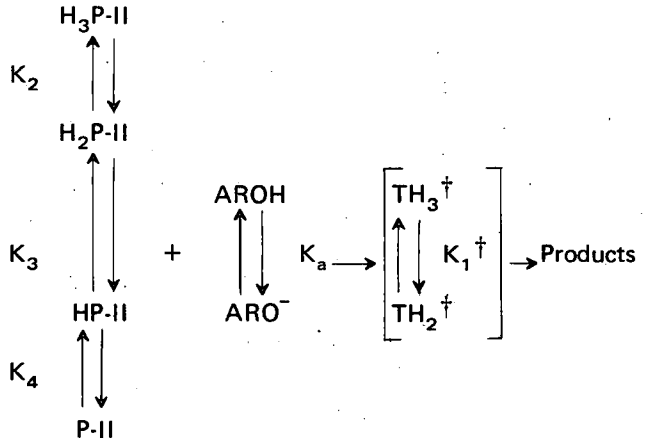
$$k_{obs} = \frac{k' f_n^{\dagger}}{f_i^{E} f_0^{A} f_k^{B} \ldots . f_Z^{Z}} \qquad (24)$$

where k' is a pH-independent rate constant, the f's are Michaelis functions, E is the enzyme, A,B, ... Z are reactants, and the subscripts refer to particular states of ionization of the enzyme, substrates, and transition state. For example, the corresponding equation for the simple reaction shown above is:

$$k_{obs} = k' \frac{(1 + K^{\dagger}/[H^+])}{(1 + K/[H^+])} \qquad (25)$$

For the oxidation of p-cresol by lactoperoxidase compound II, Figure 6 shows a single kinetically important transition-state ionization of $pK^{\dagger}$ value about 6 (increase of slope with increasing pH, from −1 to 0), so that the reaction may be represented by:

Scheme II



where the species in square brackets represent transition-state complexes in different stages of protonation, $K_1^{\dagger}$ is the associated transition-state acid-dissociation constant, and the other symbols have been defined for Scheme I. The resulting kinetic expression is:

$$k = \frac{k' (1 + K_1^{\dagger}/[H^+])}{\left(\frac{[H^+]}{K_2} + 1 + \frac{K_3}{[H^+]} + \frac{K_3 K_4}{[H^+]^2}\right)\left(1 + \frac{K_a}{[H^+]}\right)} \qquad (26)$$

where k' is the pH-independent rate constant associated with the pH-independent part of the curve between pH 3 and pH 5. This equation gives the same results as eq. 23.

The significance and advantages of this method of treating pH-rate effects in terms of transition-state acid-dissociation constants are discussed in more detail by Critchlow and Dunford (1972), but the readily apparent advantage of simplicity can be seen by comparing eqs. 23 and 26.

Another example of the use of the Critchlow-Dunford treatment is shown in Figure 7. Pocker and Meany (1965) claim that the slight perturbation apparent around pH 7.4 in the plot of k vs pH for the hydration of acetaldehyde by carbonic anhydrase is real rather than an artifact, since approximately 50 runs were made to determine the exact curvature in this region. We suggest that there is a ground-state pK at pK 7.2 (downward bend with increasing pH) and a transition-state $pK^{\dagger}$ at pH 7.4 (upward bend with increasing pH). These two values are quite close to each other, which may refer to protonation of the same group; if this is so, protonation of the group would exert only a slight accelerating influence on the rate of the reaction, indicating that the group is positioned close to the active centre of reaction, but plays little part in the

catalytic process. This seems reasonable compared to the large difference in rates between pH 5 and 8 brought about by the group of pK 7.

