An Investigation of the Trypsin Catalyzed Hydrolysis of p-Nitrophenyl Acetate Employing The Stopped-Flow Method

by

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Preface

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It has become more and more evident that nature may have a 'common pattern' in her construction of the active center in an enzyme. Analysis of that portion of the enzyme which is believed to contain the 'site' has led to the same amino acid residue sequence in no less than eight instances. Despite the progress in this direction, the actual mechanism employed by the reactive groups of an enzyme to fracture a substrate bond is by no means completely understood. However, in recent years there has been a mild trend toward what might be called the 'two complex' scheme. Certain enzyme systems give evidence that the initial Michaelis complex converts to a second complex with the release of one product, and this complex then releases the second product. A particular system that behaves in this fashion is the chymotryptic hydrolysis of p-nitrophenyl acetate. It was decided to extend this work to the enzyme trypsin with the intention of not only explaining some conflicting results in the field, but also of weighing and compare the merits of the postulated mechanisms.

In order to conveniently study reactions of the above type it was necessary to construct a stopped-flow mixing device for rapidly mixing enzyme and substrate. This was undertaken with a desire to simplify the stopped-flow technique, to the extent that commercial spectrophotometric components could be employed and still retain the high sensitivity required by the method. It was further hoped to design a mixing apparatus based on existing principles, which would lead to easier methods of construction and moreover make it less complicated for the operator to use.

The main topics of original research covered by this thesis have been rewritten for publication in the Canadian Journal of Chemistry.

I am indebted to the Department of National Health and Welfare of the Government of Canada for granting me a leave of absence on part salary for the purpose of doing this research at Ottawa University. In this respect, Drs. L.I. Pugsley, R.A. Chapman, and J.H. Mahon of that Department were more than generous with their recommendations and kind suggestions. The National Research Council of Canada is worthy of thanks for their financial aid in support of the program.

I am especially grateful to Prof. L. Ouellet, my research supervisor, who supplied much of the guidance and fortitude that were necessary to inspire me to attempt the research presented. A token of strong appreciation is also extended to Prof. K.J. Laidler, who in some respects instigated the project and was indeed helpful thereafter. Considerable credit is due to Mr. F. Giacobbi for his pains in the construction of the mixing apparatus

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Abstract

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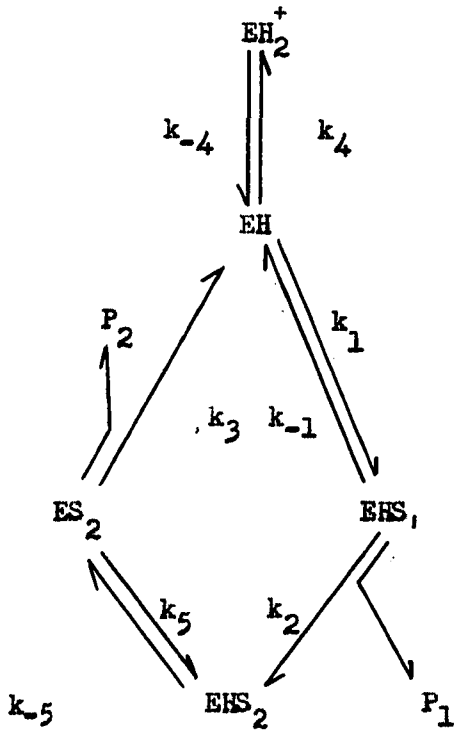
A theoretical treatment is worked out for the kinetic scheme



in which the appearance of P_1 is followed. The steady-state and transient phase equations are obtained subject to the condition that the substrate concentration is considerably greater than the enzyme concentration. The conditions under which evidence in favour of this mechanism can be derived from experimental data are discussed. When k_2 is greater than k_3 all the constants may be evaluated except k_1 and k_{-1}. This is done for the systems involving the enzymic hydrolysis of p-nitrophenyl acetate with trypsin and chymotrypsin.

A spectrophotometric method is described which enables the production of P_1 (p-nitrophenoxide-ion) to be studied at the onset of reaction. For

this purpose a stopped-flow mixing device is designed and adapted to a Beckman Model DU spectrophotometer. The reactant solutions are conveniently injected into the mixing apparatus by means of two removable storage syringes. The operations of filling the reaction syringes, mixing the reactants, and standardization are achieved with T-bore stopcocks. Provision is made in the design for interchangeable mixing chambers and observation cells. The temperature is controlled by the circulation of water through the spectrophotometer housing and an enclosure surrounding the reaction syringes. A zero suppression method is devised to record the extent of reaction.

Employing the above apparatus the stopped-flow technique is used to secure the experimental evidence on which the proposed mechanism is based. The hydrolysis of p-nitrophenyl acetate is investigated during the early stages of the reaction, and the influence of pH on the initial rate suggests competitive inhibition at the active site of the enzyme by hydrogen-ions. The dissociation constant of the enzyme obtained from the kinetics of this reaction indicates possible catalysis by an amino group (pK 7-8) or an imidazole group (pK 6-7) in the enzyme. As a model to the enzymic site, lysine methyl ester and isopropyl alcohol in the presence of each other proportionally influence the rate of hydrolysis of p-nitrophenyl acetate. Some infrared spectroscopic evidence is obtained which suggests that p-nitrophenyl acetate probably acetylates the free α-amino group of the amino acid. The results are described in terms of the assumed mechanism and the nature of the catalytic site is discussed.

Introduction

A. Nature and Scope of the Investigation

The work of Michaelis-Menten presented in 1913 has resulted in the most successful theory yet to describe the reaction mechanism of enzymic systems. This theory involves the basic idea that the enzyme combines with the substrate to form a complex, which in turn undergoes an internal rearrangement to produce products. Several difficult questions concerning the details of this mechanism are slowly in the process of being answered. For instance, although enzymes are protein-like substances, where similar intramolecular amino acid sequences are usually repeated, they frequently possess only a single active center. Progress is now being made in the identification of the group or groups (amino acid residues) responsible for this activity, and a most striking result has evolved. It appears that the amino acid sequence of at least one part of the active site is the same for several enzymes, even though their specificity requirements are different. To answer this question work is being directed toward the elucidation of the intricate mechanism by which these groups function to stimulate the breakdown of a substrate molecule.

A considerable portion of the research reported on this subject concerns the proteolytic enzymes chymotrypsin and trypsin, which also possess the ability to act as esterases. One particular phase of the work involves the use of nitrophenol esters as the substrate, since this approach enables the role normally played by the alkyl part of the ester to be studied. Previously, only the acyl part of the ester was investigated because the alkyl group (methyl, ethyl, etc.) did not lend itself to any direct means of analysis. As a consequence of this research with nitrophenol esters

there is a strong indication that enzymic action may be based on a three step rather than a two step mechanism. The first step involves the formation of the classical Michaelis enzyme-substrate complex which is followed by unknown interaction to produce products. Since these products are usually two in number (i.e. as for hydrolysis), and they are probably released from the enzyme independently rather than simultaneously, it becomes necessary to postulate a second complex. The result is, a three step process is required to account for enzymic action.

In the case of nitrophenyl esters the formation of the first complex is too rapid to be studied by present methods. The second stage, however, which involves the second complex (acylation), is only moderately fast and may be investigated using rapid mixing techniques. The deacylation or third stage is slow and requires only conventional methods for analysis. Most of the work published has been performed with the enzyme chymotrypsin and the substrate p-nitrophenyl acetate. The results obtained from these studies, and especially those relating the hydrogen-ion effect to hydrolysis, somewhat disagree with the mechanism generally accepted by workers in this field.

Because of the existence of similar reaction properties of the two enzymes chymotrypsin and trypsin, it was argued that trypsin would also probably catalyze the hydrolysis of p-nitrophenyl acetate. And if this was the case, then a study of this reaction might yield significant results to aid in developing a suitable explanation of the anomalous behaviour of p-nitrophenyl acetate with chymotrypsin.

Before undertaking this investigation it was first necessary to show that trypsin catalyzed the hydrolysis of p-nitrophenyl acetate in the same

manner as chymotrypsin. Secondly, special apparatus had to be acquired for the study of the rapid second stage of the reaction. Finally, the existing theoretical treatment of reactions involving two complexes had to be expanded to take into account the mechanism suggested by the results.

B. Previous Experimental Work

Hartley and Kilby (1,2) first reported that the enzyme chymotrypsin was capable of catalyzing the hydrolysis of p-nitrophenyl acetate. From their results they concluded that the mechanism consisted of three distinct stages:

$$[\,a\,]\quad E + AB \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{\text{I}}{EAB} \xrightarrow{k_2} \underset{\text{II}}{EA + B} \xrightarrow{k_3} \underset{\text{III}}{E + A}$$

where E = enzyme

AB = substrate (nitrophenyl ester)

EAB = enzyme-substrate complex

EA = acylated enzyme

B = nitrophenol

A = acylate

and k_1, k_{-1}, k_2, and k_3 are the appropriate rate constants.

Stage I of mechanism [a] is extremely rapid (Gutfreund and Sturtevant (3) have estimated $k_1 \geq 2 \times 10^6$ liters moles^{-1} sec^{-1}) and concerns the combination of enzyme with substrate. Stage II is moderately rapid and involves the liberation of nitrophenol and the acetylation of the enzyme, while the third and final stage which is slow, liberates acylate with the consequent regeneration of enzyme.

(a) Esterase Specificity

The ability of several different nitrophenyl acetates to acylate chymotrypsin has been illustrated by the work of Balls (4,5). However,

of greater interest is the reactivity of the nitrophenyl esters of acids other than acetic. McDonald and Balls (6) investigated various types of acids ranging from methyl-branched aliphatics to the aromatics. In general, their results indicate that the relative rate of hydrolysis for an amino acid ester > aromatic acid ester > aliphatic acid ester > methyl-branched aliphatic acid ester.

It now appears that trypsin behaves toward esters in an analogous fashion to chymotrypsin. This has been verified by Dixon and Neurath (7), and independently in this laboratory, at least to the extent of p-nitrophenyl acetate reactivity. The result is rather to be expected in the light of the work of Nord (8,9), who has made a detailed study of the acetylation of trypsin in organic solvents.

(b) Kinetics

The effect of hydrogen-ion on the hydrolysis of 2,4-dinitrophenyl acetate (3) and p-nitrophenyl acetate (10) as catalyzed by chymotrypsin has been studied by Gutfreund and Sturtevant. The behaviour of these two compounds was decidedly different in that the rate constant concerned with acetylation was pH-dependent in the case of 2,4-dinitrophenol but pH-independent in the case of p-nitrophenol. The first instance is consistent with the findings for other substrates, viz. the amino acid esters, while the second does not conform with any previous result.

Dixon and Neurath have investigated the acetylation and deacetylation reactions of chymotrypsin with p-nitrophenyl acetate (11). Both stages are reported to be pH-dependent and can be described by a simple dissociation curve for a single group having dissociation constants 6.0×10^{-7} and 1.1×10^{-7} for acetylation and deacetylation respectively. The

result at least for acetylation is not in agreement with the findings of Gutfreund who reported pH-independence. It is quite possible that this may be ascribed to the different experimental conditions. In order to slow down the rapid acetylation reaction Dixon and Neurath worked at 3°C, also the substrate and enzyme concentration were of the same order of magnitude and the reaction was plotted according to second-order reaction kinetics. They have since extended this work to trypsin (7) which they claim behaves similarly to chymotrypsin in every respect except that acetylation is 40 times slower.

(c) Characterization of the Active Center

In the three-step mechanism of hydrolysis the second complex is actually the acylated form of the enzyme. Thus a knowledge of the properties of this form should be valuable in understanding the character of the active center. Although no intensive research has been performed on the properties of acetyl-trypsin, the behaviour of acetyl-chymotrypsin will be cited because of its close relationship.

Monoacetylated chymotrypsin is stable in aqueous solution below pH 5 to 6, but hydrolyzes at above pH 7 to 8 yielding acetate and free enzyme. Besides hydrolysis, the acylated enzyme can be reactivated by transesterification to hydroxylamine (5) or as in the case of trimethyl-acetylation to n-butanol (6). Dixon and Neurath (11) give evidence that the acylation of chymotrypsin with acid anhydrides and acid chlorides at pH 5 leads to the acylation of the active center of the enzyme. This evidence is based on the facts that after acylation the enzyme is no longer capable of being acetylated by p-nitrophenyl acetate, and that if the enzyme is acylated by p-nitrophenyl acetate or acid anhydrides or acid

chlorides, the catalytic activity toward the specific substrate acetyl tyrosine ethyl ester is abolished until the acyl group is spontaneously removed by hydrolysis. There is an indication from urea denaturation studies that the reactivity of the group in question is functionally related to the specific structure of the native protein. In the presence of 8M urea, no acetylation by p-nitrophenyl acetate occurs (12,13) and the reactivity of acetyl chymotrypsin toward hydroxylamine is lacking. However, when denaturation is reversed by dilution of the urea, deacylation by hydroxylamine or by hydrolysis is restored (12).

It has now been well established that a number of organophosphorus compounds, and in particular diisopropyl phosphorofluoridate, inhibit enzymic activity (14). Evidence obtained by treating several esterases with labelled diisopropyl phosphorofluoridate (P^{32}) and performing degradation studies indicates that each of these enzymes possess a unique serine residue which may be responsible for activity (15,16). In each instance the labelled phosphorus had condensed on the hydroxyl group of a serine residue that was neighboured by aspartic acid on one side and glycine on the other. The sequence is;------- glycine, aspartic, serine, glycine, --- for trypsin (17,18,19,20,21), chymotrypsin (17,19,22,23), true-cholinesterase, pseudo-cholinesterase, red-cell ali-esterase, liver ali-esterase (24), thrombin and phosphoglucomutase (25).

C. Methods for Studying Rapid Reactions

The acetylation of chymotrypsin or trypsin by p-nitrophenyl acetate may be studied spectrophotometrically by measuring the coloured p-nitrophenoxide-ion released during the early stages of the reaction. Unless advantage is taken of the fact that the rate is diminished at low temperature or pH, special equipment is necessary to mix rapidly the enzyme and substrate, and measure the fast acetylation stage. This has been done for chymotrypsin as mentioned previously using the stopped-flow technique (3,10).

The stopped-flow method was originally conceived in 1940 by Chance (26,27,28) for the rapid mixing of small quantities of enzyme and substrate. Up until this time large volumes of reagents were required, i.e. liters, to investigate fast reactions by the constant-flow method which had been employed in principle since 1923 (29).

The first successful method for measuring the velocity of rapid chemical reactions with a half-time less than 10 seconds was devised by Hartridge and Roughton in 1923. In that year they published a paper (30) describing the constant-flow or steady-flowing technique which could be used to follow reactions in the liquid phase with half-periods down to one millisecond. The principle of their method is as follows. The two reactant solutions were placed in separate pressurized vessels and driven by pressure into a special mixing chamber. The composition of the emerging fluid was then determined by optical, thermal, electrical or other means of analysis at various points along the observation tube. If d is the distance in centimeters from the mixing chamber to the point

of observation, and $\bar{u}$ is the average linear velocity (calculated from the bore of the tubing and the volume of discharge per second) in centimeters per second, then the average time that the reaction has proceeded before reaching the place of observation is $d/\bar{u}$ seconds. Alternatively, using the same apparatus, observations could be made at a fixed distance from the mixing chamber and the velocity of fluid flow varied as in Millikan's procedure (31). Other versions of the constant-flow method have also been described (32), the most recent of which is that by Dalziel (33).

The main advantages of the constant-flow method are: (a) no recording mechanism is necessary (b) measurements can usually be made on a commercial spectrophotometer (c) high sensitivity (d) the type of method of observation (optical, thermal, electrical, etc.) is not restricted. In spite of these pertinent advantages, however, and besides the usual difficulties involved in achieving constant flow, the technique has a major drawback in that sufficient quantities, i.e. in the neighbourhood of liters, of the reactants must be available.

Because of the above disadvantage, Chance in 1940 developed the stopped-flow and accelerated-flow methods, both of which require only limited quantities of reactants, i.e. less than a cubic centimeter per determination. Since that time he has applied these methods to the investigation of enzymatic reactions (34) with continuing success (35), where the materials are not only short in supply but also expensive.

In both the stopped-flow and accelerated-flow methods the reactants are manually driven by a plunger mechanism from syringes into an observation cell via a mixing chamber. In the stopped-flow method the reaction is recorded after the termination of the plunge, while in the

accelerated-flow technique it is observed during the plunge. In this case the flow rate is recorded simultaneously with the extent of reaction by means of a voltage divider (potentiometer) connected by a pulley arrangement to the plunger mechanism. The stopped-flow technique has become the more popular method, since the results are more convenient to interpret.

Besides minimal material requirements, the stopped-flow technique has several additional advantages over the constant-flow method. These are: (a) because the fluid is stopped, it is independent of the rate and character of flow, (b) a permanent record can be obtained of the progress of the reaction for a period starting from a few milliseconds after mixture extending as long as desired, and (c) it is free from the distorting effect of mechanical disturbances.

It is obvious from the foregoing discussion that the stopped-flow method is the most suitable means of rapid mixing for the study of the acetylation of trypsin. However, when the stopped-flow method is employed it is usually necessary to design and construct special optical and electronic components. In this respect Chance (36,37,38) has designed packaged amplifiers using subminiature tubes in order to attain a desirable level of sensitivity, i.e. 1×10^{-4} optical density units, and a rapid response time, i.e. <0.05 seconds. Although Chance used commercial optical components, such as the Coleman 10-S double monochromator (37) or the Beckman DU monochromator (38,39), Gibson and Roughton (40) found that for their purposes it was necessary to construct a filter monochromator. On the other hand, Beers (41) managed to adapt his elaborate four-channel mixer to a Beckman DU spectrophotometer with little modification, but he

did not achieve the high sensitivity and response speed of Chance. Recently, Sirs has published two papers (42,43) describing the rapid mixing method when it is used in conjunction with electrometric rather than optical components as the means of measurement.

In light of the foregoing discussion it was our intention to utilize with as little modification as possible a commercial spectrophotometer and still attain the high sensitivity and response speed of Chance (37), and also to develop a stopped-flow apparatus based in principle on Chance's original design (28).

D. Review of Theoretical Considerations

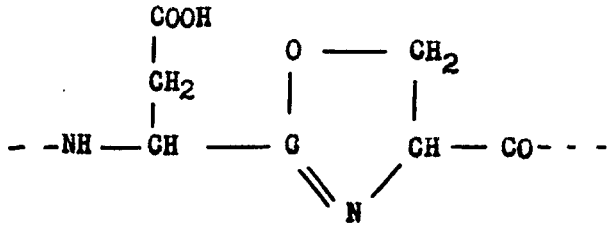
(a) Proposed Mechanisms of Hydrolysis

Even though degradation studies on the inactive diisopropyl phosphoryl derivatives of chymotrypsin (17,19,22,23), trypsin (17,18,19,20,21), and cholinesterase (24) had shown that the hydroxyl group of a serine residue was phosphorylated, this group was not believed to be the initial focal point (44). On the contrary, there was other evidence to indicate that the imidazole group of a histidine residue is the primary site involved in catalytic action (10,45). This evidence was, the pH-dependence of inhibition with diisopropyl phosphorofluoridate and the enzymic activity could each be described by a group with a pK of 5 to 7 (46); histidine and tyrosine were able to catalyze in the model hydrolysis of diisopropyl phosphorofluoridate while serine was not (47); and compounds containing the imidazole group were found to be effective in increasing the rate of hydrolysis of p-nitrophenyl acetate (48,49). Cunningham (15) has since re-examined these findings, and his recently proposed mechanism not only takes them into full account, but also postulates the hydroxyl group of serine as the primary site.

