

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UMI[®]

32

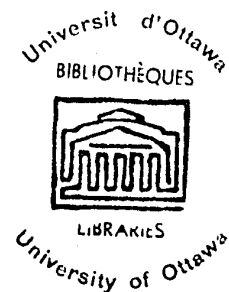
KINETICS AND MECHANISM OF
TRYPSIN CATALYSIS

by

H.P. Kasserra, M.A.Sc.

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
in the
Department of Chemistry
University of Ottawa
Ottawa, Canada

June 1969



K.J. Laidler
Professor of Chemistry
Research Supervisor

H.P. Kasserra
Ph.D. Candidate

UMI Number: DC52363

INFORMATION TO USERS

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleed-through, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.



UMI Microform DC52363

Copyright 2007 by ProQuest LLC

All rights reserved. This microform edition is protected against unauthorized copying under Title 17, United States Code.

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Discovery consists of seeing what everybody
has seen and thinking what nobody has thought.

Albert Szent-Györgyi

PREFACE

Prodigious efforts have been devoted during recent years to the elucidation of enzymatic mechanisms and the special characteristics of enzymes which make them such efficient catalysts. Our understanding of these systems has been advanced substantially with the establishment, by X-ray diffraction studies, of the detailed three-dimensional structures of some enzyme molecules. Although this information is not yet available for trypsin, many features of this enzyme have been explained on the basis of its close relationship to chymotrypsin for which a wealth of evidence, including its X-ray structure, has been accumulated. It was the object of this research to examine certain aspects of trypsin-catalyzed reactions directly and to incorporate these with other experimental data on the enzyme into an overall mechanism of catalysis.

This thesis is divided into four chapters.

Chapter I deals with recent advances in the activation process of trypsinogen to trypsin and describes in general the molecular and enzymatic properties of the enzymes. In Chapters II and III the experimental work with

a new substrate of trypsin is reported for the pre-steady state and steady-state phases of the catalytic process. Chapter IV presents a review of the structural and mechanistic evidence available for trypsin and chymotrypsin which leads to a general reaction mechanism for the two enzymes.

Part of the work described in this thesis has already been submitted for publication, and a third manuscript is in preparation.

1. pH Effects in Trypsin Catalysis, H.P. Kasserra and K.J. Laidler, Can. J. Chem., in press.
2. The Mechanisms of Action of Trypsin and Chymotrypsin, H.P. Kasserra and K.J. Laidler, Can. J. Chem., in press.
3. Transient-Phase Kinetics of Trypsin-Catalyzed Reactions, H.P. Kasserra and K.J. Laidler, manuscript in preparation.

ACKNOWLEDGEMENTS

The author wishes to express his sincere appreciation to Professor K.J. Laidler under whose direction this work was done. He is especially grateful to him for the time devoted to discussions and the stimulating freedom provided in the course of this research.

The author would also like to acknowledge the receipt of an NRC Studentship and a Province of Ontario Fellowship.

Finally, special thanks are due to my wife for her help in the preparation of this manuscript and for her moral support.

TABLE OF CONTENTS

	<u>Page No.</u>
PREFACE	iii
ACKNOWLEDGEMENTS	v
TABLE OF CONTENTS	vi
LIST OF TABLES	x
LIST OF FIGURES	xi
ABSTRACT	1

CHAPTER I

GENERAL INTRODUCTION	5
Activation of Trypsinogen	6
Molecular Structure of Trypsin	8
Active Center	12
Specificity and Stereospecificity	15
Mechanism of Catalysis	17

CHAPTER II

STEADY-STATE KINETICS OF TRYPSIN-CATALYZED HYDROLYSES	
INTRODUCTION	20
Theoretical Principles	20
General Reaction Scheme	23

	<u>Page No.</u>
Significance of the $\tilde{k}_c/\tilde{K}_m$ Ratio	27
Kinetic Studies	28
Optical Rotation Studies	30
EXPERIMENTAL DETAILS	32
Materials	32
Kinetic Procedure	34
Methods of Computation	37
Optical Rotation Measurements	39
RESULTS	42
pH Dependence of Kinetic Parameters	42
Effect of pH on Optical Rotation	51
DISCUSSION	58
Ionizations in Acidic Solution	59
Ionizations in Basic Solution	60
 <u>CHAPTER III</u>	
THE TRANSIENT PHASE OF TRYPSIN-CATALYZED HYDROLYSES	
INTRODUCTION	67
pH Dependence of the Acylation Process	67
Reaction Order of the Acylation Process	69

	<u>Page No.</u>
Solvent Effects	73
Flow Techniques for the Study of Rapid Reactions	74
TRANSIENT-PHASE THEORY	78
Three-Step Mechanism	78
(1) High Enzyme Concentration	79
(2) High Substrate Concentration	89
Effect of Hydrogen-Ion Concentration	89
COMPUTER ANALYSIS	93
Guggenheim Method	93
Least-Squares-Fit Method	95
Computer Program Tests	98
EXPERIMENTAL DETAILS	100
Materials	100
Stopped-Flow Spectrophotometer	101
Kinetic Procedure	103
Methods of Computation	105
RESULTS	109
pH Dependence of the Acylation Process	109
Solvent Effects	117

	<u>Page No.</u>
DISCUSSION	127
pH Dependence of Kinetic Parameters	127
Michaelis-Complex Formation	129
Solvent Effects	130
 <u>CHAPTER IV</u>	
GENERAL DISCUSSION	136
Structural Evidence	136
Mechanistic Evidence	140
(1) Complex Formation	141
(2) Acylation	147
(3) Deacylation	151
Conformational Changes	154
Summary	158
 <u>APPENDIX A</u>	
LEAST-SQUARES-FIT COMPUTER PROGRAM	161
 <u>APPENDIX B</u>	
DIGITAL READ-OUT FOR STOPPED-FLOW SPECTROPHOTOMETER	167
CLAIMS TO ORIGINAL RESEARCH	170
REFERENCES	173

LIST OF TABLES

<u>Table No.</u>		<u>Page No.</u>
1.	Amino Acid Sequences of Chymo- trypsinogen A and B and Trypsinogen	9
2.	Rate Constants for the Hydrolysis of BAME	44
3.	Comparison of Rate Constants for the Hydrolysis of BAME by Commercial Trypsin and TPCK-treated Trypsin	49
4.	Experimental Data and Computer Results for the Hydrolysis of CANE	111
5.	Rate Constants for the Hydrolysis of CANE	114
6.	Rate Constants for the Hydrolysis of CANE in 1-20% (v/v) Isopropyl Alcohol	118
7.	Evidence Relating to Complex Formation of Trypsin and Chymotrypsin	142
8.	Evidence Relating to Acylation of Trypsin and Chymotrypsin	148
9.	Evidence Relating to Deacylation of Trypsin and Chymotrypsin	152

LIST OF FIGURES

<u>Figure No.</u>		<u>Page No.</u>
1.	Schematic Diagram of the Titration Assembly	35
2.	Plot of k_{OH} against $1/[H^+]$ for the Alkaline Hydrolysis of BAME	38
3.	Schematic Illustration of Dixon's Method for obtaining pK Values	40
4.	Plots of Rates of Hydrolysis of BAME against $[S]$	43
5.	Variation of $\log_{10} \tilde{k}_c$ with pH	45
6.	Variation of $p\tilde{K}_m$ with pH	46
7.	Variation of $\log_{10} \tilde{k}_c / \tilde{K}_m$ with pH	47
8.	Plots of Specific Rotation against pH for Trypsin, DIP-Trypsin and Trypsinogen	52
9.	Plot of $\log_{10} \Delta[\alpha] / (\Delta[\alpha]_{max} - \Delta[\alpha])$ against pH	54

<u>Figure No.</u>		<u>Page No.</u>
10.	Plots of Specific Rotation against pH for Trypsin in the Absence and Presence of Inhibitors	56
11.	Qualitative Plot of the Rate of Production of Product p_1	85
12.	Block Diagram of the Stopped-Flow Spectrophotometer System	102
13.	Oscilloscope Trace of the Transient-Phase Hydrolysis of CANE	106
14.	Plots of Rate of Change of Absorbance for the Hydrolysis of CANE	110
15.	Lineweaver-Burk Plots for the Hydrolysis of CANE	113
16.	Variation of $\log_{10} \tilde{k}_2$ with pH	115
17.	Variation of $p\tilde{K}_m$ with pH	116
18.	Plot of $\log_{10} \tilde{k}_2 / \tilde{K}_m$ against $100/D$	120
19.	Plot of $\log_{10} \tilde{k}_2$ against $100/D$	121

<u>Figure No.</u>		<u>Page No.</u>
20.	Plot of $\log_{10} \tilde{K}_m$ against $100/D$	122
21.	Plot of $\tilde{K}_m/\tilde{k}_2$ against the Square of Isopropanol Concentration	123
22.	Plot of $1/\tilde{k}_2$ against the Square of Isopropanol Concentration	124
23.	Approximate Arrangement of Amino Acid Residues in the Active-Center Region of α -Chymotrypsin	137
24.	Approximate Arrangement of Amino Acid Residues in the Active-Center Region of Trypsin	139
25.	Ionizations in Trypsin and Chymotrypsin	144
26.	Reaction Mechanism for the α -Chymotrypsin-catalyzed Hydrolysis of N-Acetyl-L-Tyrosine Ethyl Ester	145
27.	Reaction Mechanism for the Trypsin-catalyzed Hydrolysis of N-Benzoyl-L-Arginine Ethyl Ester	146

Figure No.

Page No.

- | | | |
|-----|--|-----|
| 28. | Proton Transfer Processes during
Acylation of Trypsin and Chymotrypsin | 149 |
| 29. | Block Diagram of the Digital Read-out
for the Stopped-Flow Spectrophoto-
meter | 169 |

ABSTRACT

A steady-state kinetic study has been made of the trypsin-catalyzed hydrolysis of N-benzoyl-L-alanine methyl ester, at pH values ranging from 6-10. From the rates were calculated, at each pH, values of $\tilde{k}_c/\tilde{K}_m$, $\tilde{k}_c$ and $\tilde{K}_m$ ($=\tilde{k}_c(\tilde{k}_{-1}+\tilde{k}_2)/\tilde{k}_1\tilde{k}_2$). The pH profiles of these parameters provide pK values for the groups that ionize in the free enzyme and in the acylenzyme. The kinetic results on the acid side imply that there is a group of $pK \approx 7$ in trypsin, presumably the imidazole function of a histidine residue, and that this group is involved in acylation and deacylation, both of which can only occur if it is unprotonated. The behaviour on the basic side revealed a decrease in $\tilde{k}_c$ at high pH corresponding to a value of $pK \approx 9.5$, whereas $\tilde{k}_c/\tilde{K}_m$ showed sigmoid pH dependence. This pK was related to the α -amino group of the N-terminal isoleucine residue.

The specific levorotation of trypsin and trypsinogen has been measured as a function of pH over the pH range 5-11. The change in specific rotation of trypsin follows the ionization of a single group with a $pK(\text{app})$ of 9.4 which was not revealed by the corresponding curve for trypsinogen. At pH 11, the specific rotation of trypsin, its zymogen and its

phosphorylated derivative were approximately the same, suggesting similar conformations for all three forms of the protein. The pH-dependence curves of the specific rotation of trypsin in the presence of reversible competitive inhibitors were found to be displaced to a more alkaline pH. This was interpreted to indicate a conformational stabilization as the result of enzyme-inhibitor complex formation with a consequent increase in the $pK(\text{app})$ of the group revealed by the free enzyme.

An interpretation of the kinetic and optical rotary results that is consistent with all available information is that the group of $pK \approx 9.5$ (presumably the $-\text{NH}_3^+$ function of the terminal isoleucine residue) controls the conformation and thereby the activity of the enzyme in alkaline solution.

The transient-phase kinetics of the trypsin-catalyzed hydrolysis of N-carbobenzoxy-L-alanine-p-nitro-phenyl ester have been studied using a stopped-flow technique. Under conditions of excess enzyme concentration, the pH-dependencies of $\tilde{k}_2$ and $\tilde{K}_m$ ($=(\tilde{k}_{-1} + \tilde{k}_2)/\tilde{k}_1$) have been obtained without the need to make any assumptions as to the relative magnitude of $\tilde{k}_3$. From the results it was concluded that a group of $pK = 6.9$ participates in acylation and that the same group is not involved in Michaelis-complex formation. On the

basis of the steady-state work, this group has already been postulated to be the imidazole function of the histidine residue. A least-squares-fit computer program was developed to analyze the kinetic rate measurements directly and was used in place of the traditionally employed Guggenheim method.

The effect of organic solvent on the pre-steady state parameters has been investigated for solvent concentrations varying from 1-20% (v/v) isopropyl alcohol. The changes in $\tilde{k}_2$, $\tilde{K}_m$ and $\tilde{k}_2/\tilde{K}_m$ could be formally correlated with the reciprocal of the dielectric constant of the solvent mixture, indicating an increase in electrostatic interactions. It was concluded that the observed decrease in both binding and the rate of hydrolysis of N-carbobenzoxy-L-alanine-p-nitrophenyl ester can be justified by a loss in conformational integrity of the enzyme. This is the result of increased free-energy contributions due to electrostatic and hydrophobic interactions with little or no effect due to hydrogen bonding.

The chemical and mechanistic evidence for reactions catalyzed by trypsin and chymotrypsin has been reviewed in some detail and is considered with reference to the structure of chymotrypsin as determined by X-ray studies. Conclusions

have been drawn about the detailed nature of the processes of Michaelis-complex formation, acylation and deacylation which are incorporated in a concerted reaction mechanism. Although there are significant differences between the two enzymes as far as specificity and pH effects are concerned, it has been concluded that their mechanism of catalysis is the same.

CHAPTER I

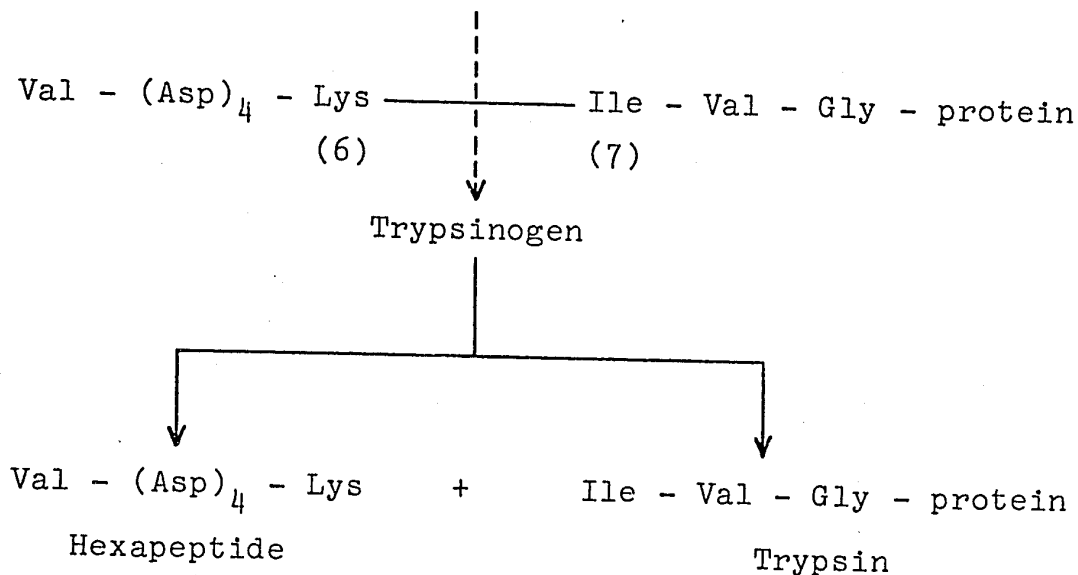
GENERAL INTRODUCTION

Trypsin is one of several enzymes concerned with the intestinal digestion of proteins. It is biosynthesized by the acinous cells of the pancreas in the form of an inactive precursor, trypsinogen, and is carried as such by the pancreatic juice into the duodenum where it is activated to form trypsin. The main role of this enzyme in the body is to catalyze the hydrolysis of peptide bonds during the process of protein digestion initiated in the stomach by pepsin. Closely related to trypsin is another pancreato-peptidase, chymotrypsin. Specific similarities between the two enzymes include (i) site of origin, (ii) rate of biosynthesis, (iii) endopeptidase activity, (iv) molecular weight, (v) isoelectric point, (vi) amino acid composition and (vii) nature of the catalytic processes (1-3). It is on this basis that frequent reference will be made to chymotrypsin in the subsequent discussion of trypsin mechanism and kinetics.

Activation of Trypsinogen

It is self-evident that during the biosynthesis of proteolytic enzymes the protoplasmic proteins of pancreatic cells must be protected against autodestruction. The protective device adopted by the digestive organs is to manufacture such enzymes in the form of inactive precursors which are stored in zymogen granules and ultimately released at the free surface of the cells. Some representative zymogens other than trypsinogen are chymotrypsinogen, pepsinogen and procarboxipeptidase.

The conversion of trypsinogen to trypsin can be catalyzed by (i) trypsin itself, (ii) enterokinase (enteropeptidase), a proteolytic enzyme secreted by the duodenal mucosa and (iii) certain mold proteinases (4). Physiologically, enterokinase seems to be the all-important activating enzyme, since no free trypsin can be found in freshly collected pancreatic secretions (5). Activation involves cleavage of a hexapeptide from the N-terminal end of the molecule (6,7) as depicted below:



MacDonald and Kunitz (8) have shown that the presence of calcium ions decreases the formation of large quantities of inert proteins which are produced in the absence of the cation at the expense of active trypsin. At the same time, the rate of proteolysis is enhanced (9) and appears to be restricted to the single peptide bond between lysine-6 and isoleucine-7, all other lysyl and arginyl bonds being resistant to tryptic cleavage. It has been proposed that trypsinogen possesses two different binding sites for calcium (10). The first site, presumably composed of aspartate or glutamate residues, has a high affinity for one calcium ion and the binding induces a conformational change which protects the molecule against the formation of

inert proteins. This site also exists in trypsin. The second site has a lower affinity for calcium and is probably located at the four adjacent N-terminal aspartyl residues. This binding induces no conformational change, but causes an acceleration of the hydrolysis of the lysine-6-iso-leucine-7 bond and appears to be responsible for the observed specificity during the normal activation process of trypsinogen (11,12).

Molecular Structure of Trypsin

The amino acid sequence of trypsinogen has been compared to that of chymotrypsinogen A and B in Table 1 (3). Trypsin is a single-chain peptide with six disulphide bonds, while α -chymotrypsin consists of three peptide chains held together by five disulphide bonds. Four of these have been found to be homologous with those of trypsin. It has also been demonstrated (15) that the two disulphide bridges of trypsin which do not coincide with those of α -chymotrypsin can be built into the chymotrypsin model (13,14,16,17) without any significant distortion of the chains. Table 1 indicates that 40% of the non-polar residues in trypsin and chymotrypsin are homologous and a further 11% are quite similar. The fact that a histidine and a serine residue,

Table 1

Amino acid sequences of bovine chymotrypsinogen A, chymotrypsinogen B and trypsinogen according to reference (3). Asn-103 in chymotrypsinogen has been changed to Asp-103 (13) and Asn-192 in trypsinogen has been changed to Asp-192 (14). The solid lines enclose regions of non-polar residues common to all three proteins. Disulphide bridges in chymotrypsinogens A and B are at identical positions as follows:

I-V (residues 1-123); III-IV (residues 42-58); VII-XII (residues 137-204); IX-X (residues 169-183); XI-XIII (residues 194-223). Disulphide bridges in trypsinogen are II-VIII (residues 22-158); III-IV; VI-XIV (residues 129-236); VII-XII; IX-X; XI-XIII.

[illegible]

separated by 138 residues in the primary structure, are in juxtaposition at the active sites of both enzymes, indicates one common three-dimensional parameter. These similarities have led several authors to predict that the three-dimensional conformation of trypsin may be strikingly similar to that of α -chymotrypsin, at least in the major aspects of its chain folding (3,15).

An interesting variation between the primary sequences of trypsin and chymotrypsin is to be pointed out. Table 1 reveals that in less than 30% of the positions homology exists in residues with a carboxylic acid side chain such as aspartic and glutamic acid. It appears likely that this difference can be associated with the well-known calcium requirements of trypsin (in contrast to chymotrypsin), both for increased stability and activity at pH values where the -COOH groups are ionized. Relatively minor variations in structure such as these and their effects upon enzymatic behaviour may be resolved when the three-dimensional structure of trypsin has been determined.

Maroux et al. (18) have recently published a preliminary report on the autolysis of trypsin between

arginine-105* and valine-106 which gives rise to a new trypsin consisting of two chains held together by disulphide bonds. This species still retains all its activity and does not contain any inactive molecules as shown by titration of the number of catalytic sites. It is interesting to speculate whether this process bears any analogy to the autocatalytic cleavage of the leucine-13*-serine-14 bond in π -chymotrypsin to yield δ -chymotrypsin (20); this process does not affect enzymatic activity either. A different two-chain form of trypsin containing an intrachain split between lysine-131 and serine-132 has been identified by Schroeder and Shaw (21). This species, isolated during the tryptic activation of trypsinogen under conditions which limit autolysis, exhibits a different specific activity from the single-chain form. The authors have pointed out that some significance may be attached to the fact that the split in trypsin at lysine-131 is within one residue of the split in α -chymotrypsin at tyrosine-146.

*

The present numbering for trypsin and chymotrypsin corresponds to consecutive numbering in trypsinogen (19) and chymotrypsinogen (16) respectively. The order of amino acids, however, is as given in Table 1.

Active Center

The term "active center" refers to those sites of the enzyme which are directly involved in the binding and hydrolysis of the substrate. Since synthetic substrates are usually small compared to the entire enzyme, it has been suggested in the past that the active center consists of only a small fraction of the total enzyme. This view has been supported by the fact that several hydrolytic enzymes have been reduced to smaller units with little or no loss of enzymatic activity (22-24).

In the case of trypsin, it has been possible to identify some components of the active center by the use of specific reagents which selectively attack functional groups. Diisopropyl fluorophosphate reacts with trypsin in a 1:1 stoichiometric reaction to give a product which contains one gram atom of phosphorous per mole of enzyme, and which is completely inactive enzymatically (25). When the phosphorylated enzyme is degraded, the phosphoryl group is found to be attached to the hydroxyl group of a serine residue (26,27) which was later identified as serine-183. More recently Lawson et al. (28) have alkylated the same serine residue with 3-(bromoacetamido) propylguanidine

nitrate causing irreversible inhibition of the enzyme towards hydrolysis of the specific substrate N-benzoyl-L-arginine ethyl ester. Both trypsin and chymotrypsin react stoichiometrically with p-nitrophenyl acetate yielding at low pH a stable inactive monoacyl enzyme which at higher pH hydrolyses into active enzyme and acetate ion (29). It is believed, as for acyl chymotrypsin (30), that the acetyl group in trypsin is linked to the same serine residue to which the phosphoryl group was found to be attached. The evidence from rate studies coupled with that from degradative studies, indicates that trypsin possesses one active center per molecule which contains the amino acid serine-183.

The kinetic importance of an ionizable group in trypsin of $pK \approx 7$ was first reported by Gutfreund (31) and has later been substantiated by many worker (32-35). This pK was associated with the imidazole ring of a histidine residue, since this is the only group in the enzyme with a pK close to 7. Shaw et al. (36) have demonstrated that trypsin reacts stoichiometrically with the substrate analog of lysine, TLCK (L-1-chloro-3-tosylamido-7-amino-2-heptanone), to form an inactive derivative in which one histidine residue has been alkylated. This was later identified to be histidine-46

(37,38). The same group also reacts with bromoacetone with consequent inactivation of the enzyme as measured by the rate of hydrolysis of N-benzoyl-L-arginine ethyl ester (39). Furthermore the introduction of an acetyl group into histidine-46 abolishes the unusual reactivity of serine-183, and this provides chemical evidence in support of the hypothesis that interaction between these residues is essential for the catalytic function of trypsin. In summary, the chemical and kinetic evidence for the participation of an imidazole group is well established.

There is less concrete information available concerning the binding loci on the enzyme which are an essential part of the active center. The best evidence is derived from inhibition studies with amidines and guanidines by Mares-Guia and coworkers (40,41). They have concluded that the "specificity site" of trypsin is composed of an anionic site to which substrates or inhibitors bind electrostatically through their positive charge. Located in line with, and between the anionic and catalytic sites, is a hydrophobic binding site in the form of a slit or crevice which binds the fully extended carbon side chain of the substrates or inhibitors. Blow,

on the basis of his X-ray crystallographic studies with α -chymotrypsin, has proposed aspartic acid-177 as the anionic binding site of trypsin (14). Binding of inhibitors structurally similar to the basic side chain of specific trypsin substrates appears to induce a change in conformation of the active center which results in an increase in reactivity of the histidyl residue (or residues) in the area of the catalytic site (42,43). Some evidence has recently been presented by Sanborn et al. (44,45) which suggests that trypsin possesses two binding sites, one to which charged molecules and one to which neutral molecules preferentially bind. These sites are in reasonable proximity to one another and allosteric interactions can be observed. However, both sites orient substrates so that the same enzyme residues are operative in the catalytic mechanism for the two types of compounds.

Specificity and Stereospecificity

Trypsin exhibits a marked specificity for hydrolyzing "basic" bonds, that is to say, bonds which link the carboxyl group of the basic amino acids arginine and lysine to the amino group of another amino acid (46). Schwert et al. (47) have demonstrated that in addition to

acting upon peptide linkages, trypsin also hydrolyzes "basic" amides and esters; the action is in fact much more rapid on esters than on suitable peptides and amides. Furthermore, trypsin does not split all basic bonds of a given class of substrates at the same rate; the chemical environment of the bond and the length and nature of the positively-charged side chain are important factors.

Peptide bonds adjacent to an unmasked α -amino or α -carboxyl group are hydrolyzed very slowly (48,49). A change in side-chain length by one methylene group causes a marked reduction in the catalytic rates of esters (50-54).

Substitution of the side-chain amino group entirely prevents the hydrolysis of some substrates (55) but only reduces the rates of others (56,57), whereas substitution on the α -amino group always facilitates the action (48).

There is increasing evidence however, that trypsin and chymotrypsin possess a certain degree of cross-specificity (33,58,59). The "chymotryptic" activity of trypsin definitely cannot be ascribed to contamination by small amounts of chymotrypsin which are usually present in commercial trypsin preparations (59). Furthermore trypsin has been found to hydrolyze compounds such as p-nitrophenyl acetate (35) and fatty acid esters of m-hydroxybenzoic acid (60)

which do not at all resemble the normal substrates. This behaviour might be related to the existence of an auxiliary binding site on trypsin as suggested by Sanborn and Hein (44) on the basis of their kinetic studies with neutral substrates and modifiers.

Trypsin exhibits stereospecificity for the L-forms of arginine and lysine derivatives (61). However, if the side chain is neutral and short or is non-existent, the D-antipodes of some esters can apparently take up an orientation which places the side-chain group in a position normally occupied by the α -hydrogen atom of the L-antipode and hydrolysis takes place at 1/10-1/20 of the rate observed for the L-esters (62). No information has as yet been obtained about the absolute configuration of trypsin-bound substrates.

Mechanism of Catalysis

A variety of mechanisms have been suggested for catalysis by α -chymotrypsin which, owing to the similarities between chymotrypsin and trypsin, have been tacitly assumed to apply to trypsin as well. Cunningham (2) has presented a detailed review of these proposals which give rise to

the conclusion that the imidazole group of a histidine residue acts as a general base in the acylation and deacylation processes, with serine being the nucleophile in acylation and water in deacylation. More recently Hess and coworkers (63,64) have implicated the N-terminal isoleucine residue of α -chymotrypsin in the control of enzyme conformation at alkaline pH and thereby in activity. The proximity of an aspartic acid residue to the imidazole ring of histidine-57 in α -chymotrypsin has prompted Blow et al. (13) to suggest a "charge relay system" between the aspartate group, histidine-57 and serine-195; this might explain the unusual degree of nucleophilicity of the serine residue. Such a feature is in accord with a mechanism postulated by Wang and Parker (65) which involves a pre-transition state protonation of the substrate. Unfortunately their treatment, which is applicable to the chymotrypsin-catalyzed hydrolysis of peptides, amides and anilides, does not seem to be valid for substituted anilides or ester substrates.

There are as yet several aspects to the mechanism of trypsin hydrolysis which require clarification before a mechanistic analogy between trypsin and chymotrypsin can be safely drawn. Trypsin kinetics at low substrate

concentrations have not been adequately investigated and the observed behaviour at high substrate concentrations differs from that of chymotrypsin. The possible involvement of the N-terminal isoleucine group in trypsin has not been established. It is the objective of this thesis to critically examine these and other problems and to reconcile the available experimental evidence in the form of a concerted reaction mechanism.

CHAPTER II

STEADY-STATE KINETICS OF TRYPSIN-CATALYZED HYDROLYSES

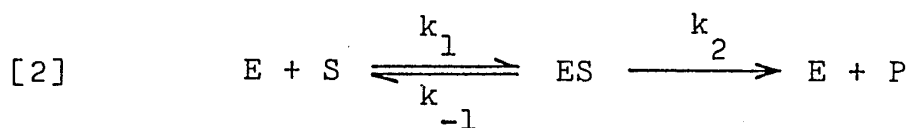
INTRODUCTION

Theoretical Principles

Many enzyme-catalyzed reactions, in particular reactions catalyzed by the hydrolytic enzymes, follow the modified Michaelis-Menten law

$$[1] \quad v = \frac{\tilde{k}_c [E]_o [S]}{\tilde{K}_m + [S]}$$

where $[E]_o$ and $[S]$ are the total enzyme and substrate concentrations and $\tilde{k}_c$ and $\tilde{K}_m$ are constants at a given pH; the symbol $\sim$ indicates that they are usually pH-dependent quantities. The simplest reaction sequence consistent with equation [1] is



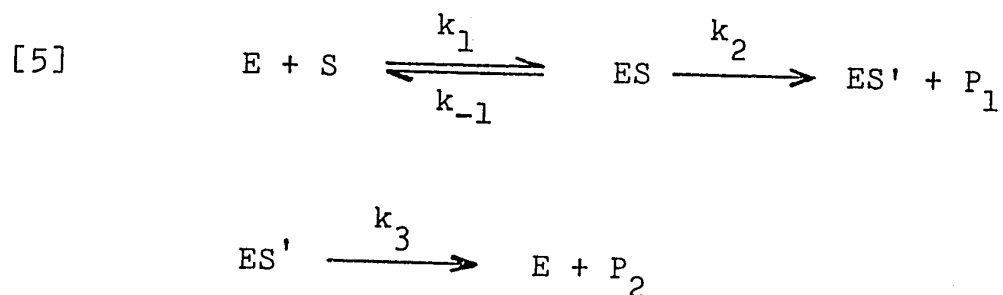
For this case

$$[3] \quad \tilde{k}_c = \tilde{k}_2$$

$$[4] \quad \tilde{K}_m = \frac{\tilde{k}_{-1} + \tilde{k}_2}{\tilde{k}_1}$$

The pH effects for the mechanism described by equation [2] have been discussed in detail by Dixon and Webb (55), Laidler (66) and Peller and Alberty (67).

For many enzymes, however, such as the serine proteinases, trypsin, chymotrypsin, elastase and thrombin and the thiol proteinases, papain, ficin and bromelain the formation of a second intermediate has been demonstrated (68). Similar behaviour has also been indicated for other hydrolytic enzymes such as cholinesterase (69), ribonuclease (70), α -amylase (71) and α -lytic protease (72). The mechanism is then



where ES and ES' are two transient intermediates and P₁ and P₂ are products of the reaction. The steady-state parameters are now

$$[6] \quad \tilde{k}_c = \frac{\tilde{k}_2 \tilde{k}_3}{\tilde{k}_2 + \tilde{k}_3}$$

$$[7]^* \quad \tilde{K}_m = \frac{\tilde{k}_{-1} + \tilde{k}_2}{\tilde{k}_1} \frac{\tilde{k}_3}{\tilde{k}_2 + \tilde{k}_3}$$

If, as can usually be assumed, $k_{-1} \gg k_2$

$$[8] \quad \tilde{K}_m = \frac{\tilde{k}_3 \tilde{K}_s}{\tilde{k}_2 + \tilde{k}_3}$$

*

K_m is used in this thesis in the sense recommended by the Enzyme Commission of the International Union of Biochemistry (73), i.e. as equal to the substrate concentration at which the initial (steady-state) velocity is $\frac{1}{2} V_{\max}$. A common current practice is to designate the parameter specified by equation [7] as the "apparent Michaelis constant" $K_m(\text{app})$. The Michaelis constant itself is then defined as by equation [4]. The present nomenclature emphasizes the point that K_m as given by equation [7] must not be equated to the equilibrium constant K_s , when the reaction sequence has more than one intermediate.

$$[9] \quad \frac{\tilde{k}_c}{\tilde{K}_m} = \frac{\tilde{k}_2}{\tilde{K}_s}$$

where $\tilde{K}_s$ is the substrate-binding constant.