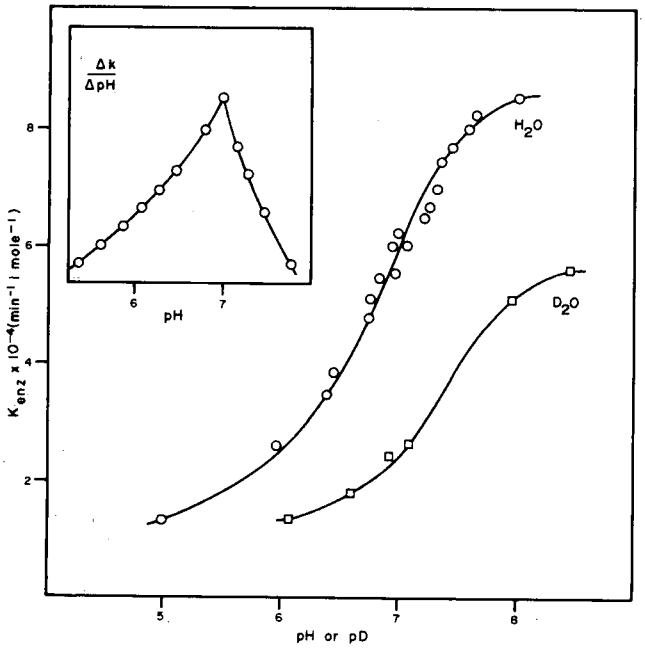


Figure 7. The bovine carbonic anhydrase-catalyzed hydration of acetaldehyde as a function of pH and pD in 0.002 *M* phosphate buffer at 0° (Pocker and Meany, 1965). Reproduced by permission of the American Chemical Society from Biochemistry, 4, 2535-41 (1965).

The method of Critchlow and Dunford is easier to apply than that of Dixon, and also it is possible to extract more information. In general, however, both methods yield at least the desired values of the pK's of catalytically important ionizing groups in the active centre of the enzyme. We are then left to deduce the identity of the groups. Various side chain groups ionize at different values of pH, e.g., carboxylic acids at pH 4, imidazole groups at pH 6.5-8, amino groups at pH 8-10, and sulfhydryl groups at pH 9-10. Further information may be deduced from, for example, the variation of the pK value with temperature. Different groups show different temperature dependence. Once the groups involved in the catalytic process are identified, it is possible to comment on the mechanism of the reaction.

INHIBITION OF ENZYME-CATALYZED REACTIONS IN THE STEADY STATE

It is commonly found that foreign substances influence the rates of enzyme-catalyzed reactions. A reduction of rate is called inhibition, and the substance bringing about the reduction is called an inhibitor (the rate is sometimes increased by an added substance; this is called activation).

Classically the study of inhibitory effects on isolated enzymic reactions and on metabolic sequences in general has been extremely important in establishing the nature of the free reactants, the nature of their binding site on the enzyme, and the specificity and mechanism of the reaction. In the cell, inhibition of key reactions by substances that may be products of the reaction itself or of the same metabolic sequence provides a ready and delicately poised control mechanism for the maintenance of a relatively constant intracellular environment and for its response to alterations in the external milieu.

Three different kinds of inhibition occur in enzyme systems, depending on the way in which the degree of inhibition, i, is related to the velocity of the inhibited reaction, v, and that of the uninhibited reaction, v_0. It is defined as the reduction in velocity, $v_0 - v$, brought about by a given amount of inhibitor divided by the velocity of the uninhibited reaction:

$$i = \frac{v_0 - v}{v_0} \qquad (27)$$

The three kinds of inhibition are:

(i) the degree of inhibition is unaffected by the concentration of substrate; in this case one speaks of *non-competitive inhibition.*

(ii) the degree of inhibition depends upon the amount of substrate present. Usually the degree of inhibition is reduced as the substrate concentration is increased; in this case, one says that the inhibition is *competitive.*

(iii) the degree of inhibition increases as the substrate concentration increases; this is called *anti-competitive,* or *uncompetitive,* inhibition.

The use of the terms competitive, non-competitive, and anti-competitive may have been somewhat unfortunate, since the terms imply conclusions about mechanisms that are not necessarily justified. The term competitive, for example, was used because it was thought that when there was a reduction of degree of inhibition with substrate concentration, the inhibitor and substrate molecules were competing with one another for a site on the enzyme surface; and when the degree of inhibition was independent of substrate concentration, it was thought that no such competition occurred, the substrate and inhibitor molecules being able to become simultaneously attached to the enzyme. Such conclusions are only justified if the mechanism is of the simple Michaelis type, with a single

intermediate. If a second intermediate is involved, or there is more than one substrate, no simple correlation exists between the behaviour observed and the competition for enzyme sites; a given mechanism can, in fact, lead to different types of behaviour depending upon the relative magnitudes of rate constants. In spite of this, it is convenient to use the terms competitive, non-competitive, and anti-competitive to describe the behaviour observed, with no implication as to mechanisms.

Equations describing the three simple types of inhibition, together with their associated Lineweaver-Burk plots, are shown in Figure 8. The three types can be distinguished graphically.