A mechanism has also been presented by Laidler (50) that does not consider imidazole to be the initial substrate-binding group. This simplified mechanism is based on some results obtained from a study of the effect of hydrogen-ion on the kinetic constants which describe the chymotrypsin catalyzed hydrolysis of methyl hydrocinnamate (51). The results show that no ionizable groups in imidazole or amino pK region are involved in substrate binding. This in itself is strong evidence

in favour of a mechanism which treats imidazole as secondary.

The most recent scheme proposed to explain esterase activity has been published by Porter, Rydon, and Schofield (52,53). Their rather complicated mechanism suggests that ring formation takes place at the active site between the serine residue and aspartic acid or glycine to form a Δ^2-oxazoline,



Hydrolysis is then brought about by a mechanism that requires the opening and closing of this ring.

(b) Mathematical Formulation

The three-step mechanism proposed to account for the enzymatic hydrolysis of p-nitrophenyl acetate (2) as outlined on page 16, reaction [a], requires some knowledge of transient phase kinetics. This is primarily because the acylated enzyme is an intermediate, and in order to study the acylation step it is necessary to investigate the reaction during its initial stages.

The first theoretical treatment of the transient phase kinetics of enzymes dates back to 1923, when Briggs and Haldane (54) derived equations based on the formation of an enzyme-substrate complex (55) to describe the relationship between the complex and reaction products. However, it remained until 1943 for Britton Chance (56) to extend and successfully

apply these relationships. In his work on peroxidase-peroxide he clearly demonstrated that the formation of an enzyme-substrate complex during enzymic action was a valid assumption. The same author has since reported the existence of a similar complex for the catalase-peroxide system (57,58). The mechanism of this system involves two consecutive reactions linked by an intermediate complex (59), and the steady-state (60) and pre-steady state kinetics (61) have been discussed in detail.

If the formation of an enzyme-substrate complex is a plausible criterion, then by studying the production of a product at the very beginning of the reaction there should be the appearance of an induction period. This induction period is due to the lower rate of product formation until the concentration of complex has attained steady-state conditions. The magnitude of this period should, therefore, be related to the rate constants governing the accumulation of complex, and its measurement could provide an alternative method for investigating the role of the complex in enzymic action. This is especially true when it is not possible to follow directly the rate of formation of complex by some physical means. The formulation of the induction period for a scheme based on a single complex has been accomplished by Roughton (62) and Gutfreund (46). Unfortunately, however, of those systems investigated experimentally (3,46,63), the equipment employed was not sensitive enough to more than estimate this period in a qualitative manner.

Laidler (64) has treated the steady-state and transient phase kinetics of the general Michaelis-Menten scheme in a thorough fashion. Expressions are arrived at in terms of both the products and the complex, and also for

the system where the complex reacts with a second substrate to yield products. The catalase-peroxide system is given as a practical illustration of the latter with a slight modification, that the complex reacts with a second molecule of the initial substrate.

Within the past few years several papers have appeared which suggest that enzymatic action may involve the sequential formation of two complexes (2,3,10,65,66). The mathematical treatment of schemes based on this idea has been given for some special cases (10,46,67). Gutfreund and Sturtevant (10) have derived expressions for the steady-state and transient phase kinetics of the chymotrypsin p-nitrophenyl acetate system, where a product is released when the initial Michaelis-Menten complex transforms to a second complex. However, the complete ramifications of the formulae are not presented nor their constants fully defined. Gutfreund (46) has also considered the possibility where the first complex converts to a second complex without the release of a product. This was done mainly from the standpoint of induction periods which may appear under special conditions. On the other hand, Ouellet and Laidler (67) have considered this type of system in complete detail.

Theoretical

A. Kinetics of the Formation of Products by an Enzyme System Involving Two Enzyme-Substrate Complexes

In cases such as the hydrolysis of phenyl ester by chymotrypsin (2) or by trypsin there are strong indications that the release of products involves at least two steps, one for the release of phenol and the other for the release of acid. When it is possible as for the above reaction to follow the rate of appearance of the first product (phenol), significant information concerning the mechanism of esterase action may be acquired. The main purpose of this section, therefore, will be to develop more fully the mathematical formulation of this system which is given by:

$$[b] \quad \underset{(e_o - y_1 - y_2)}{E} + \underset{(s_o)}{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{(y_1)}{ES_1}$$

$$[c] \quad \underset{(y_1)}{ES_1} \xrightarrow{k_2} \underset{(y_2)}{ES_2} + \underset{(p_1)}{P_1}$$

$$[d] \quad \underset{(y_2)}{ES_2} \xrightarrow{k_3} \underset{(e_o - y_1 - y_2)}{E} + \underset{(p_2)}{P_2}$$

If the initial concentration of substrate s_o is much larger than the concentration of enzyme e_o at the outset of the reaction, then s_o

may be considered constant, at least during the transient phase. This makes it possible to utilize the following differential equations that describe the system:

$$[1] \qquad \dot{y}_1 = k_1 s_o (e_o - y_1 - y_2) - y_1 (k_{-1} + k_2)$$

$$[2] \qquad \dot{y}_2 = k_2 y_1 - k_3 y_2$$

$$[3] \qquad \dot{p}_1 = k_2 y_1$$

$$[4] \qquad \dot{p}_2 = k_3 y_2 \quad .$$

(a) Steady-State

The steady-state equations are obtained by setting $\dot{y}_1$ and $\dot{y}_2$ equal to zero. This leads to:

$$[5] \qquad y_1 = \frac{k_3}{k_2} y_2$$

$$[6] \qquad y_2 = \frac{k_1 k_2 s_o e_o}{k_1 s_o (k_2 + k_3) + k_3 (k_{-1} + k_2)}$$

and

$$[7] \qquad \dot{p}_1 = \dot{p}_2 = \frac{k_1 k_2 k_3 e_o s_o}{k_1 s_o (k_2 + k_3) + k_3 (k_{-1} + k_2)} \quad .$$

Dividing the numerator and denominator of equation [7] by $k_1 (k_2 + k_3)$ we obtain:

$$[8]\quad \dot{p}_1 = \frac{\dfrac{k_2 k_3 e_o s_o}{(k_2 + k_3)}}{s_o + \dfrac{k_3 (k_{-1} + k_2)}{k_1 (k_2 + k_3)}}$$

which reduces to the classical Michaelis and Menten (55) form

$$[9]\quad \dot{p}_1 = \frac{V_{max}\, s_o}{K_m^* + s_o}$$

where V_{max}, the maximum rate is given by

$$[10]\quad V_{max} = \frac{k_2 k_3 e_o}{k_2 + k_3}$$

and K_m^*, the experimental Michaelis constant is

$$[11]\quad K_m^* = \frac{k_3}{k_2 + k_3} \cdot \frac{k_{-1} + k_2}{k_1} .$$

If k_3 is very much larger than k_2, as appears to occur frequently in enzymic action, then

$$[12]\quad K_m^* = \frac{k_{-1} + k_2}{k_1} = K_m$$

and corresponds to the case of the single complex.

(b) Transient Phase

The transient phase kinetics of the system involving the formation of products from two complexes may be derived by the differentiation of equation [1]

$$[13] \quad \ddot{y}_1 = -\dot{y}_1 (k_1 s_o + k_{-1} + k_2) - \dot{y}_2 k_1 s_o \ ,$$

and eliminating y_2 and $\dot{y}_2$ using equations [1] and [2]. The following linear second order differential equation is obtained with constant coefficients:

$$[14] \quad \ddot{y}_1 + (k_1 s_o + k_{-1} + k_2 + k_3) \dot{y}_1 + [k_1 s_o (k_2 + k_3) + k_3 (k_{-1} + k_2)] y_1 - k_1 k_3 e_o s_o = 0 \ .$$

This can be rewritten simply as,

$$[15] \quad \ddot{y}_1 + P\dot{y}_1 + Qy_1 - R = 0$$

where

$$[16] \quad P = k_1 s_o + k_{-1} + k_2 + k_3$$

$$[17] \quad Q = k_1 s_o (k_2 + k_3) + k_3 (k_{-1} + k_2)$$

and

$$[18] \quad R = k_1 k_3 e_o s_o \ .$$

It has been shown previously (67) that if the discriminant $P^2 - 4Q$

is negative, i.e. the roots of the auxilary equation are imaginary, then periodic solutions are obtained which are not particularly interesting because the period is longer than the transient phase. However, in those cases where $P^2 - 4Q$ is zero or positive the solution of equation [15] is

$$[19] \qquad y_1 = R/Q + Me^{Ft} + Ne^{Gt},$$

where

$$[20] \qquad F = \tfrac{1}{2}\left[- P + (P^2 - 4Q)^{\frac{1}{2}}\right]$$

$$[21] \qquad G = \tfrac{1}{2}\left[- P - (P^2 - 4Q)^{\frac{1}{2}}\right].$$

The boundary conditions are t (time) = o_1 $y_1 = 0$ and $\dot{y}_1 = k_1 e_o s_o$ (from equation [1]) producing the following values for M and N,

$$[22] \qquad M = - (R/Q) \frac{k_3 G + Q}{k_3 (G - F)}$$

$$[23] \qquad N = (R/Q) \frac{k_3 F + Q}{k_3 (G - F)} \quad .$$

When the value of y_1 is inserted into equation [3] one obtains

$$[24] \qquad \dot{P}_1 = k_2 R/Q + k_2 Me^{Ft} + k_2 Ne^{Gt} ,$$

and the integrated form is

$$[25] \qquad P_1 = k_2 Rt/Q + k_2 M/F (e^{Ft} - 1) + k_2 N/G (e^{Gt} - 1)$$

after applying the boundary conditions of $t = 0$ and $p_1 = 0$.

Since F and G are always negative, the terms e^{Ft} and e^{Gt} become negligible when t is sufficiently large, so that

$$[26] \quad P_1 = k_2 Rt/Q - k_2 M/F - k_2 N/G.$$

This is the integrated form of equation [7] for an open system where s_o is maintained constant.

A qualitative plot of the function represented by equation [25] and [26] is shown in Figure 1, assuming $|G| > |F|$. The initial exponential rise is due to the term $(k_2 N/G)(e^{Gt} - 1)$, and the approach to steady state with decreasing velocity is due to $(k_2 M/F)(e^{Ft} - 1)$. Still of greater interest, however, are the intercepts on the time and concentration axes obtained by extrapolating steady state conditions. These two quantities τ' and Π are secured by setting $p_1 = 0$ and $t = 0$ respectively, in equation [26].

This equation describes the steady state condition of the system. After substitution of the appropriate quantities for R, Q, M, N and F, and making $p_1 = 0$, τ' becomes

$$[27] \quad \tau' = \frac{k_3^2 - k_1 k_2 s_o}{k_1 k_3 (k_2 + k_3)(s_o + K_m^*)}$$

In actual practice the relative magnitudes of k_2 and k_3 will be the important factor. For instance, if k_3 is much larger than k_2, viz. the fleeting existence of the complex ES_2, then equation [27] reduces to

$$[28] \quad \tau' = 1/k_1 (s_o + K_m) - k_2 s_o/k_3^2 (s_o + K_m).$$

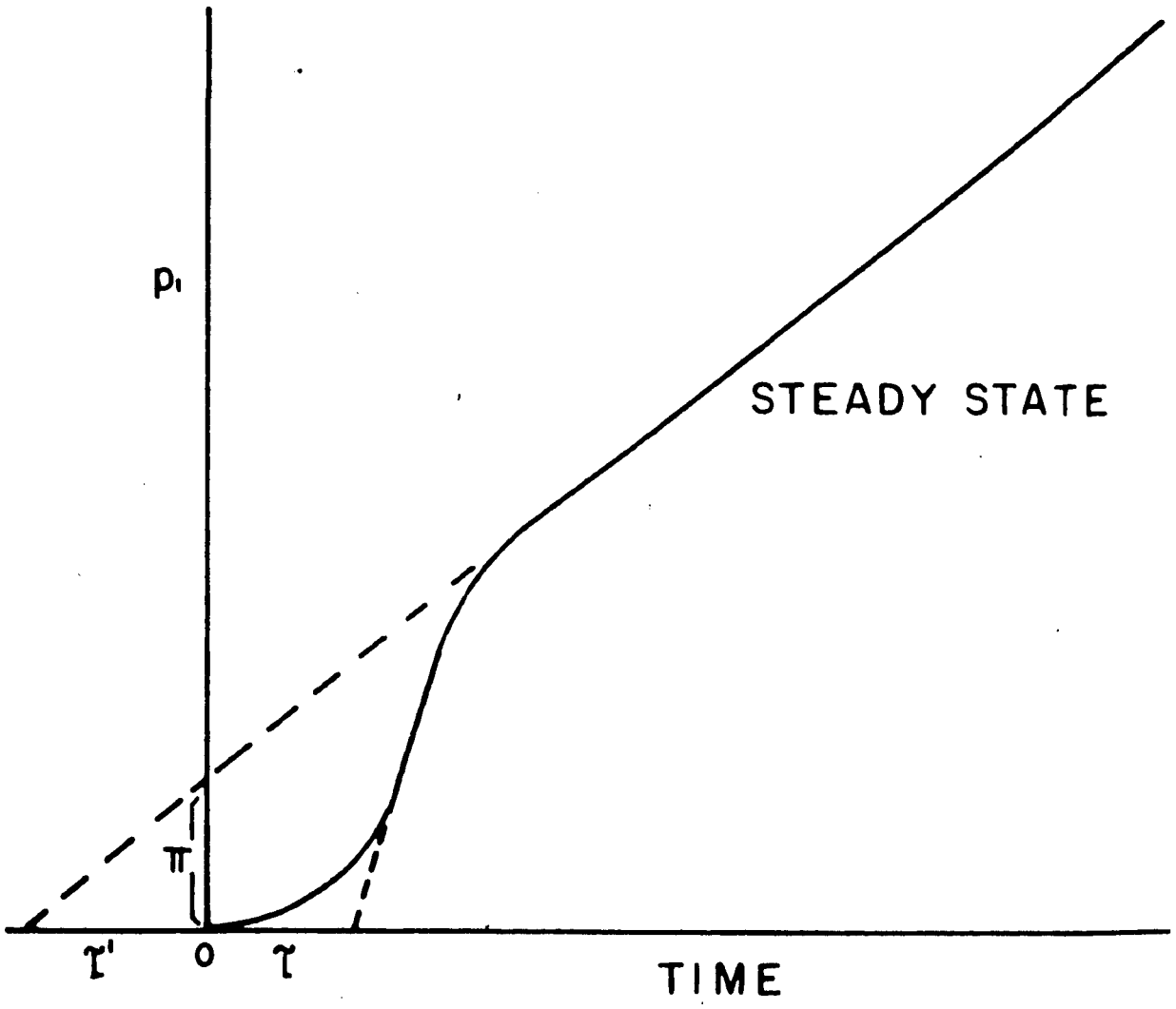


Fig. 1 A PLOT OF THE PRODUCTION OF PRODUCT (P_1) AGAINST TIME.

The first term of this expression is identical to the case for a single complex (46), while the second part may be considered as a correction term. Provided k_3^2 is of the same order of magnitude as k_1 this term will be small in comparison to the first especially since $k_2 s_o$ is usually much less than unity. In the reverse case, where k_2 is greater than k_3, equation [27] becomes

$$[29] \quad T' = - s_o/k_3 (s_o + K_m^*) + k_3/k_1 k_2 (s_o + K_m^*) .$$

However, since k_1 is usually large and k_3/k_2 small by hypothesis, equation [29] may be written simply as

$$[30] \quad T' = - s_o/k_3 (s_o + K_m^*)$$

or

$$[31] \quad 1/T' = - k_3 - k_3 K_m^*/s_o .$$

Therefore, a plot of $-1/T'$ against $1/s_o$ should produce a straight line of slope $k_3 K_m^*$ and having an intercept of k_3. Since a value for $k_3 e_o$ can readily be obtained from a study of the conventional kinetics, i.e. equation [10] under the condition that $k_2 >> k_3$, a value for the initial enzyme concentration e_o can be calculated. The quantity e_o obtained in this manner is actually an equivalent weight, so that the number of active centers in a molecule of enzyme could be estimated if the molecular weight is determinable by another method.

Under certain conditions the intercept on the concentration axis also supplies information concerning the equivalent weight of the enzyme.

According to equation [26] the extrapolated value of p_1 for t = 0 is

$$[32] \quad \Pi = [k_2^2 e_o s_o^2 / (k_2 + k_3)^2 (s_o + K_m^*)^2] - k_2 k_3^2 e_o s_o / [k_1 (k_2 + k_3)^2 (s_o + K_m^*)^2] .$$

Once again it is necessary to consider the two extreme cases one where k_3 is greater than k_2 and the other where k_2 is greater than k_3. In the first instance Π could become negative, depending on the relative magnitudes of the other constants, but it would be small compared to e_o. In the other case, where k_2 is large and k_3 small, Π is given by

$$[33] \quad \Pi = e_o s_o^2 / (s_o + K_m^*)^2 - k_3^2 e_o s_o / k_1 k_2 (s_o + K_m^*)^2 .$$

The second portion of this relationship can be disregarded unless $k_1 s_o$ is smaller than k_3^2, a somewhat improbable situation. Thus equation [33] may be rewritten as

$$[34] \quad \Pi = e_o s_o^2 / (s_o + K_m^*)^2 ,$$

and at high substrate concentration, i.e. $s_o^2/(s_o + K_m^*)^2 = 1$, reduces even further to

$$[35] \quad \Pi = e_o .$$

The experimental work of Hartley and Kilby (2) lends strong support to this simple relationship. They have reported that the extrapolation of the concentration of p-nitrophenol produced during the chymotrypsin catalyzed hydrolysis of p-nitrophenyl acetate to zero time gives the molar concentration

of enzyme at the outset of the reaction. Such an observation could be interpreted as positive evidence that chymotrypsin possesses one active site per molecule.

The examination of equation [32] indicates that the intercept on the p_1 axis is negligible unless k_2 and $k_1 s_o$ are very much greater than k_3. Furthermore, if these conditions are not fulfilled, equation [25] describing the transient phase has little consequence as steady-state conditions are approached very rapidly, i.e. the influence of the exponential terms is small. For the above reasons, the discussion of equation [25] in reference to the transient phase will be limited to those instances where k_2 and $k_1 s_o$ are much larger than k_3. Applying these conditions equations [16] and [17] condense to

$$[36] \quad P = k_1 (s_o + K_m)$$

and

$$[37] \quad Q = k_1 k_2 (s_o + K_m^*),$$

respectively, where K_m and K_m^* are

$$[38] \quad K_m = (k_{-1} + k_2) / k_1$$

$$[39] \quad K_m^* = k_3 K_m / k_2$$

With this in mind a series development of $(P^2 - 4Q)^{\frac{1}{2}}$ reduces equations [20] and [21] to

$$[40] \quad F = - Q/P - \ldots = - k_2 (s_o + K_m^*)/(s_o + K_m) - \ldots\ldots$$

$$[41] \quad G = -P + Q/P - \ldots\ldots\ldots$$

$$= - k_1 (s_o + K_m) + k_2 (s_o + K_m^*)/(s_o + K_m) - \ldots\ldots\ldots$$

Since K_m^* involves k_3/k_2 which is assumed to be a small quantity, a further simplification may be obtained in F and G, especially where experimental conditions are chosen so that s_o is much greater than K_m^*. This, therefore, leaves

$$[42] \quad F = - k_2 s_o/s_o + K_m$$

and

$$[43] \quad G = - k_1 (s_o + K_m) + k_2 s_o/(s_o + K_m),$$

as the other terms of the series expansion can be shown to be only of very minor importance.