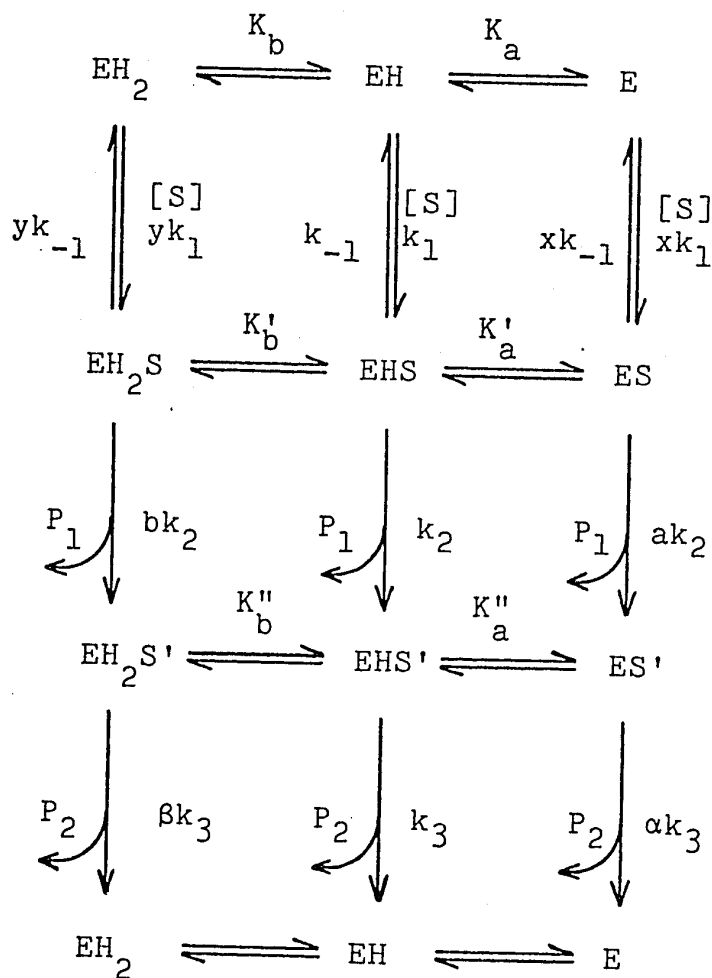
Some of the kinetic consequences of the three-step reaction sequence have been considered by Zerner and Bender (74). Krupka and Laidler (75) in particular have discussed the pH dependence of the overall rates, in terms of the nature of the ionizations of E, ES and ES', taking into consideration the various possible rate-determining steps. Krupka (76) and Webb (77) have extended this treatment to deal with cases in which the substrates or inhibitors are charged and their effects on the different ionizations.

General Reaction Scheme

A general reaction mechanism involving two intermediates and two ionizing groups on the enzyme is shown in Scheme I.

SCHEME. I

General Mechanism for pH Dependence



K_a and K_b are both dissociation constants for the free enzyme, and represent the ionization constants of an acid and basic group respectively. K'_a , K'_b , K''_a and K''_b represent

the ionization constants of the same groups in the Michaelis complex and acyl enzyme. The processes of acyl-enzyme formation and breakdown are shown to be irreversible, since under initial conditions the concentrations of P_1 and P_2 are so small that the reverse reactions may be neglected. The scheme allows for the breakdown of different ionization states of the free enzyme and both complexes.

Kaplan and Laidler (78) have described the conditions under which an ionizing group on an enzyme is kinetically detected. If, for example, $b=1$, then that group is not essential for activity, but if $b=0$, it is essential and a fall-off in the acylation rate constant $\tilde{k}_2$ at low values of pH is observed.

An approximate steady-state treatment of the mechanism described in Scheme I leads to the following expressions for the kinetic parameters:

$$[10] \quad \tilde{k}_c = \frac{k_2}{1 + \frac{K'_a}{[H]} + \frac{[H]}{K'_b} + \frac{k_2}{k_3} \frac{1 + \frac{K''_a}{[H]} + \frac{[H]}{K''_b}}{1 + \frac{\alpha K'_a}{[H]} + \frac{b[H]}{K'_b} + \frac{k_2}{k_3} \frac{1 + \frac{\alpha K''_a}{[H]} + \frac{\beta[H]}{K''_b}}}$$

$$[11] \quad \tilde{K}_m = \frac{\frac{k_{-1} + k_2}{k_1} \frac{1 + \frac{K_a}{[H]} + \frac{[H]}{K_b}}{1 + \frac{aK'_a}{[H]} + \frac{b[H]}{K'_b}}}{\frac{1 + \frac{K'_a}{[H]} + \frac{[H]}{K'_b}}{1 + \frac{aK'_a}{[H]} + \frac{b[H]}{K'_b}} + \frac{k_2}{k_3} \frac{1 + \frac{K''_a}{[H]} + \frac{[H]}{K''_b}}{1 + \frac{\alpha K''_a}{[H]} + \frac{\beta[H]}{K''_b}}}$$

$$[12] \quad \frac{\tilde{k}_c}{\tilde{K}_m} = \frac{k_1 k_2}{k_{-1} + k_2} \frac{1 + \frac{aK'_a}{[H]} + \frac{b[H]}{K'_b}}{1 + \frac{K_a}{[H]} + \frac{[H]}{K_b}}$$

It should be noted that under steady-state conditions the constants x and y in Scheme I may take any value between zero and unity, if it is assumed that the ionization processes are much more rapid than reversible complex formation between substrate and the three ionization forms of the free enzyme (79).

Significance of the $\tilde{k}_c/\tilde{K}_m$ Ratio

The ratio $\tilde{k}_c/\tilde{K}_m$ is related to the kinetic behaviour at low substrate concentrations (c.f. equ. [1]). The way in which this ratio varies with pH is shown by equation [12], and the following three cases are of special interest:

(1) The Michaelis complex ionizes in the same way as does the free enzyme (i.e. $K'_a = K_a$ and $K'_b = K_b$) and a and b are unity. This is the case of non-essential ionizing groups where $0 < x \leq 1$ and $0 < y \leq 1$. Even if these groups are involved in subsequent reactions (e.g. in deacylation) they will not be revealed in studies at low $[S]$.

(2) If a and b are zero, studies at low $[S]$ reveal K_a and K_b for the ionization of the free enzyme. If $a = 0$ but b is not zero, there will be pH dependence of $\tilde{k}_c/\tilde{K}_m$ on the basic side giving a value of K_a , and conversely if $b = 0$ but a is not zero, the pH dependence on the acid side will reveal K_b . Again it is true that $0 < x \leq 1$ and $0 < y \leq 1$.

(3) If either $K'_a = 0$ or $K'_b = \infty$, it means that the group is not free to ionize in the Michaelis complex.

Studies at low $[S]$ will then reveal either K_a or K_b for the ionization of the free enzyme and either $x = 0$ or $y = 0$. This is the case of an ionizing group which is essential to the binding of the substrate.

It follows that when one obtains K_a and K_b values from studies at low $[S]$, one can conclude that the corresponding ionizing groups not only ionize in the free enzyme but are essential to the subsequent reaction of the enzyme-substrate complex. However they are not necessarily essential to the formation of the enzyme-substrate complex. The same groups may also be involved at a later stage, e.g. deacylation, but this must be investigated at high substrate concentrations when the pH dependence of the overall rate is that of $\tilde{k}_c$ and is given by equation [10].

Kinetic Studies

Relatively few kinetic studies have been made of the influence of pH on the trypsin-catalyzed hydrolysis of esters (31,32,47,80,81) and all of these were done at substrate concentrations which are high relative to the Michaelis constant; this constant for the specific

amino acid ester substrates is of the order of $10^{-5}M$ or less. Under these conditions the pH dependence of the turn-over rate constant $\tilde{k}_c$ has revealed values of $pK_a'' \approx 10$ and pK_b'' varying from 6.02 to 7.35 depending on the substrate and the experimental conditions employed. Due to the small value of K_m , however, no work has been done at low substrate concentrations. It is evident from the arguments in the preceding section that a study of the pH variation of $\tilde{k}_c/\tilde{K}_m$ permits one to determine the ionizing groups involved in acylation (unless x and/or y are zero), even using a substrate for which deacylation is rate limiting. The present investigation was therefore undertaken to obtain a consistent set of pH profiles under the conditions that $[S] \ll \tilde{K}_m$. The choice of N-benzoyl-L-alanine methyl ester as substrate made this possible. This compound is a non-specific substrate of trypsin, differing from specific substrates in that it lacks a positive charge on the side chain and indeed only has a methyl side chain. There is good reason to believe, however, that the mechanisms of hydrolysis are the same for both specific and non-specific substrates on the basis that both classes of compounds exhibit the same pH dependence for the deacylation process (33,47,82).

Optical Rotation Studies

In view of the proposed indirect participation in Michaelis-complex formation of the N-terminal isoleucine residue of α -chymotrypsin (63,64), it appears likely that the N-terminal group in trypsin performs a similar function. It was therefore considered desirable to support the expected kinetic evidence in basic solution by optical rotation measurements as a function of pH. There is ample evidence that the optical rotation of proteins is highly sensitive to structural changes, and attempts have been made to correlate changes in optical rotation with the configuration of polypeptide chains and proteins (83-85). While there is as yet no theory which unequivocally relates the optical rotary behaviour of protein molecules to their shape and internal structure, changes in optical rotation can be interpreted qualitatively in terms of conformational alterations. This method has been applied in this investigation to trypsin, to some of its derivatives, and to trypsin in the presence of inhibitors, in order to elucidate the role of the N-terminal isoleucine residue and to examine the conformational stability of the enzyme in alkaline solution. McConn et al. (86) have recently published a detailed study on the

conformation of the high-pH form of chymotrypsin using the techniques of optical rotary dispersion and circular dichroism. The results support their earlier postulate concerning the conformation-controlling role of the N-terminal isoleucine group. In this connection it is of interest that two related serine proteinases, elastase (homologous with trypsin) and α -lytic protease, appear not to be subject to such control by their N-terminal α -amino group, since their kinetic and optical rotary properties show no indication of pH-sensitive conformational changes up to pH 10 (72,87,88).

EXPERIMENTAL DETAILS

Materials

N-Benzoyl-L-alanine methyl ester (BAME) was prepared essentially according to the method used by Kaplan and Laidler (89). An improvement in yield and ease of purification was achieved by carrying out the reaction in an ice-water bath which caused the crude product to precipitate as a solid rather than a liquid. The yield after two recrystallizations was 66.4% of BAME, m.p. 57.5-58°C, $[\alpha]_D^{22} + 30.1^\circ$ (c, 5 in sym-tetra-chloroethane). This agrees well with previously reported results (89,90). Guanidine hydrochloride (Highest Purity) was obtained from the Fisher Scientific Co. Phenylguanidine sulfate was a generous gift of Dr. E.N. Shaw of the Brookhaven National Laboratory.

Trypsin (twice crystallized, salt-free, Lot No. TRL 7EC) was obtained from Worthington Biochemical Corporation and used without further purification for most of the kinetic work. Enzyme concentrations, calculated on the basis of a molecular weight of 23,800 (91), ranged from 2 to 4×10^{-5} M. One gram of

trypsin (same lot as above) was treated with L-(1-tosyl-amido-2-phenyl) ethylchloromethyl ketone (TPCK) according to the method described by Kostka and Carpenter (92). After dialysis and lyophilization, the yield of treated enzyme was 84%. This enzyme in which all chymotrypsin activity is irreversibly inhibited was used as a control for some kinetic runs at pH 8.0. TPCK was purchased from Calbiochem (Lot No. 53227).

Three of the proteins used for optical rotation studies were obtained from Worthington Biochemical Corporation; trypsin (twice crystallized, salt-free, Lot No. TRL 8CA), trypsin treated with diisopropyl fluorophosphate (DIP-trypsin, twice crystallized, Lot No. TDIP 7AA) and trypsinogen (crystallized, Lot No. TG 7JH). Acetyltrypsin (Cat. No. AC 131) was purchased from Schuchardt Chemische Fabrik, Germany.

Water was doubly distilled, deionized and free of carbon dioxide. The titrant used for pH-stat work was 0.02 N NaOH (0.05N at pH 10.0) periodically restandardized with Fisher Certified hydrochloric acid.

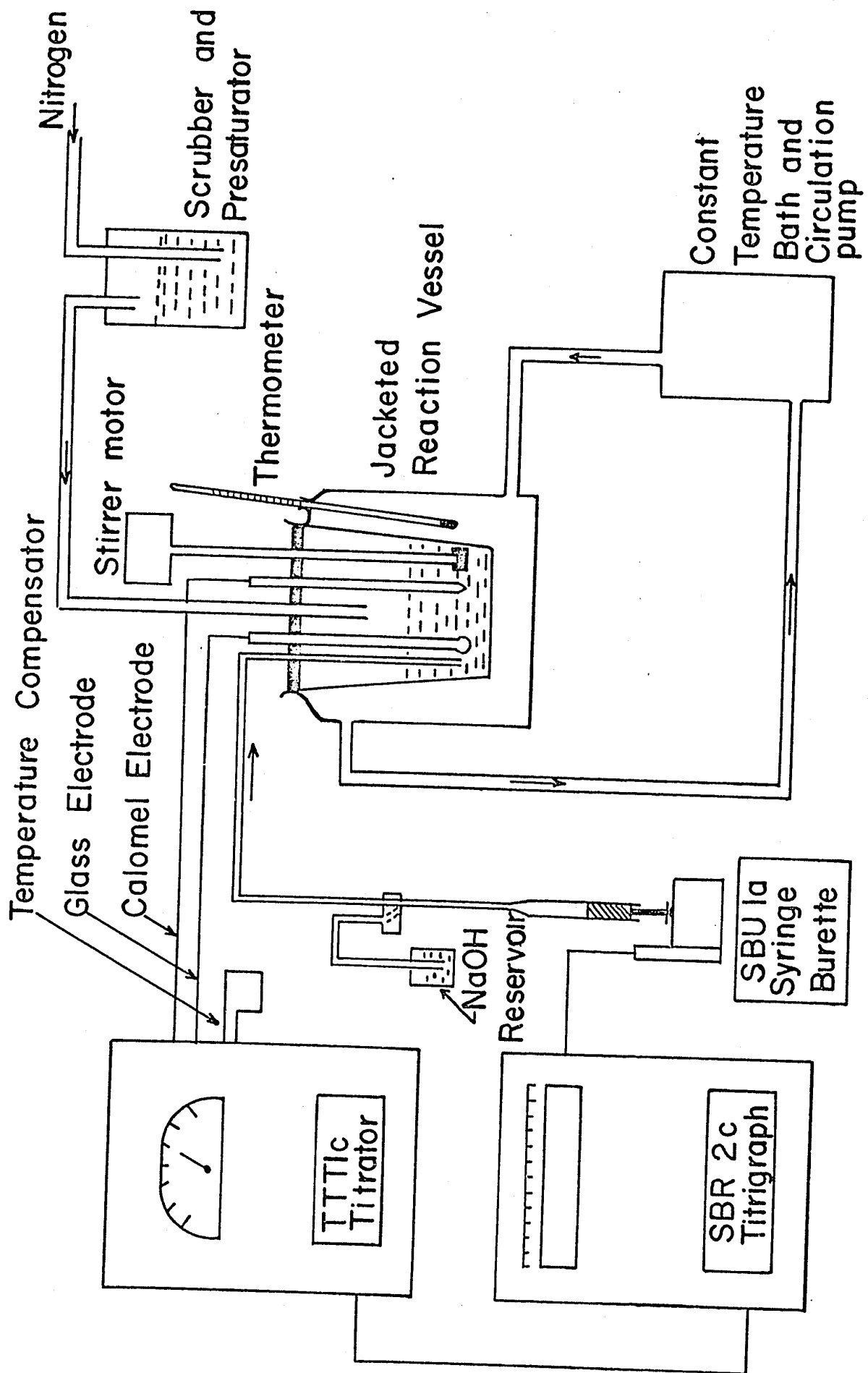
Kinetic Procedure

Rates of hydrolysis were determined by the pH-stat method with a Radiometer automatic titrator (Model TTT1c) and titrigraph (Model SBR2c). A schematic diagram of the titration assembly is shown in Fig. 1. The commercially supplied reaction vessel which consisted of a sealed, jacketed unit was redesigned to permit removal of the reaction cell itself from the outer jacket. Three reaction cells were used; two were employed exclusively for enzyme experiments and the other for buffer solutions during the standardization of the titrator. The two enzyme vessels were carefully conditioned prior to use, since it has been shown that trypsin is adsorbed on glass (93) causing difficulties with achieving experimental reproducibility (94). Conditioning was found to eliminate this source of error.

The reaction vessel was maintained at constant temperature by circulating water through the jacket at $25.0 \pm 0.05^{\circ}\text{C}$. A calibrated thermometer in the thermostatted jacket served as a continuous check on the temperature control. Inserted into the cell were a glass electrode (Radiometer G202B), a saturated calomel reference

Figure 1

Schematic diagram of the titration assembly.



electrode (Radiometer K401), a stirrer, and leads for sodium hydroxide solution and nitrogen. The nitrogen was washed with acid and alkali and then with water before being passed in a slow stream over the surface of the reaction mixture to keep the system free of carbon dioxide. The titrant was delivered by a Radiometer syringe burette (Model SBULa) into the cell containing 15 ml of solution.

Stock solutions of trypsin were made up in 0.033M CaCl_2 ($I=0.10$) and stored frozen for no longer than three days. A stock solution of BAME in water could be maintained indefinitely in the refrigerator. Substrate concentrations ranged from 0.167 to $4.33 \times 10^{-2}\text{M}$. In a typical run, substrate solution, water and 1 ml of 1.4M sodium chloride solution were mixed in the cell to make up 14 ml ($I=0.10$) and allowed to stand for five minutes to come to temperature equilibrium. The pH was adjusted to the desired value with 1N sodium hydroxide delivered from a 1 cc syringe by means of a micrometer screw gauge. In this manner the volume change during pH adjustment was kept negligible. The reaction was then started by the addition of 1.0 ml of enzyme solution and usually followed to 5 - 10% completion.

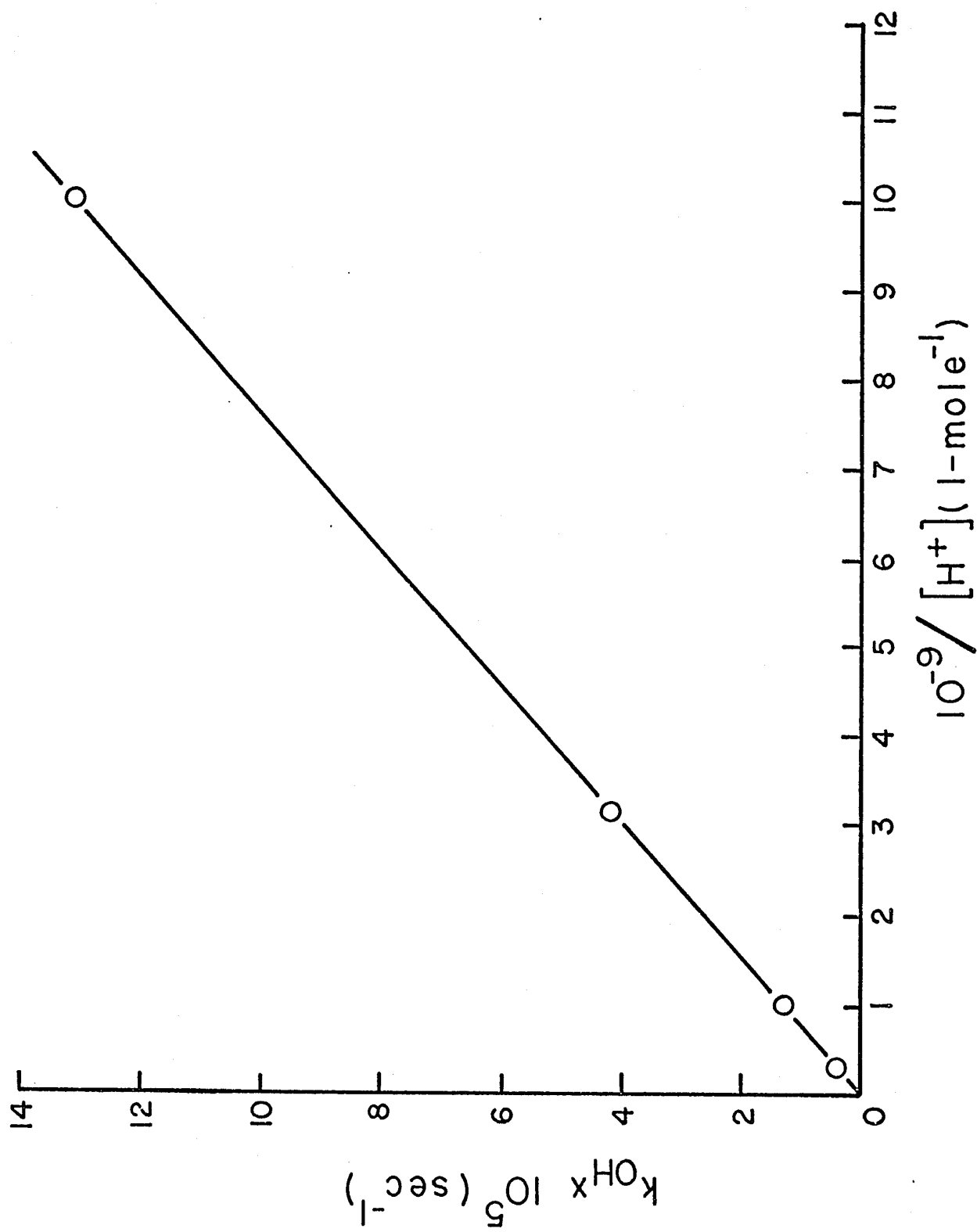
Methods of Computation

Reaction rates were calculated by measuring the initial slopes of the ΔA versus time curves recorded by the Radiometer titrigraph, where ΔA is the fractional amount of alkali used up. The recordings were practically linear for the first ten minutes, and this allowed an accurate initial slope to be taken. The gearing of the titrigraph was adjusted so that the initial slopes obtained varied between 0.7 and 1.8. At higher pH values a correction to the experimentally measured rates had to be applied for the alkaline hydrolysis of BAME. The first-order rate constant of this process ($k_{OH} = v/[S]$) was determined at various pH values and plotted against $1/[H^+]$ as in Fig. 2. The correction could then be directly calculated at all other pH values.

Throughout this work the v against $[S]$ plots gave a non-zero intercept which (after correction for alkaline hydrolysis of BAME) could only be accounted for by an "enzyme-blank" reaction. This phenomenon has previously been observed and discussed (95,96) and suitable corrections were made. In this study all experimental data have been analyzed and the kinetic constants computed on an IBM 360/65 computer using a slightly modified version of the

Figure 2

Plot of first-order rate constant for the
alkaline hydrolysis of BAME in water
(0.1 M NaCl, 0.033 M CaCl₂) at 25.0°C
against the reciprocal of the hydrogen-ion
concentration.



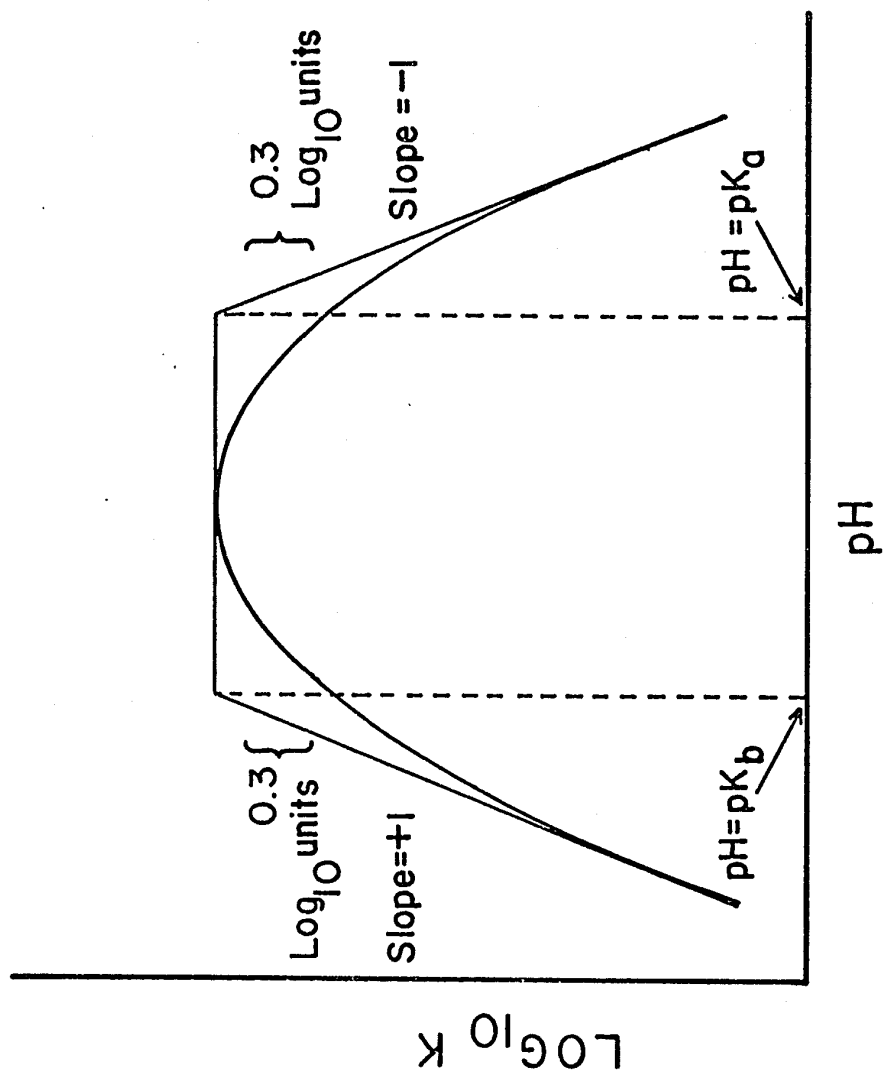
program written by Hanson, Ling and Havir (97). The program fits experimental data directly to the Michaelis-Menten equation (c.f. equ. [1]) according to the method described by Bliss and James (98). From an analysis of the standard errors in $\tilde{K}_m$ and $\tilde{k}_c$, the "enzyme-blank" correction could be deduced, assuming that the "enzyme-blank" rate was proportional to the total enzyme concentration. The correction amounts to 3-25% depending on rate, pH and enzyme concentration. The method of Dixon (99) was used to obtain ionization constants from the variation of the kinetic parameters with pH. The method is schematically illustrated in Fig. 3.

Optical Rotation Measurements

Protein solutions of approximately 1 mg per ml concentration were made up in 0.033M CaCl_2 except those containing guanidine hydrochloride and phenylguanidine sulphate where the CaCl_2 concentration was 0.019M to obtain a final ionic strength of $I=0.10$. The solution of trypsinogen was centrifuged at 6000 r.p.m. in a Sorvall Superspeed RC2-B automatic refrigerated centrifuge to remove water-insoluble impurities. Protein concentrations were determined on a Perkin Elmer Model

Figure 3

Schematic illustration of Dixon's
method (99) for obtaining pK values.



350 Spectrophotometer at 280 m μ with values of extinction $E_{1\text{ cm}}^{1\%} = 14.4$ for trypsin and DIP-trypsin, 13.9 for trypsinogen (6) and 14.4 for acetyltrypsin (100). Since complete acetylation of trypsin adds 14 acetyl groups of molecular weight 43 to the enzyme, a molecular weight of 24,400 was assumed for the acetylated protein. This is based on the report of Labouesse and Gervais (100) that during acetylation of trypsin, the N-terminal α -amino group remains free leaving 14 lysine residues to be acetylated. Protein concentrations ranged from 3.26 to 4.20×10^{-5} M. Guanidine hydrochloride and phenylguanidine sulfate were added in 1000-fold molar excess to the trypsin solution prior to use. Samples of each solution were adjusted to the desired pH with 1N NaOH and placed in a 1-dm polarimeter tube with water jacket maintained at 23°C. A Perkin Elmer Model 141 Polarimeter was used to measure optical rotations at 365 m μ and 436 m μ to an average precision of $\pm 3^\circ$.

RESULTS

pH Dependence of Kinetic Parameters

Curves representing rate measurements for the hydrolysis of BAME by trypsin at three different values of pH are shown in Figure 4. The condition of low substrate concentration relative to the Michaelis constant is strictly satisfied at pH 6 and 6.5 where a plot of rate against [S] is a straight line. At higher pH values, the plot becomes slightly curved as the order of the reaction decreases. Above pH 7.0 a small extrapolation has therefore been made to obtain the second-order rate constant $\tilde{k}_c/\tilde{K}_m$. The values and standard errors of $\tilde{k}_c$, $\tilde{K}_m$ and $\tilde{k}_c/\tilde{K}_m$ are given in Table 2. The pH dependencies of these parameters are shown in Figures 5-7. The kinetic parameters are average values based on computed results from three to five runs covering the full range of substrate concentrations from 0.167 to 4.33×10^{-2} M. The standard errors reported in Table 2 show that, with a few exceptions, the kinetic parameters can be considered reliable to ± 5 to 10%.

Figure 4

Plots of rates against $[S]$ for the trypsin-catalyzed hydrolysis of BAME at 25.0°C and $I = 0.10$ in CaCl_2 and NaCl . The curves have been corrected for alkaline hydrolysis and "enzyme-blank" rate. At pH 8.0, $[E]_0 = 2 \times 10^{-5}\text{M}$, at pH 9.5, $[E]_0 = 3 \times 10^{-5}\text{M}$ and at pH 6.5, $[E]_0 = 4 \times 10^{-5}\text{M}$.

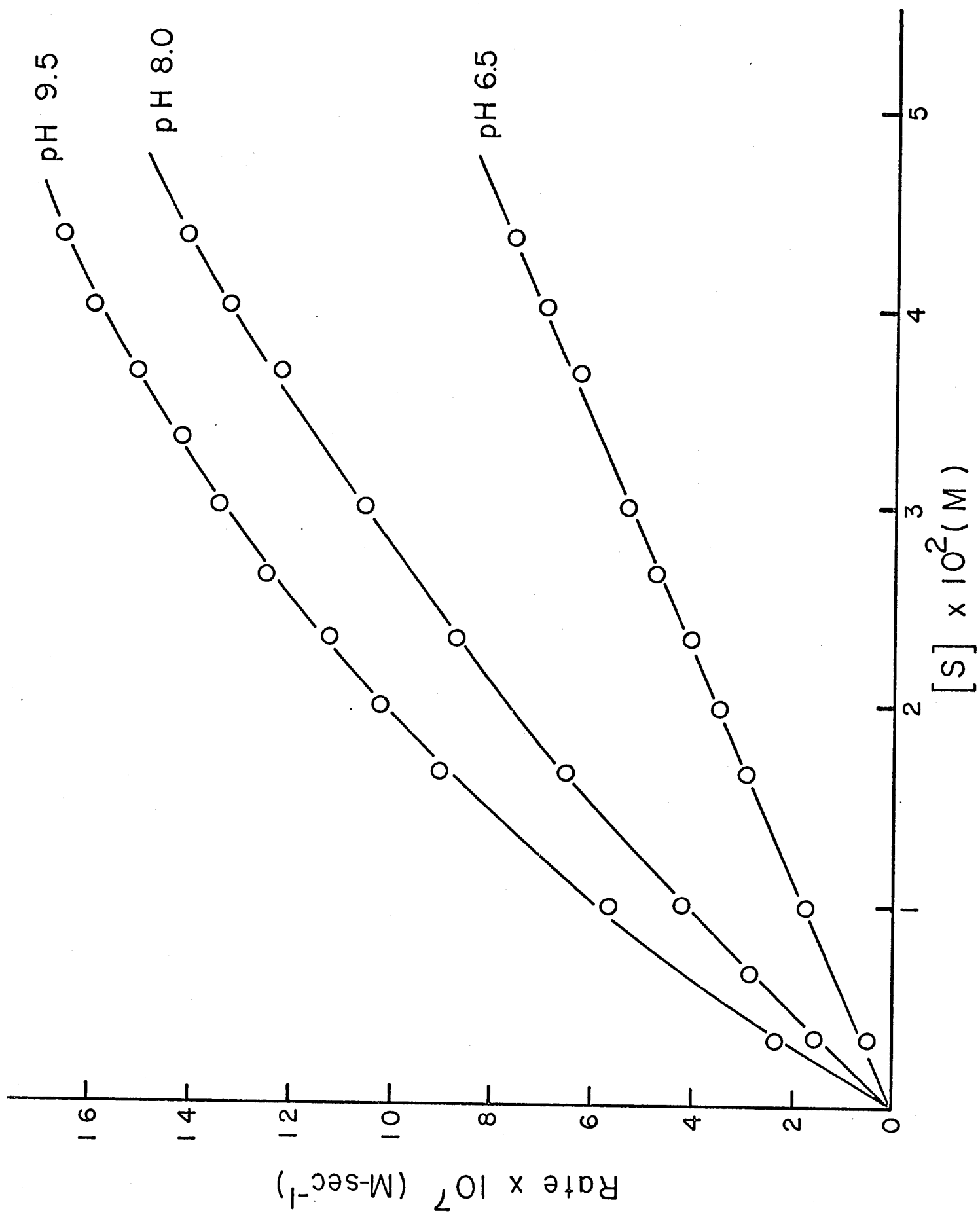


TABLE 2

Kinetic Parameters for the Trypsin-catalyzed Hydrolysis of BAME at 25.0 and I = 0.10

pH	$\tilde{k}_c (\text{sec}^{-1})$	$\tilde{K}_m (M)$	$\tilde{k}_c / \tilde{K}_m (M \text{ sec}^{-1})$
6.0	-	-	0.175 $\pm$ 0.005
6.5	0.109 $\pm$ 0.009	0.243 $\pm$ 0.010	0.448 $\pm$ 0.009
7.0	0.185 $\pm$ 0.010	0.171 $\pm$ 0.012	1.08 $\pm$ 0.07
7.5	0.232 $\pm$ 0.009	0.129 $\pm$ 0.006	1.79 $\pm$ 0.10
7.8	0.248 $\pm$ 0.008	0.112 $\pm$ 0.006	2.22 $\pm$ 0.11
8.0	0.265 $\pm$ 0.006	0.111 $\pm$ 0.003	2.40 $\pm$ 0.07
8.5	0.248 $\pm$ 0.015	0.098 $\pm$ 0.003	2.19 $\pm$ 0.21
9.0	0.184 $\pm$ 0.012	0.078 $\pm$ 0.004	2.37 $\pm$ 0.16
9.2	0.168 $\pm$ 0.008	0.077 $\pm$ 0.002	2.19 $\pm$ 0.20
9.5	0.122 $\pm$ 0.005	0.052 $\pm$ 0.003	2.35 $\pm$ 0.18
9.7	0.105 $\pm$ 0.005	0.047 $\pm$ 0.003	2.24 $\pm$ 0.17
10.0	0.070 $\pm$ 0.011	0.035 $\pm$ 0.003	1.98 $\pm$ 0.22

Figure 5

Variation of $\log_{10} \tilde{k}_c$ with pH for the trypsin-catalyzed hydrolysis of BAME at 25.0°C and $I = 0.10$ in CaCl_2 and NaCl .