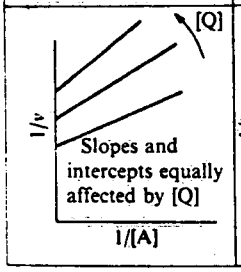
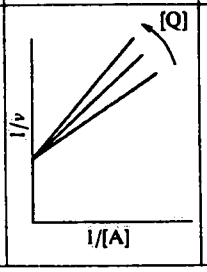
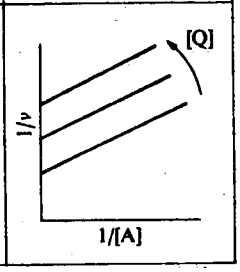
Pure non-competitive	Pure competitive	Pure anticompetitive
$v=\frac{V[A]}{(K_A+[A])\left(1+\frac{[Q]}{K_Q}\right)}$	$v=\frac{V[A]}{K_A\left(1+\frac{[Q]}{K_Q}\right)+[A]}$	$v=\frac{V[A]}{K_A+\left(1+\frac{[Q]}{K_Q}\right)[A]}$
$i=\frac{[Q]}{K_Q+[Q]}$	$i=\frac{K_A\frac{[Q]}{K_Q}}{K_A\left(1+\frac{[Q]}{K_Q}\right)+[A]}$	$i=\frac{[A]\frac{[Q]}{K_Q}}{K_A+\left(1+\frac{[Q]}{K_Q}\right)[A]}$
$\frac{V}{v}=\frac{K_A}{[A]}\left(1+\frac{[Q]}{K_Q}\right)+1+\frac{[Q]}{K_Q}$	$\frac{V}{v}=\frac{K_A}{[A]}\left(1+\frac{[Q]}{K_Q}\right)+1$	$\frac{V}{v}=\frac{K_A}{[A]}+1+\frac{[Q]}{K_Q}$
		

Figure 8. Summary of equations and plots for the three pure forms of inhibition. Q is the inhibitor and K_Q, its constant for binding to the enzyme (Laidler and Bunting, 1973, p. 93). Reproduced by permission of the Clarendon Press.

Figure 9 shows, for example, the competitive nature of ester inhibition of the carbonic anhydrase-catalyzed hydrolysis of p-nitrophenyl acetate (Pocker and Stone, 1968).

It should be mentioned that various conditions lead to *mixed* forms of inhibition. Also, inhibition in terms of schemes involving more than one intermediate is very complex, but can reduce to different simple forms under different conditions. Laidler and Bunting (1973) have given a good account of this subject.

The possibility of *irreversible* inhibition effects must also be borne in mind. Suppose, for example, that Q combined irreversibly with E but not at all with EA. The result is simply a diminution of the concentration of effective enzyme, with the extent depending on how much inhibitor is present. Addition of substrate would have no restoring effect, in contrast to the reversible case. The limiting rate V would be lowered because it contains a term in $[E]_0$, but the Michaelis constant, K_A would be unaffected. The behaviour would therefore appear to be *non-competitive.*