After substituting the appropriate simplified quantities into equation [25], the following relationship results

$$[44] \quad p_1 = \Pi + k_3 e_o t + k_2 M/F \exp [-k_2 s_o/(s_o + K_m)] t + k_2 N/G \exp [-k_1 (s_o + K_m) - k_2 s_o/(s_o + K_m)] t$$

A hypothetical plot of the equation is shown in Figure 2. This plot of p_1 against time was obtained by assuming s_o to be very much greater than K_m, and assigning the values of 100, 10, and 1 to $k_1 s_o$, k_2, and k_3, respectively. The induction period τ indicated in Figure 2, can be estimated from a series expansion of the first exponential Ft or $- k_2 s_o t/ (s_o + K_m)$ and neglecting the second exponential where k_1 is

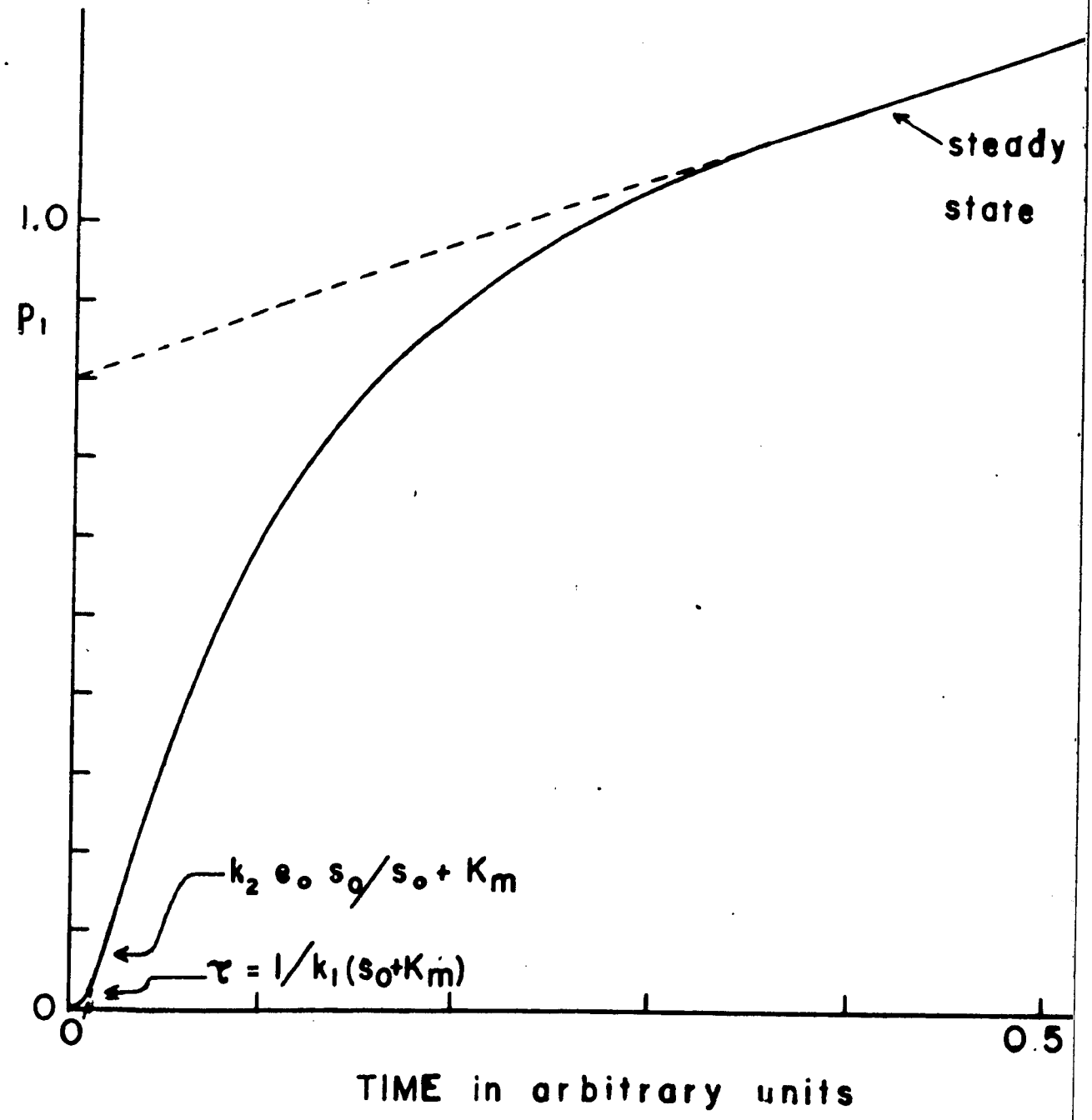


Fig. 2 A HYPOTHETICAL PLOT OF P_1 ASSUMING $s_o \gg K_m$, and $k_1 s_o$, K_2, and k_3 EQUAL TO 100, 10, AND 1, RESPECTIVELY.

considered large in comparison with k_2. With this in mind equation [25] may be written simply as

$$[45] \quad p_1 = \frac{k_2 Rt}{Q} + k_2 Mt - \frac{k_2 N}{G} ,$$

and when $p_1 = 0$

$$[46] \quad T = 1 / k_1 (s_o + K_m) .$$

This latter expression for the induction period is the same as that for the system involving a single complex (46,64,67).

Thus, provided the induction period T is measurable, the rate constant k_1 for the initial combination of enzyme and substrate could be determined. However, in those cases where the relationship is expected to apply, the induction period has unfortunately turned out to be unmeasuredly small (3,63). This result implies that the exponential involving Gt is negligible even at the beginning of the reaction. It is, therefore, possible to omit this term from equation [44], and assuming k_2 is much larger than k_3 we have

$$[47] \quad p_1 = k_3 e_o t + e_o (1 - \text{exp. } Ft)$$

$$= k_3 e_o t + e_o [1 - \text{exp. } [-k_2 s_o/(s_o + K_m)] t] .$$

If this assumption is valid, i.e. that the first complex ES_1 has reached the steady-state, then from equation [47] one can describe the initial rate of production of the first appearing product p_1. Another convenient form in which this relationship may be applied results from a

series development of the exponential term.

$$[48] \qquad p_1 = [\, k_3 + k_2 \, s_o/(s_o + K_m)\,]\, e_o t$$

Reliable values for k_2 and K_m may be obtained from this equation using initial rate data and employing the ordinary techniques of enzyme kinetics (68). It should be realized though, that k_3 must be small in comparison with the second term.

(c) Influence of Hydrogen-Ion

The theory of the influence of hydrogen-ion on the rate of enzymic reactions has been rather fully discussed by Laidler (69,70,71,72). Many of these ideas are basically applicable to the "two-complex" system where the experimental evidence indicates that the effect is not completely understood (3,10). As pointed out earlier, in the introduction, the results obtained with the different substrates 2,4-dinitrophenyl acetate (3) and p-nitrophenyl acetate (10) conflict. From the results it appears that the rate constant for the acetylation of chymotrypsin with the former is pH-dependent, while the latter is pH-independent. In order to explain this behaviour it is necessary to examine more closely the reactions which are affected by hydrogen-ion, (the substrate is taken to be unionizable). In light of the work with p-nitrophenyl acetate-trypsin that will be presented later, the following scheme is suggested by the experimental results.

$$[e] \qquad \underset{(e_o - y_1 - y_2 - y_3 - y_4)}{E} + H^+ \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \underset{(y_3)}{EH^+} \qquad K_a = \frac{k_{-4}}{k_4}$$

$$[\,f\,] \qquad \underset{(e_o-y_1-y_2-y_3-y_4)}{E} + \underset{(s_o)}{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{(y_1)}{ES}$$

$$[\,g\,] \qquad \underset{(y_1)}{ES_1} \xrightarrow{k_2} \underset{(y_2)}{ES_2} + \underset{(p_1)}{P_1}$$

$$[\,h\,] \qquad \underset{(y_2)}{ES_2} \xrightarrow{k_3} \underset{(e_o-y_1-y_2-y_3-y_4)}{E} + \underset{(p_2)}{P_2}$$

$$[\,i\,] \qquad \underset{y_2}{ES_2} + H^+ \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} \underset{y_4}{ES_2\,H^+} \qquad K_a' = \frac{k_{-5}}{k_5}$$

This scheme accounts for the discrepancy mentioned, and agrees with a mechanism proposed recently (15).

According to the above reactions ([e] to [i]) the differential equation [1] now becomes,

$$[\,1A\,] \qquad \dot{y}_1 = k_1 s_o (e_o-y_1-y_2-y_3-y_4) - y_1 (k_{-1} + k_2)$$

provided that s_o is much greater than e_o. The relationships

$$[\,49\,] \qquad y_3 = (e_o-y_1-y_2-y_3-y_4) \frac{[H^+]}{K_a}$$

and

$$[\,50\,] \qquad y_4 = y_2 [H^+] / K_a'$$

are readily obtained from the ionic reactions [e] and [i] respectively. By substituting these into equation [1A], the following result is acquired,

$$[\ 1B] \quad \dot{y}_1 = k_1 s_o \frac{K_a}{K_a + [H^+]} \left[e_o - y_1 - y_2 \frac{(K_a' + [H^+])}{K_a'} \right] - y_1 (k_{-1} + k_2).$$

Equation [2] in also modified when the effect of hydrogen-ion is considered,

$$[\ 2A] \quad \dot{y}_2 = (k_2 y_1 - k_3 y_2)\ K_a' / (K_a' + [H^+]).$$

(1) Steady-State

When the steady-state hypothesis is applied by setting equations [1B] and [2A] equal to zero, then

$$[\ 6A] \quad y_2 = \frac{k_1 k_2 s_o e_o K_a' / (K_a' + [H^+])}{k_1 s_o (k_2 + k_3') + k_3' (k_{-1} + k_2)(K_a + [H^+]) / K_a}$$

and

$$[\ 7A] \quad \dot{p}_1 = \dot{p}_2 = \frac{k_1 k_2 k_3' e_o s_o}{k_1 s_o (k_2 + k_3') + k_3' (k_{-1} + k_2)(K_a + [H^+])/K_a}$$

where

$$[\ 51] \quad k_3' = k_3 K_a' / (K_a' + [H^+])$$

Once again, equation [74] reduces to the classical Michaelis-Menten form

$$[\,9A\,] \qquad \dot{p}_1 = \frac{V'_{max}\, s_o}{s_o + K'^{*}_m}$$

However, in this instance K'^{*}_m and V'_{max} are given as

$$[\,10A\,] \qquad V'_{max} = \frac{k_2\, k'_3\, e_o}{k_2 + k_3}$$

and

$$[\,11A\,] \qquad K'^{*}_m = \frac{k'_3}{k_2 + k'_3} \cdot K'_m$$

where

$$[\,52\,] \qquad K'_m = \frac{k_{-1} + k_2}{k_1} \cdot \frac{K_a + [\,H^+\,]}{K_a} = K_m \cdot \frac{K_a + [\,H^+\,]}{K_a}$$

For those enzyme systems such as trypsin or chymotrypsin with p-nitrophenyl acetate, where k_2 and s_o are considerably greater than k'_3 and K'^{*}_m respectively, the complicated equation [7A] takes the simpler form

$$[\,53\,] \qquad \dot{p}_1 = \text{rate} = k'_3\, e_o$$

and

$$[\,54\,] \qquad \frac{1}{\text{rate}} = \frac{1}{k_3\, e_o} + \frac{1}{k_3\, e_o} \cdot \frac{[\,H^+\,]}{K'_a}$$

The latter expression may be utilized to determine k_3 and K'_a (68), provided

of course that e_o is known. Besides being the proton-inhibition constant for deacylation, K_a' bears a significant relationship to the catalytic site as will be discussed later.

(2) Transient Phase

The kinetics are derived in the same manner as before, but by differentiating equation [1B], and eliminating $\dot{y}_2$ and y_2 from the result using equation [1B] and [2A]. When this operation is performed, the following expression is arrived at

$$[\ 14A\ ] \quad \ddot{y}_1 + \left[k_1 s_o \frac{K_a}{K_a + [H^+]} + k_{-1} + k_2 + k_3' \right] \dot{y}_1 +$$

$$\left[k_1 s_o \frac{K_a}{K_a + [H^+]} (k_2 + k_3') + k_3' (k_{-1} + k_2) \right] -$$

$$k_1 k_3' e_o s_o \frac{K_a}{K_a + [H^+]} = 0$$

As before this equation takes the form of [15] which may be integrated, but the coefficients P, Q and R are now given by

$$[\ 16A\ ] \quad P = k_1 \frac{K_a}{K_a + [H^+]} (s_o + K_m') + k_3'$$

$$[\ 17A\ ] \quad Q = k_1 \frac{K_a}{K_a + [H^+]} \left(s_o k_2 + k_3' (s_o + K_m') \right)$$

$$[\ 18A\ ] \quad R = k_1 \frac{K_a}{K_a + [H^+]} \cdot k_3' e_o s_o \ .$$

Using these relationships, which now take into account the effect of hydrogen-ion on the transient phase, and under the conditions where k_3' is negligible compared to k_1 or k_2, the constants F and G become

$$[\ 42A] \qquad F = - k_2 s_o / (s_o + K_m')$$

$$[\ 43A] \qquad G = - k_1 \frac{K_a}{K_a + [\ H^+]} (s_o + K_m') + k_2 s_o / (s_o + K_m')$$

Any of the equations, i.e. [44] to [48], may be restated in terms of these new constants, but the one of primary concern is

$$[\ 47A] \quad p_1 = k_3' e_o t + e_o (1 - \exp [\ - k_2 s_o / (s_o + K_m')]\ t).$$

Under the appropriate conditions it should be possible to utilize this equation and determine k_2, k_3, K_m, K_a, and K_a', provided a method can be developed for the estimation of the exponential factor F. It is also interesting to note that this equation reduces to [53] when t is large.

(3) Guggenheim Treatment

Fortunately, equations of the type [47A] lend themselves to the Guggenheim method (10,73), which has also been extended to include second order reactions (74). This treatment is particularly useful in the study of unimolecular reactions where the initial and/or the final concentrations are unknown, and where the relative readings taken during the course of a reaction cannot be conveniently converted to concentration.

If r_1, r_2, and r_3 are any series of reading proportional to the amount of product p_1 at times t_1, $t_1 + \Delta$, and $t_1 + 2\ \Delta$ respectively, then

[55] $r_1 = At_1 + B\ (1 - \exp Ft_1)$

[56] $r_2 = A\ (t_1 + \Delta\ ) + B\ (1 - \exp F\ (t_1 + \Delta\ )\ )$

and

[57] $r_3 = A\ (t_1 + 2\ \Delta\ ) + B\ (1 - \exp F\ (t_1 + 2\ \Delta\ )\ )$

where

[58] $r = C\ p_1$

[59] $A = C\ k_3^{'}\ e_o$

[60] $B = C\ e_o$

and Δ is a fixed interval of time.

The addition of equations [55] and [57], and the subtraction of twice equation [56] leads to

[61] $r_1 + r_3 - 2r_2 = -\ B\ \exp Ft_1\ (\exp 2\ \Delta F - 2 \exp \Delta F + 1)$

or

[62] $\log\ (r_1 + r_3 - 2r_2) = \log \text{constant} + Ft_1$.

Using this linear equation it should be possible to determine F from the slope of a plot of $\log\ (r_1 + r_3 - 2r_2)$ against t_1. Since equation [42A] may be rearranged to

$$[63] \qquad -\frac{1}{F} = \frac{1}{k_2} + \frac{1}{s_o}\left(\frac{K_m}{k_2} + [H^+]\,\frac{K_m}{K_a\,k_2}\right),$$

or

$$[64] \qquad -\frac{1}{F} = \left(\frac{1}{k_2} + \frac{1}{s_o}\,\frac{K_m}{k_2}\right) + [H^+]\,\frac{K_m}{s_o\,K_a\,k_2},$$

where the slope of equation [63] is

$$[65] \qquad S = \frac{K_m}{k_2} + [H^+]\,\frac{K_m}{K_a\,k_2}$$

and the intercept of equation [64] is

$$[66] \qquad I = \frac{1}{k_2} + \frac{1}{s_o}\,\frac{K_m}{k_2},$$

appropriate plots of the data at various hydrogen-ion and substrate concentrations (68,71) should yield values for k_2, K_m, and K_a.

Finally, it should be noted that the accuracy of the Guggenheim method (73) increases with increasing values of Δ . And, if Δ is several times greater than the half-time of reaction, the accuracy should be equivalent to that attained by the conventional end-point method.

Experimental

A. Apparatus

The apparatus photographed in Figure 3 was used for the investigation of rapid enzymic reactions and is shown in Figure 4 as a block diagram. The light source, monochromator, detector (photomultiplier), and DC amplifier with recording adapter are Beckman model DU spectrophotometric equipment. Only two minor modifications were made to this equipment. The time constant of the circuit was decreased by altering the load resistance on the photomultiplier tube from 22 megohms and 0.01 microfarads to 0.68 megohms and 0.002 microfarads. Although this creates a drop in senstivity it has no adverse effect on the end result, since the sensitivity may be restored within certain limits by opening the slit of the monochromator. Secondly, the 510 ohm output resistor of the recording adapter was replaced with a 750 ohm potentiometer, so that the sensitivity may be varied or the output impedence could be matched with the recorder.

(a) Stopped-Flow Mixing Device

The apparatus to be described is in principle the same as that originally conceived by Chance (26,27,28). However, several features such as: a plunger-release mechanism, standardization, temperature control, the use of rubber "O" ring seals, removable storage syringes, and interchangeable observation tubes and mixing chambers have been added for greater ease in operation and construction.

The details of the apparatus, which is illustrated in plan view by Figure 5, and as a photograph in Figure 6, are as follows. The one ml. reaction syringes '1' are held in the plexiglass enclosure '2' which acts as a constant-temperature bath, fitted with water inlet '3' and outlet '3A'.