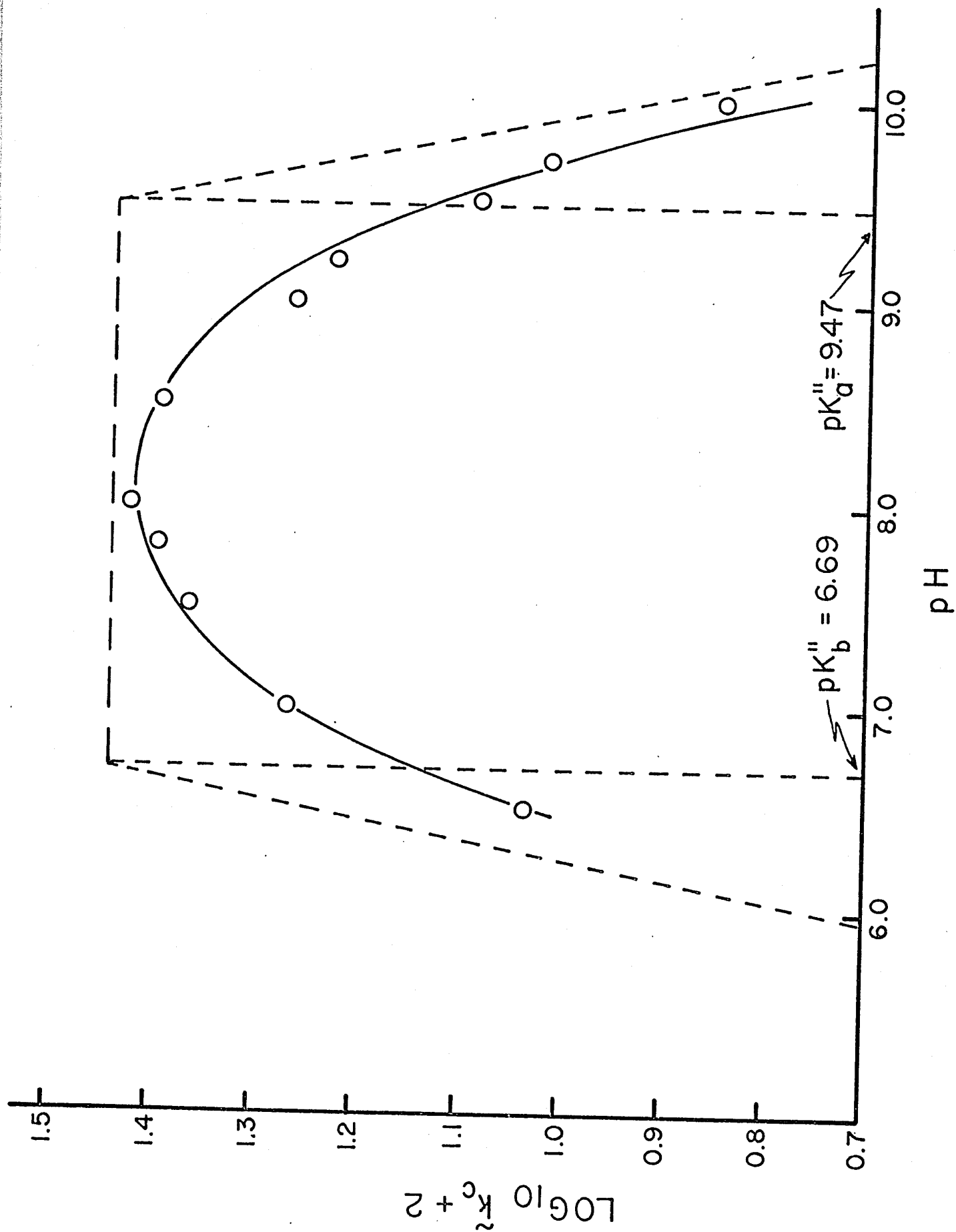


Figure 6

Variation of $p\tilde{K}_m$ with pH for the trypsin-catalyzed hydrolysis of BAME at 25.0°C and $I = 0.10$ in CaCl_2 and NaCl .

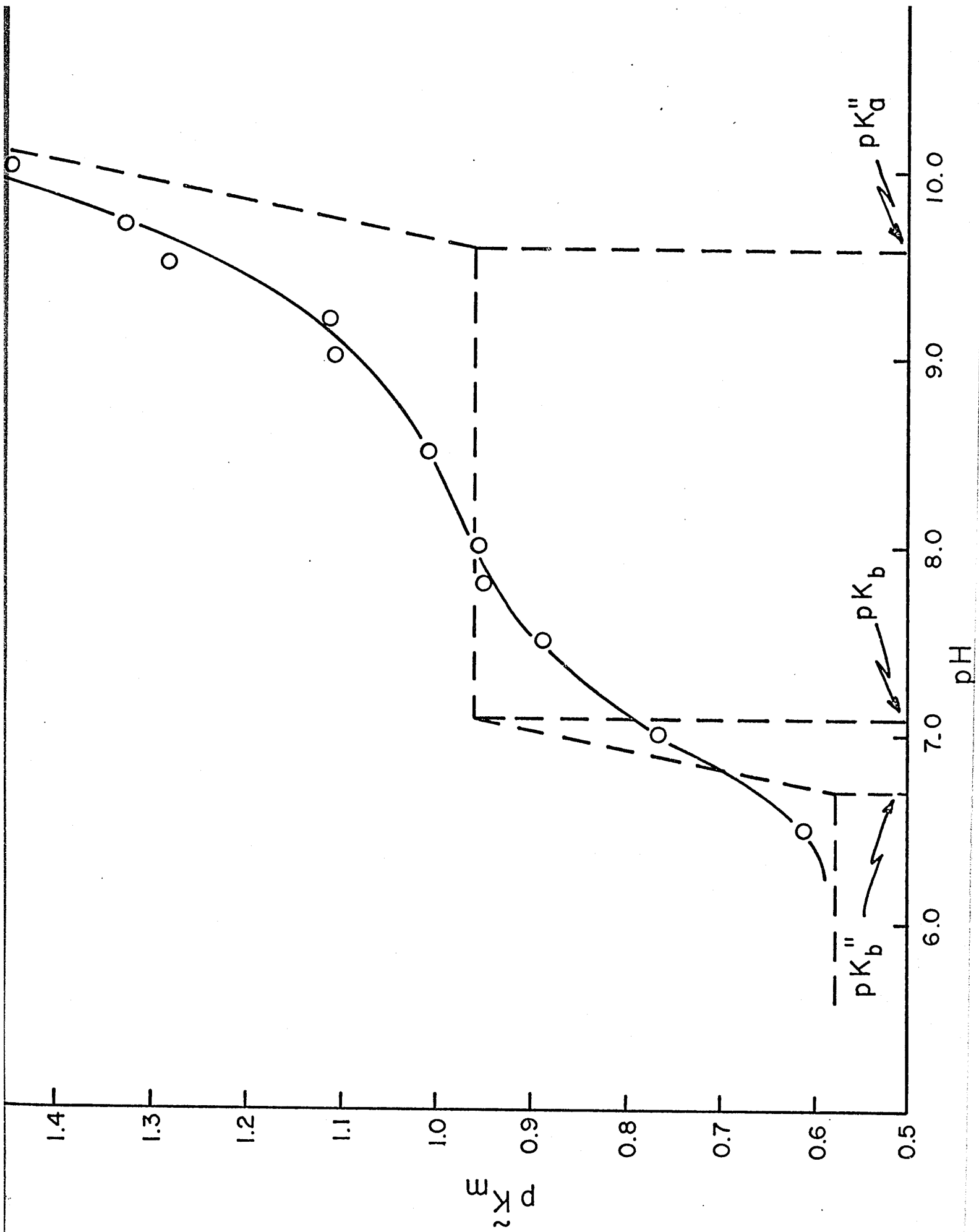
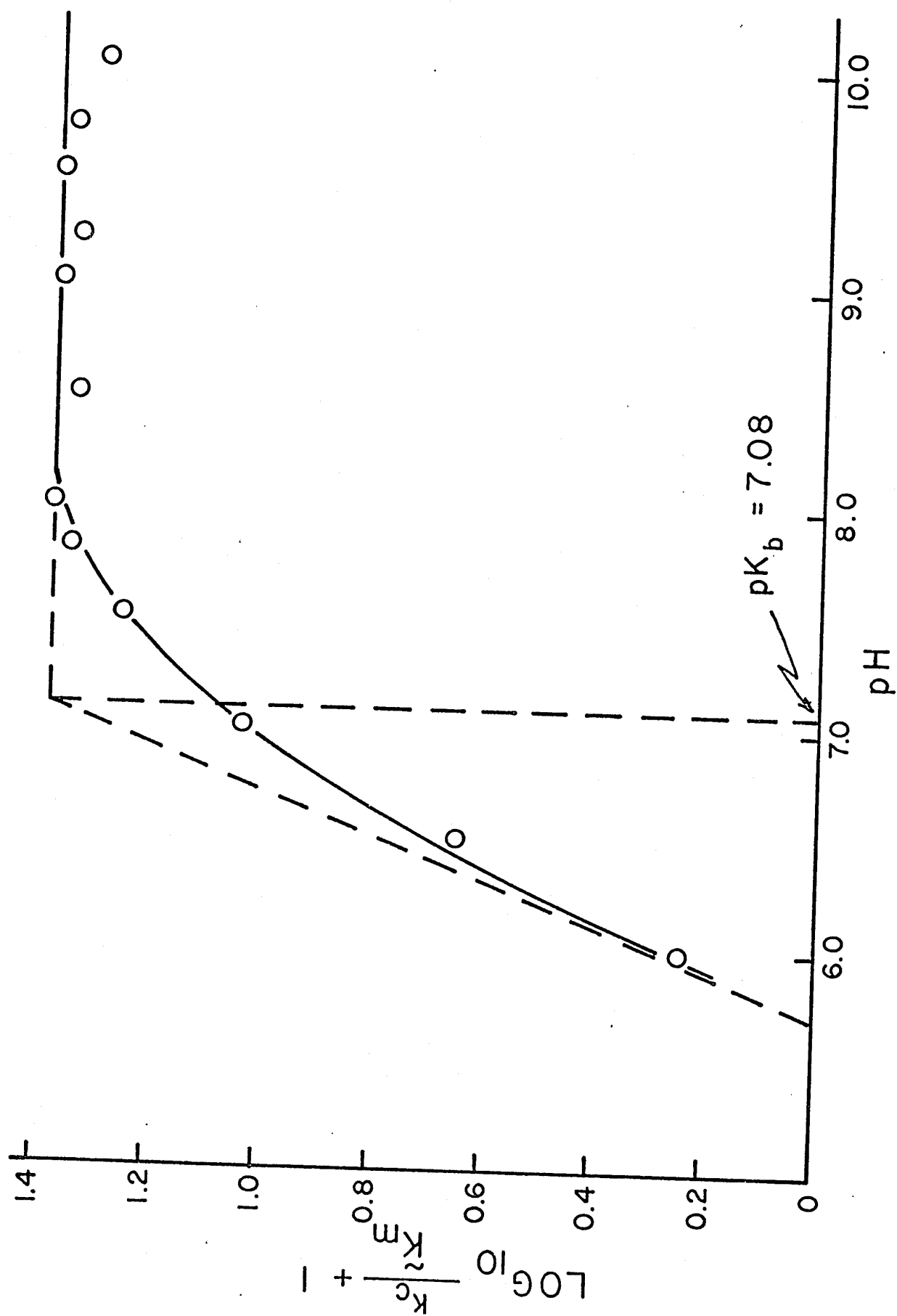


Figure 7

Variation of $\log_{10} \tilde{k}_c / \tilde{K}_m$ with pH for the trypsin-catalyzed hydrolysis of BAME at 25.0°C and $I = 0.10$ in CaCl_2 and NaCl .



Two control runs at pH 8.0 with TPCK-treated trypsin demonstrated within experimental error that the observed hydrolysis of BAME was indeed due to trypsin and not due to contaminating chymotrypsin. The results are shown in Table 3.

The curves in Figures 5 and 7 are the theoretical curves obtained by a reiterative process* which give the closest fit to the experimental points. The theoretical relationship between $p\tilde{K}_m$ and pH (c.f. Equ. 11) is too complex to permit such a fit and the curve in Fig. 6 is the best visual fit to the experimental data. The bell-shaped form of the $\log_{10}\tilde{k}_c$ -pH profile in Fig. 5 seems to suggest the participation of two groups in the catalytic reaction of the acylenzyme, $pK_b'' = 6.69$ and $pK_a'' = 9.47$. This implies that for the trypsin-catalyzed hydrolysis of BAME, deacylation is the rate-limiting step. It appears safe to make such an assumption in view of the extensive work done on α -chymotrypsin kinetics (68), as

* Knowledge of the pH-independent rate constants (c.f. Equ. 10 and 12) is not necessary. In the process of curve fitting, suitable values for these quantities can be found which give a best fit to the experimental points.

TABLE 3

Comparison of Rate Constants for the Hydrolysis of BAME by Commercial Trypsin
and TPCK-treated Trypsin at pH 8.0, T = 25.0°C and I = 0.10.

	$\tilde{k}_c (\text{sec}^{-1})$	$\tilde{K}_m (M)$	$\tilde{k}_c / \tilde{K}_m (M \text{ sec}^{-1})$
Commercial trypsin	0.265 ± 0.006	0.111 ± 0.003	2.40 ± 0.07
TPCK-treated trypsin	(1) 0.264 (2) 0.261	0.110 0.114	2.40 2.29

well as two studies on trypsin using N-trans-cinnamoyl imidazole (34) and p-nitrophenyl acetate (35).

As explained earlier, the pH profile of $\log_{10} \tilde{k}_c/\tilde{K}_m$ reveals the kinetically important groups on the free enzyme. In this case, the sigmoid-shaped curve in Fig. 7 shows that a basic group with $pK_b = 7.08$ is involved in catalysis. The $p\tilde{K}_m$ -pH profile in Fig. 6 provides an independent estimate of pK_a'' which is found to agree well with the value of 9.47 obtained from Fig. 5. The theoretical positions of pK_b and pK_b'' have also been indicated in Fig. 6.

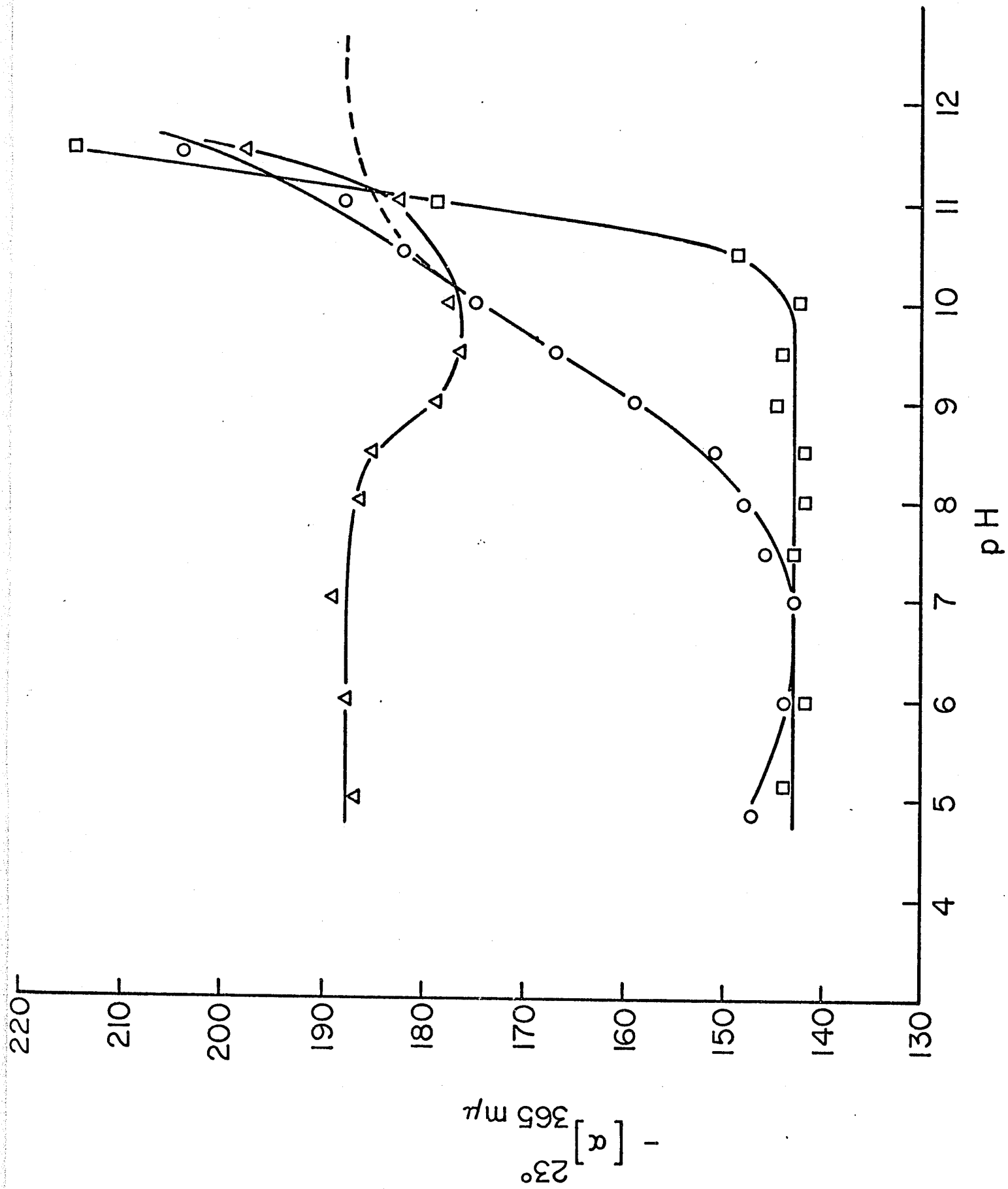
The shape of the $\log_{10} \tilde{k}_c$ -pH profile in Fig. 5 is essentially the same as that obtained by other investigators (32,47,82) for the trypsin-catalyzed hydrolysis of specific ester substrates. Similar bell-shaped behaviour of the catalytic rate constant ($\tilde{k}_c$) has been reported by Chevallier and Yon (101) for the hydrolysis of benzoyl-L-argininamide at substrate concentrations larger than K_m and 35°C. Their results qualitatively agree with those of Shukuya and Watanabe (102) and Wang and Carpenter (103). It must be remembered that for amides, acylation is considered to be the rate-limiting step (104).

Effect of pH on Optical Rotation

The pH dependence of optical rotation at 365 m μ for trypsin, DIP-trypsin and trypsinogen is shown in Fig. 8. Similar results were obtained for trypsin at 436 m μ , the overall changes in optical rotation being smaller. Samples at pH 11 were brought back to pH 8 and were found to exhibit complete reversibility in specific rotation within periods up to 20 minutes. Fig. 8 shows that the specific rotation values for DIP-trypsin and trypsinogen are significantly different from each other. The optical rotation of DIP-trypsin is pH independent between pH 5 to 10, and approximately 45° higher than that of trypsinogen which exhibits a small change in $[\alpha]$ above pH 8. The curve for trypsin has been extrapolated (dashed line) to a value of specific rotation equal to that of the inactive zymogen at neutral pH. Experimentally this value is not reached, as a result of the ionization of titratable tyrosine residues which has been related by D'Albis and Béchet (105) to a decrease in the enzyme's rotary power. The specific levorotation of all three proteins decreases sharply above pH 11, presumably because irreversible denaturation sets in. Similar results have

Figure 8

Normalized plots of specific rotation against pH at 365 mμ and 23°C. Solutions contained 3.26 to 4.20×10^{-5} M protein in 0.033 M CaCl_2 . Symbols: o, trypsin; ■, DIP-trypsin; Δ, trypsinogen.



been obtained by Neurath, Rupley and Dreyer (106) for trypsin and trypsinogen.

Using the same treatment as applied by Oppenheimer, Labouesse and Hess (107) to acetylated δ -chymotrypsin, the pH-dependent change in specific levorotation of active trypsin can be related to the specific rotation of its precursor and its phosphorylated derivative as shown in Fig. 9. The equation used as basis for this plot is

$$[13] \quad K = \frac{[E][H]}{[EH]}$$

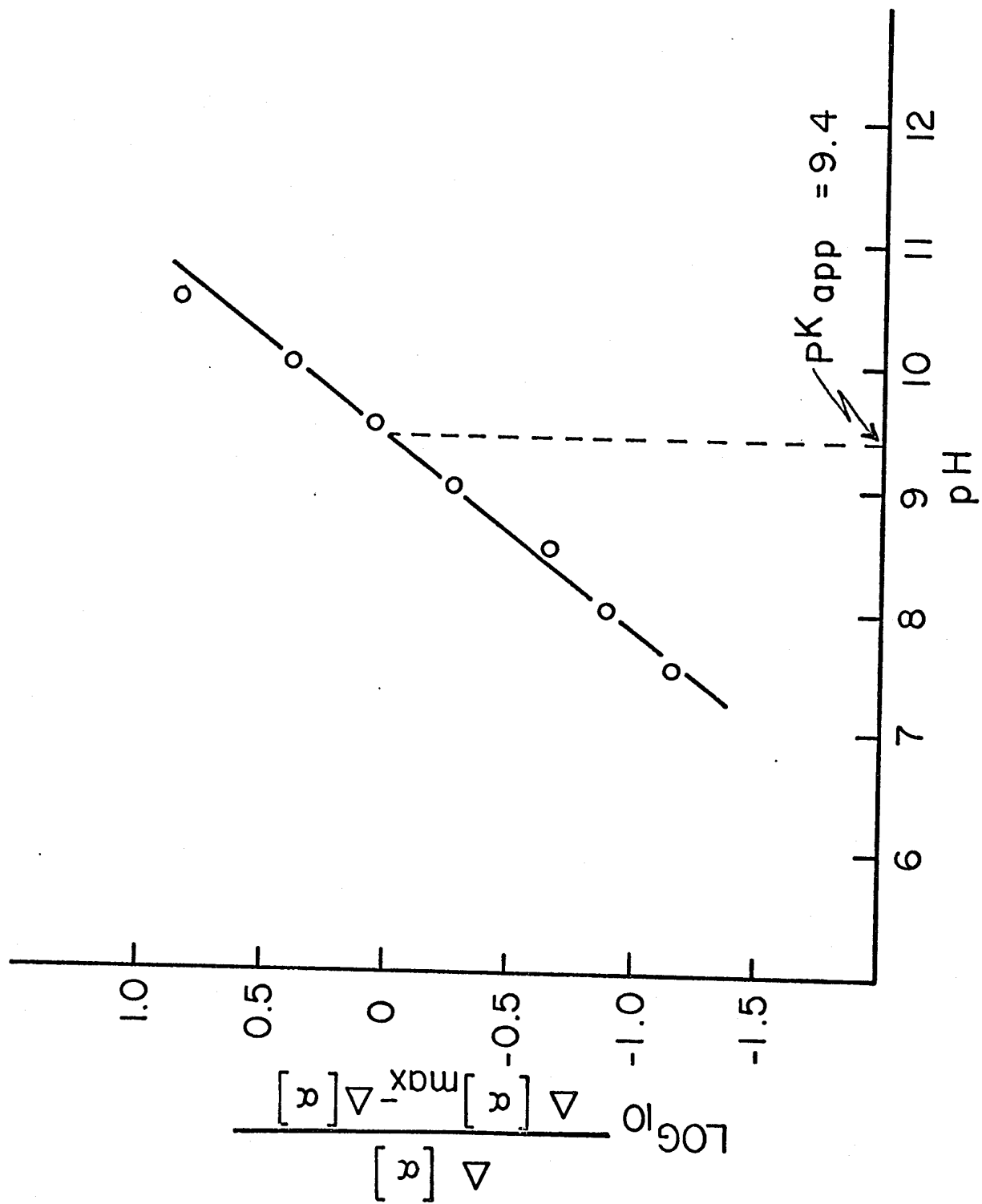
which can be rearranged into

$$[14] \quad \text{pH} = \text{pK} + \log_{10} \frac{[E]}{[EH]}$$

where E and EH represent the different ionization states of the enzyme molecule. If $\Delta[\alpha]_{\text{max}}$ is defined as the total change in specific rotation between the value at pH 7 and the limiting value given by the dashed curve in Fig. 8, and $\Delta[\alpha]$ as the change in specific rotation between

Figure 9

Plot of $\log_{10} \Delta[\alpha]/(\Delta[\alpha]_{\max} - \Delta[\alpha])$ against
pH for trypsin at 365 m μ .



pH 7 and any pH, Equation [14] becomes

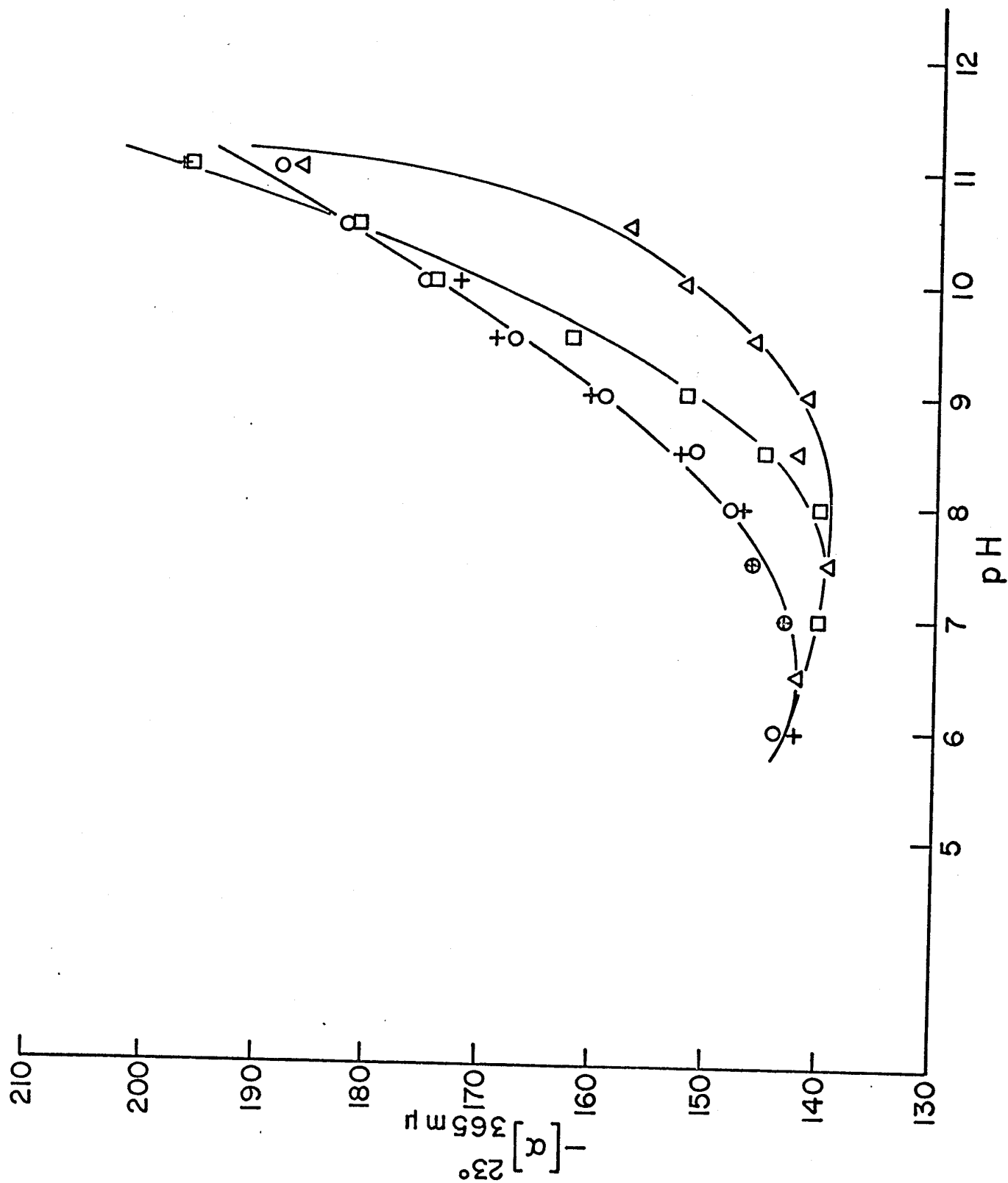
$$[15] \quad \text{pH} = \text{pK} + \log_{10} \frac{\Delta[\alpha]}{\Delta[\alpha]_{\text{max}} - \Delta[\alpha]}$$

As can be seen from the plot in Fig. 9, Equation [15] is obeyed, indicating that the specific levorotation of trypsin follows the ionization of a single group. The $\text{pK}(\text{app})$ of the group controlling the change in specific rotation may be obtained from the abscissa of the plot in Fig. 9 at the point where $\log_{10} \Delta[\alpha]/(\Delta[\alpha]_{\text{max}} - \Delta[\alpha]) = 0$. A value of $\text{pK}(\text{app}) = 9.4$ was found.

The pH dependence of specific rotation for acetyltrypsin and trypsin in the absence and presence of different inhibitors has been plotted in Figure 10. Guanidine hydrochloride and phenylguanidine sulfate are reversible competitive inhibitors of trypsin with $K_1 = 9.1 \times 10^{-3}\text{M}$ and $7.2 \times 10^{-5}\text{M}$ respectively at pH 8.15 and 15°C (40,108). They were present in the trypsin solution in 1000-fold molar excess which is still well below the concentration at which guanidine or its derivatives begin to denature trypsin. The curves for

Figure 10

Normalized plots of specific rotation against pH at 365 m μ and 23°C. Solutions contained 3.98 to 4.20 x 10⁻⁵M protein at I = 0.10. Symbols: o, trypsin; +, acetyl-trypsin; □, trypsin with guanidine hydrochloride; Δ, trypsin with phenylguanidine sulfate. Some experimental points have been omitted to avoid crowding.



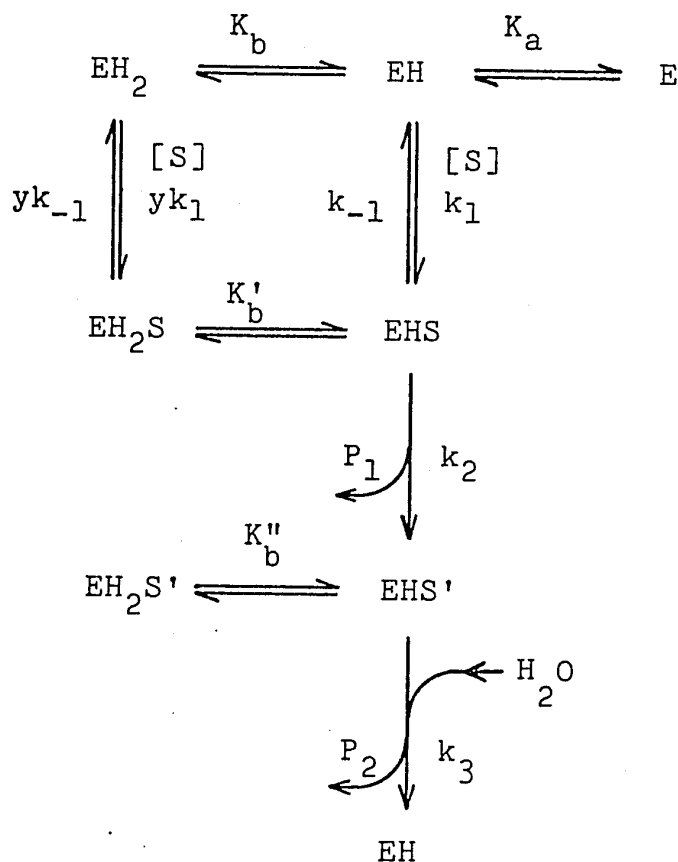
acetyltrypsin and trypsin in the absence of inhibitor are superimposable indicating that acetylation of up to 14 lysine α -amino groups does not alter the specific levorotation of the protein. The addition of an inhibitor, however, has the effect of shifting the trypsin curve by as much as two pH units to higher values of pH. The effect is strongest for phenylguanidine sulfate, the inhibitor with the lower K_i value, i.e. greater affinity for the enzyme. At pH 11 all three curves approach a common value of $[\alpha]$, suggesting that at this pH, where irreversible denaturation is expected to set in, the effect of inhibitors in retarding changes in specific rotation vanishes. D'Albis and Béchet (105) have reported that the optical rotation of trypsin at 436 m μ in the presence of butylamine is maintained constant and equal to the value in neutral solution up to pH > 11. Butylamine is a reversible competitive inhibitor which is about ten times better than guanidine (109). Their results are not directly comparable to ours, however, since their measurements were made at 10°C and butylamine is partially ionized in the upper region of their study (pK = 10.68, ref. 110) whereas the guanidine inhibitors have a pK > 12.

DISCUSSION

The pH dependencies shown in Figs. 5,6 and 7 at first sight appear to suggest a different mechanism of catalysis by trypsin as opposed to that by chymotrypsin. Much evidence has been accumulated for chymotrypsin (68, 86,89), and it has been incorporated in a reaction mechanism as shown in Scheme II.

SCHEME II.

Reaction Mechanism for Trypsin and Chymotrypsin Catalysis



We propose that the same mechanism is in fact applicable to trypsin on the basis that the observed kinetic behaviour of the enzyme is related to changes in conformation with pH. The relationship between the scheme and the catalytic action of trypsin is best discussed separately for the acid and basic sides.