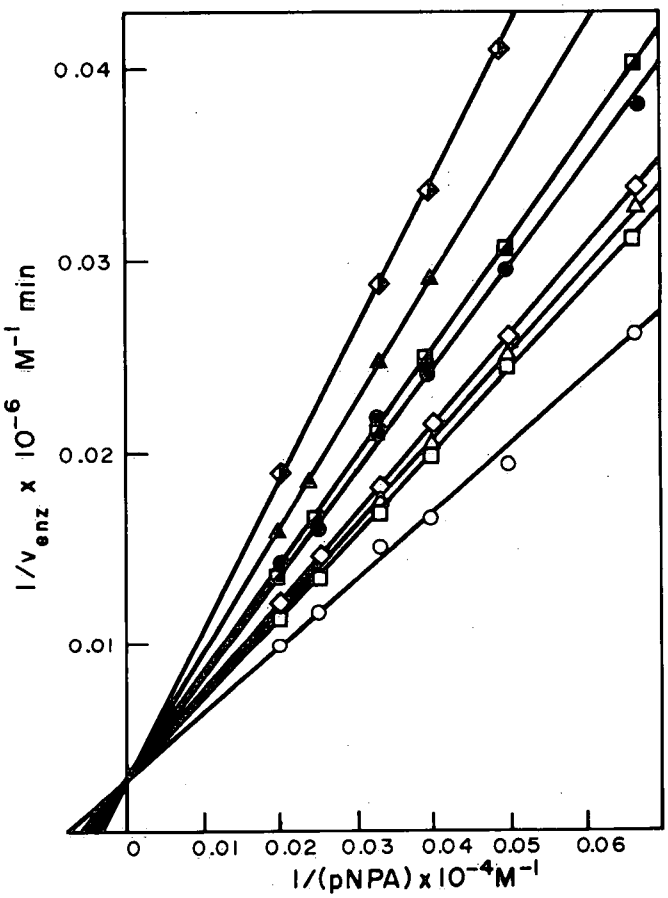


Figure 9. Lineweaver-Burk plots of the carbonic anhydrase-catalyzed hydrolysis of p-nitrophenyl acetate with added ester inhibitor at pH 7.6 and 25.0°, 10% (v/v) acetonitrile-phosphate buffer and an ionic strength of 0.09, [BCA] = 2.2×10^{-6} *M*. (O) no inhibitor added; (□) p-nitrophenyl trimethylacetate, 10^{-4} *M*; (△) m-nitrophenyl acetate, 3.2×10^{-3} *M*; (◇) β-naphthyl acetate, 3.2×10^{-4} *M*; (●) p-nitrophenyl n-hexanoate, 1.5×10^{-4} *M*; (⧄) ethyl acetate, 0.33 *M*; (▲) o-nitrophenyl acetate, 3.2×10^{-3} *M*; (◆) phenyl acetate, 1.6×10^{-2} *M* (Pocker and Stone, 1968). Reproduced by permission of the American Chemical Society from Biochemistry, 7, 3021-31 (1968).

INHIBITION OF ENZYME-CATALYZED REACTIONS IN THE TRANSIENT PHASE

As we have seen above, it is possible to determine values of individual rate constants in enzyme reaction schemes from transient-phase studies. Several theoretical treatments of transient-phase kinetics have been given. The particular treatment presented here has been developed recently by Laidler and co-workers (e.g., Maguire *et al.*, 1974). It will be applied to a kinetic study of an enzyme-catalyzed reaction that is uninhibited and competitively inhibited; the reaction is the hydrolysis of p-nitrophenyl acetate by α-chymotrypsin, and the competitive inhibitor is indole.

The mechanism of the hydrolysis of p-nitrophenyl acetate by α-chymotrypsin proceeds through a two-intermediate reaction scheme in which the first product is p-nitrophenol, EA′ is the acyl enzyme, and the second product is acetic acid. It was of interest to determine values of individual rate constants for the uninhibited reaction and the rate constants for competitive inhibition by indole as a test of the theory.

This analysis is confined to the case in which substrate and inhibitor concentrations are greatly in excess of the concentration of the enzyme. The mechanism is written as:

$$\begin{array}{ccccccc} & & & k_1 & & k_2 & k_3 \\ E & + & A & \longrightarrow & EA & \longrightarrow & EA' \longrightarrow E + Y \\ & & & \longleftarrow & m & \searrow & n \qquad y \\ e_0 - m - n & & a_0 & & k_{-1} & X & \\ & & & & & x & \end{array} \qquad (28)$$

in which the lower case k's are the rate constants and the other lower case letters refer to the concentration of the appropriate species at any time. The differential rate equations for the system are:

$$\frac{dm}{dt} = k_1 a_0 (e_0 - m - n) - \bar{k}\, m \qquad (29)$$

$$\frac{dn}{dt} = k_2 m - k_3 n \qquad (30)$$

$$\frac{dx}{dt} = k_2 m \qquad (31)$$

where $\bar{k} = k_{-1} + k_2$. Replacement of the differentials by operators, P, according to the Laplace-Carson transform method leads to:

$$m = \frac{k_1 a_0 e_0 (P + k_3)}{(P + \lambda_1)(P + \lambda_2)} \qquad (32)$$

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

$$P^2 + P(k_1 a_0 + \bar{k} + k_3) + k_3 \bar{k} + k_1 a_0 (k_2 + k_3) = 0 \qquad (33)$$

and the following relations are obeyed by the roots:

$$\lambda_1 + \lambda_2 = k_1 a_0 + \bar{k} + k_3 \qquad (34)$$

$$\lambda_1 \lambda_2 = k_1 a_0 (k_2 + k_3) + \bar{k}\, k_3 \qquad (35)$$

In this study we were interested in the variation of X as a function of time, i.e., we monitored the rate of production of p-nitrophenol spectrophotometrically. Thus the original for m is:

$$m = \frac{k_1 k_3 a_0 e_0}{\lambda_1 \lambda_2} - \frac{k_1 a_0 e_0 (k_3 - \lambda_1) \exp(-\lambda_1 t)}{\lambda_1 (\lambda_2 - \lambda_1)} - \frac{k_1 a_0 e_0 (k_3 - \lambda_2)}{\lambda_2 (\lambda_1 - \lambda_2)} \exp(-\lambda_2 t) \qquad (36)$$