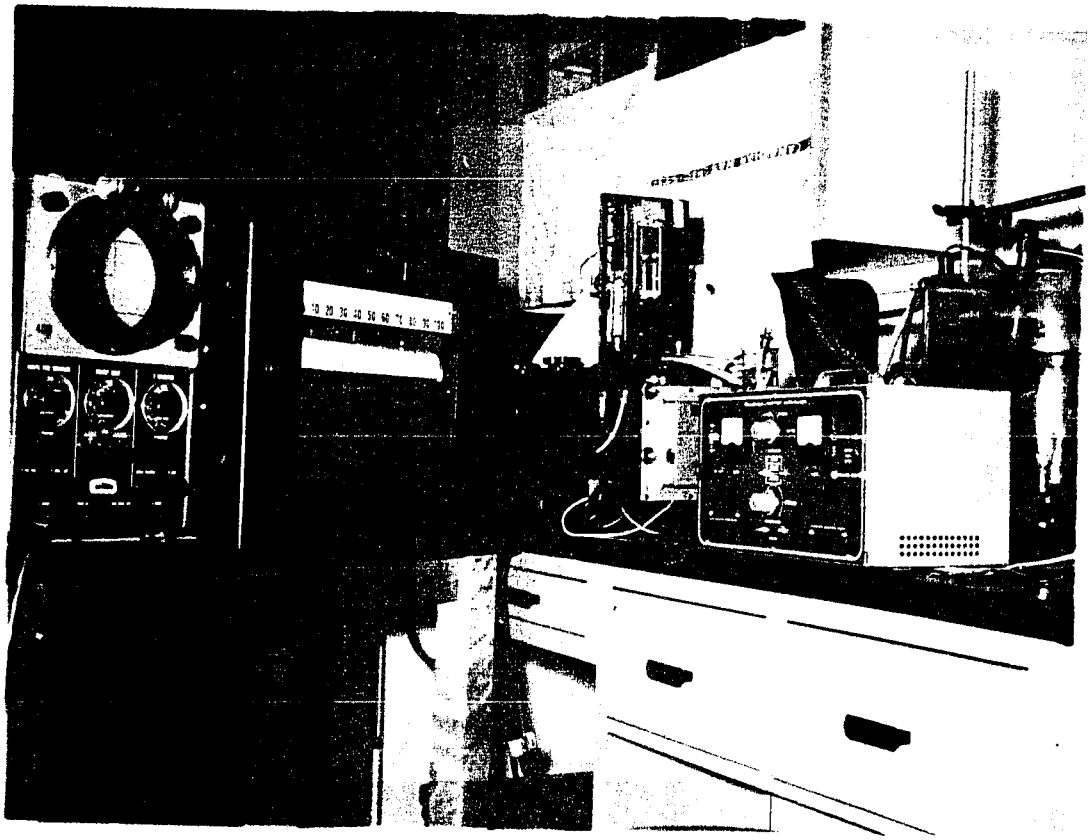


Fig. 3 A PHOTOGRAPH OF THE APPARATUS

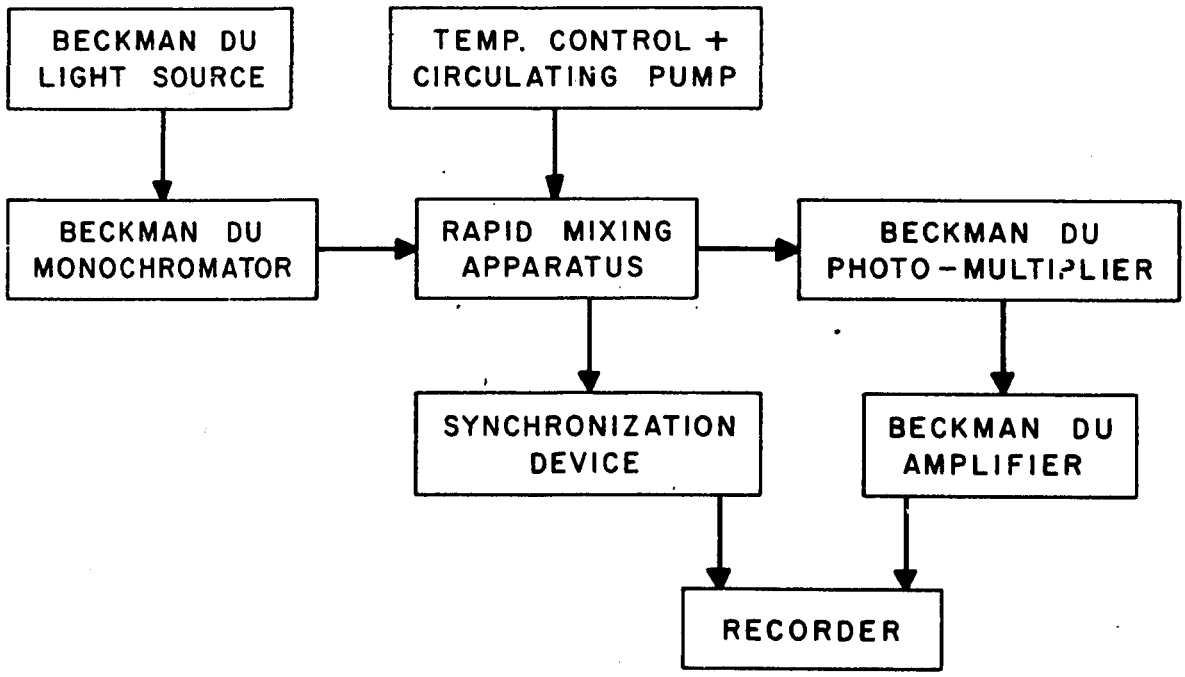


Fig. 4 A BLOCK DIAGRAM OF THE APPARATUS

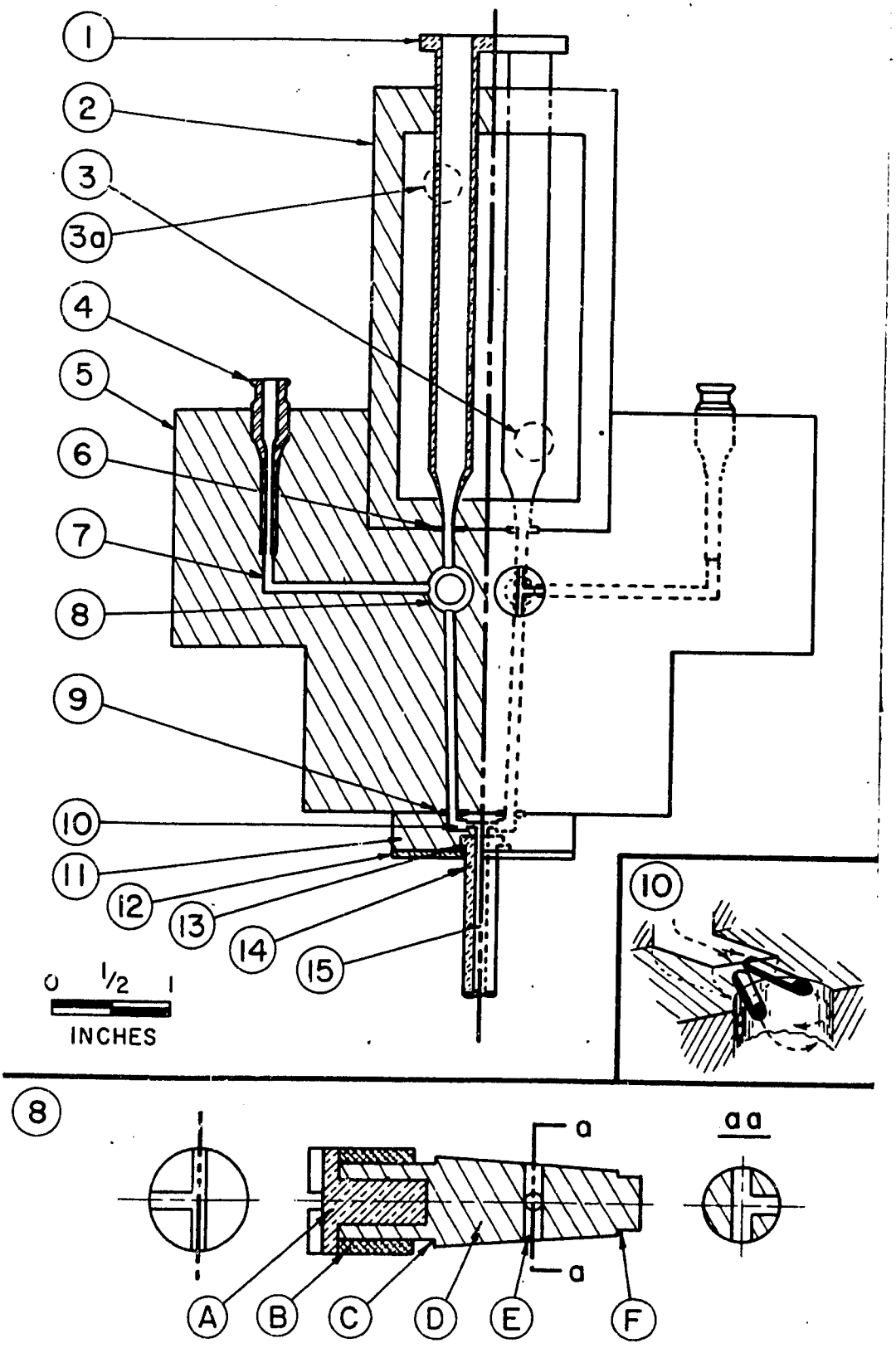


Fig.5 The Stopped-Flow Mixing Device.

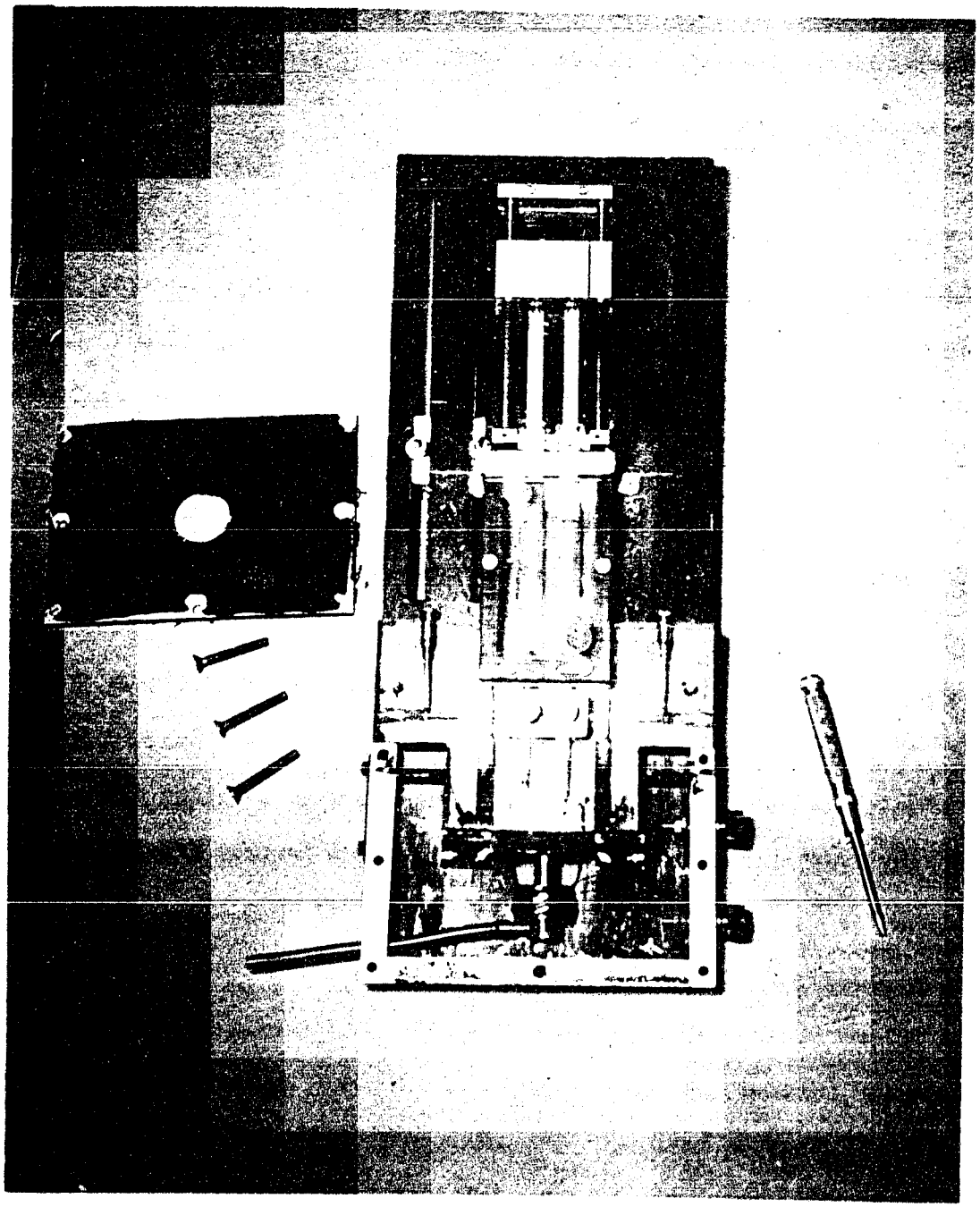


Fig. 6 A PHOTOGRAPH OF THE STOPPED-FLOW MIXING DEVICE, DISMANTLED

The reaction syringes are filled from removable storage syringes located on hypodermic needles '4' embedded in the plexiglass section '5'. The lower insert in Figure 5 gives the details of the T-bore plug of the stopcock '8', which connects the veins '7' from storage syringe to reaction syringe, reaction syringe to mixing chamber, and storage syringe to mixing chamber. The T-slotted head of brass core 'A' is positioned to coincide with T-bore 'E' to aid in the alignment of the bore with the veins. An aluminum sleeve 'B' secures the plexiglass plug 'D' to brass core 'A'. The external leakage of liquid is prevented by rubber "O" ring seals located at 'C' and 'F', which are held in position by pressure plates (not shown).

A three-dimensional sketch of the mixing chamber '10' is illustrated in the right-hand insert of Figure 5. Here, each reactant enters through a pair of 0.5 mm jets, that are located between the tangent and diameter of the chamber to prevent streamline and turbulent flow respectively (29). The mixed reactants pass into the observation cell '15' where the change in absorbance is measured photoelectrically. The 2 mm observation cell was constructed of plexiglass, but provision has been made in the design for the use of quartz or pyrex observation tubes. These could be sealed in the plexiglass block '11' by a rubber "O" ring and pressure plate '12'.

The plexiglass sections '2', '5', and '11' are fastened together with vertical screws, and external leakage at the joints is prevented by rubber "O" ring seals at '6' and '9'. The apparatus is attached to an aluminum plate which replaces the cell compartment of a Beckman DU spectrophotometer as illustrated in Figure 7. This aluminum plate also

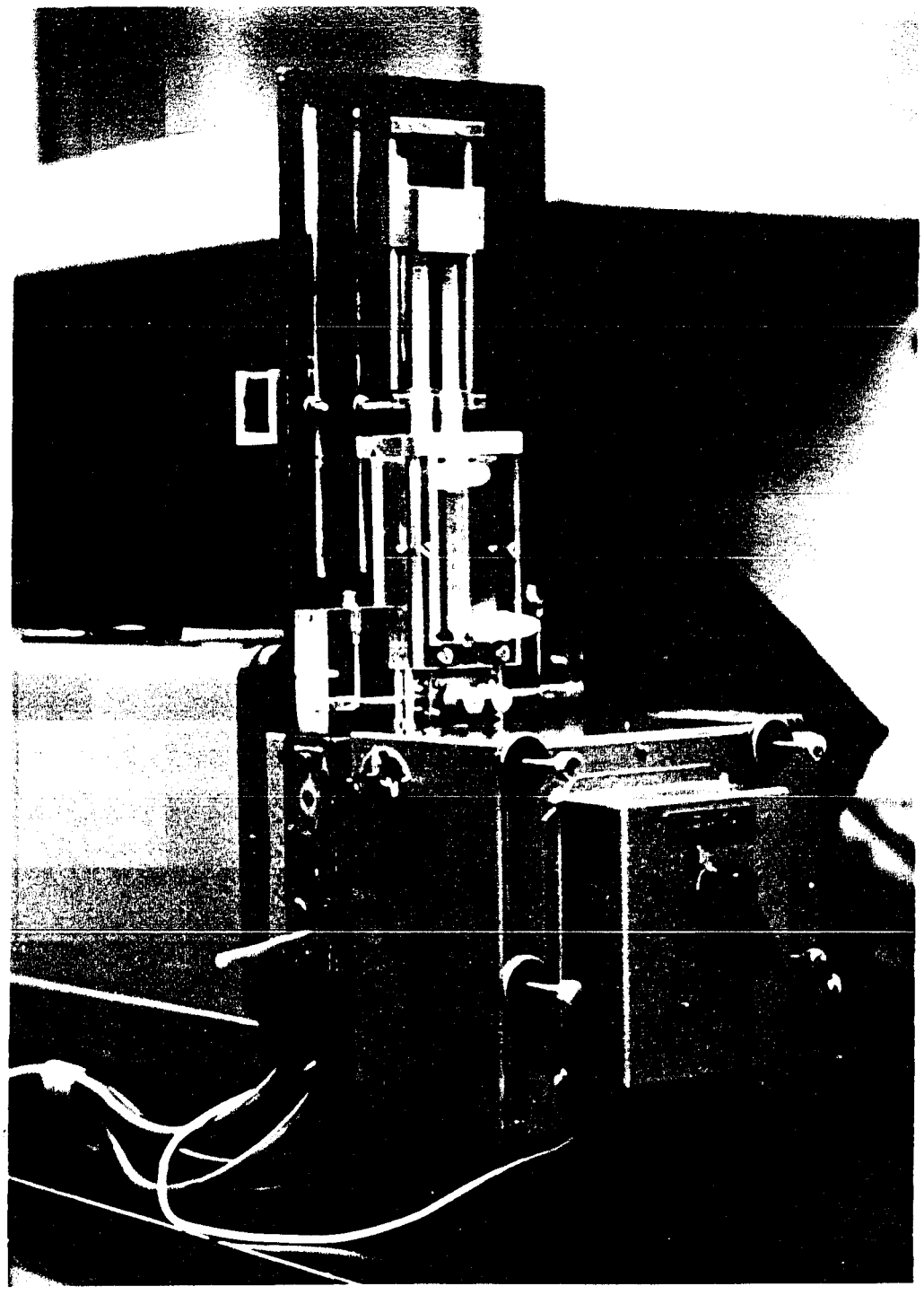


Fig. 7 A PHOTOGRAPH OF THE STOPPED-FLOW MIXING DEVICE MOUNTED IN THE BECKMAN DU

supports the plunger and synchronization mechanism.

(b) Plunger-Holder and Release Mechanism

Equal portions of the two reactants are driven manually from the reaction syringes into the observation cell by the mechanism shown in Figure 8. A track consisting of two verticle rods carries a brass block that drives the plungers. The downward motion of the plungers is restrained by a plunger-release plate which is hinged only to one side of the track so that the plungers may be freed. This allows the reaction syringes to be filled by pressure from the storage syringes, or the plungers to be removed for the elimination of residual air by completely filling the syringes.

(c) Synchronization Mechanism

The synchronization mechanism in Figure 9 is used to synchronize the events of stopped flow and the commencement of recording. This is accomplished by a brass arm on the carriage of the plunger mechanism (Figure 8), which activates the "L" leaf of a snap-action switch when it reaches the crossbar of the case surrounding the switch (Figure 9). Synchronization is adjusted by a screw located in this crossbar, and can be regulated, if desired, to start recording before flow stoppage.