Ionizations in Acidic Solution

The ionizations on the acid side are the same for trypsin and chymotrypsin, the entire pattern of kinetic behaviour on the acid side being very similar. Figure 7, which relates to low substrate concentrations, shows that there is a decrease in rate on the acid side corresponding to a pK_b value of 7.08. This undoubtedly relates to the ionization of the imidazolyl side chain of histidine-46. The fact that this residue is revealed in the $\log_{10} \tilde{k}_c/\tilde{K}_m$ plot indicates not only that the group is free to ionize in the free enzyme, but also that it is involved in the acylation process; in other words, acylation requires the function to be in the unprotonated form and cannot occur if it is protonated. It is for this reason that (as with chymotrypsin) the scheme shows no arrow between EH_2S and EH_2S' .

Figure 5, which relates to high substrate concentrations, provides information about the process of deacylation, which for esters is slower than that of acylation. The decrease in rate at low pH values corresponds to a pK_b'' value of 6.69. This undoubtedly can be attributed to the same imidazole group, and the fact that there is a falling-off of rate shows that this group is essential to deacylation, which can only occur if the function is unprotonated. The conclusion that the imidazole residue is essential to deacylation was first proposed by Gutfreund (31), who obtained $pK_b'' = 6.25$ with benzoyl-L-arginine ethyl ester. B  chet (32), on the other hand, found $pK_b'' = 7.35$ using tosyl-L-arginine methyl ester. Similar variations in pK values with different substrates are not uncommon.

Ionizations in Basic Solution

The situation on the basic side appears to be very different for trypsin from that with chymotrypsin. It is convenient to consider first the enzymatic behaviour at high substrate concentrations, which relates to the rate-determining deacylation process. It is seen from Fig. 5 that (in contrast to chymotrypsin) there is a

significant decrease in $\tilde{k}_c$ in basic solution, corresponding to the ionization of a group of pK_a'' equal to 9.47. In the case of chymotrypsin there seems to be no doubt (63,107) that one of the active residues in that enzyme is the $-NH_3^+$ group of the terminal isoleucine-16, and it is reasonable to suggest that in trypsin the observed pK relates to the corresponding $-NH_3^+$ group of the terminal isoleucine-7. Himoe, Parks and Hess (63) propose, however, that this group is not catalytically active in chymotrypsin, but controls an equilibrium between two major conformations of the enzyme and thereby the enzymatic activity.

Similar evidence to ours has been obtained by Béchet (80) with tosyl-L-arginine methyl ester as substrate; he obtained $pK_a'' = 10.1$. It is significant in this connection that the reaction of nitrous acid with the α -amino group of the terminal isoleucine residue causes inactivation of trypsin. Scrimger and Hofmann (111) have suggested that this is due to a complete destruction of the active site, and not to a change in the kinetic parameters, since the ability of the enzyme to react with diisopropyl fluorophosphate is lost at the same rate as the catalytic activity towards benzoyl-L-arginine ethyl

ester. We suggest therefore that the observed fall-off in the rate of deacylation by trypsin in alkaline solution is the result of a conformational change in the acylenzyme and does not reflect the participation of an acidic group in the catalytic process. Ionization of the group with pK_a'' equal to 9.47 induces a catalytically inactive conformation of the acylenzyme and thereby controls the rate of deacylation.

Such an interpretation would also explain the observed shifts with pH in the bell-shaped deacylation curves of different trypsin substrates (Fig. 5,33,80,82), if it is assumed that different substrates stabilize the acylenzyme to a different extent and thus affect the pK of the conformation-controlling group. This is in contrast to most forms of acylchymotrypsin which do not exhibit a fall-off in $\tilde{k}_c$ up to pH 10 (68,89). The specific rotation curve for DIP-trypsin (c.f. Fig. 8) shows that even the irreversibly inhibited enzyme undergoes a conformational change at a lower pH (above pH 10) than does acetylated DIP- δ -chymotrypsin (above pH 11) (107).

The work at low substrate concentrations (c.f. Fig. 7) shows no significant decrease in the second order rate constant up to pH $\approx$ 10. This result seems to suggest

that the ionizing group, observed in deacylation, is free but not involved in complex formation (in contrast to the situation with chymotrypsin) or in the acylation process. The participation of the terminal isoleucine residue in the catalytic mechanism of trypsin is discussed in detail in the last chapter. It appears likely, that the proximity of two carboxylate residues, aspartate -177 and -182, contributes to the increased acidity (i.e. $pK_a > 10$) of the N-terminal α -amino group in trypsin relative to chymotrypsin. In addition the ionization of this group seems to be very sensitive to the presence of reversibly or irreversibly bound modifiers in the active site region of trypsin as explained below.

The interpretation of the optical rotation studies presented in Figure 8 is that the conversion of catalytically inactive trypsinogen to active trypsin is accompanied by the appearance of a new ionizing group which controls the conformation of the enzyme. Evidence that a conformational change accompanies the activation of zymogen to enzyme has been previously published (106). We have shown that the specific levorotation of trypsin varies with pH and that the change in specific rotation

follows the ionization of a single group with $pK(\text{app})$ of 9.4 (c.f. Figs. 8 and 9). Since the specific rotation of trypsin at pH 11 is approximately the same as that of trypsinogen, the indication is that increasing alkalinity causes the enzyme conformation to revert to that of the zymogen. The curve for DIP-trypsin suggests that phosphorylation of the reactive serine residue has the effect of preserving the neutral-pH conformation of the enzyme almost up to the value of pH where irreversible denaturation is known to set in.

Figure 10 indicates that the formation of a trypsin-inhibitor complex displaces the $pK(\text{app})$ of the group associated with changes in enzyme conformation to a higher pH. In other words, at alkaline pH, complex formation due to non-covalent bonding alone, has the effect of stabilizing the enzyme structure in a conformation more closely resembling that in neutral solution. This stabilizing effect appears to be related to the K_i of the inhibitor and is completely neutralized under conditions where trypsin is subject to irreversible denaturation. A possible reason for this would be that denaturation alters the binding site of trypsin in such

a way that the enzyme-inhibitor complex can no longer form. Related evidence is provided by the work of Hopkins and Spikes (112). On the basis of differences in the stability to urea denaturation between trypsin, trypsinogen, DIP-trypsin and chymotrypsin, chymotrypsinogen and DIP-chymotrypsin, as measured by tryptophan fluorescence, these authors propose that the conformational changes involved in the catalytic mechanism of trypsin may be quite different from those of chymotrypsin. This suggestion is borne out by our kinetic and optical rotary results when compared to those for chymotrypsin.

It is of passing interest to note that acetylation of up to 14 lysine ϵ -amino groups appears to cause no conformational change in trypsin, as reflected by the pH dependence of specific rotation (c.f. Fig. 10), even though the charge distribution of the acetylated protein is expected to be quite different from that of the native enzyme. Similar behaviour has been reported for δ -chymotrypsin (107) which leads to the somewhat unexpected suggestion that the lysine residues are not directly involved in maintaining the conformation of the active enzymes.

The experimental results obtained in this work together with some facts presented below are consistent with the hypothesis that both kinetic and optical rotation studies in basic solution reveal the same group in the enzyme, namely the terminal isoleucine-7 residue. These facts are that (a) activation of trypsinogen to trypsin results in isoleucine-7 as the new NH_2 -terminal residue (6,7), (b) reaction of nitrous acid with the isoleucine group causes inactivation of the enzyme (111), and (c) acetylation of all lysyl ϵ -amino groups and exposed tyrosyl groups of trypsin does not cause reduced enzymatic activity (100,113). In contrast to chymotrypsin (89), however, conformational changes induced by ionization of the NH_2 -terminal group do not become apparent in the kinetics of Michaelis-complex formation between trypsin and the non-specific ester substrate BAME. The existence of inactive forms of the enzyme in subsequent stages of the catalytic process is revealed by a decrease in the kinetic constant ($\tilde{k}_c$) for both the amidase and esterase activity of trypsin.

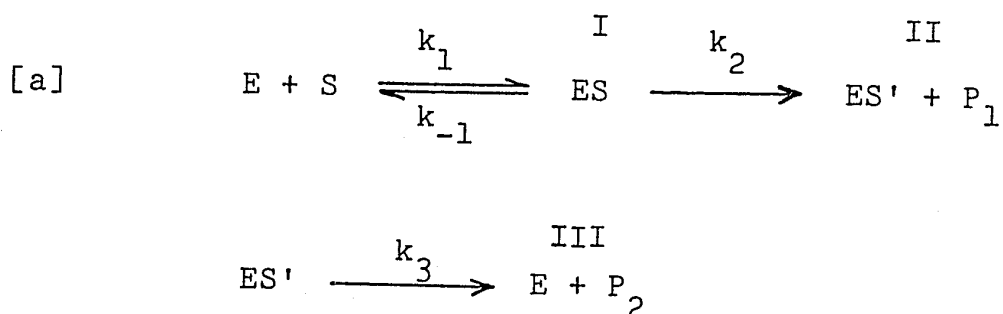
CHAPTER III

THE TRANSIENT PHASE OF TRYPSIN-CATALYZED HYDROLYSES

INTRODUCTION

pH Dependence of the Acylation Process

Hartley and Kilby (114,115) first reported that the enzyme chymotrypsin catalyzes the hydrolysis of p-nitrophenyl acetate. From their results they concluded that the mechanism consisted of three distinct steps:



where k_1 , k_{-1} , k_2 and k_3 are rate constants. Step I was extremely rapid and involved the combination of enzyme (E) with p-nitrophenyl acetate (S) to form the classical Michaelis complex ES (116). Gutfreund and Sturtevant (104) later estimated that $k_1 \geq 2 \times 10^6 \text{ l-mole}^{-1}\text{-sec}^{-1}$.

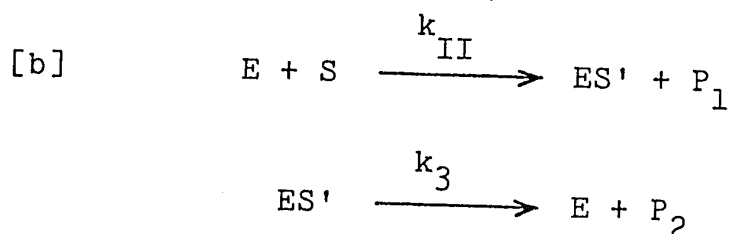
Steps II and III could be studied spectrophotometrically and consisted of an initial rapid liberation of approximately one mole of p-nitrophenol (P_1) per mole of chymotrypsin, followed by a slow (zero-order) release of p-nitrophenol and acetate ion (P_2). This suggested the existence of an intermediate acylenzyme (ES') which was isolated by Balls and Aldrich (117) by treating chymotrypsin with p-nitrophenyl acetate at low pH. The pre-steady state kinetics of this system have been studied in some detail (118-121) under a variety of conditions. It was finally established, contrary to the original results of Gutfreund and Sturtevant (118), that the acylation rate constant k_2 was pH dependent with a $pK \approx 6.8$.

Dixon and Neurath (29) later reported that p-nitrophenyl acetate was also hydrolyzed by trypsin in a manner similar to chymotrypsin, and the mechanism of hydrolysis was investigated in detail by Stewart and Ouellet (35). These authors interpreted their results in terms of competitive inhibition by hydrogen ions during complex formation and found the first-order rate constant k_2 to be pH independent. This appeared to be in accord with the evidence presented by Bender et al. (34,122)

for the kinetics of hydrolysis of N-trans-cinnamoylimidazole by α -chymotrypsin and trypsin. A similar analysis as a function of pH has not been carried out for a specific substrate of trypsin, largely owing to the fact that the acylation rates of such substrates are much too fast to be measured by conventional stopped-flow techniques. Bender, Kézdy and Feder (123), in an effort to establish that the three-step mechanism shown by sequence [a] could also be applied to trypsin-catalysis, did obtain a value of $k_2 = 0.395 \text{ sec}^{-1}$ at pH 2.66 for the specific substrate α -N-carbobenzoxyl-L-lysine-p-nitrophenyl ester hydrochloride. In view of the apparent discrepancies with regard to the pH dependence of the acylation process of trypsin and chymotrypsin, it was deemed desirable to apply the stopped-flow method to a suitable trypsin system in order to resolve this problem.

Reaction Order of the Acylation Process

In 1966, Faller and Sturtevant (124) reported that the acylation reaction of α -chymotrypsin by p-nitrophenyl acetate followed second-order kinetics rather than Michaelis-Menten kinetics, i.e. the complete reaction sequence would be



In order to understand this situation it is necessary to realize that previous investigators had analyzed their experimental results on the basis of a theoretical treatment of the pre-steady state which was subject to the following conditions:

(1) $k_2 \gg k_3$, i.e. deacylation is considered to be the rate-limiting step for p-nitrophenyl acetate.

(2) $[\text{S}] \approx [\text{S}]_0$, i.e. the substrate concentration must remain approximately constant throughout the period of the transient phase under consideration and hence $[\text{S}]_0$ should greatly exceed the total enzyme concentration $[\text{E}]_0$; unfortunately, p-nitrophenyl esters are not very soluble in aqueous solvents, and this sets a low upper limit for $[\text{E}]_0$ or necessitates the use of organic solvent mixtures.

(3) $[\text{S}]_0 \gg K_s$ (c.f. equ. [8]) and it follows from equation [8] that if $k_2 \gg k_3$, then K_s greatly exceeds K_m . This condition is unlikely to be satisfied when K_m

is large and the substrate is not very soluble. Failure to meet condition (3) has the consequence that conventional stopped-flow data cannot distinguish reaction sequence [a] from reaction sequence [b], i.e. the concentration of ES becomes experimentally undetectable and k_{II} is now a second-order rate constant. As derived by Bender and Marshall (125)

$$[16] \quad k_{II} = \frac{k_2}{K_s + [S]_0}$$

Hence if $[S]_0 \ll K_s$, then $k_{II} \approx k_2/K_s$, a ratio which under certain conditions is also given by steady-state data (c.f. equ. [9]).

Faller and Sturtevant (124) have found, in fact, that the pre-steady state results of earlier investigators for the hydrolysis of p-nitrophenyl acetate by chymotrypsin and trypsin could be adequately represented by second-order kinetics. Most recently, Milstien and Fife (126) have reported apparent second-order kinetics for the acylation of α -chymotrypsin by various p-nitrophenyl esters. The only results presently available which can be interpreted to indicate Michaelis-Menten kinetics

for the chymotrypsin-p-nitrophenyl acetate system are those of Kézdy and Bender (120). In their experiments, in which $[S]_0 \ll [E]_0$, the assumptions referred to above were legitimate and the data, although not as extensive as might be desired, are definitely better described by reaction sequence [a] with $K_S \approx 10^{-3}M$ than with $K_S \gg [S]_0$.

The usefulness of p-nitrophenyl acetate for testing the applicability of the three-step mechanism [a] to enzymes such as trypsin and chymotrypsin has frequently been questioned. This ester is a highly non-specific substrate and is hydrolyzed among others by such diverse enzymes as α -lytic protease (a bacterial peptidohydrolase) (72), carbonic anhydrase (carbonate hydro-lyase) (127) and D-glyceraldehyde-3-phosphate dehydrogenase (a NAD oxidoreductase) (128). An enzyme-like mechanism of catalysis has also been suggested for the hydrolysis of p-nitrophenyl acetate by a vinyl copolymer containing carboxyl and phenol groups (129) as well as by a copolymer of L-tyrosine and L-glutamic acid (130).

In view of these facts it was decided to study the pre-steady state kinetics of trypsin hydrolysis with

an amino acid ester for which the acylation process could be measured by conventional stopped-flow methods and for which the enzyme exhibited some specificity. The substrate N-carbobenzoxy-L-alanine-p-nitrophenyl ester was found to be suitable for this purpose. The work was carried out under the conditions of excess enzyme concentration, so that the existence of an intermediate Michaelis-Menten complex could be verified.

Solvent Effects

Faller and Sturtevant (124), who made a careful study of the kinetics of the α -chymotrypsin-catalyzed hydrolysis of p-nitrophenyl acetate in organic solvent-water mixtures, reported a substantial decrease in k_2/K_m with increasing solvent concentrations. The conditions of their experiments did not permit them, however, to separate the effect of solvent on k_2 and K_m individually. It has been claimed (131) that changes in solvent composition largely affect K_m in a manner that can be accounted for by combining the effects of competitive inhibition and of changing dielectric constant on electrostatic interactions. Most solvent studies have been concerned with the effects on the overall catalytic

constants, but because of the very complexity of these constants (c.f. equ. [6] and [7]), quantitative interpretations in terms of dielectric constant and/or inhibition must be treated with some caution (81,131,132). In particular, the use of methanol-water mixtures may have led in some cases to ambiguous results, since methanol has been shown to participate directly in the deacylation process (133,134).

During the last few years it has generally been recognized that the solvent plays an exceedingly important role in determining and stabilizing the characteristic structure of a protein molecule in solution (135). It was therefore felt to be of interest to investigate the effect of an organic solvent on the individual constants k_2 and K_m and to correlate, if possible, their variation with predicted changes in enzyme conformation.

Flow Techniques for the Study of Rapid Reactions

The first successful method for measuring the velocity of rapid chemical reactions with a half-time of less than 10 seconds was devised in 1923 by Hartridge and Roughton (136). They developed a continuous-flow

apparatus which could be used to follow reactions in the liquid phase and originally applied it to the rapid reactions of haemoglobin with oxygen and other ligands. The principle of their method was 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 photometric, calorimetric, electrical or other means of analysis at various points along the flow tube which was attached to the mixing chamber. In general such observations were made during constant rate of flow which permitted the use of reading devices requiring some considerable time to obtain a value for a particular point. 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 the procedure described by Millikan (137).

Subsequent developments of the flow method (138) resulted in improvements in two respects due to modern electronic equipment giving high resolution in time and signal: (1) greater fluid economy and (2) sensitivity due to novel sensing elements for various physical properties. In spite of this, the technique has a major

drawback in that relatively large quantities of reactants are required. Chance in 1940 therefore developed the accelerated-flow machine (139) to obtain economy in material and was the first to apply rapid-reaction techniques to the study of enzyme kinetics. The type of apparatus at present most widely used is the stopped-flow device developed by Gibson (140).

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 device. In the stopped-flow method the spectral changes accompanying the reaction are recorded photoelectrically after termination of the plunge, while in the accelerated-flow technique they are observed during the plunge. In the latter 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. Besides minimal material requirements, the stopped-flow technique has several advantages over the constant-flow method:

- (1) it is independent of the rate and character of flow in the observation cell,

(2) a permanent record can be obtained of the progress of the reaction over a period starting at a few milliseconds and extending as long as desired, and

(3) it is free from the distorting effect of mechanical disturbances.

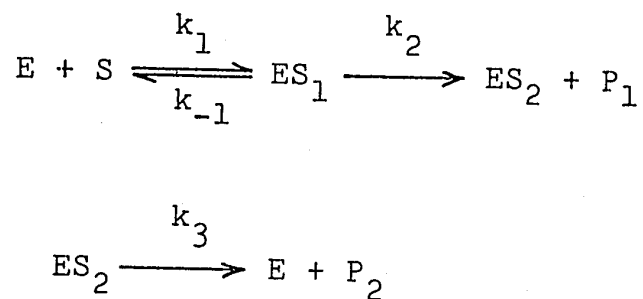
On the other hand, the stopped-flow method is often less sensitive and the methods of observation are more restricted. Other special techniques for the study of rapid enzymatic reactions, in particular relaxation techniques (141), have been summarized by Gutfreund (142).

TRANSIENT-PHASE THEORY

A general treatment of the transient phase of an enzyme-substrate system involving one enzyme-substrate complex or two consecutive enzyme-substrate complexes with products appearing as a result of the decomposition of the last complex has been given by Laidler and Ouellet (143,144). Special cases of these two schemes have also been treated by Gutfreund (145). Gutfreund and Sturtevant (118) have summarized solutions for the steady-state and transient-phase kinetics of the two-intermediate system (114) where the first product is released when the Michaelis-Menten complex transforms to a second complex. The complete formulation of this scheme has been published by Ouellet and Stewart (146). Peller and Alberty (147) and more recently Darvey (148) have discussed the approach to steady state for a fully reversible reaction sequence.

Three-Step Mechanism

The scheme of reactions applicable to the single-substrate system involving two intermediates is as follows:



Let e_o be the total concentration of enzyme, s_o the total concentration of substrate, and let the concentrations of ES_1 , ES_2 , P_1 and P_2 at time t be y_1 , y_2 , p_1 and p_2 respectively. Then at time t the concentration of free enzyme is

$$[17] \quad [E] = e_o - y_1 - y_2$$

and the concentration of free substrate is

$$[18] \quad [S] = s_o - y_1 - y_2 - p_2$$

The reaction is considered under conditions when p_1 and p_2 are sufficiently small for the second and third step to be treated as irreversible.

(1) High Enzyme Concentration

When the enzyme concentration is much greater than the substrate concentration, steady-state kinetics.

are non-existent. This will be proven mathematically, but in essence the reason is that in a catalytic reaction steady state can only exist if the concentration of intermediates is always much less than that of the substrate. When $e_o \gg s_o$, it is possible that the two concentrations approach one another and hence steady state can never be attained. Under these conditions one can make the assumption that

$$[19] \quad [E] \approx e_o$$

and write the following differential equations describing the above system:

$$[20] \quad \dot{y}_1 = k_1 e_o (s_o - y_1 - y_2 - p_2) - y_1 (k_{-1} + k_2)$$

$$[21] \quad \dot{y}_2 = k_2 y_1 - k_3 y_2$$

$$[22] \quad \dot{p}_1 = k_2 y_1$$

$$[23] \quad \dot{p}_2 = k_3 y_2$$

Differentiation of equation [20] gives

$$[24] \quad \ddot{y}_1 = k_1 e_o (-\dot{y}_1 - \dot{y}_2 - \dot{p}_2) - \dot{y}_1 (k_{-1} + k_2)$$

and elimination of $\dot{y}_2$ and $\dot{p}_2$ by use of equations [21] and [23] gives

$$[25] \quad \ddot{y}_1 + \dot{y}_1(k_1 e_o + k_{-1} + k_2) + y_1(k_1 k_2 e_o) = 0$$

This is a linear second-order differential equation which can be rewritten in the simplified form

$$[26] \quad \ddot{y}_1 + P\dot{y}_1 + Qy_1 = 0$$

where P and Q are defined as

$$[27] \quad P = k_1(e_o + K_m)$$

$$[28] \quad Q = k_1 k_2 e_o$$

$$[29] \quad K_m = \frac{k_{-1} + k_2}{k_1}$$

The general solution of equation [26] is, if the roots are real,

$$[30] \quad y_1 = Me^{Ft} + Ne^{Gt}$$

It has been shown for other systems (144) that if the roots of the auxiliary equation are imaginary, i.e. $(P^2 - 4Q)$ is negative, periodic solutions are obtained; these are not particularly interesting, because the period is longer than

the transient phase. Hence

$$[31] \quad F = \frac{1}{2}[-P + (P^2 - 4Q)^{\frac{1}{2}}]$$

$$[32] \quad G = \frac{1}{2}[-P - (P^2 - 4Q)^{\frac{1}{2}}]$$

The constants M and N in equation [30] are determined from the boundary conditions that at $t = 0$, $y_1 = 0$ and therefore

$$[33] \quad \dot{y}_1 = k_1 e_o s_o$$

This leads to the following values for M and N

$$[34] \quad M = -N = \frac{k_1 e_o s_o}{F-G}$$

A binomial series expansion of $(P^2 - 4Q)^{\frac{1}{2}}$ using the first two terms only reduces equations [31] and [32] to

$$[35] \quad F = -\frac{Q}{P}$$

$$[36] \quad G = \frac{Q}{P} - P$$

The series converges so rapidly that the other terms of the expansion become negligible. Substitution of equation [30] into equation [22], and integration with the boundary conditions that at $t = 0$, $p_1 = 0$, gives the variation of

p_1 as a function of time:

$$[37] \quad p_1 = \frac{k_2 M}{F} (e^{Ft} - 1) + \frac{k_2 N}{G} (e^{Gt} - 1)$$

Since F and G are always negative, the terms e^{Ft} and e^{Gt} become negligible when t is sufficiently large, so that equation [37] reduces to

$$[38] \quad p_1 = - \frac{k_2 M}{F} - \frac{k_2 N}{G}$$

Substitution of the appropriate values for M , N , F and G into equation [38] gives

$$[39] \quad p_1 = s_o$$

which is expected from the condition that $e_o \gg s_o$. Equations [37] and [38] show that for a system in which the enzyme concentration is in excess and therefore constant, steady state is non-existent, since p_1 exponentially approaches a constant value at large t .

A qualitative plot of the function represented by equation [37] is shown in Figure 11. The initial exponential rise is due to the term $(k_2 N/G)(e^{Gt} - 1)$

followed by an exponential fall-off to zero rate due to the term $(k_2 M/F)(e^{Ft} - 1)$. The individual behaviour of the two terms is also shown in Figure 11. Their relative magnitude depends on the relative magnitudes of F and G . As all the quantities involved are positive, G will usually be larger than F , and e^{Gt} will approach zero more rapidly than e^{Ft} , while the coefficient of e^{Gt} will be smaller than the coefficient of e^{Ft} . This condition suggests that the e^{Ft} term will be easier to detect, experimentally, than the e^{Gt} term.

Since the roots of equation [30] must be real, the value of Q/P never exceeds the value of $P/4$, and is probably always less than $P/4$. Therefore equation [36] can be reduced to

$$[40] \quad G = -P$$

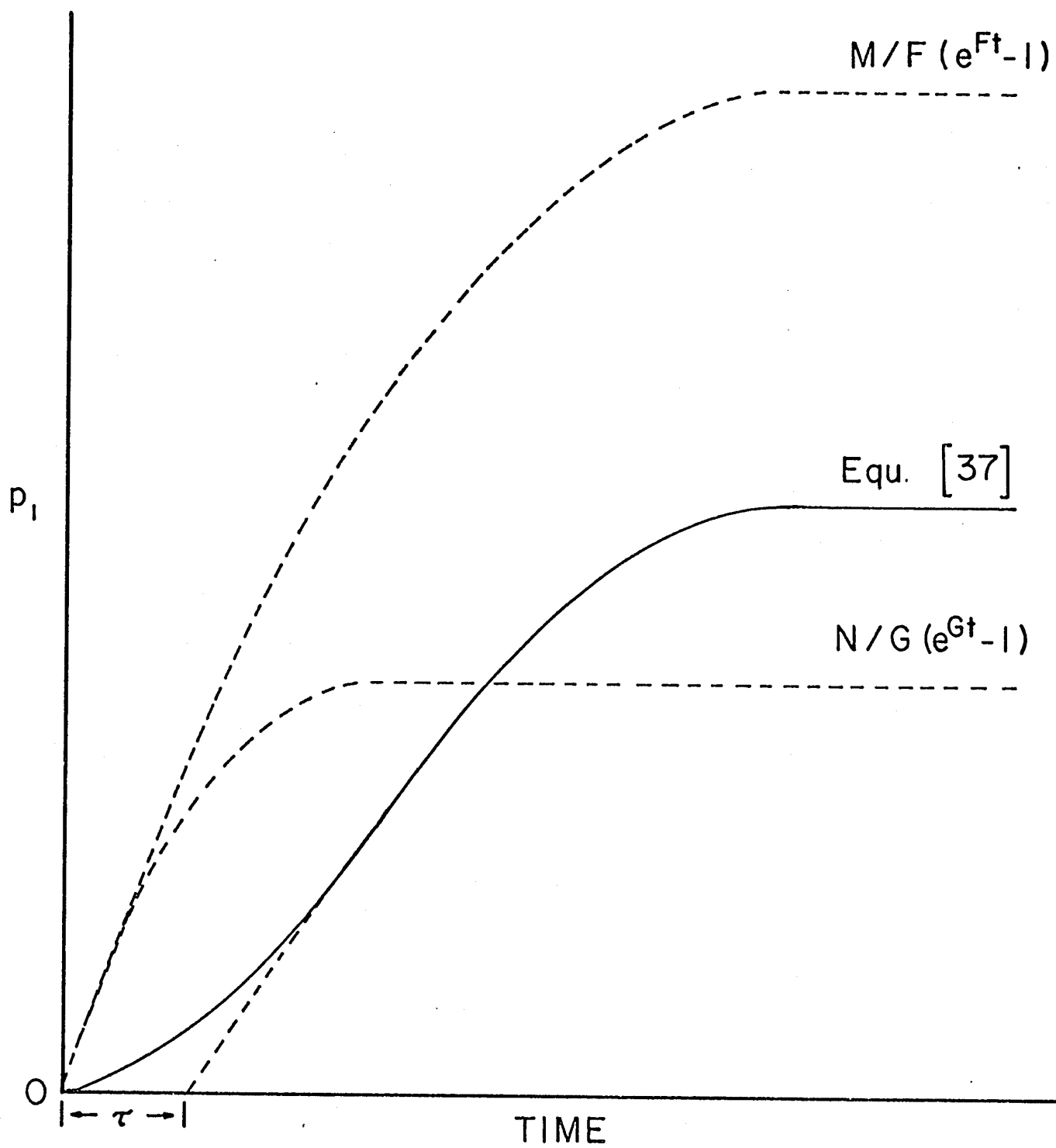
This means that

$$[41] \quad FG = Q$$

which is also the case when equations [31] and [32] are considered without simplification and therefore justifies the above assumption.

Figure 11

Qualitative plot of the rate of production
of product p_1 according to equation [37].



Extrapolation of the portion of the curve in Figure 11 which is due to the term in F (c.f. equ. [37]) to the time axis yields a quantity τ which may be conveniently referred to as the induction period of the reaction. For a certain period of time $e^{Gt} \approx 0$ while e^{Ft} is not. A Maclaurin series expansion of e^{Ft} , using the first two terms only since t is small at the beginning of the reaction, reduces equation [37] to

$$[42] \quad p_1 = k_2 M t - \frac{k_2 N}{G}$$

When $p_1 = 0$, substitution for the value of G using equation [40] yields the expression

$$[43] \quad \tau = \frac{1}{k_1(e_0 + K_m)}$$

This expression for the induction period is the same as that for the system involving a single intermediate (143-145) with e_0 in place of s_0 . Thus provided τ is measurable, the rate constant k_1 (and k_{-1} if k_2 is known) for the reversible formation of the Michaelis complex could be determined. However, in those cases where the three-step mechanism could apply, the induction period appears to be immeasurably small (104,149). This result

implies that the term e^{Gt} is negligibly small even at the beginning of the reaction.

Substitution of the appropriate values of P and Q into equations [35] and [40] gives

$$[44] \quad F = - \frac{k_2 e_o}{e_o + K_m}$$

$$[45] \quad G = -k_1(e_o + K_m)$$

Together with the values for M and N from equation [34], the complete expression of equation [37] becomes

$$[46] \quad p_1 = \frac{s_o}{\frac{k_2 e_o}{k_1(e_o + K_m)^2} - 1} (e^{Ft} - 1) + \frac{s_o}{\frac{k_1(e_o + K_m)^2}{k_2 e_o} - 1} (e^{Gt} - 1)$$

It is reasonable to assume that $k_1 \gg k_2$, and for the condition that e_o is very large, keeping in mind that e^{Gt} approaches zero very rapidly, equation [46] reduces to the simple expression

$$[47] \quad p_1 = s_o \left[1 - \exp\left(-\frac{k_2 e_o}{e_o + K_m} t\right) \right]$$

If therefore p_1 or a physical property proportional to p_1 can be measured, k_2 and K_m can be evaluated from the values of $k_2 e_o / (e_o + K_m)$ at different concentrations of enzyme. This can be done using the Guggenheim technique (150) or directly fitting the constants to equation [47] by a least-squares method.

At the beginning of the reaction, the series development of the exponential term in equation [47] would give a good approximation of the function. The initial rate of production of the product p_1 is then described by

$$[48] \quad p_1 = \frac{k_2 e_o}{e_o + K_m} s_o t$$

and approximate values of k_2 and K_m can be obtained from initial rate data using the ordinary techniques of enzyme kinetics (66).

(2) High Substrate Concentration

More conventionally, the three-step mechanism is studied under the condition that the substrate concentration is much greater than the enzyme concentration rather than the reverse. The kinetic theory of such a system has been worked out in detail by Ouellet and Stewart (146) who obtained the following simplified expression for the variation of p_1 with time:

$$[49] \quad p_1 = k_3 e_o t + e_o \left[1 - \exp\left(-\frac{(k_2 + k_3)s_o + k_3 K_m}{s_o + K_m} t\right) \right]$$

The equation becomes useful for the determination of k_2 and K_m only, when the further assumptions are made that $k_2 \gg k_3$ and $s_o \gg k_3 K_m / (k_2 + k_3)$. Faller and Sturtevant (124) have shown that earlier investigators incorrectly applied these assumptions to the system chymotrypsin-p-nitrophenyl acetate.

Effect of Hydrogen-Ion Concentration

The significance of hydrogen-ion effects on steady-state constants as derived from classical enzyme kinetics has been discussed in detail by Laidler (66) and

Dixon and Webb (55). Many of these concepts are basically applicable to the two-complex system, parts of which have been treated theoretically by different investigators (78, 120). Stewart and Lee (151) have recently presented a complete theoretical treatment of pH effects as applied to the three-step mechanism shown in Scheme I.