and substitution of eq. 36 into 31 and integration with respect to the relevant boundary conditions leads to the following equation for the variation of x with time:

$$x = \frac{k_1 k_2 k_3 a_0 e_0 t}{\lambda_1 \lambda_2} + \frac{k_1 k_2 a_0 e_0 (k_3 - \lambda_1)}{\lambda_1^2 (\lambda_2 - \lambda_1)} (\exp(-\lambda_1 t) - 1) + \frac{k_1 k_2 a_0 e_0 (k_3 - \lambda_2)}{\lambda_2^2 (\lambda_1 - \lambda_2)} (\exp(-\lambda_2 t) - 1) \qquad (37)$$

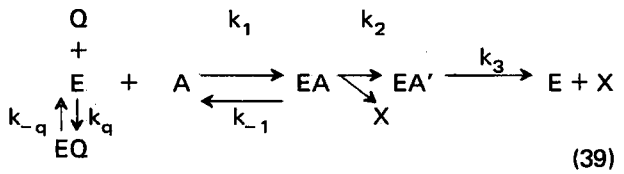
The important point to note about this equation is that it describes a *biphasic* exponential approach to the steady state, which is represented by the term linear in t. Equation 37 can be rearranged to one of the form:

$$-x + vt + \beta = \beta_1 \exp(-\lambda_1 t) + \beta_2 \exp(-\lambda_2 t) \qquad (38)$$

The data were analyzed with reference to eq. 38. The quantities β and v can be determined from the variation of x with time as seen in Fig. 10. A plot of $\ln(-x + vt + \beta)$ vs time will consist, to a good approximation (if λ_1 and λ_2 are not too close together), of two straight regions from which λ_1 and λ_2 can be determined. A plot of $\lambda_1 + \lambda_2$ against a_0 (cf. eq. 34) is linear, with a slope of k_1 and an intercept of $k_{-1} + k_2 + k_3$. A plot of $\lambda_1 \lambda_2$ against a_0 (cf. eq. 35) is also linear with a slope of $k_1 (k_2 + k_3)$ and an intercept of $(k_{-1} + k_2) k_3$. The individual constants k_1, k_{-1}, k_2, and k_3 can then be separated.

Hijazi and Laidler (1973) have used this method of integration and have obtained the equations for the

transient phase in the presence of a competitive inhibitor when the mechanism is:



(39)

The variation of X with time was found to have *triphasic* exponential dependence,

$$-x + vt + \beta = \beta_1 \exp(-\lambda_1 t) + \beta_2 \exp(-\lambda_2 t) + \beta_3 \exp(-\lambda_3 t) \quad (40)$$

where v, β, and the β_i's are, of course, different from those for the uninhibited reaction, and:

$$\lambda_1 + \lambda_2 + \lambda_3 = k_1 a_0 + k_{-q} + \bar{k} + k_3 + k_q q_0 \quad (41)$$

$$\lambda_1\lambda_2\lambda_3 = \bar{k}\, k_3 (k_q q_0 + k_{-q}) + k_1 k_3 k_{-q} q_0 + k_1 k_2 k_{-q} a_0 \quad (42)$$