In the "on" position the switch applies an electrical potential to initiate the sweep circuit of a cathode-ray oscillograph, the chart drive of a pen recorder, or the operation of a time-marker device. In those cases where more than one potential is to be synchronized a fast acting four-pole-double-throw relay may be incorporated in the circuit.

(d) Temperature Control

Water from a thermostated bath capable of control to $\pm$ 0.1°C is

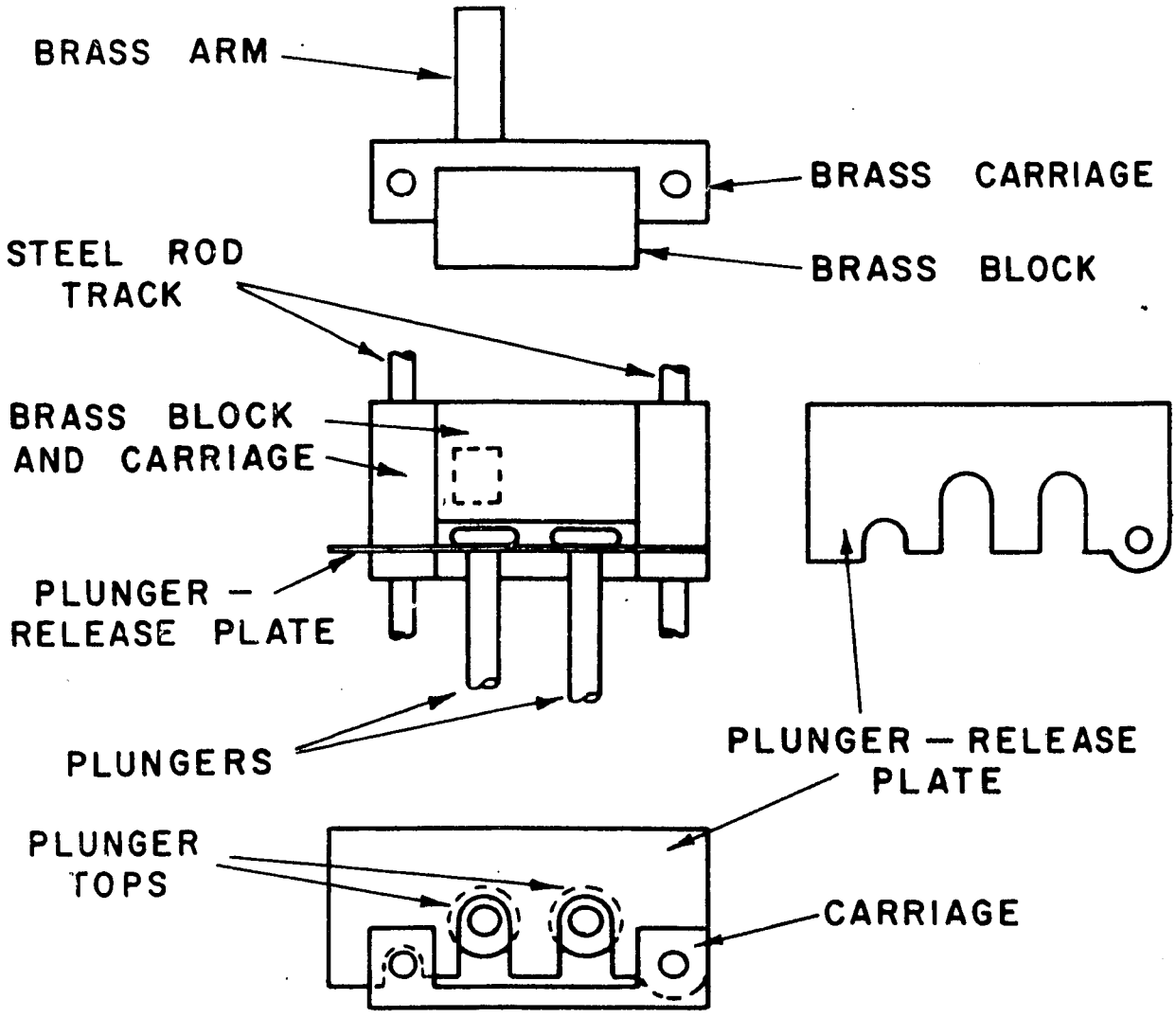


Fig. 8 THE PLUNGER-HOLDER AND RELEASE MECHANISM

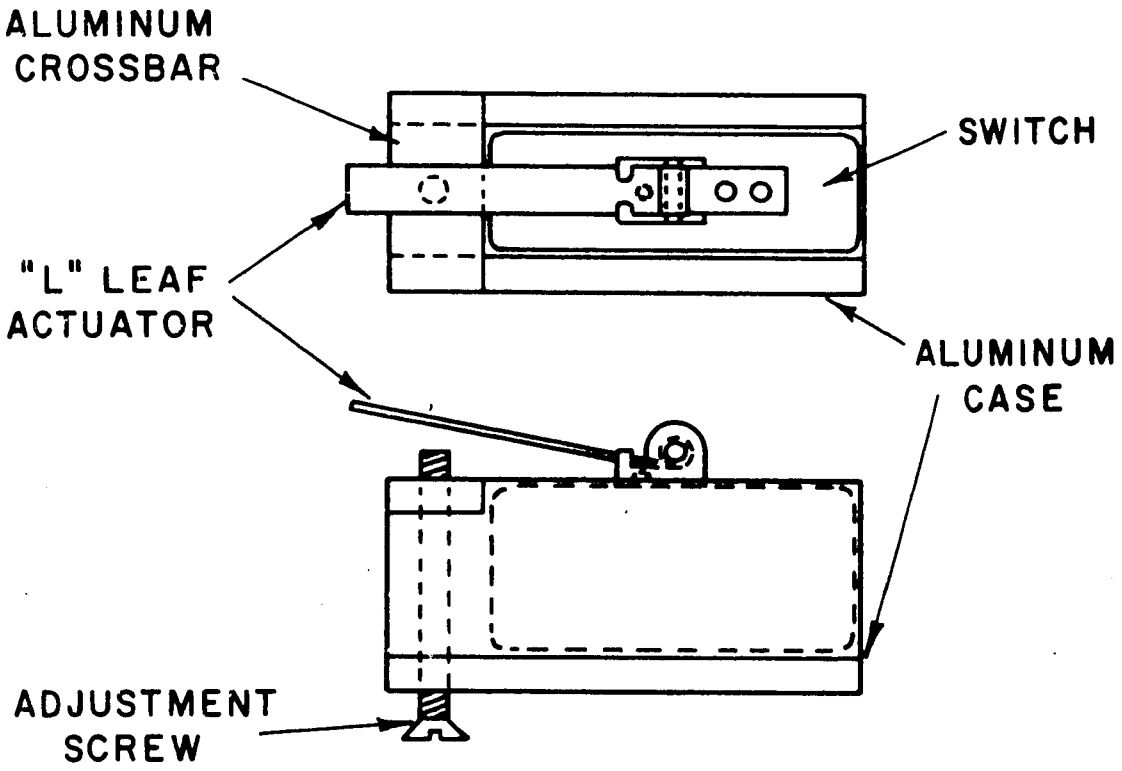


Fig. 9 THE SYNCHRONIZATION MECHANISM

circulated through, the enclosure around the reaction syringes, Beckman thermospacers, and the lamp housing of the spectrophotometer.

(e) Recorders

The cathode-ray oscillograph, the pen oscillograph, and the high-speed strip-chart recorder are all suitable for studying rapid reactions, each having its own particular advantage. The cathode-ray oscillograph is useful when a response of < 0.01 sec. is required, while the pen oscillograph could be commended above this range because of its direct-writing feature. However, in the case of certain reactions, and especially those which have been studied for this thesis, the high-speed strip-chart recorder with a response of 0.25 sec. for a 10 in. deflection was found to be sufficient.

B. Mode of Operation

Reactants kept in a constant temperature bath were transferred to the reaction syringes by means of the storage syringes. The reaction was recorded by manually driving the plunger mechanism, which forces the reactants into the mixing chamber and the observation cell. When the flow stopped, a synchronized recorder produced the reaction trace.

The following zero suppression method was found to achieve the sensitivity required by the stopped-flow technique (37). With the phototube shutter closed (0% transmittance) the output voltage of the recording adapter was given an arbitrary value (300 mv available) using the suppression and dark current adjustments. The phototube shutter was then opened, and the slit of the monochromator varied until this output was zero. Using the solvent as blank the latter may be chosen to correspond to 100% transmittance or a convenient percentage reaction with an appropriate standard solution in the observation cell. The first procedure was used in this work where the reactants are colourless and the products coloured but the second should be adapted when it is necessary to study the disappearance of coloured reactants.

All voltage measurements were made using a Tinsley, type 3184D, portable potentiometer which could be read to < 0.01 of a millivolt.

C. Application

The apparatus and techniques described have been employed in a kinetic study of the enzymic hydrolysis of p-nitrophenyl acetate with trypsin and is the main subject matter of this thesis. However, the stopped-flow method has been used in a similar instance for the study of p-nitrophenyl acetate hydrolysis with chymotrypsin (10). It was, therefore, convenient to repeat part of this work as a check on technique.

(a) Reagents

(i) Enzyme. Twice crystallized, salt free, chymotrypsin and trypsin were purchased from the Mann Research Laboratories, Incorporated, New York.

(ii) Substrate. p-nitrophenyl acetate was synthesized according to the method of Chattaway (75), and recrystallized from ethanol-water solution m.p. 78-79°; lit. m.p. 79.5-80° (76) and 77.5-78° (48).

(iii) Buffers. The appropriate buffered solutions were prepared from monosodium and disodium phosphate so that the final reactant solutions contained 0.05M phosphate.

(iv) Solvent. To improve the solubility of substrate 20% (V/V) isopropyl alcohol was employed.

(b) Results

(1) p-Nitrophenyl Acetate with Chymotrypsin.

For this system the rate of appearance of p-nitrophenol (405 mμ) was recorded at small values of t (time). These measurements were then employed to evaluate F in relationship [62] as outlined in the theoretical portion of this thesis.

Figure 10 gives the Guggenheim plot, i.e. log $(r_1 + r_3 - 2r_2)$ vs t_1, for the reaction trace in Figure 11 that was recorded with a Brown

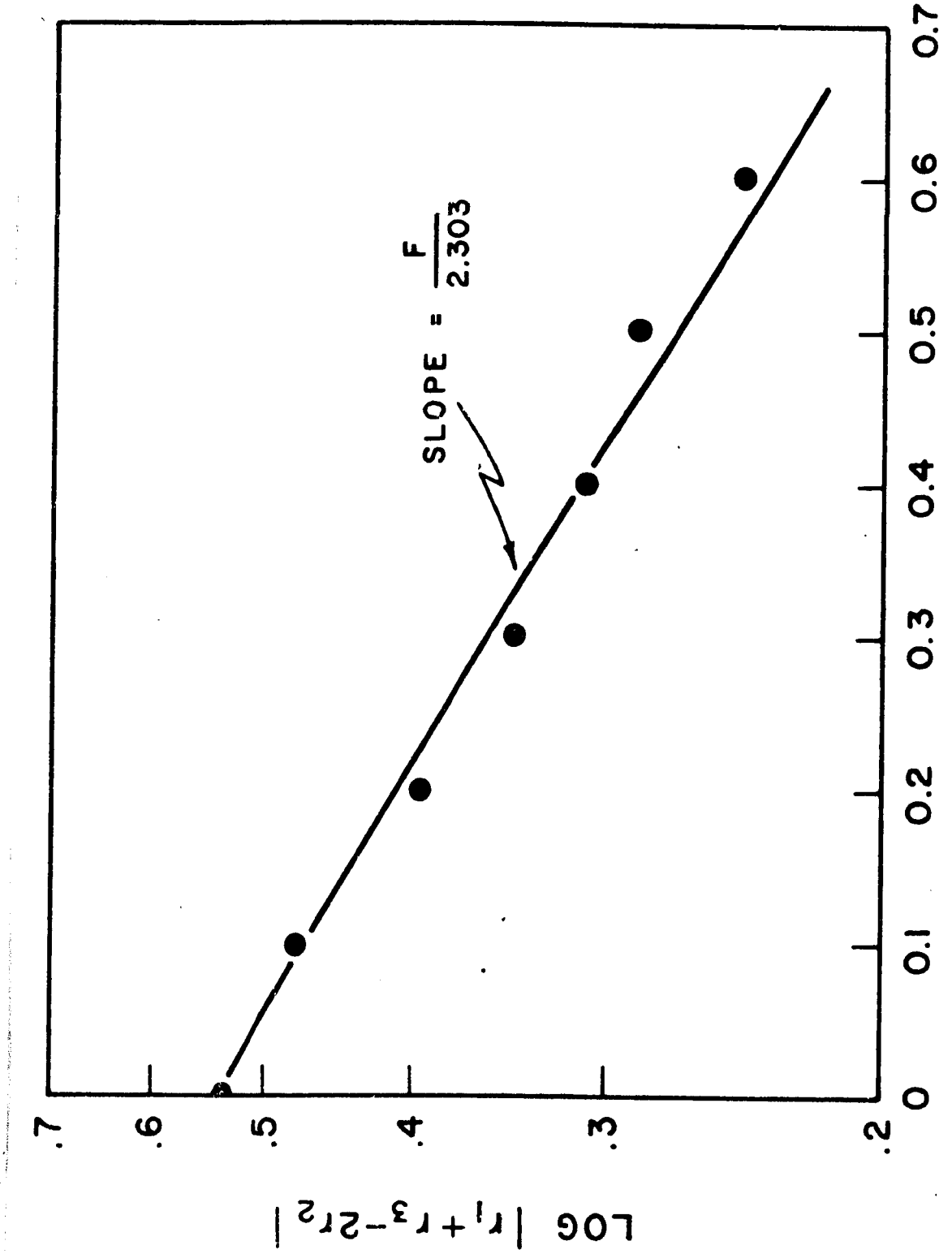


Fig. 10 A GUGGENHEIM PLOT OF THE CHYMOTRYPSIN p-NITROPHENYL ACETATE SYSTEM.

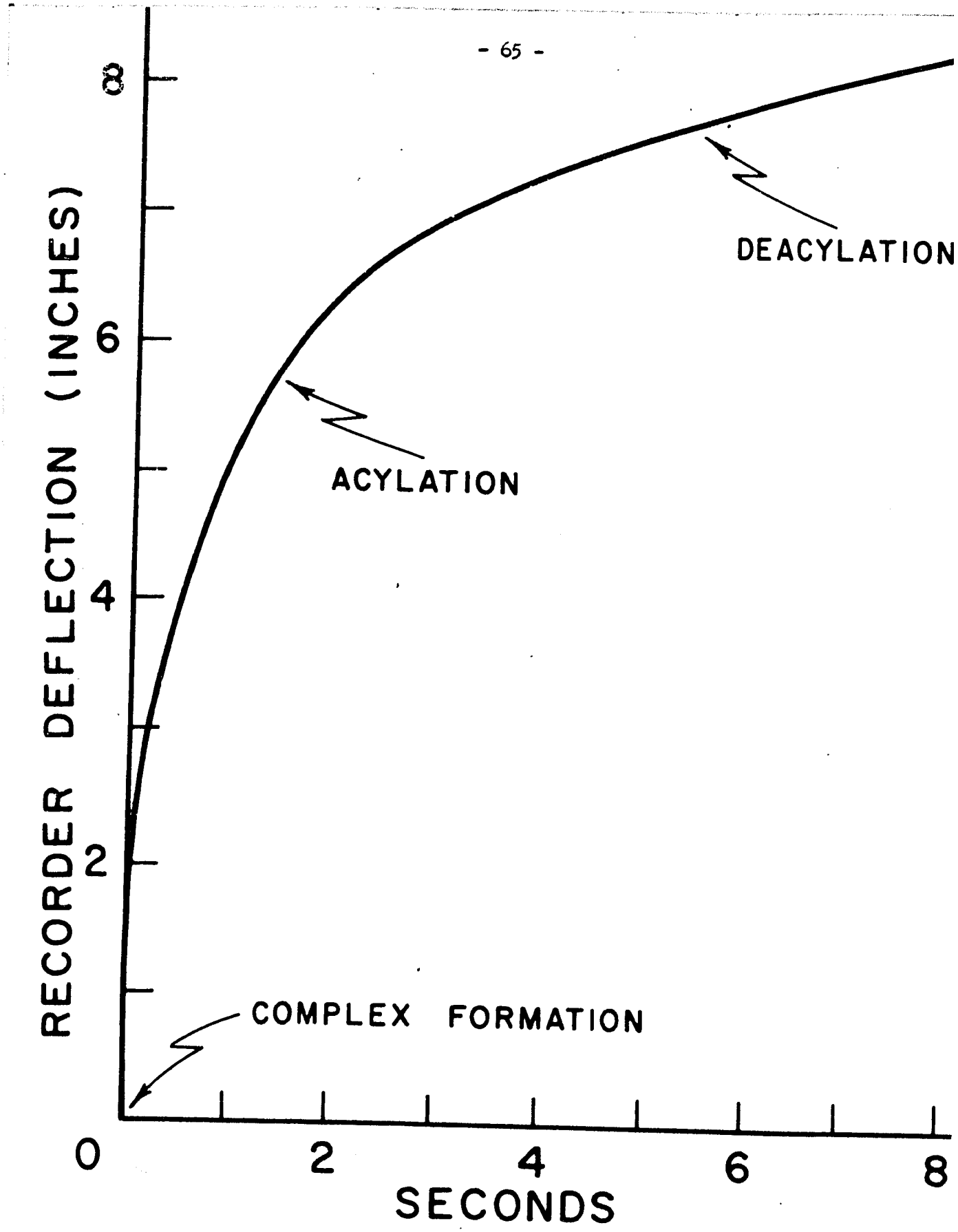


Fig. 11 A REACTION TRACE OF THE CHYMOTRYPTIC HYDROLYSIS OF p-NITROPHENYL ACETATE.

"Quarter Second" strip-chart recorder. The same reaction was also recorded by means of a Brush (BL 202) pen oscillograph and DC amplifier (BL 913), and a DuMont type 403 cathode-ray oscillograph equipped with a Polaroid Land camera type 2620. The values for F obtained from these records are listed in Table I along with the result estimated from the work reported previously (10). The mean result for a substrate concentration of 5×10^{-3} M and a pH 7.8 at 25°C, is 1.34 with a standard deviation of $\pm$ 0.02. This is well within the limit of error expected of a technique as difficult as the stopped-flow method.

(2) p-nitrophenyl Acetate with Trypsin

The rapid acetylation reaction was studied employing the stopped-flow apparatus as described, while the slow deacetylation reaction was followed with a Cary Model 11 spectrophotometer using the differential technique.

(i) Effect of pH on Acetylation. In the 'two complex' scheme of hydrolysis the acylated enzyme is a transient making it necessary to investigate the reaction during its initial stages. Since it is not possible to follow the formation of acylated enzyme, the assumption has to be made that acylation is directly related to if not simultaneous with the release of coloured p-nitrophenoxide-ion. The initial rapid production of p-nitrophenol, Figure 12, corresponding to the formation of acetyl trypsin was recorded at several substrate and hydrogen-ion concentrations. The pH values reported refer to the buffer as measured in the absence of alcohol, but the presence of enzyme, using a Beckman Model G pH meter. The instrument was calibrated using a standard Beckman

TABLE I

The Value of F Obtained with Different Types of Recorders for $S_o = 5 \times 10^{-3}$ M at pH 7.8 and 25°C.