Using the basic relationships suggested by Laidler (66), the same general expression for the transient phase production of product p_1 as that by Stewart and Lee is obtained, by simply defining the pH dependence of the rate constants in equations [46] and [49]. With reference to Scheme I, the total concentration of the Michaelis complex in its three states of ionization is given by

$$[50] \quad [ES]_T = [ES] + [EHS] + [EH_2S]$$

If K'_a and K'_b are defined as dissociation constants then

$$[51] \quad [ES]_T = [EHS] \left(\frac{K'_a}{[H]} + 1 + \frac{[H]}{K'_b} \right)$$

The overall rate of acylation may be written as

$$[52] \quad v_2 = \tilde{k}_2 [ES]_T$$

which, after substitution of equation [51] gives

$$[53] \quad v_2 = \tilde{k}_2[\text{EHS}]\left(1 + \frac{K'_a}{[\text{H}]} + \frac{[\text{H}]}{K'_b}\right)$$

The symbol $\sim$ indicates that the quantities are in general pH dependent. In terms of the three possible ionization forms of the Michaelis complex, the rate of acylation is also

$$[54] \quad v_2 = k_2[\text{EHS}] + ak_2[\text{ES}] + bk_2[\text{EH}_2\text{S}]$$

which may be expressed as

$$[55] \quad v_2 = k_2[\text{EHS}]\left(1 + \frac{aK'_a}{[\text{H}]} + \frac{b[\text{H}]}{K'_b}\right)$$

Division of equation [55] by equation [53] yields the following pH-dependent expression for the acylation rate constant:

$$[56] \quad \tilde{k}_2 = \frac{k_2\left(1 + \frac{aK'_a}{[\text{H}]} + \frac{b[\text{H}]}{K'_b}\right)}{1 + \frac{K'_a}{[\text{H}]} + \frac{[\text{H}]}{K'_b}}$$

Similarly the other Michaelis parameters are

$$[57] \quad \tilde{k}_3 = \frac{k_3 \left(1 + \frac{\alpha K''_a}{[H]} + \frac{\beta [H]}{K''_b} \right)}{1 + \frac{K''_a}{[H]} + \frac{[H]}{K''_b}}$$

$$[58] \quad \tilde{K}_m = \frac{\frac{k_{-1} \left(1 + \frac{x K'_a}{[H]} + \frac{y [H]}{K'_b} \right) + k_2 \left(1 + \frac{a K'_a}{[H]} + \frac{b [H]}{K'_b} \right)}{1 + \frac{K'_a}{[H]} + \frac{[H]}{K'_b}}}{\frac{k_1 \left(1 + \frac{x K_a}{[H]} + \frac{y [H]}{K_b} \right)}{1 + \frac{K_a}{[H]} + \frac{[H]}{K_b}}}$$

Taking account of the influence of hydrogen-ion on the rate constants, equation [47] is then written in the form

$$[59] \quad p_1 = s_o \left[1 - \exp \left(- \frac{\tilde{k}_2 e_o}{e_o + \tilde{K}_m} t \right) \right]$$

COMPUTER ANALYSIS

Guggenheim Method

Experimental results obtained for the transient phase of the three-step enzymatic process as discussed in the previous section are frequently analyzed by the Guggenheim method (35,118,150). This treatment, which has also been extended to include second-order reactions (152), is useful where the initial or final concentrations are unknown, and where the relative readings taken during the course of a reaction cannot be conveniently converted to concentrations.

Equation [59] may be expressed in the general form

$$[60] \quad p_1 = s_o(1 - e^{Ft})$$

where F is defined as

$$[61] \quad F = - \frac{\tilde{k}_2 e_o}{e_o + \tilde{K}_m}$$

If A_1 , A_2 and A_3 are any series of readings directly proportional to the amount of product p_1 at times t_1 ,

$t_1 + \Delta t$ and $t_1 + 2\Delta t$ respectively, where Δt is a fixed interval of time, then

$$[62] \quad A_1 + A_3 - 2A_2 = -s_0(e^{Ft_1})(e^{F\Delta t}-1)^2$$

and

$$[63] \quad \log (A_1 + A_3 - 2A_2) = \log \text{ constant} + \frac{Ft_1}{2.303}$$

Using this linear equation it is possible to determine F from the slope of a plot of $\log |(A_1 + A_3 - 2A_2)|$ versus t_1 .

It follows from equation [62] that the only meaningful values of the term $(A_1 + A_3 - 2A_2)$ are negative; this condition is superficially satisfied if Δt is equal to three or four times the half-time of the reaction (118). Apart from the fact that it is difficult to assume a value of Δt when the half-time of the reaction is not known, it is important to realize that the choice of Δt is also strongly dependent on the relative precision with which the experimental readings of A can be taken. This is of course directly related to the absolute magnitude of the A values and the length of time over which measurements can be made. Using experimental data

a more realistic criterion for choosing Δt is therefore the condition that for a given set of readings

(i) the term $(A_1 + A_3 - 2A_2)$ must never be positive, and

(ii) differences between successive terms should be at least equal to twice the expected percentage precision of A.

In this connection it should be noted that the mathematical manipulation of taking the logarithm of the difference terms in A may also introduce some bias into the Guggenheim analysis.

Least-Squares-Fit Method

In view of the limitations inherent in the Guggenheim technique, it was considered desirable to obtain the parameter F in equation [60] by a least-squares-fit method. The product concentration p_1 is directly related to absorbance A by the Lambert-Beer law

$$[64] \quad A = \epsilon p_1 \ell$$

where ϵ is the molar extinction coefficient of the absorbing species and ℓ is the thickness of the absorbing

medium. Base-line instability and electrical drift of the stopped-flow equipment used in this study made it necessary to introduce a correction factor ΔA into equation [60] which then reads

$$[65] \quad A - \Delta A = Q(1 - e^{-Ft})$$

where Q is defined as

$$[66] \quad Q = \epsilon s_0 l$$

Rearranging equation [65] yields

$$[67] \quad \ln \left(1 - \frac{A - \Delta A}{Q} \right) = -Ft$$

If the logarithmic term is expressed as A_{new} , then

$$[68] \quad \text{Sum of the (deviations)}^2 = \Sigma (A_{\text{new}} - Ft)^2$$

Differentiating

$$[69] \quad \frac{\delta \Sigma}{\delta F} = \Sigma [-2t(A_{\text{new}} - Ft)]$$

To satisfy the condition that $\Sigma (A_{\text{new}} - Ft)^2$ is minimized, equation [69] is put equal to zero and solved for F , which gives

$$[70] \quad F = \frac{\Sigma A_{\text{new}} t}{\Sigma t^2}$$

where A_{new} has been defined as

$$[71] \quad A_{\text{new}} = \ln \left(1 - \frac{A - \Delta A}{Q} \right)$$

The restrictions on equation [71] are that ΔA cannot be larger than the smallest value of A and that Q must be larger than the largest value of $(A - \Delta A)$.

A digital computer program involving multiple iterations with given values of Q and ΔA was written to calculate the best value of F by the method of least squares. It should be noted that two minima in $\Sigma(A_{\text{new}} - Ft)^2$ exist,

(i) the desired minimum square deviation for finite values of Q and F ,

and

(ii) a trivial solution under the conditions that $Q = \infty$ and $F = 0$.

A copy of the program and the output of a typical experimental run are shown in Appendix A.

Computer Program Tests

To establish the accuracy and reproducibility of the least-squares fit, the following tests were carried out on the program with a number of experimental runs.

(1) Using the "best" fitted results for Q , ΔA and F , values of A were recalculated and found to agree with the experimental absorbance values within $\pm 2\%$ or better. This is well within the accuracy of the oscilloscope read-out used in this study.

(2) Guggenheim analyses of a set of recalculated absorbance values using different time intervals ($\Delta t > 4t_{\frac{1}{2}}$) gave results for F which agreed with one another and with the least-squares-fit value originally used in recalculating A within less than 2% .

(3) A random 2% variation of some recalculated A values in a given run caused less than 1% change in the least-squares-fit result for F . In contrast, a Guggenheim fit on sets of data altered in a similar way, caused up to 40% changes in F . In addition the results depended on

the value of Δt . The two preceding tests taken together clearly indicate the limitations inherent in the Guggenheim technique as discussed earlier.

(4) A systematic or random reduction of data points from 18 to 9 within a given run caused in some cases an increase of up to 4% in the least-squares-fit result for F . Essentially no variation was noted when more than 12 data points were used.

(5) In most runs, F could be fitted to better than 3% precision, by using intervals of 0.0001 and 0.00001 between successive Q and ΔA values respectively for the iteration procedure. To maintain the same precision, these factors were reduced by 10 in those cases where the experimental minimum absorbance values were of the order of 0.005 units.

(6) A lower limit in the precision of fitting F was reached when the "best" fitted results for Q , ΔA and F and those adjacent to it gave recalculated values of A which agreed within $\pm 2\%$. This situation arose only when the experimental data were restricted to extremely low absorbance values or showed unusually large scatter.

EXPERIMENTAL DETAILS

Materials

N-Carbobenzoxy-L-alanine-p-nitrophenyl ester (Lot No. K-5538-R) was purchased from Cyclo Chemical Corporation and recrystallized from ethyl acetate-hexane, twice in the presence of charcoal and once without, with slight warming. The product was stored dry and frozen, since it tended to decompose on standing. After recrystallization the white fluffy material melted at 80-81°C which is slightly higher than a previously reported result (153).

Trypsin (twice crystallized, salt-free, Lot No. TRL 8CA) was obtained from Worthington Biochemical Corporation and used without further purification.

Sodium phosphate buffer solutions were prepared with "Baker Analyzed" Reagent Grade materials according to Gomori (154). Isopropyl alcohol (Fisher Certified A.C.S. Spectranalyzed) was dried and stored over Linde molecular sieves previously reactivated by heating at 350°C for several hours. Water was doubly distilled, deionized and free of carbon dioxide.

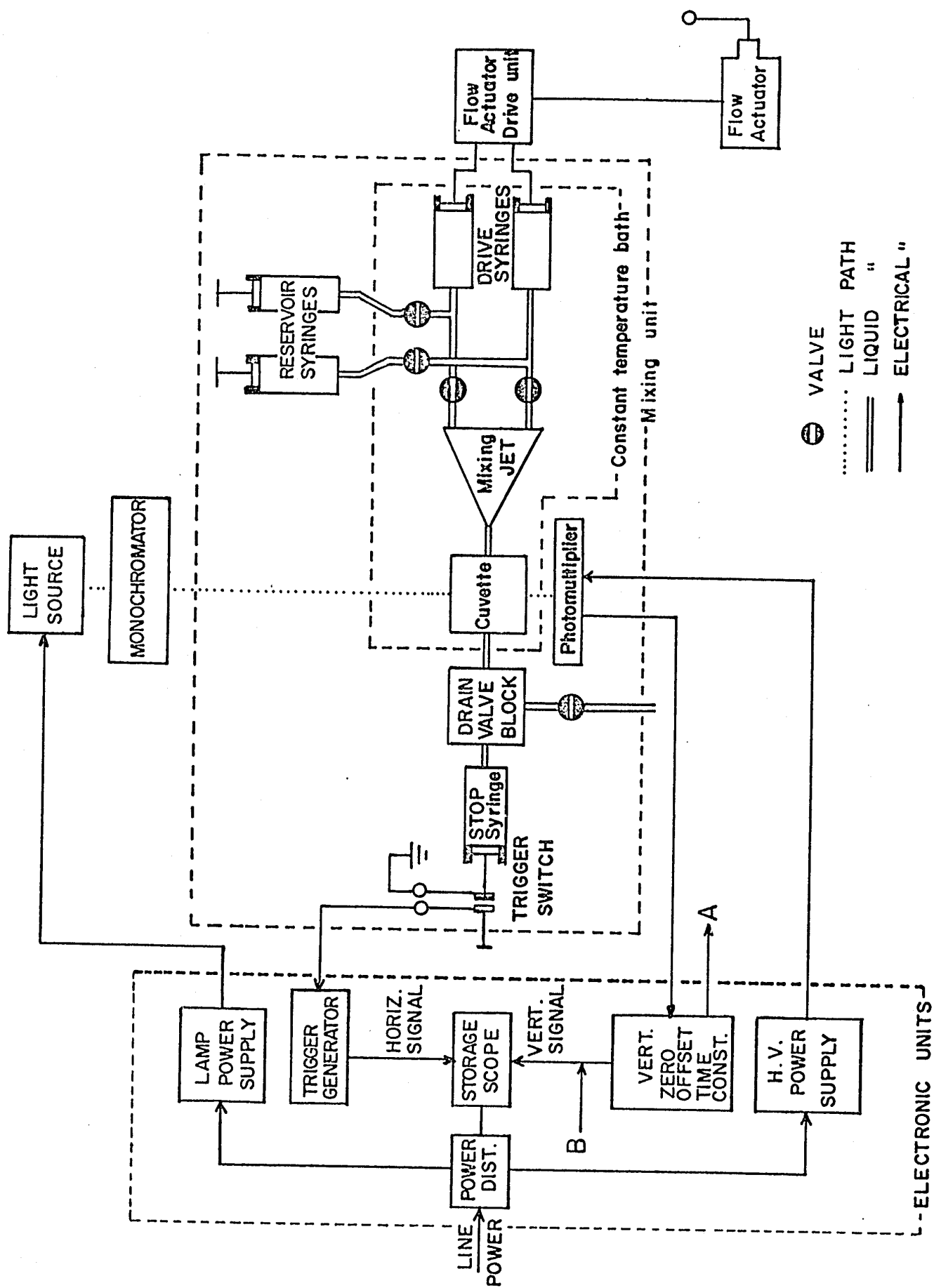
Stopped-Flow Spectrophotometer

The pre-steady state rates of hydrolysis of N-carbobenzoxy-L-alanine-p-nitrophenyl ester (CANE) were measured with a Durrum-Gibson Stopped-Flow Spectrophotometer purchased from the Durrum Instrument Corporation, Palo Alto, California. A schematic diagram of the system is shown in Fig. 12.

Equal volumes of substrate in isopropanol-water solution and enzyme in an appropriate buffer solution are hydraulically forced through the mixing jet into the cuvette and hence into the stop syringe. Just before its plunger hits a mechanical stop, it makes contact with a trigger switch which actuates the horizontal time-base sweep for the oscilloscope display. Since the flow of sample is stopped immediately after mixing, the reaction in the cuvette takes place with a minimum of interference caused by turbulence or flow artifacts. The monochromatic light obtained from the monochromator passes through the mixed solution in the cuvette and the resultant variation in light intensity is sensed by the photomultiplier tube. The photomultiplier output, proportional to the instantaneous light intensity transmitted by the reacting solution, is applied

Figure 12

Functional block diagram of the
stopped-flow spectrophotometer
system.



to the vertical axis of the oscilloscope. The display on the storage oscilloscope, which indicates percentage transmittance versus time, in general started just before the reaction began and ended according to a pre-selected time-base setting. All measurements were made at 400 mμ. The reaction system was maintained at $25.0 \pm 0.1^{\circ}\text{C}$ by means of a thermostatted water bath.

Kinetic Procedure

Stock solutions of CANE were made up in dry isopropyl alcohol and were stored in sealed bottles in the refrigerator. Just before use the alcohol solution was diluted with gas-free water to a final concentration of $2 \times 10^{-5}\text{M}$ substrate in 2-40% (v/v) alcohol as required. Dilution had to be done slowly and with cooling to avoid aeration of the solution caused by the evolution of heat during mixing. A fresh stock solution of trypsin in 0.1 M phosphate buffer was prepared every day. The enzyme solution was first centrifuged for 30 minutes at 7000 r.p.m. in a Sorvall Superspeed RC2-B refrigerated centrifuge to remove water-insoluble impurities. Protein concentrations were determined with a Perkin Elmer Model 350 Spectrophotometer at 280 mμ using a value of extinction

$E_{1\text{ cm}}^{1\%} = 14.4$ (6). The solution was then diluted with the appropriate buffer to give dilutions ranging in concentration from 0.14 to $1.4 \times 10^{-3}\text{M}$ enzyme. The pH of the trypsin solution was measured to ± 0.05 units at the beginning and end of each series of runs and was found to remain constant for that period of time.

Preceding a typical series of runs, the oscilloscope was calibrated for 100% transmittance with substrate solution and the appropriate buffer solution in the drive syringes. The calibration was repeated for different settings of amplifier gain and zero offset to give a satisfactory record of the reaction to be studied. For this purpose the zero-offset circuit of the Durrum-Gibson Spectrophotometer had been modified to include a calibrated ten-turn potentiometer. The buffer solution was then replaced by enzyme solution buffered to the same pH, and approximately 0.15 ml each of enzyme and substrate solution were injected into the cuvette. It should be noted here that this procedure effectively halved the concentration of all species in the mixture. At least 10 minutes thermostating time was allowed each time after the drive syringes had been filled with reactants.

After four or five traces reflecting the rate of hydrolysis of CANE were reproduced on the oscilloscope screen, a polaroid picture of the stored display was taken. Depending on the pH, runs lasted from 0.5 to 5 sec. The oscilloscope picture of a typical experimental run is shown in Fig. 13.

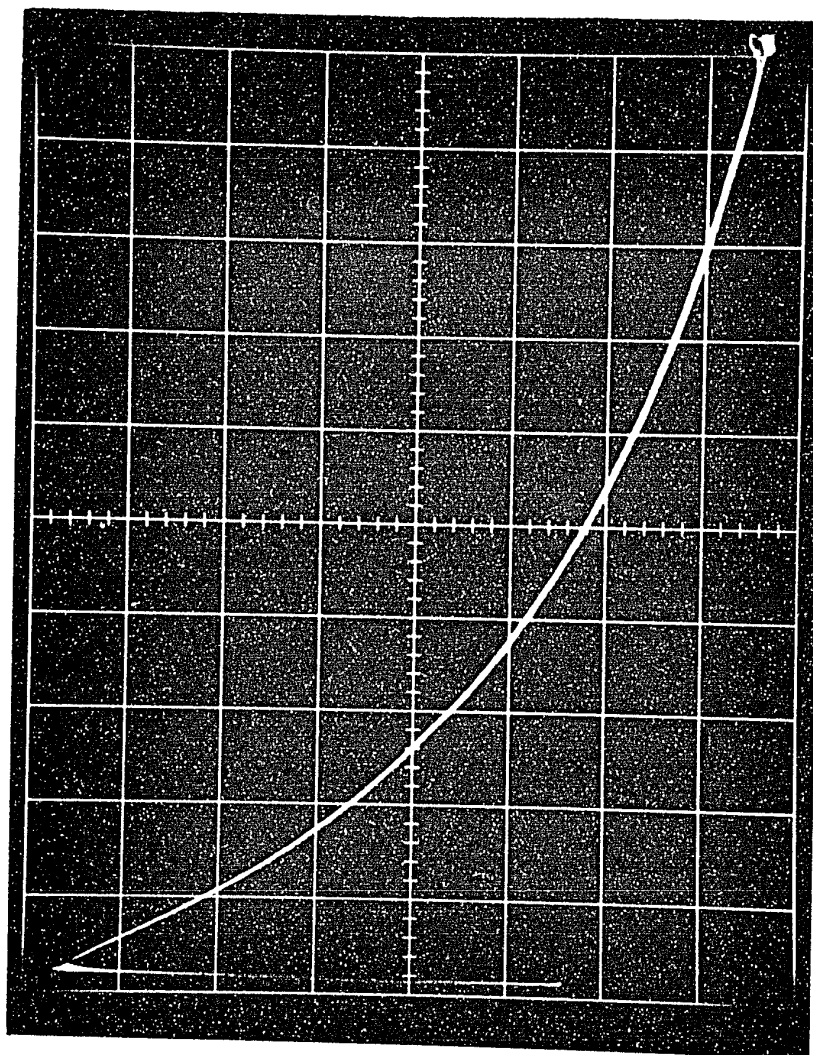
Methods of Computation

It was found that for the pH range studied in this work (pH 5.91 - 7.41) and due to the short run times, no correction for the alkaline hydrolysis of CANE was required.

Since the analysis of results depends on the fact that the Lambert-Beer law (c.f. equ. [64]) is obeyed over the full range of product concentrations at all values of pH, the relationship was tested at the two extreme pH values. Linearity between absorbance and concentration of p-nitrophenol existed up to an optical density of 2.0. In this work the highest measured optical densities did not exceed 0.1. The least-squares-fit method discussed earlier does not require conversion of the experimentally measured absorbance data to concentrations of product. It is

Figure 13

Oscilloscope trace for the trypsin-catalyzed hydrolysis of N-carbobenzoxy-L-alanine-p-nitrophenyl ester. Vertical scale: 50-100% transmittance, horizontal scale: 0.5 sec/div, time constant: 5 msec.



therefore not necessary to take account of the fact that, over the pH range studied, the product exists partially as p-nitrophenol and partially as p-nitrophenoxide ion. At 400 mμ the extinction coefficients of these two species are 81.4 and 18,330 respectively (155).

Input data for the least-squares-fit computer program (c.f. Appendix I) were obtained from enlargements of the oscilloscope pictures in arbitrary units of transmittance and time. The program was written in Fortran IV for the IBM 360/65 computer system. In general an approximate fit for F was first obtained by using intervals of 0.001 and 0.0001 between successive values of Q and ΔA in the iteration procedure. This was followed by a final fit with appropriately smaller intervals for Q and ΔA . The program includes a modification which selects and prints out for every value of Q only those values of ΔA and F which give a minimum in $\Sigma(A_{\text{new}} - F_t)^2$. The "best" values of Q, ΔA and F were then chosen from the computer print-out on the basis of the lowest minimum square deviation.

It soon became apparent that the conversion of oscilloscope traces to numerical data as described above

was both tedious and easily tended to error. A single channel data sampling system was therefore designed and later built by Instronics Techno-Products Ltd., Stittsville, Ontario which prints out the photomultiplier output signal directly in terms of millivolts at pre-selected time intervals. The system was not used in this particular study, but is briefly described in Appendix B.

RESULTS

pH Dependence of the Acylation Process

Typical curves for the pre-steady state rate of production of product p_1 in terms of rate of change of absorbance are shown in Fig. 14. Only one-half of the 18 experimental points usually used for the computer fit have been plotted. The intercept on the absorbance axis is equal to the "best" value of ΔA obtained from the least-squares-fit analysis for F . The value of ΔA can safely be assumed to be constant for the duration of an experimental run and therefore does not contribute to any absolute error in F . It does however contribute to the precision in fitting F , since it represents a third parameter in equation [65], which describes the exponential variation of absorbance with time.

The constants $\tilde{K}_m$ and $\tilde{k}_2$ corresponding to the formation of the Michaelis complex and the acylenzyme have been evaluated from Lineweaver-Burk plots (156) of the computer-fitted results for F as a function of enzyme concentration. In Table 4, three representative sets of results for the transient phase of the tryptic

Figure 14

Experimental curves of change in absorbance
with time for the pre-steady state hydrolysis
of N-carbobenzoxy-L-alanine-p-nitrophenyl
ester.

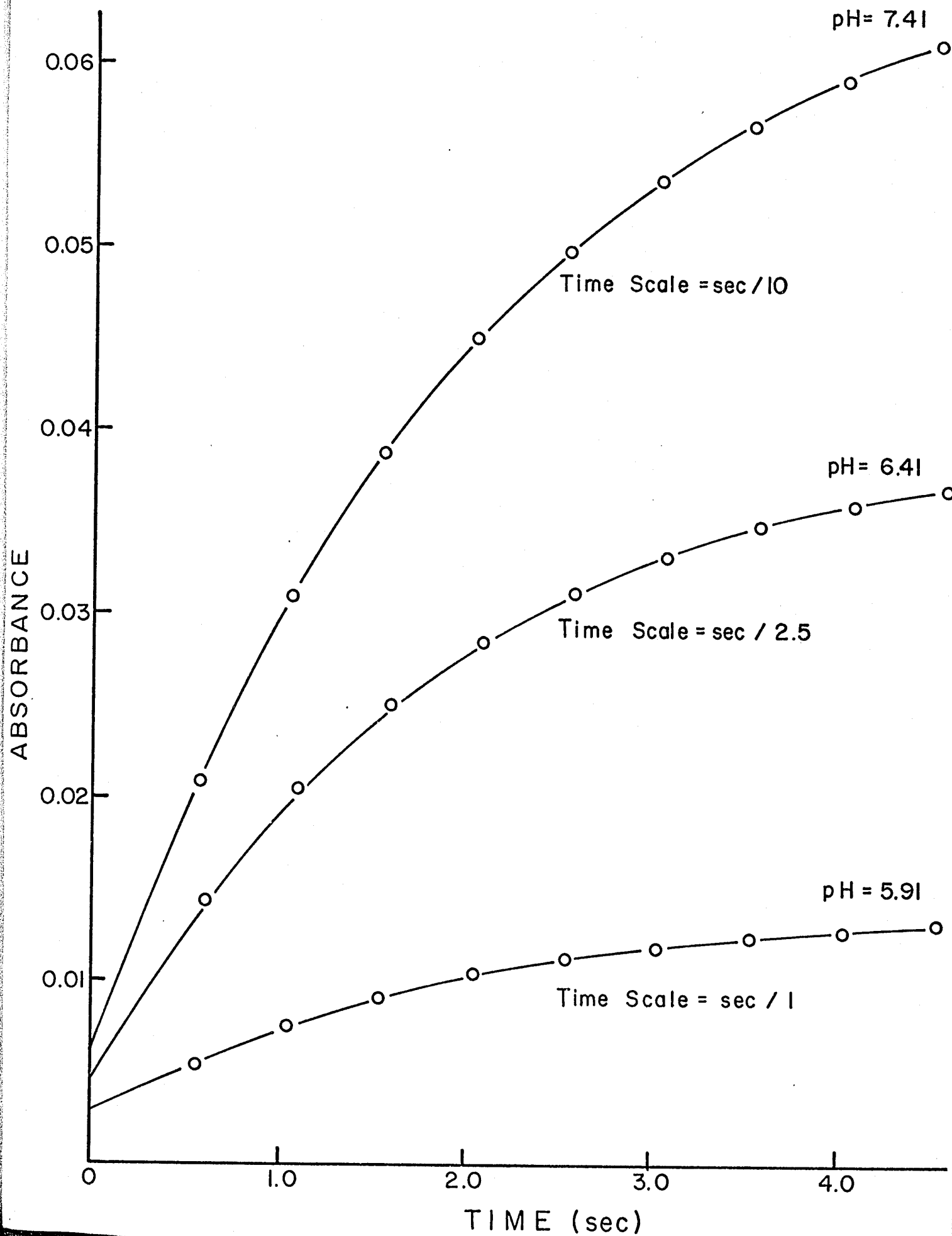


Table 4

Pre-steady State Data for the Trypsin-catalyzed Hydrolysis
of CANE in 1% (v/v) Isopropanol and 0.05M Phosphate Buffer
at 25.0°C and $[S]_0 = 1 \times 10^{-5} M$

$[E] \times 10^4$ (M)	$1/[E] \times 10^{-4}$ (M ⁻¹)	pH					
		5.91	6.41	7.41			
		F(sec ⁻¹)	1/F(sec)	F(sec ⁻¹)	1/F(sec)	F(sec ⁻¹)	1/F(sec)
0.70	1.43	0.286	3.50	0.705	1.42	2.45	0.408
0.91	1.10	0.367	2.72	0.816	1.23	2.95	0.339
1.19	0.84	0.399	2.50	0.910	1.10	3.13	0.319
1.75	0.57	0.483	2.07	1.10	0.908	3.82	0.262
3.15	0.32	0.543	1.84	1.34	0.746	4.50	0.222
4.90	0.20	-	-	1.48	0.674	4.90	0.204
7.00	0.14	0.678	1.47	1.59	0.628	5.66	0.177

1
11
1

hydrolysis of CANE are reported at different values of pH. These data are plotted in Fig. 15. For this as for all other linear plots in this study the intercepts and slopes have been obtained by a least-squares fit. The values of $\tilde{k}_2$ and $\tilde{K}_m$ obtained in this way from the Lineweaver-Burk plots are given in Table 5. The kinetic parameters are average values based on three runs covering the full range of enzyme concentrations. The pH dependencies of these parameters are shown in Figures 16 and 17.

The curve in Figure 16 is the theoretical curve obtained by a reiterative process which gives the closest fit to the experimental points. It fits the equation

$$[72] \quad \tilde{k}_2 = \frac{k_2}{1 + \frac{[H]}{K'_b}}$$

which is obtained from equation [56] under the conditions that $b = 0$ and $[H] \gg K'_a$. The best fit results when a value of $k_2 = 7.5 \text{ sec}^{-1}$ is assumed for the pH-independent rate constant of the acylation process. The shape of the $\log_{10} \tilde{k}_2$ -pH curve in Figure 16 suggests the participation of a single basic group in the process of

Figure 15

Lineweaver-Burk plots for the pre-steady state hydrolysis of N-carbobenzoxy-L-alanine-p-nitrophenyl ester at excess enzyme concentration.

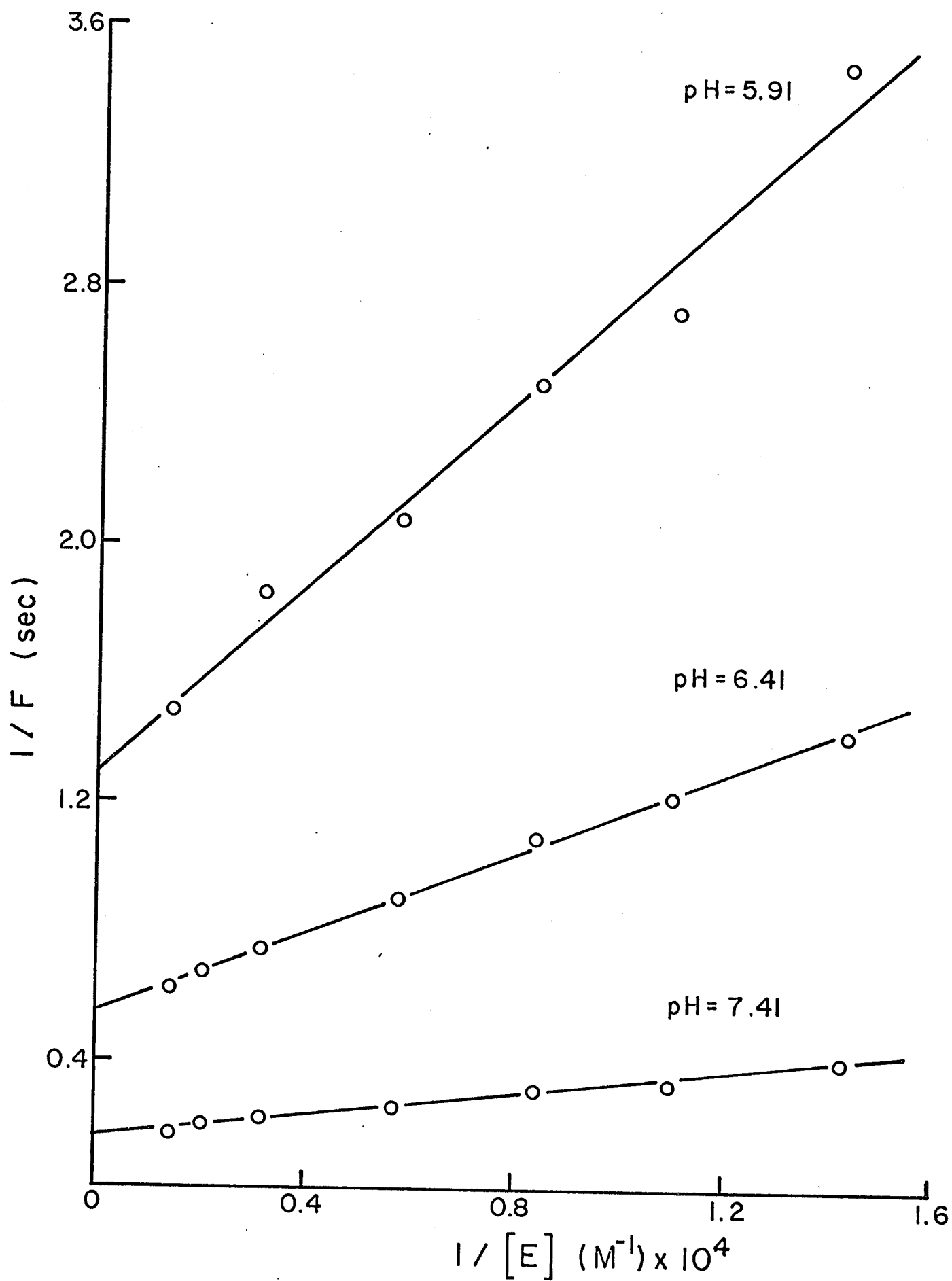


Table 5

Kinetic Parameters for the Trypsin-catalyzed Hydrolysis
of CANE in 1% (v/v) Isopropanol and 0.05M Phosphate Buffer
at 25.0°C

pH	$\tilde{k}_2(\text{sec}^{-1})$	$\tilde{K}_m \times 10^4 (\text{M})$
5.91	0.698	0.976
6.08	1.12	1.17
6.41	1.81	1.10
6.62	2.30	1.07
6.90	3.55	1.19
7.10	4.88	1.48
7.41	5.76	1.00
Mean Value		1.14 ± 0.12

Figure 16

Variation of $\log_{10} \tilde{k}_2$ with pH for the
pre-steady state hydrolysis of CANE in
0.05M phosphate buffer at 25.0°C.