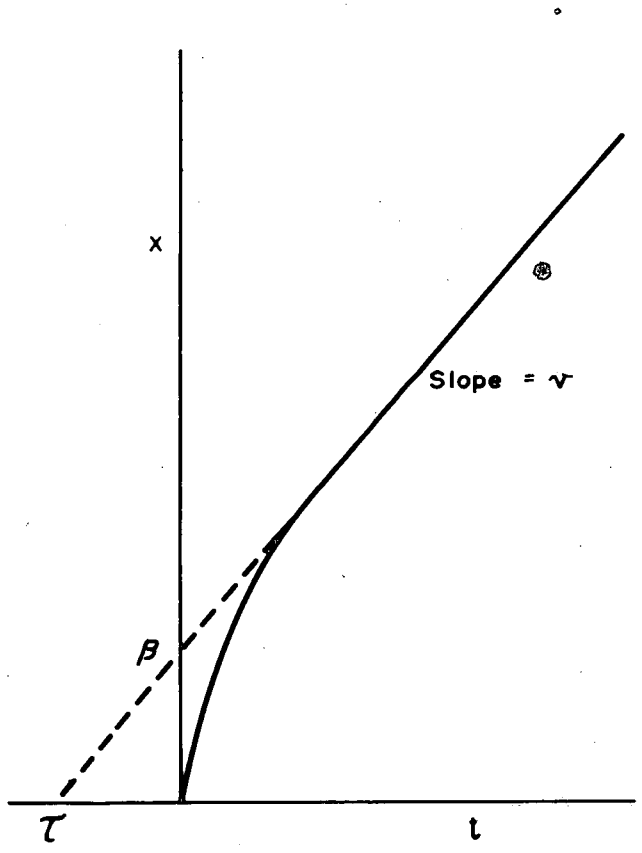


Figure 10. The transient-phase and the steady-state rates of appearance of product; v is the steady-state velocity, and β is obtained from an extrapolated intercept on the ordinate axis (Maguire *et al.*, 1974). Reproduced by permission of Biochimica et Biophysica Acta.

Analysis of data with reference to eq. 40 can be carried out as described above for the uninhibited case. Plots of ln $(-x + vt + \beta)$ against time will consist of three straight regions from which λ_1, λ_2, and λ_3 may be determined (if λ_1, λ_2, and λ_3 are not too close together). The λ_i's follow the relations in eqs. 41 and 42, and plots of $\lambda_1 + \lambda_2 + \lambda_3$ against q_0 can lead directly to values of k_q. The ratio $k_{-q}/k_q = K_q$ is known from steady-state analysis, so that k_{-q} can be calculated. On the other hand, plots of $\lambda_1\lambda_2\lambda_3$ against a_0 are linear with a slope of $k_1 k_2 k_{-q}$, and since k_1 and k_2 are known, k_{-q} can be calculated, and hence k_q (no additional advantage is to be gained by using the equation for $\lambda_1\lambda_2 + \lambda_1\lambda_3 + \lambda_2\lambda_3$).

In the present work the hydrolysis of p-nitrophenyl acetate by α-chymotrypsin was studied because the kinetics and mechanism of this reaction are well documented, and the reaction is slow enough that the transient phase can be observed conveniently with a stopped-flow apparatus. We observed the rate of release of p-nitrophenol, which is x in the reaction scheme shown above. Indole was used in the inhibition studies because it has been shown to be a competitive inhibitor.

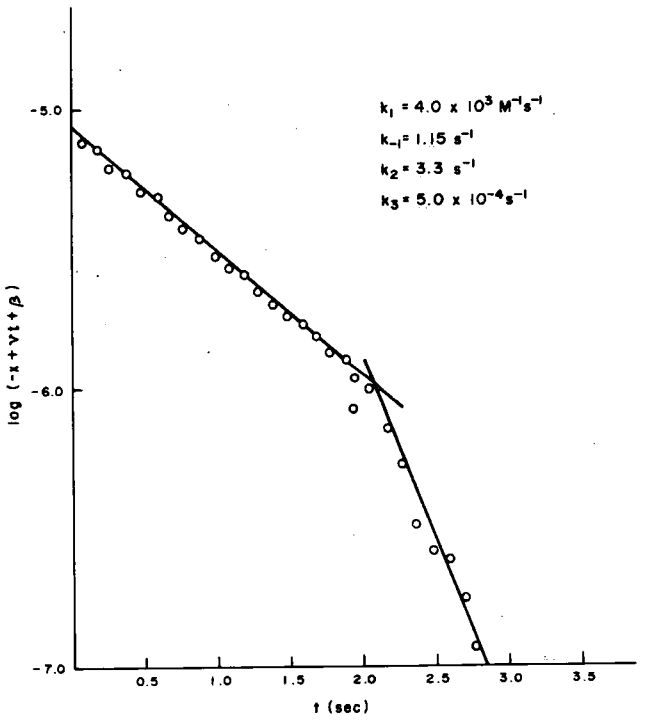


Figure 11. A plot of log $(-x + vt + \beta)$ vs time for the uninhibited reaction, and values of the rate constants obtained (Maguire *et al.*, 1974). Reproduced by permission of Biochimica et Biophysica Acta.

Figure 11 is a plot of log $(-x + vt + \beta)$ vs time for the uninhibited reaction. All plots of the uninhibited reaction showed two slopes, indicating a biphasic exponential

approach to the steady state. The subsequent analysis yielded the values for the rate constants indicated in Figure 11. This is the first time that k_1 and k_{-1} have been determined for the hydrolysis of p-nitrophenyl acetate by α-chymotrypsin; the values of k_2 and k_3 are in agreement with values presented by Frankfater and Kézdy (1971). Figure 12 shows the triphasic behaviour observed for the competitively inhibited reaction, an observation that is in agreement with theory. Values of k_q and k_{-q} were not obtained because of experimental error.