Type of Recorder	-F
Pen Oscillograph (Brush BL201)	1.30 (10)
Pen Oscillograph (Brush BL202)	1.33 ± 0.07
Cathode-Ray Oscillograph (DuMont 403)	1.37 ± 0.12
Strip-Chart (Brown, Quarter Second)	1.35 ± 0.07
Mean	1.34
Standard Deviation	± 0.02

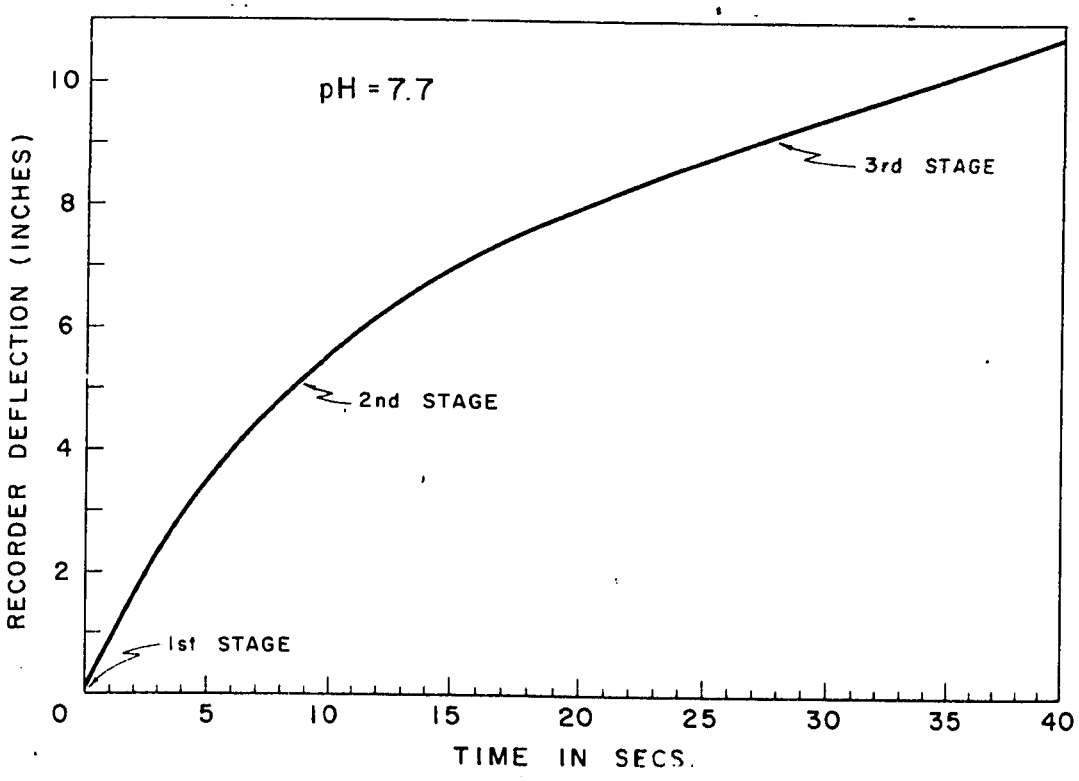


Fig. 12 A RECORD OF THE TRYPTIC HYDROLYSIS OF p-NITROPHENYL ACETATE

buffer pH 7.0. The constant F was then calculated from the reaction trace by the Guggenheim method, Figure 13, i.e. equation [62]. A typical Lineweaver-Burk plot, i.e. equation [63], of some of the results is given in Figure 14, where the extrapolation of 1/F to infinite substrate concentration yields the same value for $1/k_2$ at two widely separated pH values. The fact that seven such plots between pH 6.2 and 7.8 produced the same result for $1/k_2$ strongly suggests competitive inhibition by hydrogen-ions, see e.g. ref. 77. Furthermore the same result, though not elucidated as competitive inhibition, was obtained with chymotrypsin (10). Similar to the usual Michaelis-Menten treatment, the slopes given by the Lineweaver-Burk plot of F as in Figure 14 involve K_m/k_2. According to Laidler (71,77), the variation of K_m/k_2 with pH may provide the dissociation constants of the groups which comprise the active site of the enzyme. It should also be pointed out, however, that should the two-complex mechanisms be general for other enzyme systems, then the standard Michaelis constant may consist of a rather large number of constants.

The results in Figures 14 and 15 may be readily explained if we assume that hydrogen-ion competitively inhibits the initial combination of enzyme and substrate. Such an assumption leads directly to pH-independence for the acetylation stage (k_2), provided that complex formation renders the group or groups involved non-ionizable. This appears to be the case, and justifies the mechanism as postulated in the theoretical section.

Figure 16 was obtained by plotting the slopes of equation [63] against $[H^+]$. The resultant dissociation constant $K_a = 1.4 \times 10^{-7}$, which is related to the active site of the enzyme, was calculated from the

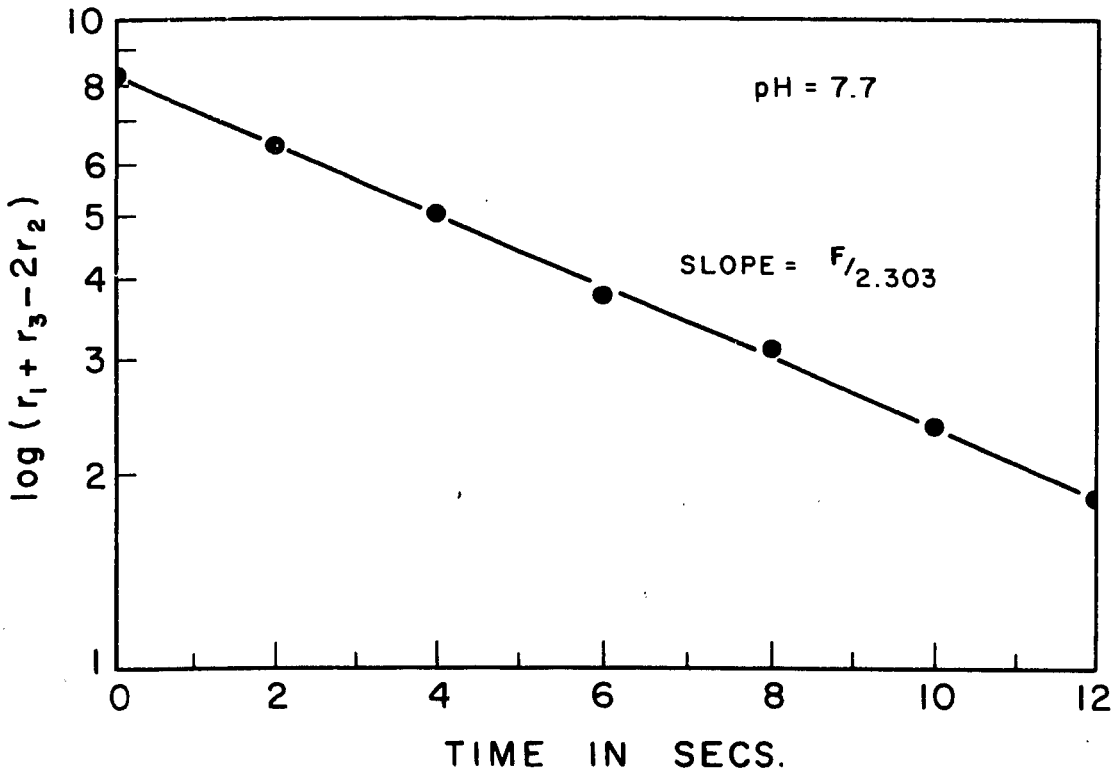


Fig. 13 THE GUGGENHEIM PLOT OF A TYPICAL REACTION TRACE FOR THE p-NITROPHENYL ACETATE-TRYPSIN SYSTEM.

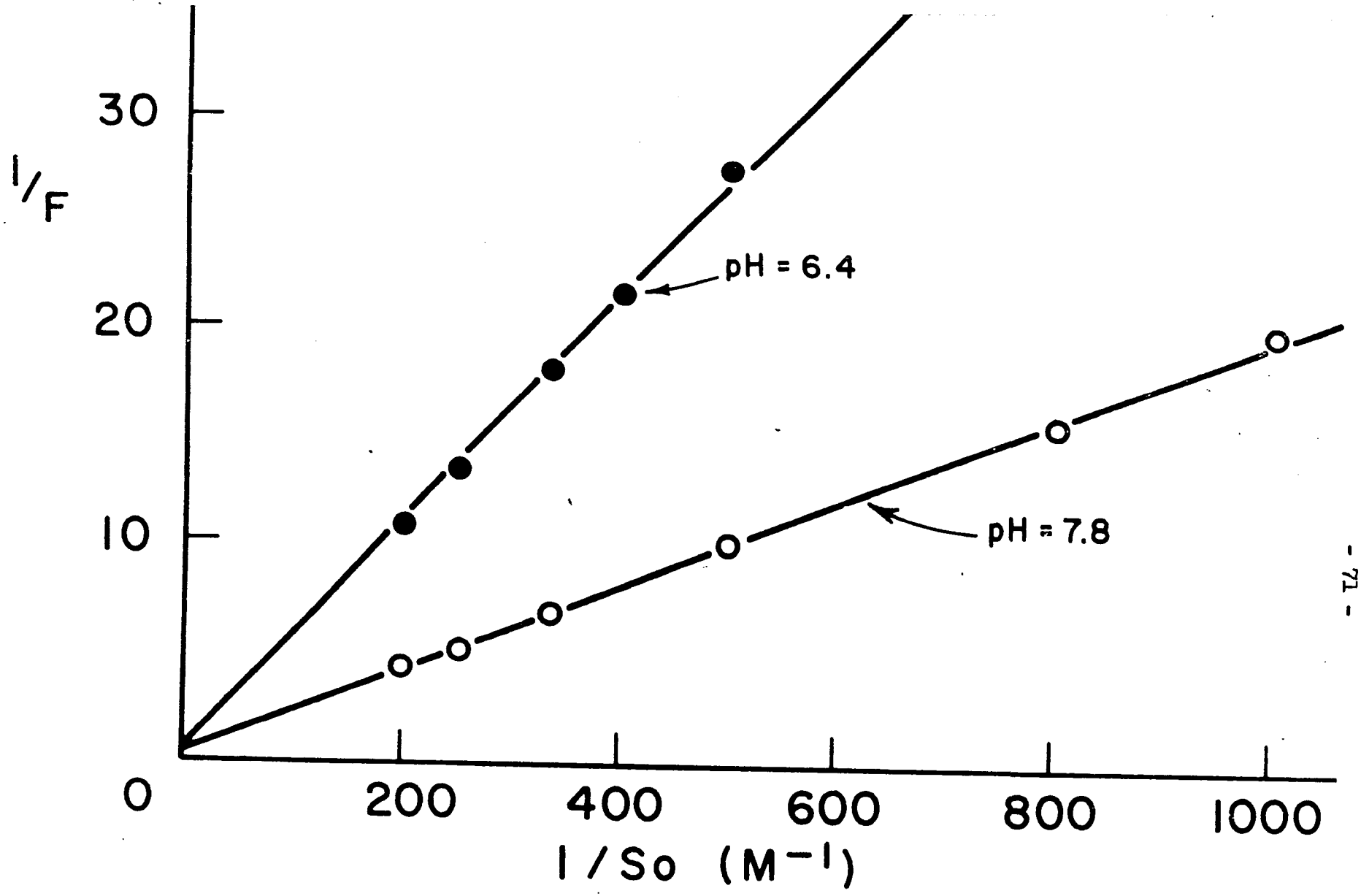


Fig. 14 LINEWEAVER - BURK TREATMENT OF THE DATA

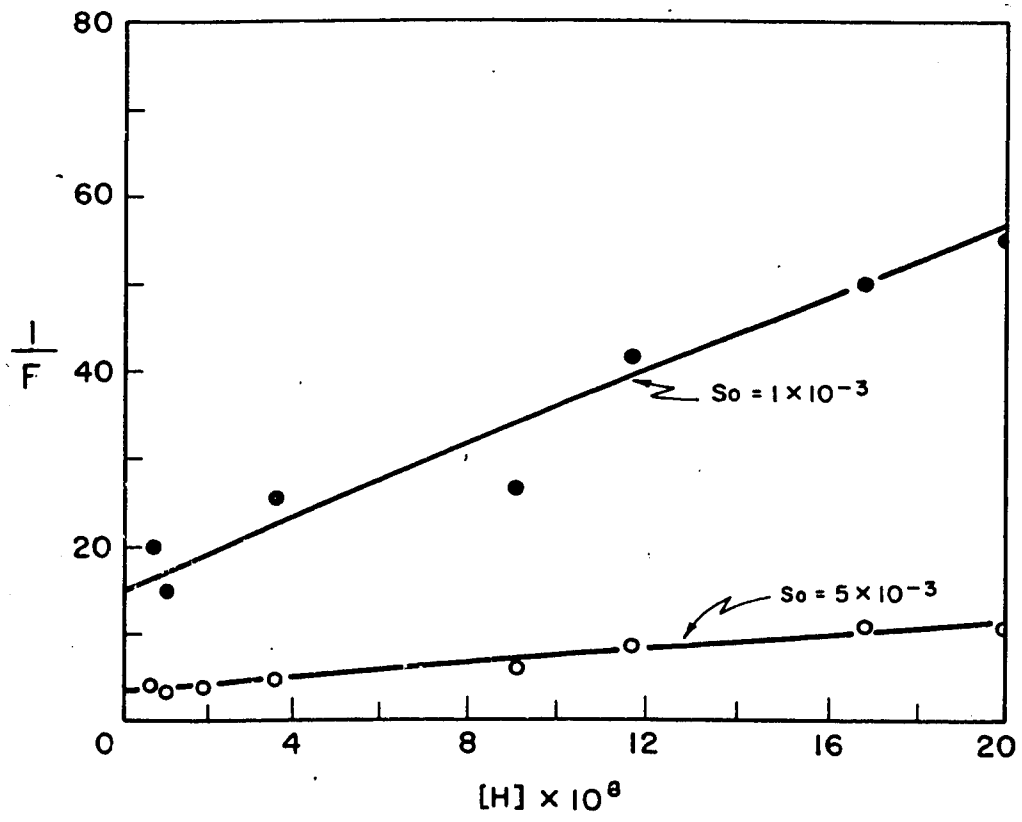


Fig. 15 A PLOT OF 1/F VS. [H^+] AT CONSTANT s_o

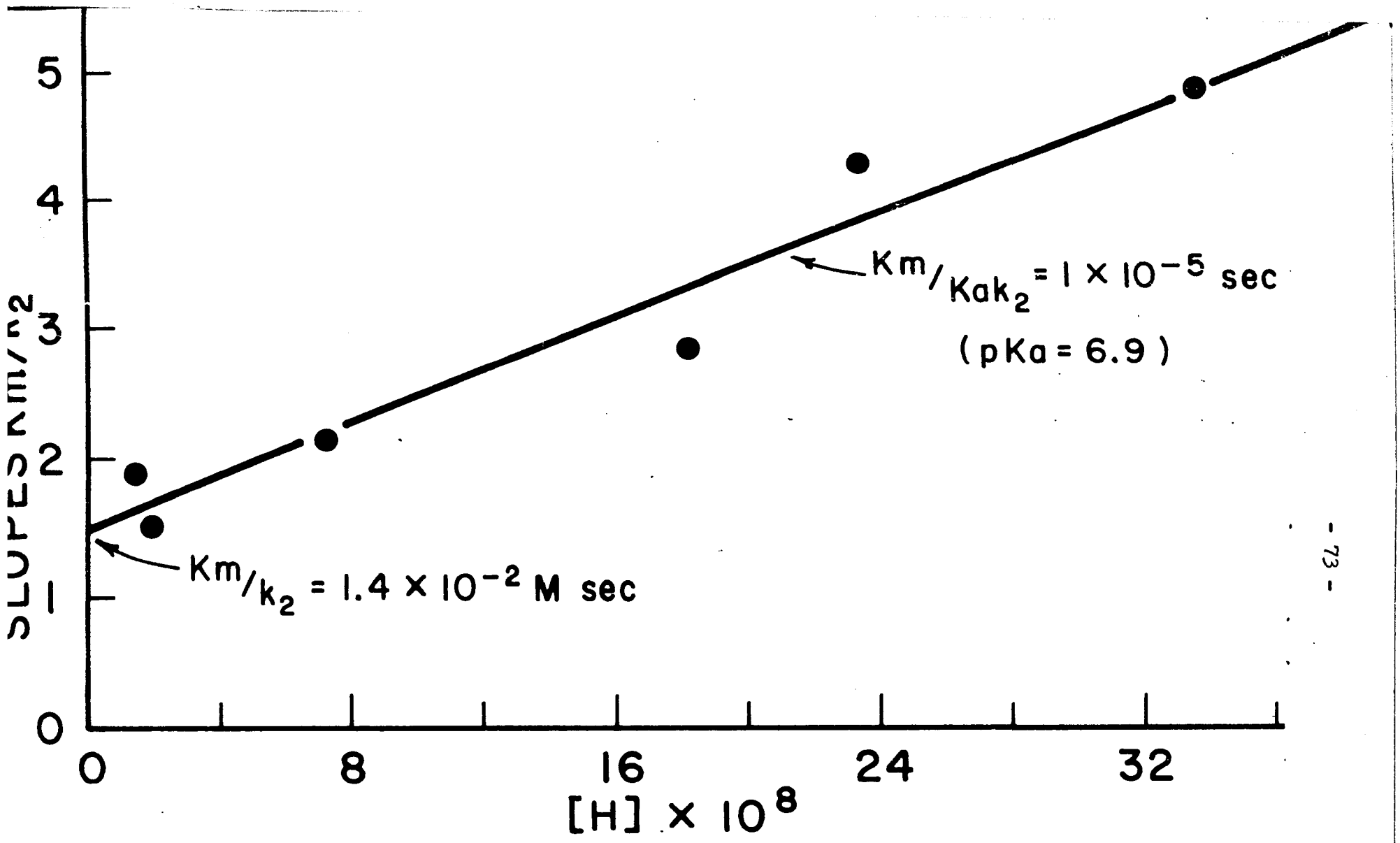


Fig. 16 THE DETERMINATION OF K_m/k_2 AND $K_m/K_a\ k_2$ FROM A PLOT OF SLOPES AGAINST $[H^+]$.

intercept K_m/k_2 and the slope $K_m/k_2 K_a$. The other constants k_2 and K_m were evaluated next by plotting the intercepts of equation [64] against $1/s_o$, as is illustrated in Figure 17. The rate constant of acetylation $k_2 = 1.5$ sec^{-1} was estimated from the intercept $1/k_2$, and allowed the Michaelis constant $K_m = 2.1 \times 10^{-2}$ M to be determined from the slope K_m/k_2.

These results for k_2, K_a, and K_m are included in Table II, where the constants for the trypsin and chymotrypsin systems are compared.