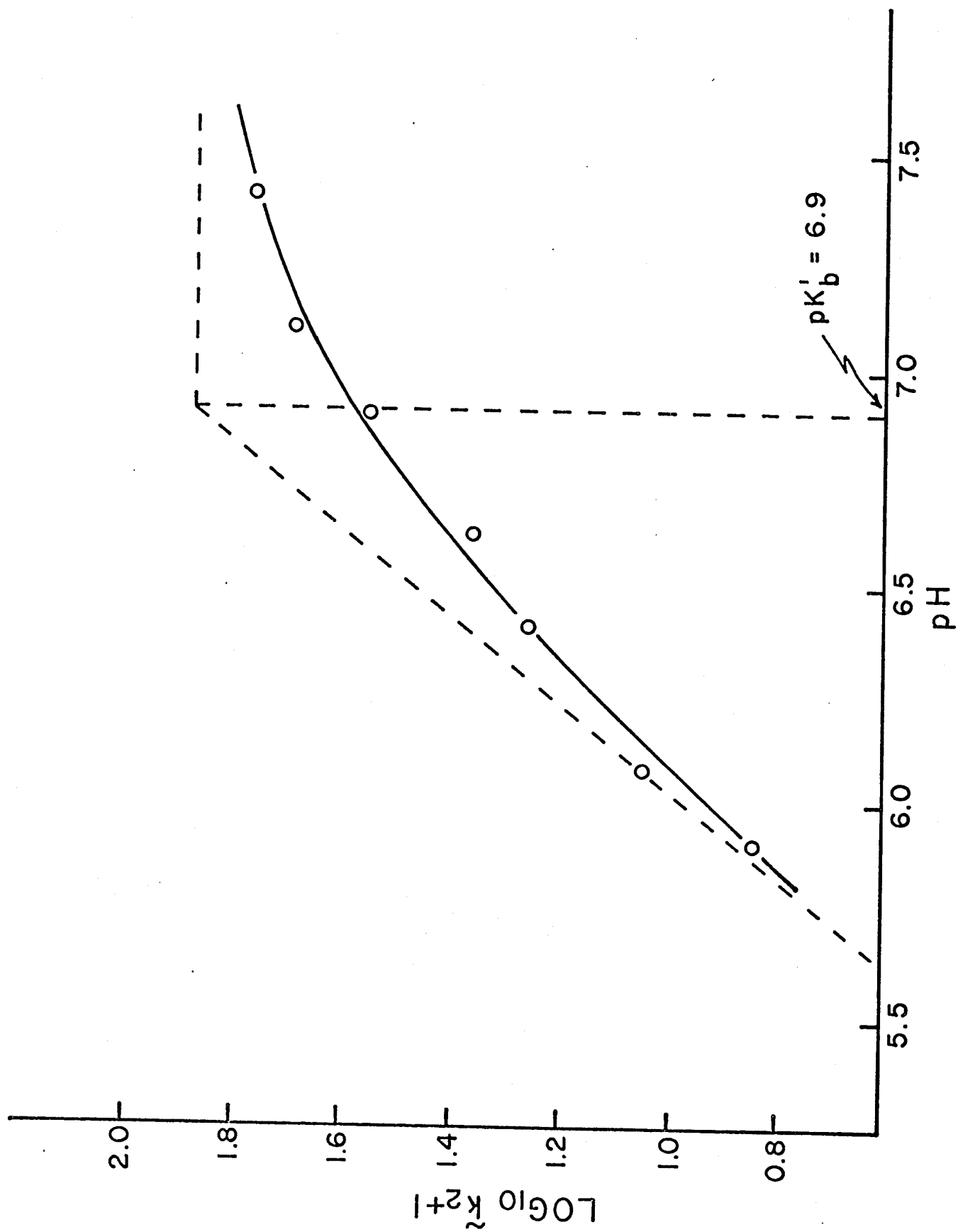
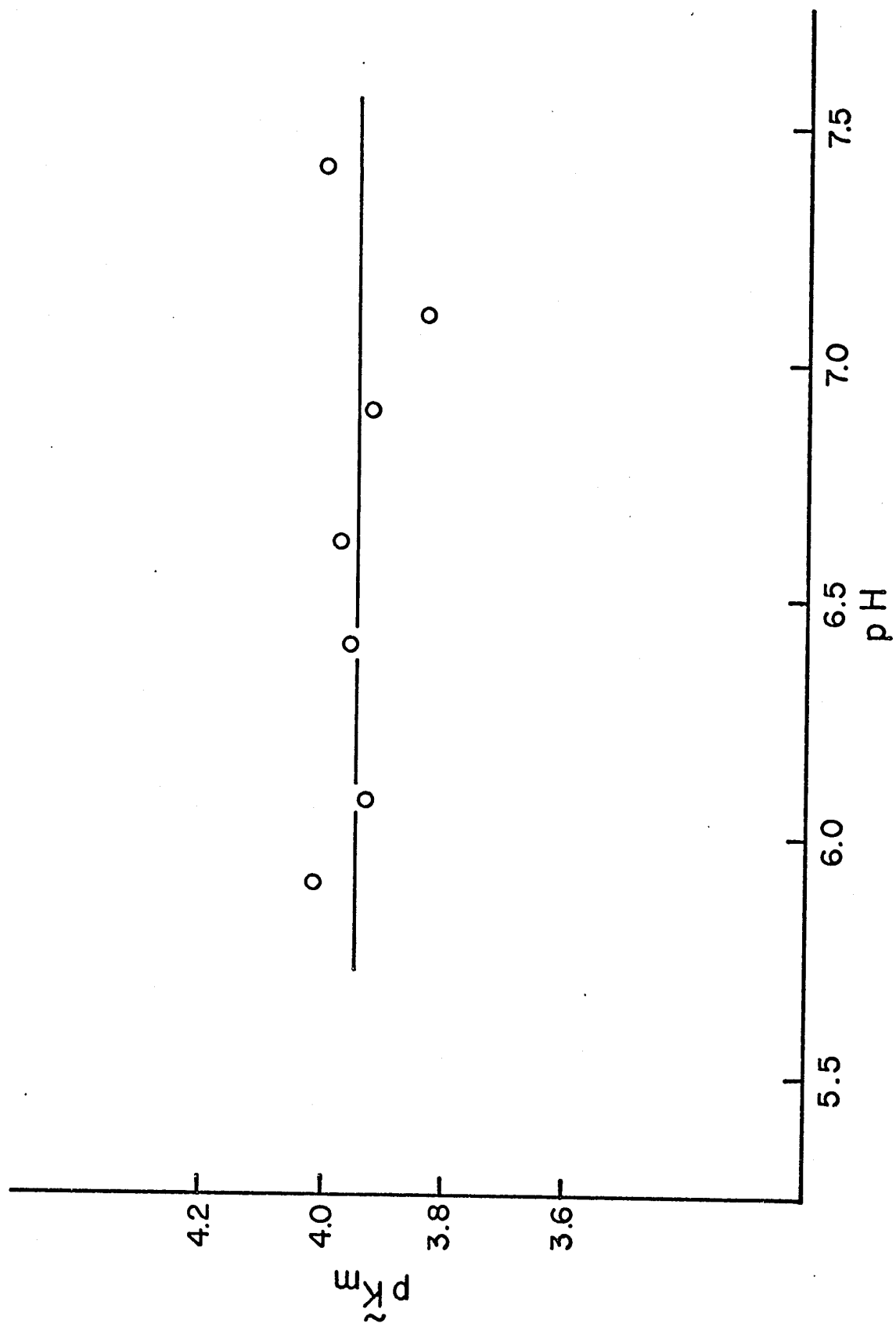


Figure 17

Variation of $p\tilde{K}_m$ with pH for the pre-steady state hydrolysis of CANE in 0.05M phosphate buffer at 25.0°C.



acylation with $pK'_b=6.9$. Such a result was already predicted on the basis of the steady-state studies on the trypsin-catalyzed hydrolysis of N-benzoyl-L-alanine methyl ester discussed in the preceding chapter.

The Michaelis constant $\tilde{K}_m$ was found to be constant over the pH range studied with an average value of $1.14 \times 10^{-4}M$. It appears therefore that both $\tilde{k}_1$ and $\tilde{k}_{-1}$ are pH-dependent quantities in this region; they are, however, too large to be measured by the stopped-flow technique.

Solvent Effects

The work at excess enzyme concentration in 1% (v/v) isopropyl alcohol was extended to 20% (v/v) alcohol to test the effect of solvent on the rate constants describing Michaelis-complex formation and the acylation process. The results at pH 6.90 are reported in Table 6. Dielectric constants were taken from the data published by Åkerlöf (157).

Both $\tilde{k}_2/\tilde{K}_m$, relating to complex formation, and $\tilde{k}_2$, relating to acylation, decreased with increasing

Table 6

Kinetic Parameters for the Trypsin-catalyzed Hydrolysis
of CANE in 0.05M Phosphate Buffer at 25.0°C and pH 6.90

[ISOPROPANOL] (%)	(M)	DIELECTRIC CONSTANT D	$\tilde{k}_2$ (sec ⁻¹)	$\tilde{K}_m \times 10^4$ (M)	$\tilde{k}_2/\tilde{K}_m$ (M ⁻¹ sec ⁻¹)
1	0.130	77.8	3.55	1.19	29,800
8	1.04	72.8	2.40	1.37	17,500
15	1.95	67.7	1.59	1.65	9,600
20	2.60	64.1	1.16	2.13	5,400
Extrapolated for water		78.5	3.66	1.14	32,300

solvent concentration, whereas $\tilde{K}_m$ was approximately doubled in 20% isopropyl alcohol. A similar effect on $\tilde{k}_2/\tilde{K}_m$ has been reported for the pre-steady state hydrolysis of p-nitrophenyl acetate by chymotrypsin (124). Plots of the logarithm of these parameters against the reciprocal of the dielectric constant were found to be essentially linear and are shown in Figures 18, 19 and 20. The apparent significance of such curves is discussed in the next section. Extrapolation of the lines in Figures 18-20 to $100/D_{H_2O}$ yields a good estimate of the rate constants in water; these cannot be obtained experimentally because of the low solubility of CANE in water. A value of $\tilde{K}_m$ close to $1.14 \times 10^{-4} M$ in water would in fact be expected from the mean value of $\tilde{K}_m = 1.14 \times 10^{-4} M$ in 1% (v/v) isopropyl alcohol.

The influence of organic solvents on enzymatic reaction rates, both in the transient-phase and steady-state regions, has occasionally been associated with inhibition effects (81,124,131). In this study, plots of the reciprocal of the first and second order rate constants against solvent concentration according to the method proposed by Dixon (99), did not yield a straight line. As shown in Figures 21 and 22, however, $\tilde{K}_m/\tilde{k}_2$ and $1/\tilde{k}_2$

Figure 18

Variation of $\log_{10} \tilde{k}_2/\tilde{K}_m$ with the reciprocal of the dielectric constant of isopropyl alcohol-water mixtures at pH 6.90 and 25.0°C.

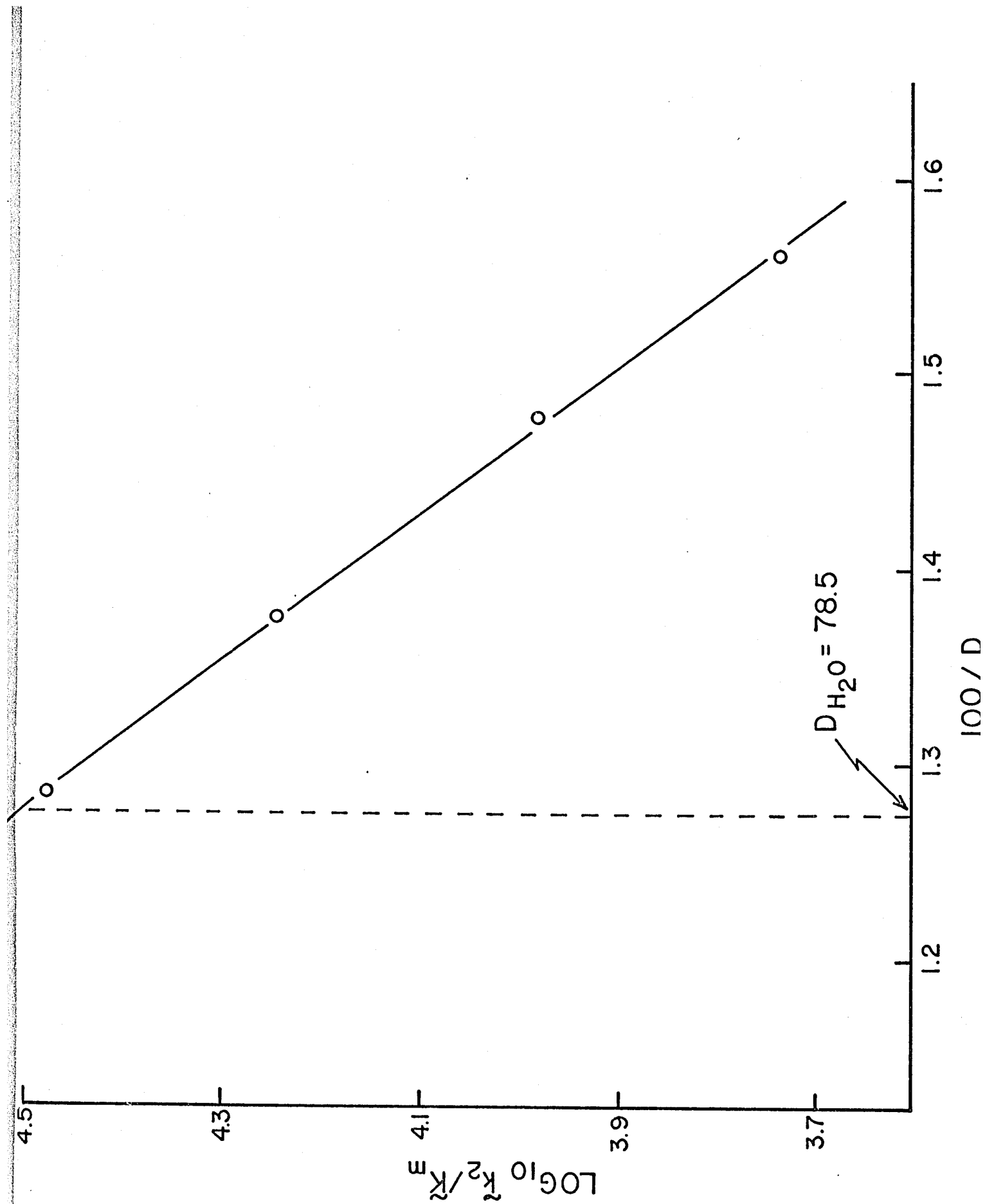


Figure 19

Variation of $\log_{10} \tilde{k}_2$ with the reciprocal of the dielectric constant of isopropyl alcohol-water mixtures at pH 6.90 and 25.0°C.

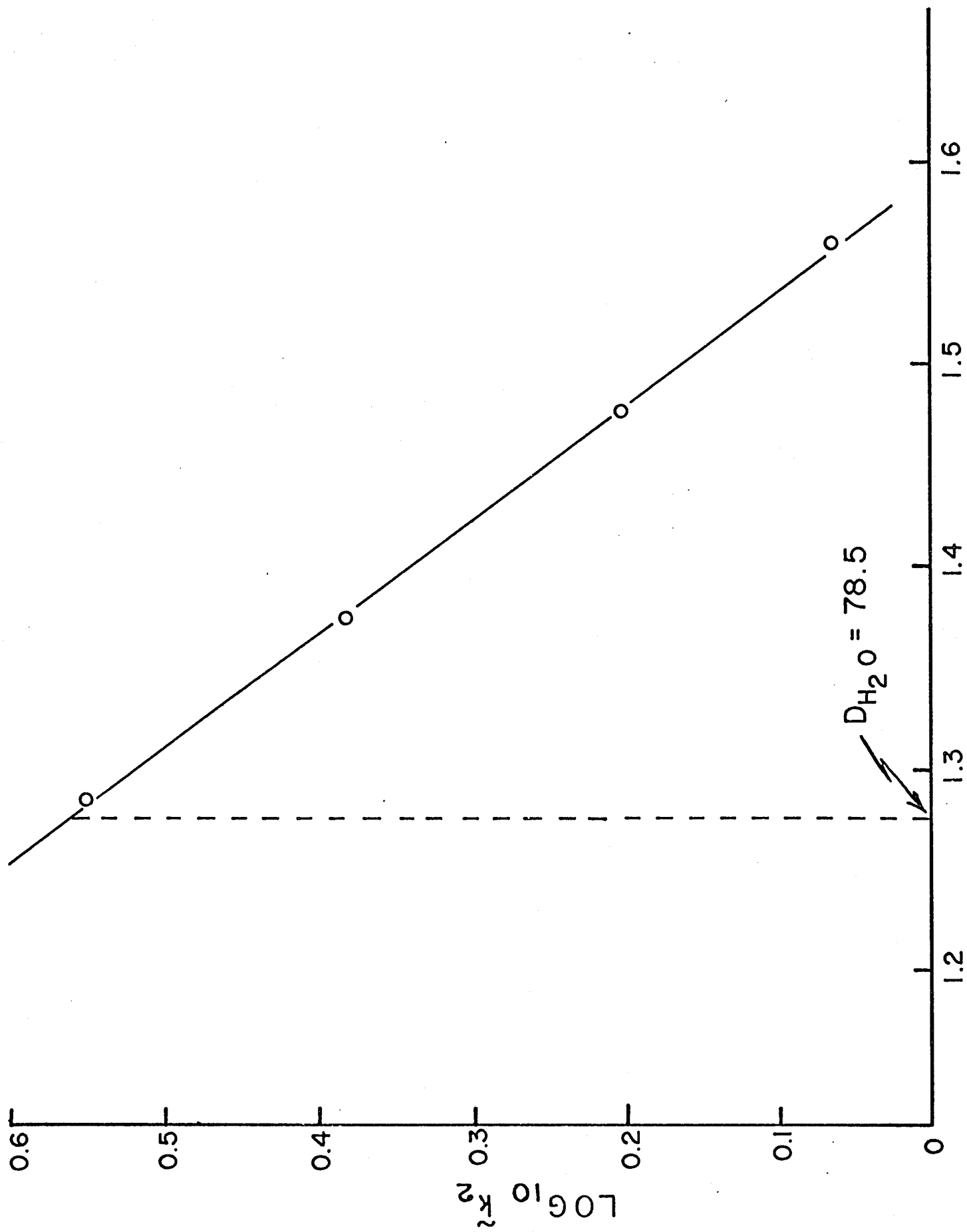


Figure 20

Variation of $\log_{10} \tilde{K}_m$ with the reciprocal
of the dielectric constant of isopropyl
alcohol-water mixtures at pH 6.90 and
25.0°C.

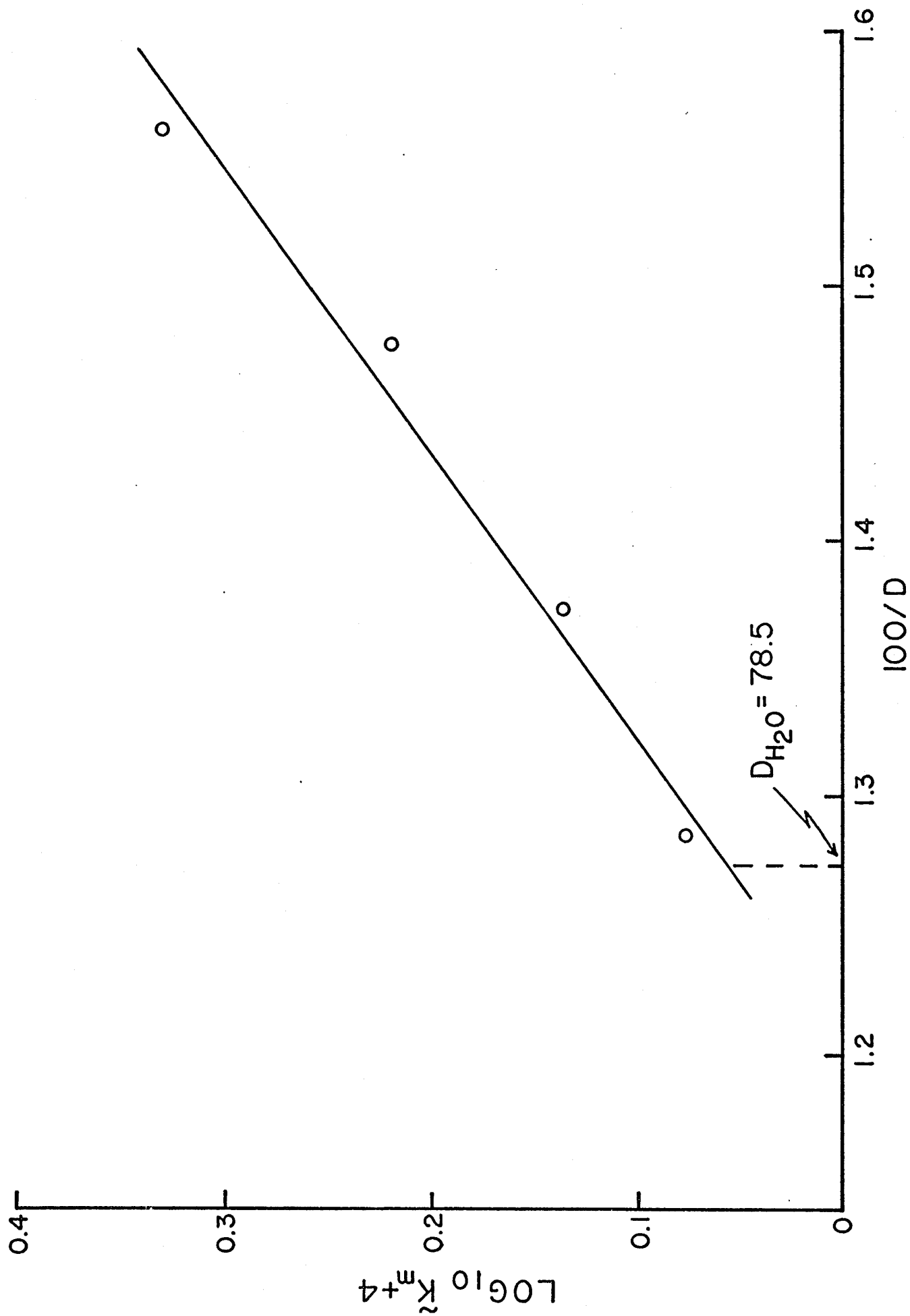


Figure 21

Variation of $\tilde{K}_m/\tilde{k}_2$ with solvent concentration squared at pH 6.90 and 25.0°C.

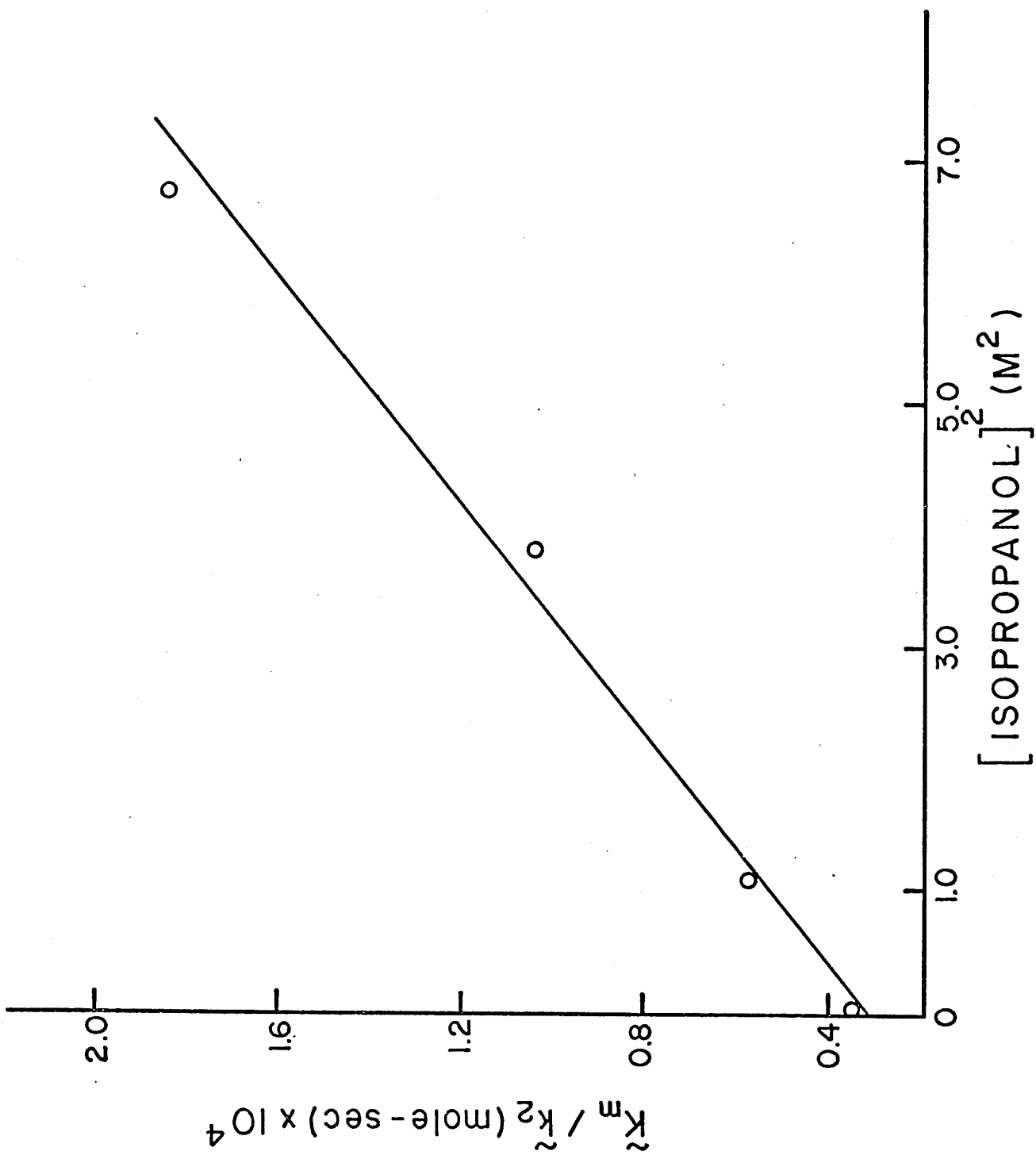
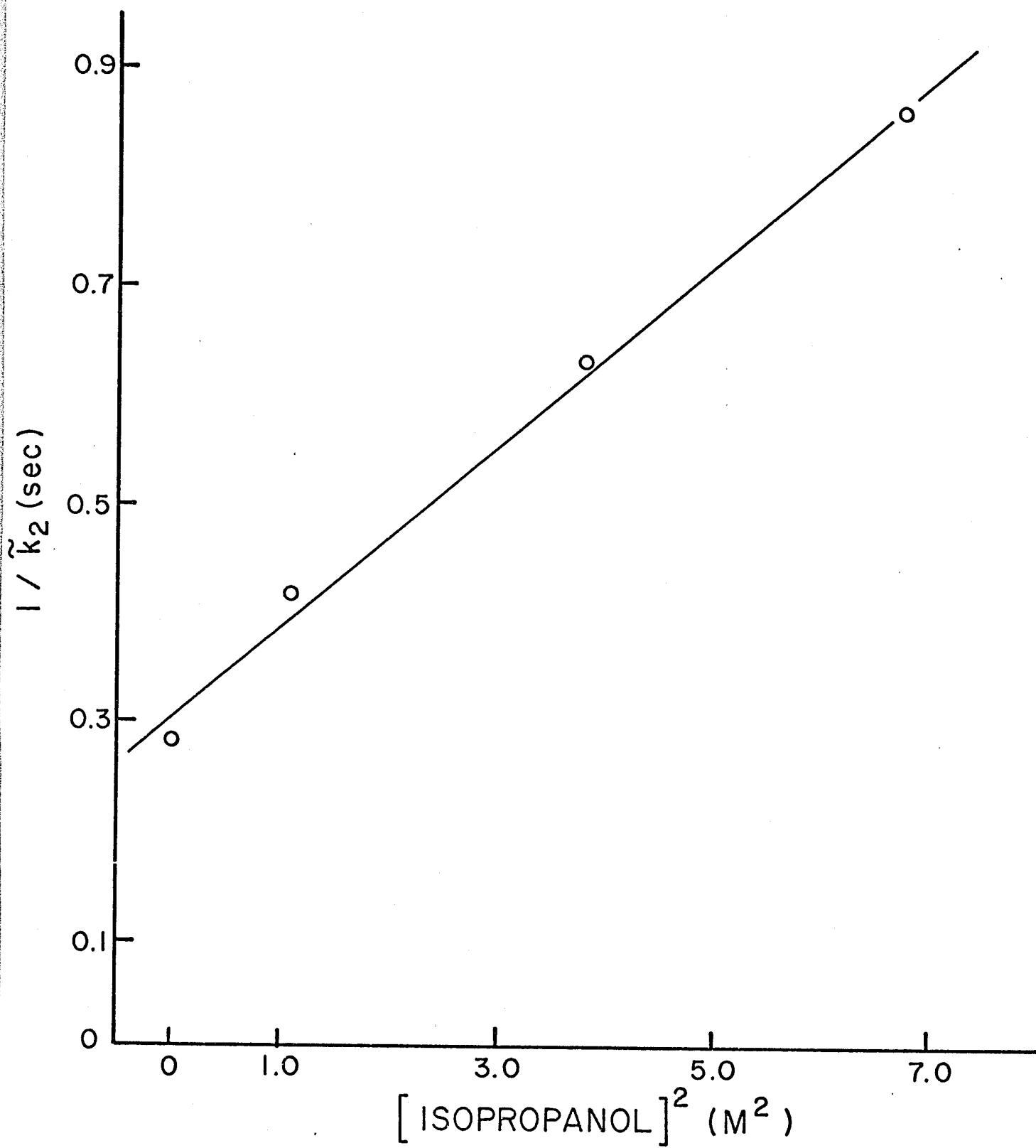


Figure 22

Variation of $1/\tilde{k}_2$ with solvent concentration squared at pH 6.90 and 25.0°C.



seemed to vary linearly with the square of solvent molarity. The intercepts at zero isopropanol concentration give rate constants in water which agree fortuitously well with those obtained from Figures 18-20; the latter are shown in Table 6. Faller and Sturtevant (124) obtained a linear relationship for the effect of solvent composition on the second-order rate constant by plotting $(K_m/k_2)^{1/2}$ against solvent molarity. The authors suggested that the observed behaviour might possibly be interpreted in terms of inhibition by solvent at two identical but independent sites on the enzyme. The concentration of active enzyme is then given by

$$[73] \quad [E]_{\text{act}} = [E]_0 \left(1 + \frac{[I]}{K_1} + \frac{[I]^2}{K_1 K_2} \right)^{-1}$$

where the dissociation constants of EI_1 and EI_2 are K_1 and K_2 respectively and $K_2 = 4K_1$. Such an interpretation would obviously be an oversimplification of the situation, since it completely disregards the effect of solvent on electrostatic interactions and enzyme conformation.

It is difficult to give a fully objective estimate of the reliability of the rate constants reported

in this section. As has been stated earlier, the precision of the computer fit was of the order of $\pm 3\%$. However, reproducibility from run to run was more difficult to attain and individual rate constants may be subject to an uncertainty of $\pm 20\%$. For purposes of internal comparison the kinetic parameters should generally be reliable to $\pm 10\%$.

DISCUSSION

pH Dependence of Kinetic Parameters

The shape of the $\log_{10} \tilde{k}_2$ - pH profile in Fig. 16 demonstrates that the rate constant relating to the acylation of trypsin by CANE is pH dependent with a $pK'_b = 6.9$. This undoubtedly corresponds to the ionization of the imidazole ring of histidine-46 and agrees with the results of the steady-state work obtained from the pH dependence of $\log_{10} \tilde{k}_c/\tilde{K}_m$; in other words, acylation requires the imidazole residue to be ionized and cannot occur if it is protonated. Similar results have been obtained for the acylation of α -chymotrypsin by p-nitrophenyl acetate (120,121) which supports the proposal that the same mechanism is applicable to the two enzymes (c.f. Scheme II). The transient-phase data reported by Stewart and Ouellet (35) for the trypsin-catalyzed hydrolysis of p-nitrophenyl acetate, which suggest an apparent pH independence of k_2 , can be interpreted in the following manner. The experimental conditions used by these authors were such that the assumption $(k_2 + k_3)s_o \gg k_3K_m$ was not valid. Small changes in k_2 with pH were therefore difficult to detect

by conventional stopped-flow analysis since K_m was relatively large. The published variation of the second-order rate constant k_2/K_m with pH, however, indicates the dissociation of a group which is involved in acylation and is only active when it is in the unprotonated form. Presumably this is the imidazole residue which Stewart and Ouellet believed to be inactive to complex formation as a result of competitive inhibition by hydrogen ions.

In agreement with other authors (101,102) for the trypsin-catalyzed hydrolysis of N-benzoyl-L-argininamide, the Michaelis constant $\tilde{K}_m$ was found to be pH independent over the range of pH 5.91 - 7.41 (c.f. Fig. 17). It must be remembered that for amides, acylation is considered to be the rate-limiting step (104). This result implies that the pH profile of $\log_{10} \tilde{k}_2/\tilde{K}_m$ would reveal the pK of a basic group ($pK_b \approx 6.9$) which is free to ionize in the free enzyme. Complex formation between trypsin and CANE can therefore take place regardless of whether the imidazolyl side chain of histidine-46 is protonated or not. With due consideration to the fact that no direct comparison can be made between the pre-steady state and the steady-state results obtained in this work, it is nevertheless noted that the data are internally consistent.

Michaelis-Complex Formation

When the enzyme concentration is much greater than the substrate concentration, conditions exist opposite to those of a normal enzymatic reaction. It has been shown in the theoretical section, however, that in such a situation one does not have to make any assumption as to the magnitude of k_3 , due to the fact that the turn-over reaction cannot affect in any manner the concentration of the enzyme, which must remain constant and equal to the original concentration during the whole reaction. This condition has been successfully employed in this study to verify the existence of a Michaelis complex ($K_m = 1.14 \times 10^{-4} M$) for the trypsin-catalyzed hydrolysis of p-nitrophenyl esters which had been placed in some doubt by the work of Faller and Sturtevant (124). Even though CANE cannot be considered a specific substrate of trypsin, since it lacks a positive charge on the side chain and indeed only has a methyl side chain, the acylating group of this molecule resembles the specific amino acid substrates more closely than the acetyl moiety of p-nitrophenyl acetate. Kézdy and Bender (120) have suggested that complications may arise from the lability of the latter substrate towards non-enzymatic catalysis at protein loci other than the active center,

such as imidazole side chains, and from substrate-activation effects. Since acylation rates cannot be directly measured at optimal pH values by stopped-flow methods using specific ester substrates of trypsin, the observed kinetic evidence with CANE helps to substantiate the postulated three-step mechanism at least as far as Michaelis-complex and acyltrypsin formation is concerned.