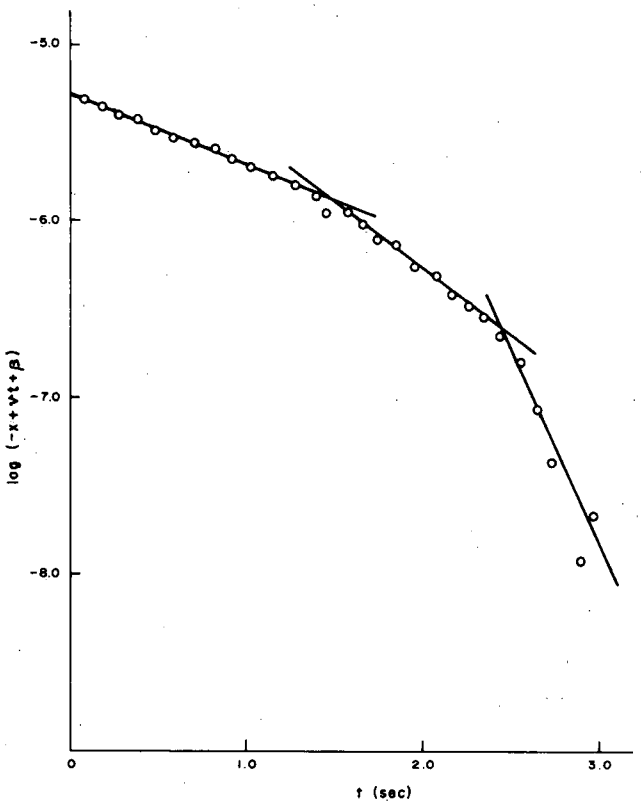


Figure 12. A plot of log (−x + vt + β) vs time for the inhibited reaction (Maguire *et al.*, 1974). Reproduced by permission of Biochimica et Biophysica Acta.

It is apparent that poor definition of slopes in plots of log (− x + vt + β) vs time can be a source of considerable error; nevertheless, it has been demonstrated that the uninhibited and competitively inhibited reactions exhibit biphasic and triphasic exponential approaches to the steady state, respectively, in agreement with theory, and that values of individual rate constants in two-intermediate reaction mechanisms may, at least in principle, be determined by this technique.

SOLID-SUPPORTED ENZYME SYSTEMS

It is now recognized that the enzymes of the living cell operate within the framework of a highly organized structure. Many enzymes are localized on or within the various membranous structures of the cell, and evidence is accumulating with respect to the more precise localization of such enzymes. Increasingly, enzymes that perform sequential reactions have been isolated either as multi-enzyme complexes or as components of the same membrane fraction. Therefore, many enzymes seem to function in a characteristic microenvironment, which may affect their mode of action. The cell as a whole may be envisaged as a microheterogeneous system in which surface effects take precedence over ordinary statistical mass-action relationships. It is thus of great interest not only to isolate a single enzyme and study its properties in aqueous solution, but also to consider how the enzyme functions in its native milieu.

In an analysis of the influence of the micro-environment on enzyme activity, one can distinguish between those effects directly attributable to the matrix in which the enzyme is embedded and those resulting from the enzymic reaction. The chemical and physical properties of the matrix, such as electric charge, dielectric constant, electric field, and lipophilic or hydrophilic nature, may affect enzymic activity. Specific interactions between a given enzyme and neighbouring protein and lipid molecules may drastically alter its mode of action. The micro-environment can also be modified as a result of the enzymic reaction, leading to local accumulation of products, such as acids, bases, and inhibitors, as well as to local depletion in the concentration of substrate.

Considerable efforts are being made to elucidate the composition, structure, and organization of subcellular structures by a variety of biological, chemical, and physical techniques. These will undoubtedly yield a better understanding of the nature of the microenvironment in which membrane-bound enzymes act *in vivo*. Because of the complexity of biological membranes and the lack of sufficiently powerful methods for determining both the precise macromolecular structures of cell membranes and the composition of the adjacent phases, our understanding of the influence of microenvironmental effects on enzyme activity is incomplete. Model systems in which enzymes are artificially immobilized by their attachment to membranes or to polymer particles are useful in studying the effect of a single microenvironmental parameter, because artificially immobilized enzymes form a new phase, the characteristics of which are determined to a large extent by the nature of the artificial carrier. Results obtained with these model systems can be used to evaluate the possible role of a given microenvironmental parameter in determining the activity of an enzyme in its native milieu.