(ii) Effect of pH on Deacetylation. The slow deacetylation reaction was studied spectrophotometrically by following the p-nitrophenoxide-ion produced after the rapid acetylation reaction had subsided. The results were conveniently corrected for the spontaneous hydrolysis of p-nitrophenyl acetate by placing the reaction solution in the sample cell and the same solution less enzyme in the reference cell of the Cary double beam spectrophotometer, which directly recorded the difference in absorbance. The reaction was started by introducing equal portions of p-nitrophenyl acetate simultaneously into both cells with hypodermic syringes. The necessary corrections were then applied to the records to take into account that the fraction of coloured species of p-nitrophenol, i.e. $pK_a = 7.15$ (78), varies with pH.

In the above manner the rates at different hydrogen-ion concentrations were obtained, and are plotted according to equation [54] as the reciprocal rate against $[H^+]$ as in Figure 18. This plot gives a dissociation constant $K_a' = 1 \times 10^{-7}$ for acetylated trypsin and compares favourably with the constant K_a. The significance of this will be discussed later. The rate constant for deacetylation, $k_3 = 1.3 \times 10^{-2}$ sec^{-1},

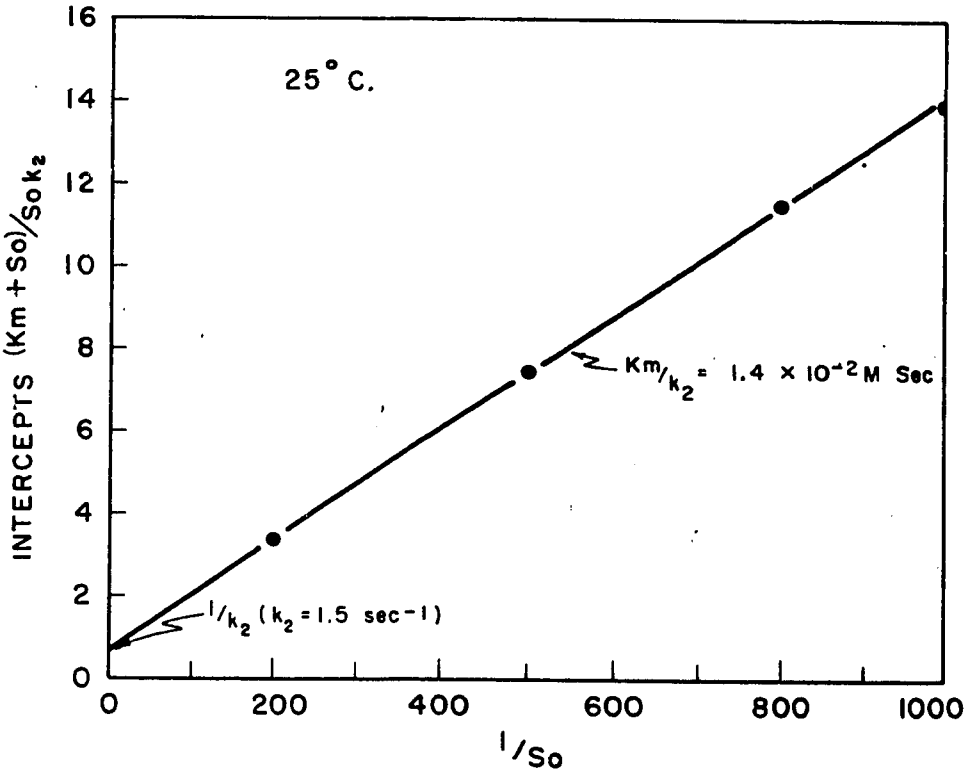


Fig. 17 THE ESTIMATION OF $1/k_2$ AND K_m/k_2 FROM A PLOT OF INTERCEPTS AGAINST THE RECIPROCAL OF THE SUBSTRATE CONCENTRATION.

TABLE II

Kinetic Constants for the Enzymatic Hydrolysis of p-Nitrophenyl Acetate

Constant	Trypsin	Chymotrypsin (10)
k_2 (Sec^{-1})	1.5	3.1
Km (M)	2.1×10^{-2}	6.8×10^{-3}
pK_a	6.9	6.6
pK_a'	7.0	7.3*
k_3 (Sec^{-1})	0.013	0.025

* 6.8 for ester hydrolysis see ref. 11.

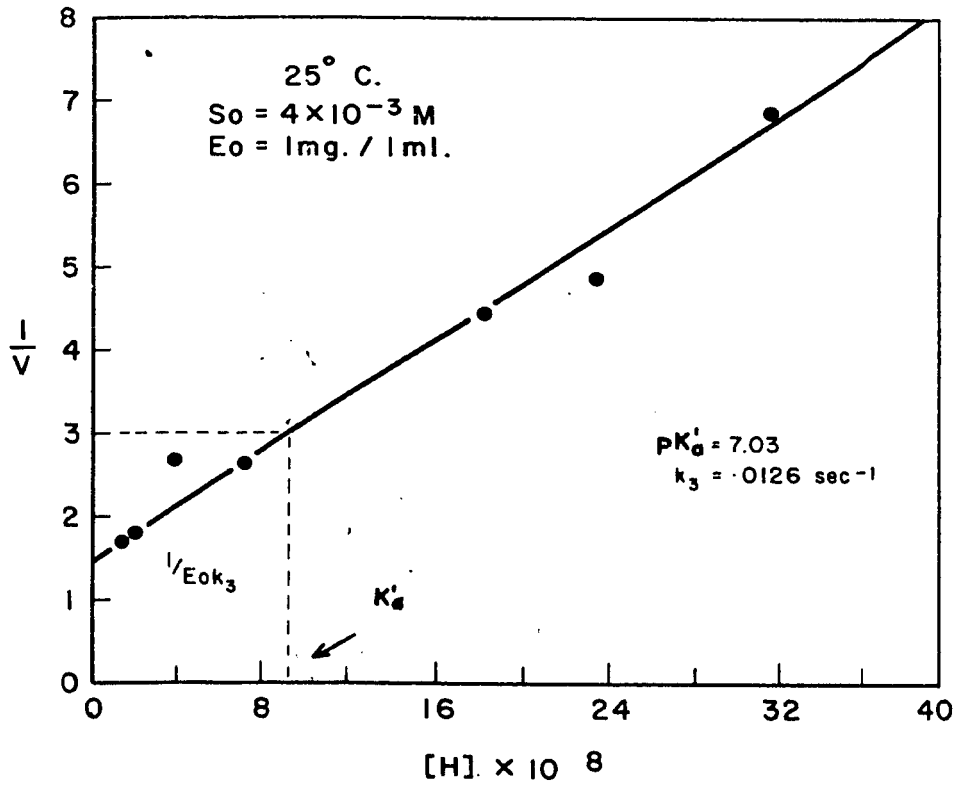


Fig. 18 A PLOT OF THE RECIPROCAL RATE OF LIBERATION OF p-NITROPHENOXIDE-ION VS $[H^+]$, FOR THE DETERMINATION OF $k_3 e_o$ AND K'_a

was determined from the intercept of Figure 18; the extinction coefficient of p-nitrophenol was taken as 1.8×10^4 (79), and the molecular weight of trypsin 21,000 (80).

Discussion

A. Mechanism of Hydrolysis

When the effect of proton inhibition is introduced, the result suggests a mechanism similar to that proposed by Cunningham (15). Quite different from former schemes his mechanism advocates the hydroxyl group as primary site rather than the imidazolyl, which is now thought to be secondary. To facilitate a comparison both mechanisms are shown in Figure 19, where the reactions [e] to [i] are represented in the scheme suggested as a result of this work. As the present experimental method was not designed to detect the acyl-transfer reaction, the absence of this transfer is the main difference in the two schemes. Thus, the rate constant k_3 for deacetylation may be a composite constant involving two stages: the transfer of acetyl to nitrogen and its subsequent release.

In the scheme as shown in Figure 19 the enzyme is inactive to complex formation unless a proton has been released from the active center. This possibly explains why inhibition is competitive rather than non-competitive as is usually found (3,11,44). The discrepancy, however, has theoretical justification. For the transient phase, the substitution of equations [16A] and [17A] into [40] results in,

$$[\,67\,] \qquad \frac{1}{F} = - \frac{s_o + K_m'}{s_o (k_2 + k_3') + K_m' k_3}$$

provided $k_1 \frac{K_a}{K_a + [H]} (s_o + K_m')$ is considerably greater than k_3'. For standard kinetics, the inversion of equation [9A] leads to

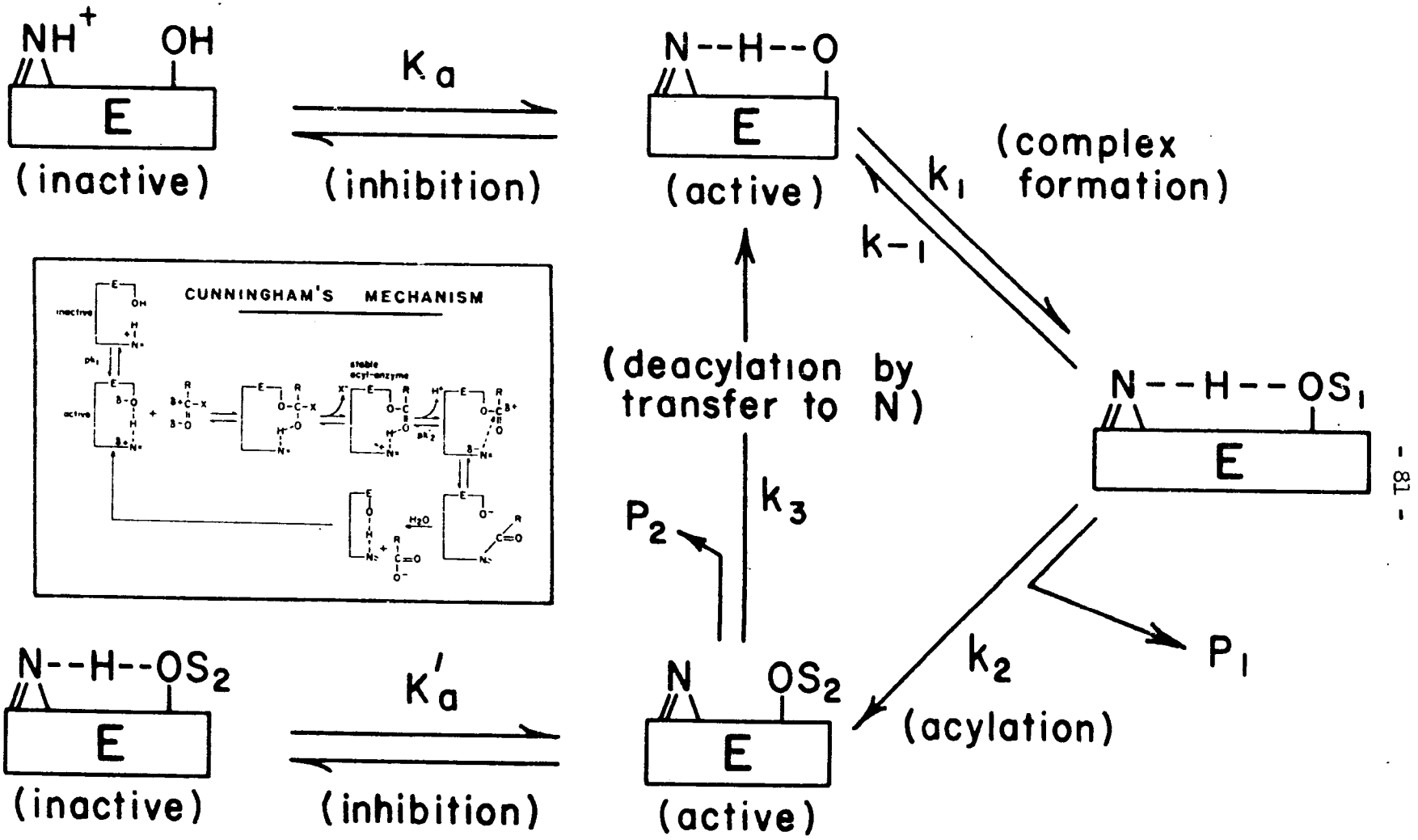


Fig. 19 THE PROPOSED MECHANISM OF ESTERASE HYDROLYSIS

$$[68] \qquad \frac{1}{V} = \frac{1}{V'_{max}} + \frac{K'^{*}_{m}}{s_o V'_{max}} \quad .$$

According to these equations, it is seen that under certain conditions competitive inhibition may not be realized. This is especially true when the rate constants of acetylation and deacetylation are of the same order of magnitude. A Lineweaver-Burk plot of the data will not produce a straight line with constant intercept and competitive inhibition may, therefore, appear to be non-competitive (81). Furthermore, the substrate concentration should be considerably greater than the enzyme concentration; this was an initial condition stipulated in the theoretical section.

The initial point of attack of substrate is the hydroxyl group of serine where the complex undergoes partial decomposition releasing p-nitrophenoxide-ion. The hydroxyl group becomes acetylated, but before deacetylation can proceed there must be a transfer of the acetyl group to the nitrogen. On the basis of this, the stability of certain acylated enzymes at low pH (4,5,11) can be explained, since transfer from the hydroxyl only takes place at high pH values when the nitrogen does not have the inhibiting hydrogen intact. Thus, this proton inhibition at the deacylation stage accounts for the pH-dependence of deacylation, and the inactivity and stability of acetyl-trypsin and chymotrypsin below a pH of 5 to 6.

A similar approach may be used to explain inhibition by certain organophosphorus compounds (1,82,83). For instance, in the case of diisopropyl phosphofluoridate the hydroxyl group of a serine residue

in the enzyme is phosphorylated (18,19,20,22,24,25,45,84,85,86), but the transfer of this phosphoryl group to the nitrogen is difficult and the result is an inactive enzyme.

The mechanism of enzymic hydrolysis outlined in Figure 19 is further supported by the relative magnitude of the ionization constants K_a and K_a', which correspond to the enzyme and acetylated enzyme respectively. If the mechanism is valid these constants should be in close agreement, but not equal. And as was found to be the case (see Table II), K_a' will appear to be slightly greater than K_a, since the acetylation of the serine will possibly lessen the tendency of the nitrogen group to ionize a proton.

The pH-independence of the rate constant k_2 over a range which includes the pK of the imidazole or other nitrogen group, is worthy evidence that acetylation involves a group with a higher pK value such as the hydroxyl of serine. It should not be overlooked, however, that a group such as imidazole may have an indirect influence on the hydroxyl group, conditioning it to acylate and deacylate in the manner necessary to hydrolyze substrate.

B. Nature of the Catalytic Site

Although the postulated mechanism of hydrolysis places the nitrogen containing group (pK = 6.9) in a secondary role, its function is two-fold. In the first instance this group, when ionized, perturbs the hydroxyl hydrogen of serine conditioning it for acylation. Secondly, at the deacylation stage it is an acceptor of the acyl group before it is finally released. This nitrogen containing group has been invariably suggested to be the imidazole group of a histidine residue (15,45,65,66), and, therefore, must be accounted for in the construction of a model active site.

As reviewed in the Introduction Section B(c), tracer experiments with P^{32} labelled diisopropyl phosphorofluoridate show the existence of a reactive serine residue in several enzymes, but no histidine (imidazole) occurs in this vicinity (19,20). According to the most recent report on trypsin (20), the P^{32} phosphorylated enzyme was degraded (by means of performic acid oxidation and tryptic hydrolysis) and several large radioactive peptides were obtained. None of these peptides, one of which had 55 amino acid residues, contained histidine. It was, therefore, concluded that if histidine occurs in the vicinity of the reactive serine it must be a structural phenomenon of the protein. In this respect Westheimer (87) presents the necessary detailed orientation of the active center of chymotrypsin that enables the imidazole and hydroxyl groups to come together by folding. Some spectrophotometric evidence also has been published to show that the acetyl-imidazole linkage is an intermediate when acetyl-chymotrypsin decomposes (88). Several papers (48,89,90,91)

have now appeared which are concerned with the ability of imidazole and its related compounds to catalyze the hydrolysis of phenyl and nitrophenyl acetates. In the examination of a number of these compounds Bruice and Schmir (90) concluded that both the neutral and ionic species contributed to catalysis. Furthermore, when reactive groups other than imidazole (such as hydroxyl or α-amino) were present in the molecule they also contributed. In general, the rate passed through a minimum for those derivatives where the pK of the imidazole group occurred in the region of 4 to 5. The rate constants for the hydrolysis of p-nitrophenyl acetate with several catalysts including imidazole are compared in Table III.

The lack of histidine in the peptide chain containing the phosphorylated serine, and the differences in the pK values and the degree of activity of trypsin and chymotrypsin (see Table II) has led to the idea that other types of nitrogen groups might function in partnership with the hydroxyl group to constitute an active center. This group in chymotrypsin is less basic than in trypsin and has a greater influence on the rate of hydrolysis of p-nitrophenyl acetate. As for model hydrolysis (89), this fact presents the possibility that the degree of basicity of the nitrogen group might be an important factor. For example, since the pK of a free α-amino group in an amino acid ester is in the range of 7 to 8 (92), lysine methyl ester was tested and found to influence the rate of hydrolysis of p-nitrophenyl acetate under conditions comparable to enzymic hydrolysis. The effect is demonstrated in Figure 20, where the rate of hydrolysis is shown to be directly proportional to the

TABLE III

Rate Constants for the Formation of p-Nitrophenol from p-Nitrophenyl Acetate by Various Catalysts

Catalyst	Rate	Solvent	Ref.
Hydroxide-ion	511 lm^{-1} sec^{-1}	5% Dioxane	(91)
Chymotrypsin	k_2, 3.1 sec^{-1} k_3, 0.025 sec^{-1}	5% Isopropyl Alcohol	(Table II)
Trypsin	k_2, 1,5 sec^{-1} k_3, 0.013 sec^{-1}	5% Isopropyl Alcohol	(Table II)
Insulin	0.6 lm^{-1} sec^{-1}	5% Isopropyl	(2)
DIP-Chymotrypsin	0.5 lm^{-1} sec^{-1}	5% Isopropyl	(2)
Imidazole	0.5 lm^{-1} sec^{-1}	5% Dioxane	(91)

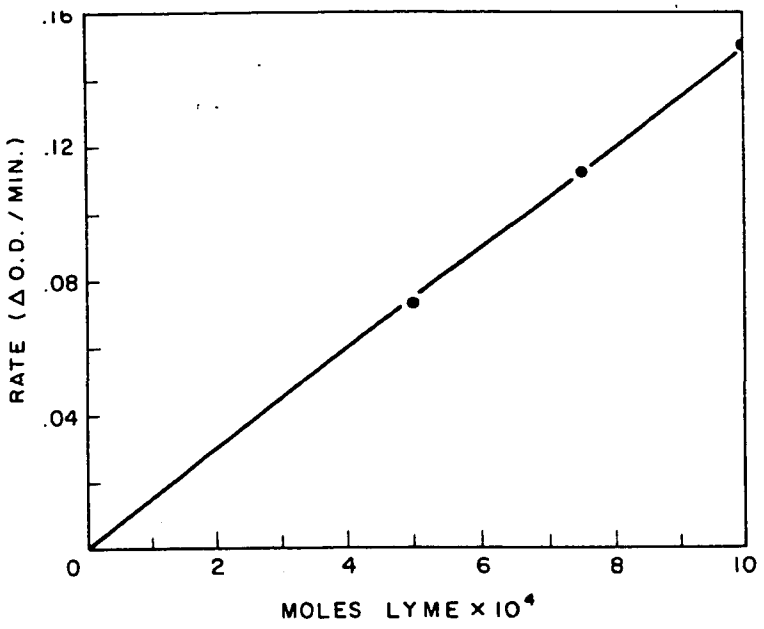


Fig. 20 INFLUENCE OF LYME (LYSINE METHYL ESTER) ON HYDROLYSIS OF NPA (20% ISOPROPYL ALCOHOL)

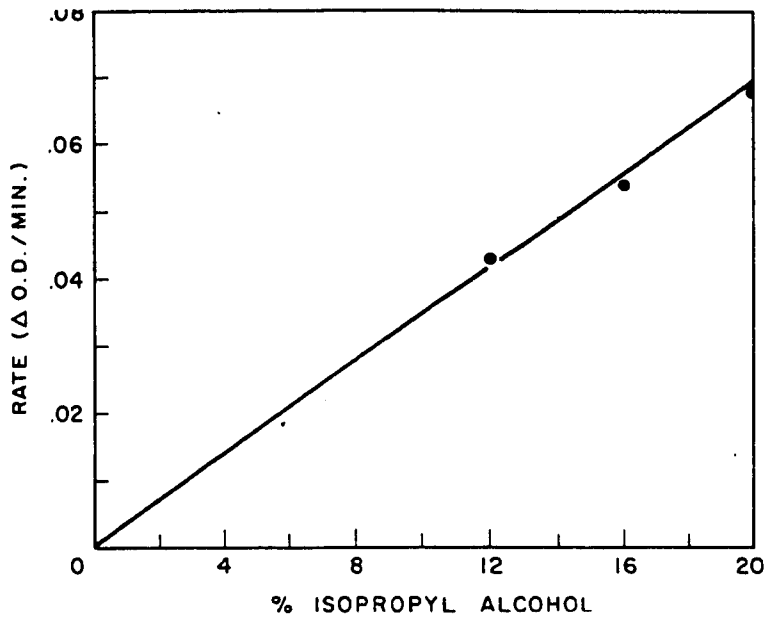


Fig. 21 INFLUENCE OF ISOPROPYL ALCOHOL ON THE HYDROLYSIS OF NPA (4×10^{-4}M LYME)

amino acid ester concentration. A similar result was also obtained when the isopropyl alcohol content was varied and the amino acid ester concentration held constant as in Figure 21. These results suggest that both the aliphatic hydroxyl and amino groups could play a significant part in the constitution of an enzymic site.