Solvent Effects

In considering the effect of organic solvent on $\tilde{k}_2$ and $\tilde{K}_m$ no allowance needs to be made for the decrease in water concentration as the concentration of isopropyl alcohol is increased. Water participates in its role as nucleophilic acceptor only in the deacylation process and any observed effects on the two preceding steps must be of a more indirect nature such as changes in enzyme conformation. The results in Table 6 indicate that both binding of substrate to enzyme and the acylation rate decrease with increasing solvent concentration. Similar effects have been previously noted in the literature (81, 124, 131, 132) although the results are often conflicting partly owing to the complexity of the rate constants being considered. Present evidence on the conformations of

protein molecules in non-aqueous solvents (85,135) suggests that three interdependent factors must be considered in determining protein conformations: (1) electrostatic interactions, (2) hydrogen bonds between peptide linkages and (3) hydrophobic interactions. In addition, conformational entropy changes may contribute to the total free energy of the protein in solution.

The electrostatic free energy F_E of a protein molecule due to its net charge and its interaction with an ion atmosphere according to the Debye-Hückel theory (158) is given by

$$[74] \quad F_E = \frac{Z^2 e^2}{2D} \left(\frac{1}{b} - \frac{\kappa}{1 + \kappa a} \right)$$

where Z is the net charge on the protein ion of radius b , e is the electronic charge, κ is the reciprocal of the thickness of the Debye-Hückel ion-atmosphere and a is the distance of closest approach of the macroion and its counterion, measured between centers. If we consider the value of F_E for the native conformation of a protein molecule in different solvents, b is constant and κ can be maintained constant; hence the effect of changing the solvent can be resolved into the effect on the values of

Z and D. Addition of up to 20% (v/v) of a weakly protic solvent such as isopropyl alcohol to trypsin which carries a large positive charge at pH 6.90 (mobility $\approx + 4 \times 10^{-5}$ cm²/v-sec, pH 6-8) and has an isoelectric point of 10.8 (159) should not cause much change in Z. Consequently the electrostatic free energy F_E of the native aqueous conformation of the enzyme will increase as D decreases, which would favour some disordering of the molecular structure.

Laidler (66) has suggested that the dielectric constant of the solvent may be correlated with the interaction between enzyme and substrate. On the basis of absolute reaction rate theory he has proposed that

$$[75] \quad \ln k_D = \ln k_{\infty} + \frac{A}{DT}$$

where k is either a rate constant such as k_2 or k_2/K_m or an "equilibrium" constant such as K_m . D is the dielectric constant of the medium, T is the absolute temperature and A is a constant. Figures 18,19 and 20 indicate that equation [75] can be formally applied to the data of this study with the constant A having a negative sign. Laidler has pointed out, however, that

this treatment is far from satisfactory in that "a considerable source of uncertainty arises from the simplification of regarding the solvent as a continuous dielectric".

It has been proposed some years ago that it is unlikely that hydrogen bonds other than those involving peptide linkages can make a major contribution to the overall conformational stability of most proteins and that, taken by themselves, such bonds can confer only marginal stabilization to the native conformation of a protein molecule in aqueous solution (160,161). It is of interest to note therefore that for the model compound N-methylacetamide, the free energy change of transferring a hydrogen-bonded dimer from an aqueous to an apolar (CCl_4) environment is essentially zero (162). Apparently the intrinsic interaction between donor and acceptor groups is not sensitive to the adjacent molecular surroundings. Only very minor changes in the optical rotary parameters b_0 and $[\alpha]_D$ have been observed for globular proteins in aqueous solvent mixtures containing less than 20% (v/v) of the organic component (163). We suggest therefore that isopropyl alcohol, which has been called an "unreactive" organic solvent (164) and has a low proton-donating capacity

relative to the N-H group, induces little alteration in intrapeptide hydrogen bonding of the trypsin molecule at least up to 20% (v/v) concentration.

If it is assumed that the native conformation of a protein molecule in aqueous solution is in considerable part stabilized by hydrophobic interactions, it follows that this stabilization should be substantially, if not completely lost on transferring that protein to almost any pure non-aqueous solvent. This has been found to be true for a variety of proteins (135). By analogy to α -chymotrypsin (13,14,16,17) it is expected that most of the non-polar residues of trypsin are located in the interior of the molecule. A reduction in hydrophobic interactions between these residues with increasing concentration of organic solvent could therefore lead to substantial unfolding of the enzyme. The total free energy (ΣF) of the native protein in aqueous solution is negative and assumed to be separable into the free energy contributions owing to conformational entropy (F_S), electrostatic interactions (F_E), hydrogen bonding (F_{HB}) and hydrophobic interactions (F_{HI}). Much evidence in the literature suggests that in non-aqueous solvents F_{HI} is so markedly increased that even a decrease in F_{HB} is

inadequate to compensate and retain ΣF as negative. In the light of the preceding discussion, the observed decrease in the first- and second-order rate constants and the increase in the Michaelis constant for the trypsin-catalyzed hydrolysis of CANE can be qualitatively justified by a loss in conformational integrity of the enzyme with increasing isopropanol concentration.

CHAPTER IV

GENERAL DISCUSSION

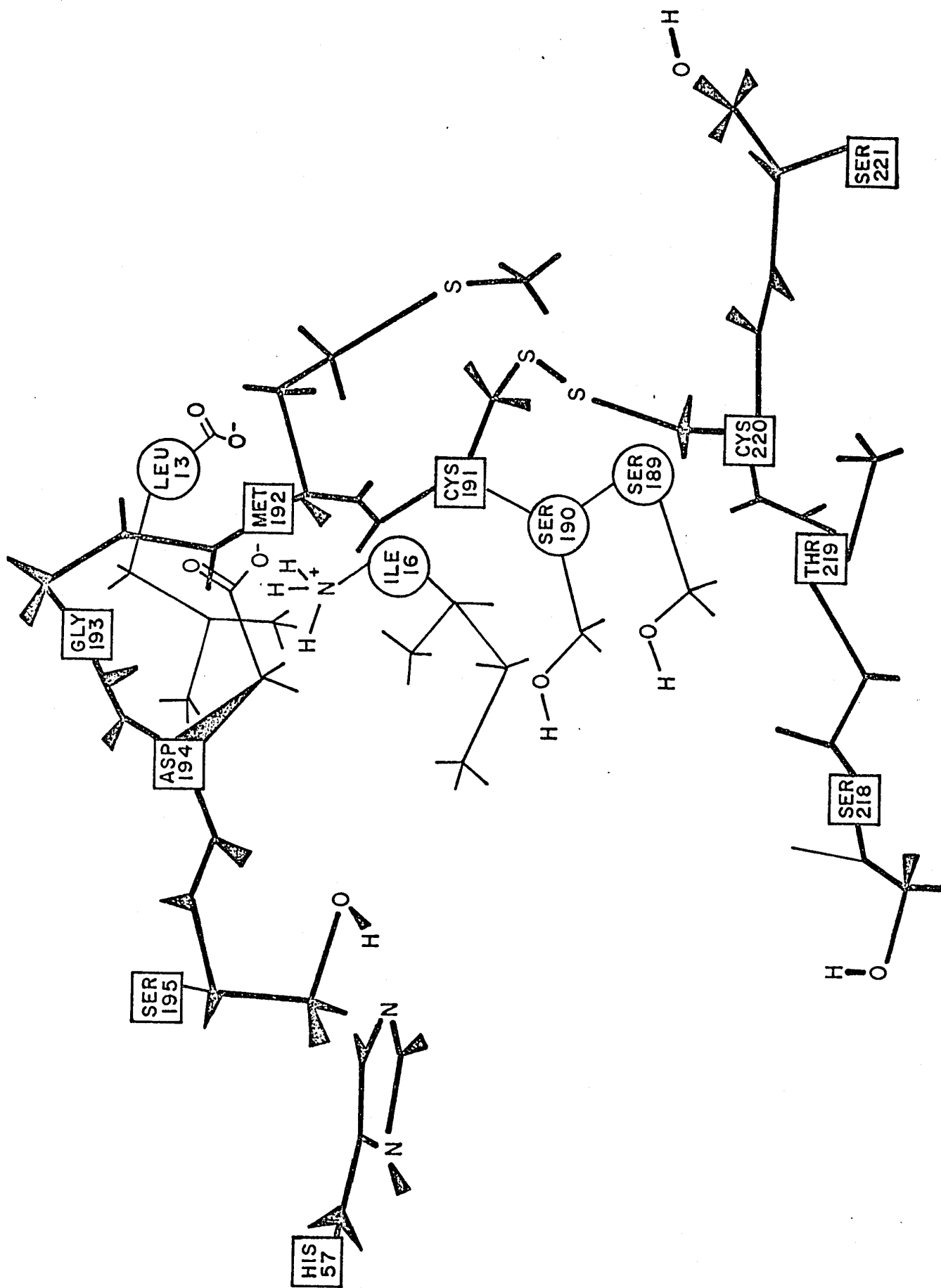
In chapters II and III we have presented evidence which shows that the kinetic behaviour of trypsin is in some aspects different from that of chymotrypsin. To support our conclusion that their catalytic mechanism is in fact the same, existing kinetic, chemical and crystallographic information on the two enzymes has been compared and contrasted.

Structural Evidence

The three-dimensional structure of α -chymotrypsin has now been established by X-ray methods (13,14, 16,17). Figure 23 has been constructed from Fig. 4 of reference 16 and Fig. 2 of reference 13, with the help of a model we have built. It shows an approximate arrangement of the groups that appear to exist in the region of the active center, and their relative positions. The histidine-57 and serine-195 groups are known from kinetic and other evidence to be catalytically important, as are the $-\text{NH}_3^+$ group of N-terminal isoleucine-16 and

Figure 23

Approximate arrangement of the more important amino acid residues in the region of the active center of α -chymotrypsin. Heavy lines indicate groups close to the surface of the enzyme, thin lines those buried more deeply within the molecule.



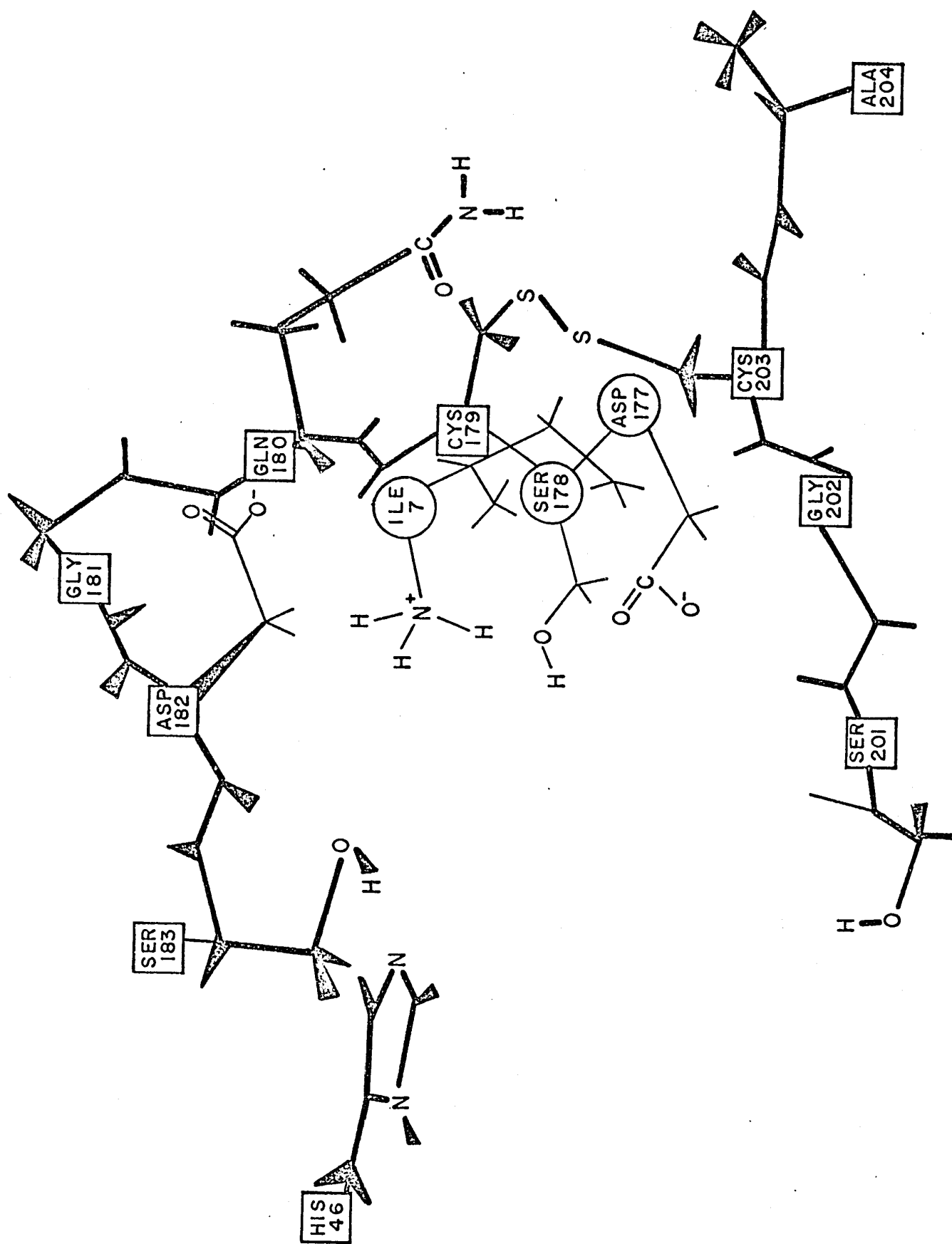
the - COO⁻ groups of aspartic acid-194 and aspartic acid-102. In the background of the diagram are a number of non-polar groups, such as the side chains of isoleucine-16, leucine-163 and isoleucine-212. Some of these groups are undoubtedly concerned with forming hydrophobic bonds with the non-polar groups on the substrate.

The detailed X-ray structure of trypsin has not yet been established. However, there is very persuasive evidence that the general conformation of the enzyme is very similar to that of chymotrypsin. There is a very close correspondence between the amino acids along the chains, and there is a particularly strong homology as far as polar and non-polar groups are concerned (3). Further support for the similarity in conformation comes from the demonstration (15) that the two extra disulphide bridges that exist in trypsin can be built into the chymotrypsin model without distortion of the structure.

Figure 24 has been prepared for trypsin on the assumption that its general structure is the same as that of chymotrypsin. It is striking that so many of the groups near the active centers of the two enzymes are the same or very similar. The absence in trypsin of any group

Figure 24

Approximate arrangement of the more important amino acid residues in the region of the active center of trypsin. Heavy lines indicate groups close to the surface of the enzyme, thin lines those buried more deeply within the molecule.



equivalent to leucine-13 of chymotrypsin is probably not significant, since this group appears to be too far away in chymotrypsin to be actively involved. We are left with the result that the only significant difference between the two enzymes is that the neutral serine-189 in chymotrypsin has become the negatively-charged aspartic acid-177 in trypsin. This strongly suggests that the different specificities of the two enzymes are largely due to the difference between these two groups; in particular, the fact that trypsin favors substrates having a positively-charged group in a suitable position (e.g. derivatives of arginine and lysine) seems to be related to the existence of the -COO^- group at position 177 in trypsin.

Mechanistic Evidence

In Tables 7, 8 and 9 we have summarized the most important mechanistic evidence for the two enzymes, classifying it according to its relevance to (1) complex formation, (2) the acylation process and (3) the deacylation process.

(1) Complex Formation

Table 7 summarizes the evidence and conclusions regarding the process of Michaelis-complex formation. The main difference between the behavior of the two enzymes lies in their pH dependence at low substrate concentrations; with chymotrypsin there is evidence for a group of $pK \sim 9$, while with trypsin there is no falling-off in rate up to $pH \sim 10$.

There is no reason to doubt that the groups ionizing at $pH \sim 7$ are histidine-57 in the case of chymotrypsin and histidine-46 in the case of trypsin; support for this is provided by the work with chymotrypsin in mixed solvents (89). The mixed-solvent work also supports the conclusion that the group of $pH \sim 9$ in chymotrypsin is the $-NH_3^+$ group of isoleucine-16, existing in the charged state as an ion-pair with the $-COO^-$ group of aspartic acid-194 (16). When this $-NH_3^+$ group loses its proton it is postulated (17) that the $-COO^-$ group of aspartic acid-194 moves to a more polar environment. It appears likely that this causes a shift in the position of the reactive serine-195 residue which, as will be discussed, has an effect on subsequent processes.

Table 7

Evidence Relating to Complex Formation of Chymotrypsin and Trypsin

<u>Kind of Evidence</u>	<u>Chymotrypsin</u>	<u>Trypsin</u>	<u>Reference</u>
Studies at low substrate concentrations	Bell-shaped curve; pK's of ~7 and ~9	Sigmoid curve; pK of ~7; no falling-off up to pH~10	89, this work
Studies in mixed solvents	Group of pK~7 is a cationic acid; group of pK~9 is a neutral or ion-pair acid	--	89
Studies at low pH	Carboxyl group (pK~4) present	Carboxyl group (pK~4) present	165, 166
Chemical and kinetic studies	Single histidine residue involved in reaction, but not in complex formation	Single histidine residue involved in reaction, but not in complex formation	2,37, 39,89, this work
Chemical and kinetic studies	N-terminal isoleucine-16 (pK~9) indirectly participates in the binding process	--	63

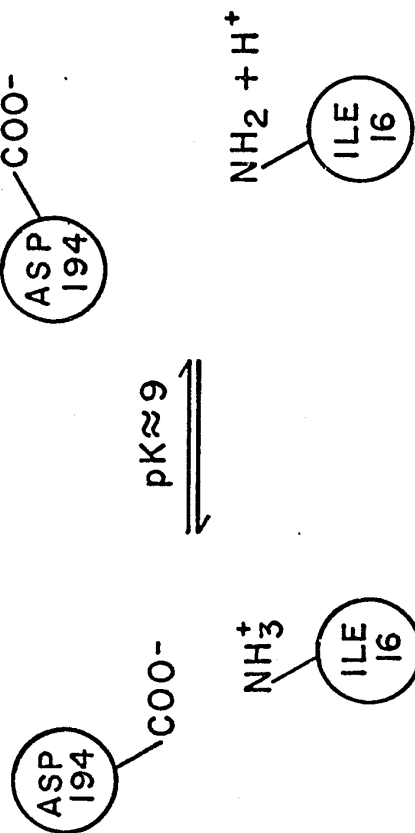
A suggestion as to why the pK_a of the $-NH_3^+$ group in trypsin is higher than that in chymotrypsin is illustrated in Figure 25. In trypsin the $-NH_3^+$ group is close to two $-COO^-$ groups, and it is therefore more difficult for the proton to leave.

The upper portions of Figures 26 and 27 present more detailed mechanisms for the ionizations of chymotrypsin and trypsin, and for the way in which Michaelis-complex formation occurs. It is postulated, in agreement with other workers (41,63,68,107,167), that binding in the complexes does not directly involve the ionizing groups, but is primarily due to hydrophobic bonding between non-polar groups on the substrate and enzyme. In addition to this, the anionic site in trypsin (aspartate-177) forms an electrostatic bond with the positively-charged side chain of specific trypsin substrates. The state of ionization of the histidine residues has no effect on complex formation. However, ionization of the $-NH_3^+$ groups of the terminal isoleucines has an indirect effect on binding by bringing about a conformational change in the enzyme molecules; support for this in the case of chymotrypsin is provided by the work of Hess and coworkers (64,107) and is discussed further with reference to trypsin in a later section.

Figure 25

Ionization scheme of some amino acid
residues in free trypsin and chymotrypsin.

CHYMOTRYPSIN



TRYPSIN

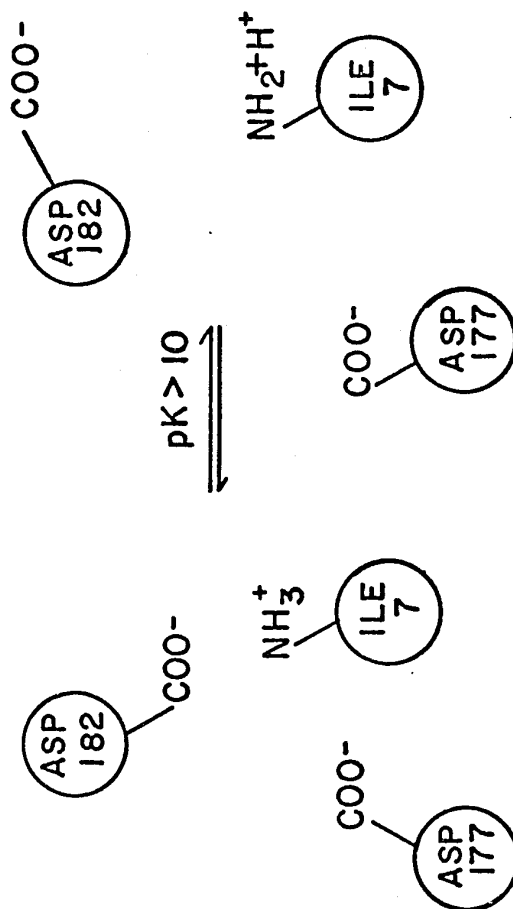


Figure 26

Reaction mechanism for the α -chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tyrosine ethyl ester (R=tyrosyl). The important groups are shown in schematic relationship to one another. Hydrogen bonds are indicated by dashed lines.

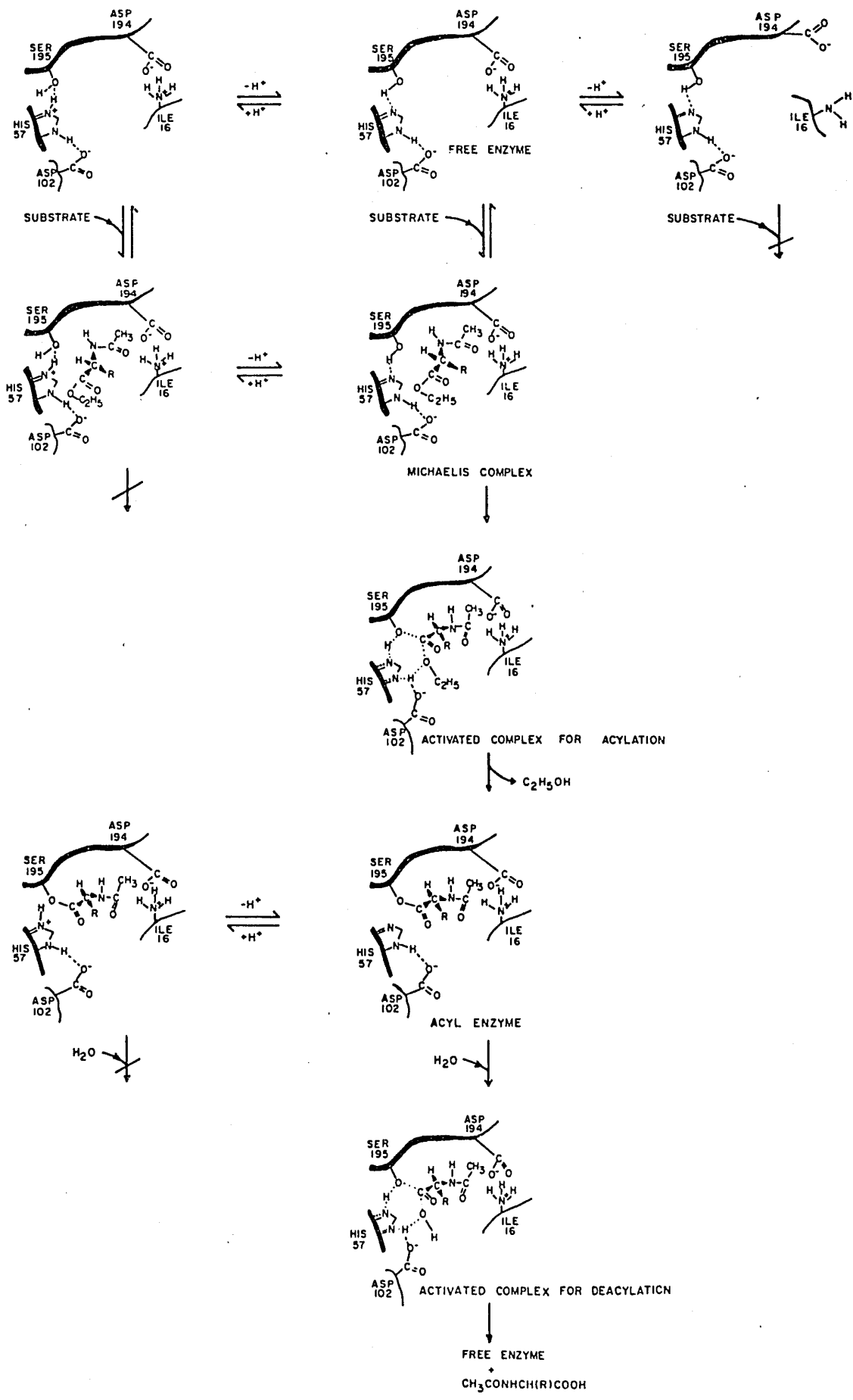
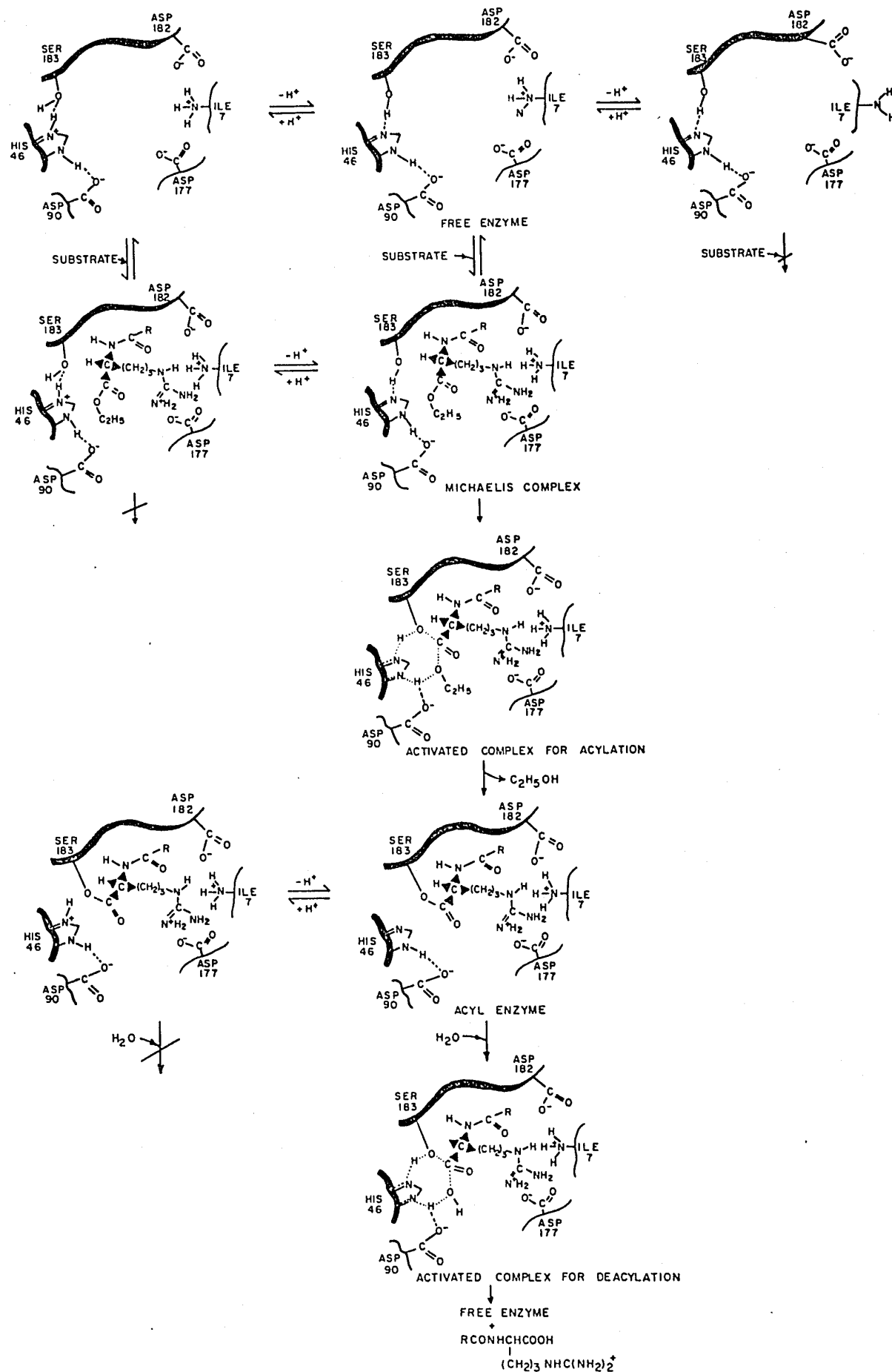


Figure 27

Reaction mechanism for the trypsin-catalyzed hydrolysis of N-benzoyl-L-arginine ethyl ester (R=benzoyl). The important groups are shown in schematic relationship to one another. Hydrogen bonds are indicated by dashed lines.



(2) Acylation

The evidence and conclusions relating to the acylation steps for the two enzymes are summarized in Table 8. It has already been seen from the results relating to complex formation that the forms of the free enzyme in which the isoleucine - NH_3^+ group has lost its proton cannot bind substrate; expressed differently, the Michaelis complex cannot lose a proton from the - NH_3^+ group as readily as can the free enzyme, and the unprotonated form is of no significance within the range of pH that has been investigated.

The fact that with both enzymes the acylation rates fall off at pH values below about 7.5 implies that the imidazole ring of histidine-57 or histidine-46 must be unprotonated. The function of the unprotonated imidazole ring is no doubt to aid in the removal of the proton from the serine hydroxyl group. At the same time a proton must be transferred to the alcoholic oxygen atom of the substrate. A way in which these processes can occur in a single stage is shown in Fig. 28. The suggested scheme makes use of the remarkable properties of the imidazole group as a proton-transfer agent. Eigen et al. (173,174), who studied this as well as other acid-base

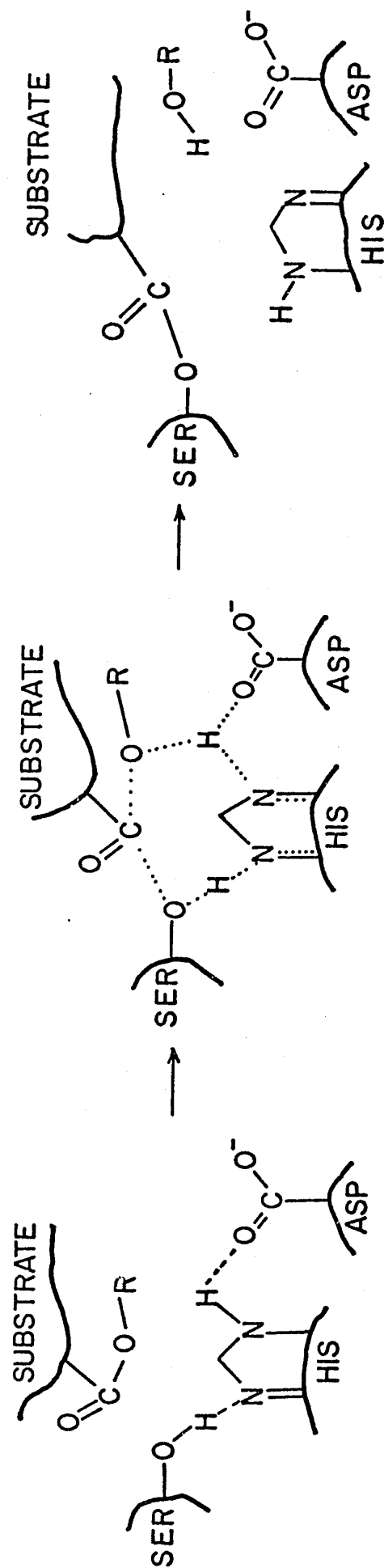
Table 8

Evidence Relating to Acylation of Chymotrypsin and Trypsin

<u>Kind of Evidence</u>	<u>Chymotrypsin</u>	<u>Trypsin</u>	<u>Reference</u>
Chemical and kinetic studies	Acylation involves the hydroxyl group of serine-195	Acylation involves the hydroxyl group of serine-183	25-28 168-170
pH Studies with amides, for which acylation is rate-limiting	Rate falls off at pH<7.5; no fall-off in alkaline solution up to pH 9.5	Rate falls off at pH<7.5; no fall-off in alkaline solution up to pH 9.5	63,101 103,167 171
Substituent effects	Proton transfer is involved in acylation	--	172
Deuterium isotope effects	Proton transfer is involved in acylation	--	171
pH Studies at low concentrations of esters, for which deacylation is rate-limiting	Both ionizing groups are apparently involved in acylation	Single ionizing group (pK=7) participates in acylation	89, this work

Figure 28

Proton transfer processes during
acylation of trypsin and chymo-
trypsin.