Many artificially immobilized enzyme systems have been described in the literature. Of particular interest are

systems consisting of enzymes immobilized within cross-linked polymeric networks, enzymes covalently bound to high molecular weight carriers and enzymes embedded in artificial membranes. Water-insoluble enzyme derivatives provide specific, easily removable reagents. They have also been used in the form of enzyme columns and enzyme membranes in continuous enzymic process, e.g., in the treatment of renal failure (Sparks *et al.*, 1969) and the destruction of asparagine-dependent tumours in leukemia (Allison *et al.*, 1972).

Four principal methods have been used for the preparation of water-insoluble derivatives of enzymes: (a) adsorption on inert carriers or synthetic ion exchange resins (e.g., McLaren and Estermann, 1957); (b) occlusion into gel lattices, the pores of which are too small to allow the escape of the entrapped protein (e.g., Chang, 1964); (c) covalent binding of proteins to a suitable water-insoluble carrier by functional groups not essential for their biological activity (e.g., Goldstein *et al.*, 1964); and (d) covalent cross-linking of the protein by an appropriate bifunctional reagent (e.g., Ashoor *et al.*, 1971). It should be noted that the activity of artificially insolubilized enzymes is generally less than that of the free enzymes in solution. For example, yeast alcohol dehydrogenase is inactive in polyacrylamide gel, and β-galactosidase, yeast alcohol dehydrogenase, and α-chymotrypsin possess virtually no activity in nylon microcapsules (J. Maguire, unpublished observations), while other enzymes may retain a substantial portion of their activity in these supports. Goldman *et al.* (1971) provide a good review of this subject.

There are four main reasons why an enzyme may behave differently when supported than when present in free solution (Laidler and Sundaram, 1971):

(1) The enzyme may be conformationally different in the supported state as compared with free solution. Enzyme conformation is critical in kinetic behaviour, certain changes leading to a complete loss of activity.

(2) In the support, the interaction between the enzyme and the substrate takes place in a different environment from that existing in free solution. Studies of enzyme reactions in different solvents have shown that environmental effects can be profound.

(3) There will be partitioning of the substrate between the support and the free solution, so that the substrate concentration in the neighbourhood of the enzyme may be different from what it is in free solution. For example, a relatively non-polar substrate will be more soluble in a support that contains a number of non-polar groups than in aqueous solution; a polar substrate will be less soluble. In addition to this, significant effects may arise if both the substrate and the support are electrically charged (Goldstein *et al.*, 1964).

(4) The reaction in the solid support may be to some extent diffusion-controlled. In homogeneous aqueous solution, even the fastest of enzyme-catalyzed reactions appear not to be diffusion-controlled, but this is no longer true when the substrate has to diffuse toward the enzyme through the solid support. Indications are that the reaction will be diffusion-controlled except when the enzyme reaction is exceedingly slow.

Little information is available at present about factors 1 and 2 for solid-supported enzymes. The kinetic effects of partitioning of substrate between support and solution have been considered by Goldman *et al.* (1968), but only for electrostatic interactions. Sundaram *et al.* (1970) have developed a general treatment of the kinetic laws applicable when an enzyme-containing membrane is present in a solution of a substrate so that the solution is in contact with the opposite faces of the membrane. Kobayashi and Laidler (1973) have developed a theory dealing with the determination of the kinetic parameters V and K_A of an enzyme-substrate system in which the enzyme is contained in spherical particles, and in which substantial diffusional effects are present. Yarev *et al.* (1972) have discussed a model of permease as a rotary carrier through membranes, and Goldman and Katchalski (1971) have discussed the kinetic behaviour of a two-enzyme membrane during a consecutive set of reactions. This exciting field of research is relatively new and a great deal of work still lies ahead.

CONCLUSIONS

Whether enzymes in their natural state are in free solution or are insolubilized, many useful techniques may be employed to assess the effects of inhibitors, or changes in physical parameters, on enzyme-catalyzed reactions. Precise methods exist for determining whether a substance is an inhibitor of a particular enzyme reaction, how the substance inhibits the reaction, and how effective it is in relation to the substrate or other inhibitors. By varying other parameters such as pH it may be possible to deduce the site of binding of the inhibitor to the enzyme. With the knowledge of the mechanisms of enzyme action, specific effects of added chemicals on living organisms can be predicted; this is of great importance in problems of water pollution. It may also be possible, through judicious use of structure-activity relationships, to design compounds that are necessary as inhibitors of certain processes, but that are harmless to other systems. This would be a substantial contribution to medicine and other environmental sciences.

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