Further evidence to support this point of view may be secured from the literature. Hartley and Kilby (2) have reported that the phenolic hydroxyl and the aliphatic amino groups may act as acceptors of the acetyl group. The work of Balls and co-workers reveals that chymotrypsin mediates the transfer of acetyl to ethanol (5) or trimethylacetyl to n-butanol (6), illustrating the importance of hydroxyl. Their findings also provide evidence for an amino group as shown by the transfer of acetyl from chymotrypsin to hydroxylamine (5) and the reactivation of certain monoacylated chymotrypsin derivatives by tyrosine ethyl ester (6). Also of importance is the discovery by Wagner-Jauregg and Hackley (47) during an investigation of the model hydrolysis of organophosphorus inhibitors, that certain amino and hydroxyl substituted pyridines, and such compounds as catechol, increased the rate of hydrolysis as compared to pyridine.

Several publications have revealed nitrophenyl esters to be acylating agents toward compounds other than enzymes (2,93). Undoubtedly one of the most useful examples is the condensation of the nitrophenyl esters of N-acyl amino acids with amino acid alkyl esters to form peptides (93,94,95,96). Because of its simplicity this reaction should become a popular method in the synthesis of polypeptides. Furthermore, N-acetyl-phenylalanine ethyl ester has been isolated from a solution of

phenylalanine ethyl ester treated with p-nitrophenyl acetate (2). The same authors also showed that the related compound tyrosine ethyl ester increased the rate of spontaneous hydrolysis of p-nitrophenyl acetate. Of interest in this respect is the finding that only tyrosine and histidine of the several amino acids investigated had the capacity to influence the hydrolysis of diisopropyl phosphorofluoridate (47). This acid contains a phenolic hydroxyl group as well as an α-amino group so that it would be of some importance to show that the α-amino group can be acetylated with p-nitrophenyl acetate. A side experiment was, therefore, designed utilizing infrared spectroscopy as a simple means of detecting acetylation. Solutions of hydrolyzed p-nitrophenyl acetate, tyrosine ethyl ester, N-acetyl tyrosine ethyl ester, and p-nitrophenyl acetate hydrolyzed in the presence of tyrosine ethyl ester were prepared with phosphate buffer pH = 7.8, and 20% ($^{v}/v$) isopropyl alcohol. These solutions were freeze-dried, and the residues mixed with powdered potassium bromide and pressed into windows (97). Figure 22 gives their infrared spectra which were recorded on a Perkin-Elmer Model 21 spectrophotometer. From these spectra it is seen that both N-acetyl tyrosine ethyl ester and the system of p-nitrophenyl acetate hydrolyzed in the presence of tyrosine ethyl ester possess appreciable absorbance in the region 1660 cm^{-1}. On the other hand tyrosine ethyl ester or hydrolyzed p-nitrophenyl acetate do not absorb in this region, thereby disclosing that tyrosine ethyl ester is N-acetylated by p-nitrophenyl acetate.

Even though it has been shown that the hydroxyl group, imidazole group, and amino group influences the rate of hydrolysis of p-nitrophenyl

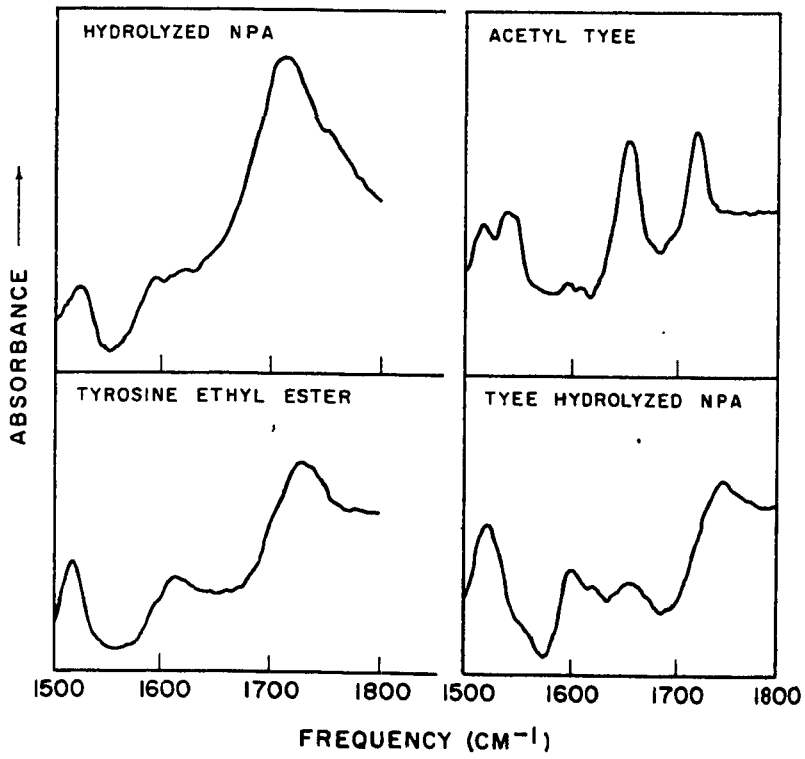


Fig. 22 THE INFRARED SPECTRA OF FREEZE-DRIED SOLUTIONS OF HYDROLYZED p-NITROPHENYL ACETATE, TYROSINE ETHYL ESTER, N-ACETYL TYROSINE ETHYL ESTER, AND p-NITROPHENYL ACETATE HYDROLYZED IN THE PRESENCE OF TYROSINE ETHYL ESTER.

acetate, further investigations will be required before definitely establishing the nature of their relationship to enzymic hydrolysis. This is particularly true in the case of the α -amino group.

Finally, from the titration curve of trypsin (98), it is interesting to notice that approximately one group ionizes in the region pH 5 to 7.

Claims to Original Research

Claims to Original Research

1. The adaptation of an improved stopped-flow mixing device, with removable storage syringes, T-bore stopcocks, and a plunger-release feature, to the Beckman Model DU spectrophotometer without due modification to the optical or electrical components.

2. The application of the zero suppression method to spectrophotometry for the recording of kinetic data where high sensitivity is required.

3. The competitive inhibition of the formation of the Michaelis complex by protons, when p-nitrophenyl acetate is hydrolyzed by chymotrypsin or trypsin.

4. The pH-independence of the acetylation of trypsin with p-nitrophenyl acetate.

5. A series of mathematical relationships to describe the pH-independence of acetylation of trypsin and chymotrypsin by p-nitrophenyl acetate.

6. The ability of isopropyl alcohol and lysine methyl ester, when both are present, to increase the rate of hydrolysis of p-nitrophenyl acetate.

7. Spectroscopic evidence that p-nitrophenyl acetate may act as an acetylation agent toward tyrosine ethyl ester in alcoholic aqueous solution.

References

References

1. Hartley, B.S. and Kilby, B.A., Biochem. J. 50, 672 (1952).

2. Hartley, B.S. and Kilby, B.A., Biochem. J. 56, 288 (1954).

3. Gutfreund, H. and Sturtevant, J.M., Proc. Nat. Acad. Sci. 42, 719 (1956).

4. Balls, A.K. and Aldrich, F.L., Proc. Nat. Acad. Sci. 41, 190 (1955).

5. Balls, A.K. and Wood, H.N., J. Biol. Chem. 219, 245 (1956).

6. McDonald, C.E. and Balls, A.K., J. Biol. Chem. 227, 727 (1957).

7. Dixon, G.H. and Neurath, H., Fed. Proc. 16, 173 (1957).

8. Vratsanos, S.M., Bier, M., and Nord, F.F., Nature 181, 414 (1958).

9. Vratsanos, S.M., Bier, M., and Nord, F.F., Arch. Biochem. Biophys. 77, 216 (1958).

10. Gutfreund, H. and Sturtevant, J.M., Biochem. J. 63, 657 (1956).

11. Dixon, G.H. and Neurath, H., J. Biol. Chem. 225, 1049 (1957).

12. Dixon, G.H., Dreyer, W.J., and Neurath, H., J. Am. Chem. Soc. 78, 4810 (1956).

13. Harris, J.I. and Hartley, B.S., Biochim. et Biophys. Acta 21, 201 (1956).

14. Dixon, M. and Needham, D.M., Nature 158, 432 (1946).

15. Cunningham, L.W., Science 125, 1145 (1957).

16. Gladner, J.A. and Laki, K., J. Am. Chem. Soc. 80, 1263 (1958).

17. Oosterbaan, R.A., Kunst, P., and Cohen, J.A., Biochim. et Biophys. Acta 16, 299 (1955).

18. Dixon, G.H., Go, S., and Neurath, H., Biochim. et Biophys. Acta 19, 193 (1956).

19. Schaffer, N.K., Simet, L., Harshman, S., Engle, R.R., and Drisko, R.W., J. Biol. Chem. 225, 197 (1957).

20. Dixon, G.H., Kauffman, D.L., and Neurath, H., J. Am. Chem. Soc. 80, 1260 (1958).

21. Dixon, G.H., Kauffman, D.L., and Neurath, H., J. Biol. Chem. 233, 1373 (1958).

22. Turba, F. and Gundlach, G., Biochem. Z. 327, 186 (1955).

23. Oosterbaan, R.A., Kunst, P., Van Rotterdam, J., and Cohen, J.A., Biochim. et Biophys. Acta 27, 556 (1958).

24. Oosterbaan, R.A., Jansz, H.S., and Cohen, J.A., Biochim. et Biophys. Acta 20, 402 (1956).

25. Koshland, D.E. and Erwin, M.J., J. Am. Chem. Soc. 79, 2657 (1957).

26. Chance, B., J. Franklin Inst. 229, 455 (1940).

27. Chance, B., J. Franklin Inst. 229, 613 (1940).

28. Chance, B., J. Franklin Inst. 229, 737 (1940).

29. Roughton, F.J.W., in Technique of Organic Chemistry. Weissberger, A., ed., Vol. VIII, Interscience, New York. 1953. p. 669.

30. Hartridge, H. and Roughton, F.J.W., Proc. Roy. Soc. A104, 376 (1923).

31. Roughton, F.J.W. and Millikan, G.A., Proc. Roy. Soc. A155, 277 (1936).

32. Roughton, F.J.W., in Technique of Organic Chemistry. Weissberger, A., ed., Vol. VIII, Interscience, New York. 1923. p. 678.

33. Dalziel, K., Biochem. J. 55, 79 (1953).

34. Chance, B., in Technique of Organic Chemistry. Weissberger, A., ed., Vol. VIII, Interscience, New York. 1953. p. 690.

35. Chance, B., Discussions Faraday Soc. 20, 205 (1955).

36. Chance, B., Rev. Sci. Instr. 18, 610 (1947).

37. Chance, B., Rev. Sci. Instr. 22, 619 (1951).

38. Chance, B., Rev. Sci. Instr. 22, 627 (1951).

39. Chance, B., Rev. Sci. Instr. 22, 634 (1951).

40. Gibson, Q.H. and Roughton, F.J.W., Proc. Roy. Soc. B143, 310 (1955).

41. Beers, R.F., Biochem. J. 62, 492 (1956).

42. Sirs, J.A., Trans. Faraday Soc. 54, 201 (1958).

43. Sirs, J.A., Trans. Faraday Soc. 54, 207 (1958).

44. Neurath, H., Ann. N.Y. Acad. Sci. 68, 11 (1957).

45. Jandorff, B.J., Michael, H.O., Schaffer, N.K., Egan, R., and Summerson, W.H., Discussions Faraday Soc. 20, 134 (1955).

46. Gutfreund, H., Discussions Faraday Soc. 20, 167 (1955).

47. Wagner-Jauregg, P. and Hackley, B.E., J. Am. Chem. Soc. 75, 2125 (1953).

48. Bender, M.L. and Turnquest, B.W., J. Am. Chem. Soc. 79, 1652 (1957).

49. Hartley, B.S. and Massey, V., Biochim. et Biophys. Acta 21, 58 (1956).

50. Laidler, K.J. The Chemical Kinetics of Enzyme Action. Oxford University Press, London 1958. p. 149.

51. Laidler, K.J. and Barnard, M.L., Trans. Faraday Soc. 52, 497 (1956).

52. Porter, G.R., Rydon, H.N., and Schofield, J.A., Nature 182, 927 (1958).

53. Rydon, H.N., Nature 182, 928 (1958).

54. Briggs, G.E. and Haldane, J.B.S., Biochem. J. 19, 338 (1925).

55. Michaelis, L. and Menten, M.L., Biochem. Z. 49, 333 (1913).

56. Chance, B., J. Biol. Chem. 151, 553 (1953).

57. Chance, B., Acta Chem. Scand. 1, 236 (1947).

58. Chance, B., Greenstein, D.S., and Roughton, F.J.W., Arch. Biochem. and Biophys. 37, 301 (1952).

59. Chance, B., J. Biol. Chem. 194, 471 (1952).

60. Beers, R.F. and Sizer, I.W., J. Phys. Chem. 57, 290 (1953).

61. Beers, R.F., J. Phys. Chem. 58, 197 (1954).

62. Roughton, F.J.W., Discussions Faraday Soc. 17, 116 (1954).

63. Gutfreund, H., Discussions Faraday Soc. 17, 220 (1954).

64. Laidler, K.J., Can. J. Chem. 33, 1614 (1955).

65. Hammond, B.R. and Gutfreund, H., Biochem. J. 61, 187 (1955).

66. Gutfreund, H., Trans. Faraday Soc. 51, 441 (1955).

67. Ouellet, L. and Laidler, K.J., Can. J. Chem. 34, 146 (1956).

68. Lineweaver, H. and Burk, D., J. Am. Chem. Soc. 56, 658 (1934).

69. Laidler, K.J., Trans. Faraday Soc. 51, 528 (1955).

70. Laidler, K.J., Trans. Faraday Soc. 51, 540 (1955).

71. Laidler, K.J., Trans. Faraday Soc. 51, 550 (1955).

72. Laidler, K.J., The Chemical Kinetics of Enzyme Action. Oxford University Press, London. 1958. p. 117.

73. Guggenheim, E.A., Phil. Mag. 2, 538 (1926).

74. Roseveare, W.E., J. Am. Chem. Soc. 53, 1651 (1931).

75. Chattaway, F.D., J. Chem. Soc. 134, 2495 (1931).

76. Kaufmann, A., Ber. 42, 3482 (1909).

77. Laidler, K.J., The Chemical Kinetics of Enzyme Action. Oxford University Press, London. 1958. p. 81.

78. Robinson, R.A. and Biggs, A.I., Trans. Faraday Soc. 51, 901 (1955).

79. Biggs, A.I., Trans. Faraday Soc. 50, 800 (1954).

80. Sedlacek, B. and Bartl, P., Chem. Listy 49, 996 (1955).

81. Laidler, K.J., The Chemical Kinetics of Enzyme Action. Oxford University Press, London. 1958. p. 82.

82. Balls, A.K. and Jansen, E.F., Advances in Enzymol. 13, 321 (1952).

83. Mounter, L.A., Tuck, K.D., Alexander, H.C., and Dien, L.T.H., J. Biol. Chem. 226, 873 (1957).

84. Schaffer, N.K., May, S.C., and Summerson, W.H., J. Biol. Chem. 202, 69 (1953).

85. Schaffer, N.K., May, S.C., and Summerson, W.H., J. Biol. Chem. 206, 201 (1954).

86. Cohen, J.A., Oosterbaan, R.A., Warringa, M.G.P., and Jansz, H.S., Discussions Faraday Soc. 20, 114 (1955).

87. Westheimer, F.H., Proc. Nat. Acad. Sci. 43, 969 (1957).

88. Dixon, G.H. and Neurath, H., J. Am. Chem. Soc. 79, 4558 (1957).

89. Bruice, T.C. and Schmir, G.L., J. Am. Chem. Soc. 79, 1663 (1957).

90. Bruice, T.C. and Schmir, G.L., J. Am. Chem. Soc. 80, 148 (1958).

91. Bender, M.L. and Turnquist, B.W., J. Am. Chem. Soc. 79, 1656 (1957).

92. Alberty, R.A., in The Proteins, Neurath, H. and Bailey, K., ed., Vol. I, Academic Press, New York. 1953. p. 465.

93. Bodanszky, M., Nature 175, 685 (1955).

94. Bodanszky, M., Szelke, M., Tomorkeny, E., and Weisz, E., Chem. Ind. 1517 (1955).

95. Elliot, D.F. and Russell, D.W., Biochem. J. 66, 49P (1957).

96. Iselin, B., Rittell, W., Sieber, P., and Schwyzer, R., Helv. Chim. Acta 40, 373 (1957).

97. Toribara, T.Y. and DiStefano, V., Anal. Chem. 26, 1519 (1954).

98. Duke, J.A., Bier, M., and Nord, F.F., Arch. Biochem. and Biophys. 40, 424 (1952).