ACTIVATED COMPLEX

catalysts, have shown that the effectiveness of this system is further increased by hydrogen bonds between the donor and acceptor groups. In addition, the tautomeric character of the unprotonated imidazole ring makes it possible for this group to accept and donate a proton at the same time. In this connection it is interesting to note the suggestion by Hofmann (175) that the tautomeric nature of imidazole appears to be associated with an intermolecular exchange of protons rather than an intramolecular proton shift. These ideas are incorporated into the mechanisms shown in Figures 26 and 27. As far as the behavior on the basic side is concerned it is suggested that, for both enzymes, the substrate has the effect of locking the enzyme into a conformation in which the -COO^- group of aspartic acid-194 or -182 is held close to the -NH_3^+ group of the terminal isoleucine residue. This makes it more difficult for the α -amino group to lose a proton, a fact that explains why there is no decrease in the acylation rates up to high pH values. With trypsin this ionization is also difficult in the free enzyme, and we have suggested that this is due to the presence of the second -COO^- group, i.e. aspartate-177. With chymotrypsin, however, the ionization is less difficult in the free enzyme, a pK of ~ 9 being observed.

(3) Deacylation

Table 9 summarizes the evidence and conclusions relating to the deacylation step. It is seen that there is again a difference between the two enzymes in their pH dependence; with trypsin there is a decrease in rate above pH ~ 9, whereas with chymotrypsin there is no falling-off up to pH ~ 10; the results with chymotrypsin are consistent with a pK of 10.5 or greater.

In both enzymes the imidazole ring of histidine-57 or -46 must be in its unprotonated form in order for deacylation to occur. The function of the unprotonated imidazole group is to aid in the splitting of a water molecule acting as the nucleophile and to facilitate the transfer of a proton to the serine oxygen atom. This again involves a prototropic change in the imidazole ring, one of the nitrogen atoms accepting a proton from the water molecule and the other donating its proton to the serine oxygen atom. This process is the reverse of that shown in Fig. 28 with R = H. The lower portions of Figures 26 and 27 show the conclusions arrived at for the deacylation process.

Table 9

Evidence Relating to Deacylation of Chymotrypsin and Trypsin

<u>Kind of Evidence</u>	<u>Chymotrypsin</u>	<u>Trypsin</u>	<u>Reference</u>
pH Studies with N- α -substituted esters, for which deacylation is rate limiting	Sigmoid curve; pK of ~ 7 ; no falling-off up to pH ~ 10	Bell-shaped curve; pK's of ~ 7 and ~ 9.5 . Apparently both groups involved in deacylation	32, 33, 68, 80, 82, 89, 171, this work
pH Studies with esters on which the α -amino group is not substituted	Bell-shaped curve; pH optimum shifted from pH ~ 8 to pH ~ 6	Bell-shaped curve; pH optimum shifted from pH ~ 8 to pH ~ 6	176-178
Effect of added nucleophiles	Deacylation is first order in the nucleophile (e.g. methanol)	--	134
Kinetic studies	No intermediate detected in deacylation	--	68
Deuterium isotope effects	Proton transfer is involved in deacylation	Proton transfer is involved in deacylation	171, 179

The behavior of trypsin on the alkaline side can be explained along the following lines. The -NH_3^+ group of isoleucine-7 splits off its proton with a pK of 9.5, and when this occurs there is a movement (probably to a more polar environment) of the -COO^- group of aspartic acid-182; this movement affects the position of the neighbouring serine residue and diminishes the ease with which the acyl portion of the substrate can be removed from the serine by the action of the imidazole group. The fact that this ionization occurs at pH 9.5, rather than at a higher value as in the free enzyme, is possibly the result of slightly different relative spatial arrangements of the -NH_3^+ and the two -COO^- groups in the acyl enzyme as compared with the free enzyme; the two -COO^- groups may be a little further away from the -NH_3^+ group in the acyl enzyme and so allow the proton to leave more readily.

The different behavior of chymotrypsin is perhaps also to be explained in terms of slight conformational changes during complex formation and acylation. It is suggested that in acyl chymotrypsin the -COO^- group of aspartic acid-194 has been brought a little closer to the -NH_3^+ group of isoleucine-16 than in the

free enzyme; this could have the effect of raising the pK from the value of ~ 9 in the free enzyme to a value of 10.5 in the acyl enzyme.

The differences in kinetic behavior on the alkaline side between trypsin and chymotrypsin suggest the possibility that during the catalytic process the trypsin molecule is subjected to a general loosening of its three-dimensional structure, while chymotrypsin undergoes a tightening in conformation. From X-ray studies (14) it is known that the chymotrypsin molecule has a relatively open structure, and the results for trypsin should prove to be of great interest.

Conformational Changes

There is a growing amount of information indicating the possibility of substrate-induced conformational changes in proteins (180-183). Lumry and Koshland, in particular, have formulated theories concerned with the concept of active-site flexibility contributing to enzyme mechanisms. Some of the evidence available for trypsin and chymotrypsin directly relating to the N-terminal α -amino group will be referred to briefly. The

X-ray studies on chymotrypsin (16) have shown that there is an ion pair between aspartic acid-194 and isoleucine-16. It has been suggested (17) that as the latter group ionizes the former moves away into a more polar environment, and this, we believe, indirectly affects the kinetic behavior. The resultant conformational change probably accounts for the decrease in catalytic properties at alkaline pH (86). In support of this suggestion, Oppenheimer, Labouesse and Hess (107) find that acetylation of the α -amino group of isoleucine-16 inactivates δ -chymotrypsin, while all other amino groups can be acetylated without loss of activity. Furthermore Karibian et al. (184) have recently shown that the change in activity and structure of acetyl- δ -chymotrypsin in the alkaline pH range is not correlated with the ionizations of titratable tyrosine residues.

It seems likely that the terminal isoleucine-7 in trypsin performs a similar function to isoleucine-16 in chymotrypsin; this implies the existence of an ion-pair bond between this residue and aspartic acid-182. Support for this is provided by the work of Scrimger and Hofmann (111), who report that the reaction of nitrous

acid with the N-terminal isoleucine group of trypsin causes inactivation of the enzyme. We have shown that the pH-dependent change in the specific levorotation of trypsin at 365 mμ can be associated with a single group having an apparent pK of 9.4. It is reasonable to suggest that this is the pK of isoleucine-7; its ionization probably causes a conformational change in the enzyme, but this does not become apparent in the kinetics of complex formation with the non-specific substrate N-benzoyl-L-alanine methyl ester. In this connection it should be noted that Wang and Carpenter (103) report a pK of 10.4 for the free enzyme using N-benzoyl-L-argininamide; this ionization can probably also be related to the N-terminal isoleucine residue.

The pK of this group appears to be strongly affected by the degree of conformational stability of the enzyme resulting from non-electrostatic, electrostatic and even steric interactions between the substrate and various portions of the enzyme. Our results with diisopropylphosphoryl trypsin, showing that the specific rotation of the enzyme is pH-independent up to pH 10, are consistent with this suggestion. Similar evidence is provided by the optical rotary studies of trypsin in the

presence of reversible inhibitors; this work indicates that complex formation causes a shift in the apparent pK of the N-terminal isoleucine residue to higher pH values.

The relatively high pK value of 9-10 observed for trypsin catalyses in alkaline solution has led previous investigators (32,185) to suggest that this ionization relates to a tyrosine or lysine ϵ -amino group. Labouesse and coworkers (100,113) have recently shown, however, that at alkaline pH homogeneous N- ϵ -acetyl-trypsin exhibits the same activity as native trypsin towards synthetic substrates. Furthermore, acetylation of the free tyrosines (4 to 5 out of 10) leads to an enzyme which, below pH 9, is even more active as an esterase than is native trypsin. As a result of the X-ray diffraction studies by Matthews et al. (16) on chymotrypsin, it is now likely that the existence of an ion-pair bond between isoleucine and aspartic acid contributes to the unusually high pK value of the isoleucine α -amino group in trypsin, compared to a pK of 8 or less in peptide terminal α -amino groups (110).

Summary

The preceding discussion has led to the conclusion that, in spite of some differences in kinetic behavior, the mechanisms of chymotrypsin and trypsin action appear to be the same. We propose that the substrates fit into the two enzymes in a similar manner, the carbonyl carbon atom of the substrate forming roughly an equilateral triangle with the imidazole nitrogen atom and the alcoholic hydrogen atom of the serine residue. The alcoholic oxygen atom of the substrate also forms roughly an equilateral triangle with these two atoms on the enzyme. Both enzymes exhibit very low specificity as far as the alcoholic function of ester substrates is concerned, and this group almost certainly remains outside the enzyme. For chymotrypsin the X-ray evidence (14) indicates that the aromatic side chain of the substrate fits into a cavity which lies to the lower right of the reactive histidine-serine pair (c.f. Fig. 23); it is presumably held there largely by hydrophobic bonding. Serine-189 is situated at the bottom of this cavity. In trypsin the positively-charged side chain of the substrate, or the corresponding grouping in the case of a non-cationic substrate, is held in a similar position, there being electrostatic attraction to the

aspartate-177 residue of the enzyme in the case of the cationic substrates. We suggest that this anionic binding site makes an important contribution to the stereospecificity of trypsin.

Various mechanisms involving the imidazole and serine hydroxyl groups have been proposed for α -chymotrypsin catalysis (2). Some of these mechanisms contained features which were later found to be inconsistent with experimental evidence, notably

(a) the existence of a large deuterium-isotope effect in the acylation and deacylation steps,

(b) the absence of an observable intermediate in the deacylation process,

(c) the presence of a single histidine group in the active center, and

(d) the conformation-controlling role attributed to the N-terminal residue.

Mechanisms involving aspartic acid or arginine residues in the catalytic process can now be eliminated in view of the additional information derived from the

three-dimensional structure of α -chymotrypsin (13,14,16,17). The association of specific methionine (186,187), tyrosine (185,188) and tryptophan (189,190) residues with some aspects of the catalytic mechanism has also been studied. Sigler et al. (17) have recently reported that in crystalline chymotrypsin, methionine-192 and tyrosine-146 are involved in intermolecular interactions, the exact nature of which is not always clear. Present evidence suggests that none of these residues directly participates in the catalytic mechanism of trypsin or chymotrypsin.

APPENDIX A

LEAST-SQUARES-FIT COMPUTER PROGRAM

The digital computer program which calculates F in the pre-steady state rate equation

$$[65] \quad A - \Delta A = Q(1 - e^{Ft})$$

is given. The output of a typical experimental run is also shown.


```

READ (1,14) (DA(MM),MM=1,M)
READ (1,15) (JJ(MM),MM=1,M)
READ (1,15) (KK(MM),MM=1,M)
DO 13 MM=1,M
  SQDEV1=100.0
  SQDEV2=1000.0

```

C
C
C

CALCULATION OF ABSORBANCE AND TIME

```

READ (1,1) B,C,N,TITLE
READ (1,31) TSCALF,HRO
READ (1,9) (VR(I),I=1,N)
WRITE (3,10) TITLE,B,C,N
WRITE (3,32) TSCALF,HRO
WRITE (3,19)
WRITE (3,11) (VR(I),I=1,N)
DO 12 I=1,N
  TRANS(I)=100.0-(8*VR(I)/C)
  A(I)=ALOG10(100.0/TRANS(I))
  WRITE (3,20)
  WRITE (3,11) (TRANS(I),I=1,N)
  TIMEQ=(HRO/C)*TSCALE
  T(1)=1.5*TSCALE-TIMEQ
DO 29 I=2,N
  T(I)=T(I-1)+0.5*TSCALE
  WRITE (3,21)
  WRITE (3,11) (T(I),I=1,N)
  WRITE (3,22)
  WRITE (3,11) (A(I),I=1,N)

```

C
C
C

CALCULATION OF LEAST SQUARES FIT PARAMETERS

```

WRITE (3,23)
WRITE (3,24)
JJJ=JJ(MM)
DDD=DA(MM)
DO 4 J=1,JJJ
  Q(MM)=Q(MM)+1.0E-04
  KKK=KK(MM)
  DA(MM)=DDD
DO 7 K=1,KKK
  DA(MM)=DA(MM)+1.0E-05
SUMANT=0.0
SUMT2=0.0

```

```
DO 3 I=1,N
  DIFF(I)=1.0-(A(I)-DA(MM))/Q(MM)
  IF(DIFF(I))26,26,27
026 WRITE(3,25)
    GO TO 13
027 AN(I)=ALOG(DIFF(I))
  SUMANT=SUMANT+AN(I)*T(I)
003 SUMT2=SUMT2+T(I)*T(I)
  F=SUMANT/SUMT2
  SQDEV=0.0
DO 5 I=1,N
  DEV=AN(I)-F*T(I)
005 SQDFV=SQDEV+DEV*DEV
  IF (SQDEV2-SQDEV1) 16,17,17
016 IF (SQDEV2-SQDEV) 18,17,17
018 WRITE (3,6) Q1,D1,F1,SQDEV2
017 SQDEV1=SQDEV2
  SQDEV2=SQDEV
  Q1=Q(MM)
  D1=DA(MM)
  F1=F
007 CONTINUE
004 CONTINUE
013 CONTINUE
  RETURN
END
```

PSIN-CANE, RUN NO.20L, PH=6.90

1.25C C= 65.0 N= 18

ALE= 0.10 HRC= 34.0

VERTICAL READINGS			
135.0000	179.0000	217.0000	254.0000
316.5000	343.0000	368.0000	391.5000
434.0000	453.5000	470.0000	486.0000
514.0000	526.0000	538.0000	286.5000
			414.0000
			500.5000

PER CENT TRANSMISSION			
97.4038	96.5577	95.8269	95.1154
93.9135	93.4038	92.9231	92.4711
91.6538	91.2788	90.9615	90.6538
90.1154	89.8846	89.6538	94.4904
			92.0385
			90.3750

TIME READINGS (SEC.)

0.0977	0.1477	0.1977	0.2477	0.2977
0.3477	0.3977	0.4477	0.4977	0.5477
0.5977	0.6477	0.6977	0.7477	0.7977
0.8477	0.8977	0.9477		

ABSORBANCE

0.0114	0.0152	0.0185	0.0217	0.0246
0.0273	0.0296	0.0319	0.0340	0.0360
0.0378	0.0396	0.0411	0.0426	0.0440
0.0452	0.0463	0.0474		

LEAST SQUARES FIT PARAMETERS

Q	DA	F	MIN. SQDEV
0.561000E-01	0.351000E-02	-0.159615E 01	0.769850E-03
0.562000E-01	0.350000E-02	-0.159149E 01	0.651572E-03
0.563000E-01	0.350000E-02	-0.158605E 01	0.545961E-03
0.564000E-01	0.350000E-02	-0.158065E 01	0.452489E-03
0.565000E-01	0.351000E-02	-0.157450E 01	0.370621E-03
0.566000E-01	0.351000E-02	-0.156919E 01	0.299769E-03
0.567000E-01	0.351999E-02	-0.156313E 01	0.239432E-03
0.568000E-01	0.353999E-02	-0.155633E 01	0.189076E-03
0.569000E-01	0.354999E-02	-0.155037E 01	0.148115E-03
0.570000E-01	0.356999E-02	-0.154368E 01	0.116028E-03
0.571000E-01	0.358999E-02	-0.153706E 01	0.922656E-04
0.572000E-01	0.360999E-02	-0.153048E 01	0.763205E-04
0.573000E-01	0.362999E-02	-0.152397E 01	0.676817E-04
0.574000E-01	0.365999E-02	-0.151675E 01	0.658110E-04
0.575000E-01	0.368999E-02	-0.150960E 01	0.702687E-04
0.576000E-01	0.371999E-02	-0.150251E 01	0.805616E-04
0.577000E-01	0.374999E-02	-0.149548E 01	0.962519E-04
0.578000E-01	0.378999E-02	-0.148778E 01	0.116869E-03
0.579000E-01	0.382999E-02	-0.148014E 01	0.142056E-03
0.580000E-01	0.385999E-02	-0.147331E 01	0.171394E-03
0.581000E-01	0.389999E-02	-0.146581E 01	0.204521E-03
0.582000E-01	0.393999E-02	-0.145838E 01	0.241075E-03
0.583000E-01	0.397999E-02	-0.145102E 01	0.280742E-03
0.583999E-01	0.402997E-02	-0.144302E 01	0.323195E-03
0.584999E-01	0.406997E-02	-0.143579E 01	0.368165E-03
0.585999E-01	0.410996E-02	-0.142862E 01	0.415381E-03
0.586999E-01	0.415995E-02	-0.142083E 01	0.464628E-03
0.587999E-01	0.419995E-02	-0.141380E 01	0.515589E-03
0.588999E-01	0.423994E-02	-0.140682E 01	0.568131E-03
0.589999E-01	0.428994E-02	-0.139923E 01	0.622017E-03

APPENDIX B

DIGITAL READ-OUT FOR STOPPED-FLOW
SPECTROPHOTOMETER

The unit has been specifically designed to digitize the analog signal received from the photomultiplier tube of the stopped-flow spectrophotometer. The information digitized consists of a rapidly rising and exponentially decaying dc voltage. The system is triggered by the rising voltage and samples the signal at preset time intervals ranging from 2.5 msec to 5 sec. The present maximum is 20 samples per run. Input sensitivity can be varied from 50 mv to 1 v.

The system samples information from one channel only, converts and stores this information and prints the data out after a suitable time interval. An intermediate memory is required to buffer between the input sampling speed and the teletype output speed. The unit consists of five basic blocks as follows:

Analog Section

- 1) Input amplifier and offset control
- 2) Analog to digital converter

Digital Section

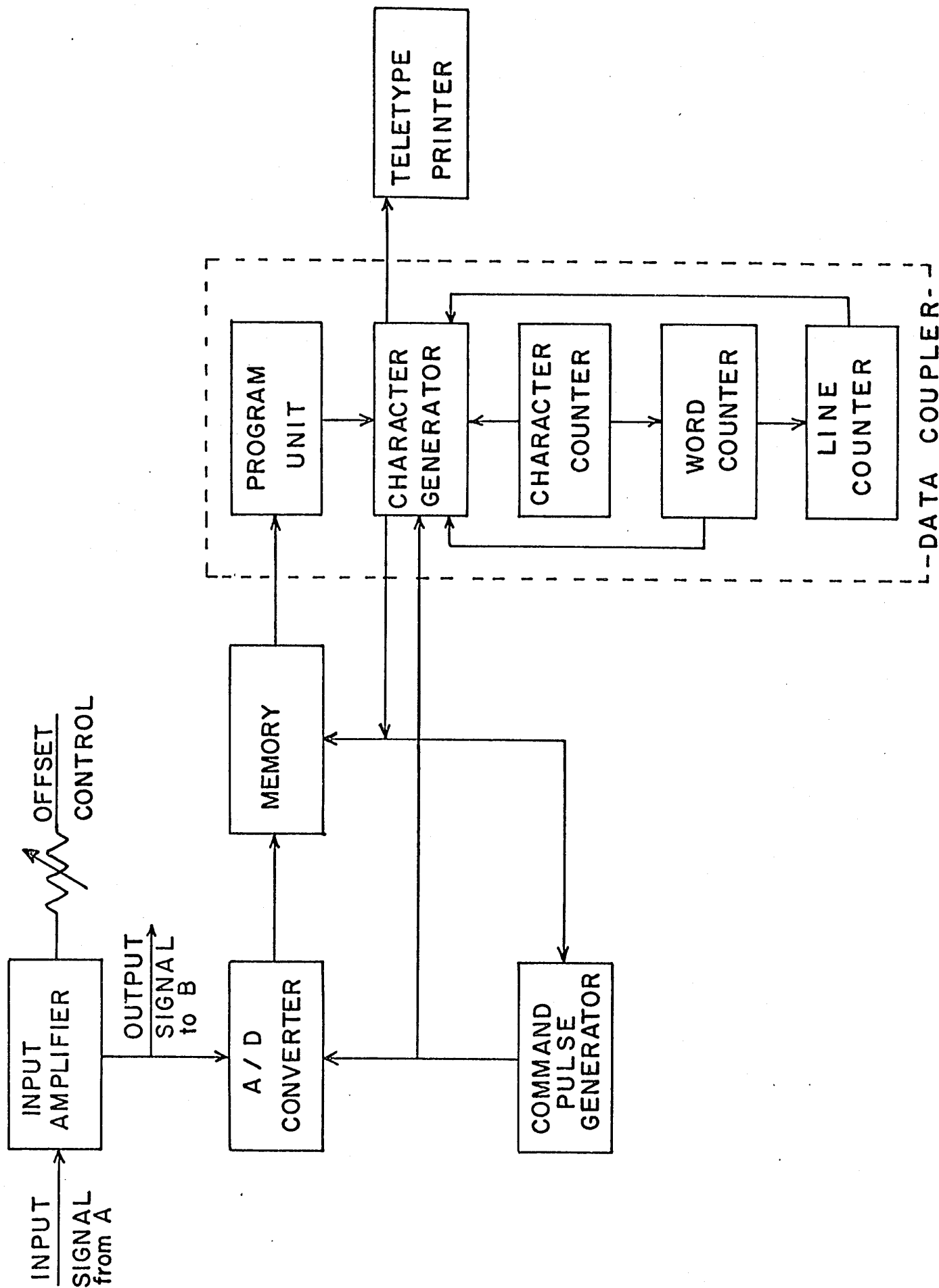
- 3) Memory
- 4) Data coupler
- 5) Teletype printer

A manually-operated selector switch is also provided which permits the print of an indentifying number from 0 - 99 at the start of each run.

The storage oscilloscope of the stopped-flow spectrophotometer system can be operated in series with the digital read-out if desired. The negative voltage at the output of the time-constant coupling circuit of the oscilloscope is amplified and compensated by the operational amplifiers of the digital read-out. The signal is then applied to the A/D converter and/or to the positive input of the differential amplifier of the storage oscilloscope. These positions are marked A and B respectively in Figures 12 and 29.

Figure 29

Block diagram of the digital read-out
for the stopped-flow spectrophotometer.



CLAIMS TO ORIGINAL RESEARCH

1. A new substrate, an ester derivative of N- α -substituted-L-alanine, has been found for trypsin.
2. The steady-state kinetics of the trypsin-catalyzed hydrolysis of N-benzoyl-L-alanine methyl ester have been studied at low substrate concentrations. This has not been possible in the past with specific ester substrates of trypsin. It was shown that a single group with $pK \approx 7$ (presumably the imidazole group of a histidine residue) is free to ionize in the free enzyme, but participates in the acylation and deacylation processes. It was concluded that the group with $pK \approx 9.5$, which was only revealed in the deacylation step, is not catalytically involved in this process.
3. The specific levorotation of trypsin as a function of pH has been shown to follow the ionization of a single group with $pK(\text{app}) = 9.4$. With reference to the known activation process of trypsinogen and its optical rotary behaviour, this pK has been related to the α -amino group of the N-terminal isoleucine residue of trypsin.

4. From the variation of specific rotation with pH for trypsin and for reversibly and irreversibly inhibited trypsin, it was concluded that ionization of the group with $pK \approx 9.5$ is very sensitive to modifier-induced conformational changes in enzyme structure.

5. Equations describing the pH dependence of the transient phase of enzyme reactions involving two intermediates have been formulated for the condition of excess enzyme concentration.

6. A digital computer program has been developed to evaluate transient-phase rate data directly by a reiterative least-squares-fit technique.

7. The transient-phase kinetics of the trypsin-catalyzed hydrolysis of N-carbobenzoxy-L-alanine-p-nitrophenyl ester were studied at excess enzyme concentration. The existence of an intermediate Michaelis complex in the three-step mechanism was confirmed. It was shown that a group with $pK=6.9$ is involved in the breakdown of the Michaelis complex but not in its formation.

8. An investigation has been made of the effect of isopropanol concentration on the transient-phase parameters for the trypsin-catalyzed hydrolysis of N-carbobenzoxy-L-alanine-p-nitrophenyl ester. It has been concluded that the observed decrease in binding and rate of hydrolysis can be justified by a loss in conformational integrity of the enzyme resulting from enhanced electrostatic and hydrophobic interactions.

9. A new mechanism of action was proposed for trypsin-catalyzed reactions. A single histidine residue is postulated to act as a proton-transfer agent during acylation and deacylation, being particularly suited for this purpose on account of the tautomeric nature of its imidazole ring. A serine hydroxyl group is the nucleophile in acylation, a water molecule in deacylation. It is suggested that the N-terminal isoleucine residue contributes to control of the enzyme conformation at alkaline pH and thereby to its activity.

REFERENCES

1. P. Desnuelle, "The Enzymes", P.D. Boyer, H. Lardy and K. Myrbäck (eds.), Vol. 4, Academic Press Inc., New York, 1960, p. 93.
2. L. Cunningham, "Comprehensive Biochemistry", M. Florkin and E.H. Stotz (eds.), Vol. 16, Elsevier, Amsterdam, 1965, p. 85.
3. L.B. Smillie, A. Furka, N. Nagabhushan, K.J. Stevenson and C.O. Parkes, Nature, 218, 343 (1968).
4. M. Kunitz, J. Gen. Physiol., 22, 293, 429, 447 (1939).
5. J.H. Northrop, M. Kunitz and R.H. Herriott (eds.), "Crystalline Enzymes", 2nd ed., Columbia University Press, New York, 1948, Chap. 6.
6. E.W. Davie and H. Neurath, J. Biol. Chem., 212, 515 (1955).
7. P. Desnuelle and C. Fabre, Biochim. Biophys. Acta, 18, 29 (1955).
8. M.R. MacDonald and M. Kunitz, J. Gen. Physiol., 25, 53 (1941).
9. C. Gabeloteau and P. Desnuelle, Arch. Biochem. Biophys., 69, 475 (1957).
10. M. Delaage and M. Lazdunski, Biochem. Biophys. Res. Commun., 28, 390 (1967).

11. M. Delaage, P. Desnuelle and M. Lazdunski, *Biochem. Biophys. Res. Commun.*, 29, 235 (1967).
12. T.M. Radhakrishnan, K.A. Walsh and H. Neurath, *J. Am. Chem. Soc.*, 89, 3059 (1967).
13. D.M. Blow, J.J. Birktoft and B.S. Hartley, *Nature*, 221, 337 (1969).
14. D.M. Blow, Biochemical Conference on Protein Structure and Function, Ste. Marguerite, Quebec, March 2-7, 1969.
15. H. Neurath, K.A. Walsh and W.P. Winter, *Science*, 158, 1638 (1967).
16. B.W. Matthews, P.B. Sigler, R. Henderson and D.M. Blow, *Nature*, 214, 652 (1967).
17. P.B. Sigler, D.M. Blow, B.W. Matthews and R. Henderson, *J. Mol. Biol.*, 35, 143 (1968).
18. S. Maroux, M. Rivery and P. Desnuelle, *Biochim. Biophys. Acta*, 140, 377 (1967).
19. O. Mikeš, V. Tomášek, V. Holeyšovský and F. Šorm, *Collect. Czechoslov. Chem. Commun.*, 32, 655 (1967).
20. F.R. Bettelheim and H. Neurath, *J. Biol. Chem.*, 212, 241 (1955).
21. D.D. Schroeder and E. Shaw, *J. Biol. Chem.*, 243, 2943 (1968).

22. G.E. Perlman, Nature, 173, 406 (1954).
23. R.L. Hill and E.L. Smith, Biochim. Biophys. Acta, 19, 376 (1956).
24. R.L. Hill and E.L. Smith, J. Biol. Chem. 235, 2332 (1960).
25. A.K. Balls and E.F. Jansen, Advan. Enzymol. 13, 32 (1952).
26. N.K. Schaffer, R.P. Lang, L. Simet and R.W. Drisko, J. Biol. Chem., 230, 185 (1958).
27. G.H. Dixon, D.L. Kauffman and H. Neurath, J. Biol. Chem., 233, 1373 (1958).
28. W.B. Lawson, M.D. Leafer, Jr., A. Tewes and G.J.S. Rao, Hoppe-Seyler's Z. Physiol. Chem., 349, 251 (1968).
29. G.H. Dixon and H. Neurath, Fed. Proc., 16, 173 (1957).
30. R.A. Oosterbaan and M.E. van Adrichem, Biochim. Biophys. Acta, 27, 423 (1958).
31. H. Gutfreund, Trans. Faraday Soc., 51, 441 (1955).
32. J.-J. B  chet, J. Chim. Phys., 61, 584 (1964).
33. C.J. Martin, J. Golubow and A.E. Axelrod, J. Biol. Chem., 234, 1718 (1959).
34. M.L. Bender and E.T. Kaiser, J. Am. Chem. Soc., 84, 2556 (1962).

35. J.A. Stewart and L. Ouellet, Can. J. Chem., 37, 751 (1959).
36. E. Shaw, M. Mares-Guia and W. Cohen, Biochemistry, 4, 2219 (1965).
37. E. Shaw and S. Springhorn, Biochem. Biophys. Res. Commun., 27, 391 (1967).
38. V. Tomášek, E.S. Severin and F. Šorm, Biochem. Biophys. Res. Commun., 20, 545 (1965).
39. J.G. Beeley and H. Neurath, Biochemistry, 7, 1239 (1968).
40. M. Mares-Guia and E. Shaw, J. Biol. Chem., 240, 1579 (1965).
41. M. Mares-Guia, E. Shaw and W. Cohen, J. Biol. Chem., 242, 5777 (1967).
42. T. Inagami and H. Hatano, J. Biol. Chem., 244, 1176 (1969).
43. T. Inagami and S.S. York, Biochemistry, 7, 4045 (1968).
44. B.M. Sanborn and G.E. Hein, Biochemistry, 7, 3616 (1968).
45. B.M. Sanborn and W.P. Bryan, Biochemistry, 7, 3624 (1968).
46. M. Bergmann, J. Fruton and H. Pollok, J. Biol. Chem., 127, 643 (1939).
47. G.W. Schwert, H. Neurath, S. Kaufman and J.E. Snoke, J. Biol. Chem., 172, 221 (1948).

48. H. Neurath and G.W. Schwert, Chem. Revs., 46, 69 (1950).
49. S.G. Waley and J. Watson, Biochem. J., 57, 529 (1954).
50. K. Kitagawa and N. Izumiya, J. Biochem. (Japan), 46, 1159 (1959).
51. N. Izumiya, H. Okazaki, I. Matsumoto and H. Takiguchi, J. Biochem. (Japan), 46, 1347 (1959).
52. N. Baines, J. Baird and D. Elmore, Biochem. J., 90, 470 (1964).
53. J. Baird, E. Curragh and D. Elmore, Biochem. J., 96, 733 (1965).
54. D. Elmore, D. Roberts and J. Smyth, Biochem. J., 102, 728 (1967).
55. M. Dixon and E.C. Webb, "Enzymes", Academic Press Inc., New York, 1958, p. 116, 243.
56. L. Benoiton and J. Deneault, Biochim. Biophys. Acta, 113, 613 (1966).
57. M. Gorecki and Y. Shalitin, Biochem. Biophys. Res. Commun., 29, 189 (1967).
58. H.A. Ravin, P. Bernstein and A.M. Seligman, J. Biol. Chem., 208, 1 (1954).
59. T. Inagami and J.M. Sturtevant, J. Biol. Chem., 235, 1019 (1960).

60. B.H.J. Hofstee, *Biochim. Biophys. Acta*, 24, 211 (1957).
61. B.F. Erlanger, N. Kokowski and W. Cohen, *Arch. Biochem. Biophys.*, 95, 271 (1961).
62. H.P. Kasserra, unpublished results.
63. A. Himoe, P.C. Parks and G.P. Hess, *J. Biol. Chem.*, 242, 919 (1967).
64. J. McConn, E. Ku, C. Odell, G. Czerlinski and G.P. Hess, *Science*, 161, 274 (1968).
65. J.H. Wang and L. Parker, *Proc. Natl. Acad. Sci. U.S.*, 58, 2451 (1967).
66. K.J. Laidler, "The Chemical Kinetics of Enzyme Action", Oxford University Press, London, 1958, p. 63, 117, 205.
67. L. Peller and R.A. Alberty, *J. Am. Chem. Soc.*, 81, 5907 (1959).
68. M.L. Bender and F.J. Kézdy, *Ann. Rev. Biochem.*, 34, 49 (1965).
69. I.B. Wilson, "The Enzymes", P.D. Boyer, H. Lardy and K. Myrback (eds.), Vol. 4, Academic Press Inc., New York, 1960, p. 501.
70. D.M. Brown and A.R. Todd, "The Nucleic Acids", Vol. I, E. Chargaff and J.N. Davidson, (ed.), Academic Press Inc., New York, 1955, p. 409.

71. K. Hiromi, Y. Takasaki and S. Ono, Bull. Chem. Soc. Japan, 36, 563 (1963).
72. H. Kaplan and D.R. Whitaker, Can. J. Biochem., 47, 305 (1969).
73. Report of the Commission on Enzymes of the International Union of Biochemistry, Pergamon Press, New York, 1961, p. 13.
74. B. Zerner and M.L. Bender, J. Am. Chem. Soc., 86, 3669 (1964).
75. R.M. Krupka and K.J. Laidler, Trans. Faraday Soc., 56, 1467 (1960).
76. R.M. Krupka, private communication.
77. J.L. Webb, "Enzyme and Metabolic Inhibitors", Vol. I, Academic Press Inc., New York, 1963, p. 701.
78. H. Kaplan and K.J. Laidler, Can. J. Chem., 45, 539 (1967).
79. K.J. Laidler, Trans. Faraday Soc., 51, 528 (1955).
80. J.-J. Béchet, J. Chim. Phys., 62, 1095 (1965).
81. T. Inagami and J.M. Sturtevant, Biochim. Biophys. Acta, 38, 64 (1960).
82. P. Benmouyal and C.G. Trowbridge, J. Chim. Phys., 62, 576 (1965).
83. P. Urnes and P. Doty, Advan. Protein Chem., 16, 402 (1961).

84. W.F. Harrington, R. Josephs and D.M. Segal, Ann. Rev. Biochem., 35, 599 (1966).
85. H.A. Scheraga, "Protein Structure", Academic Press Inc., New York, 1961, p. 177.
86. J. McConn, G.D. Fasman and G.P. Hess, J. Mol. Biol., 39, 551 (1969).
87. H. Kaplan and H. Dugas, Biochem. Biophys. Res. Commun., 34, 681 (1969).
88. G.M. Paterson and D.R. Whitaker, Can. J. Biochem., 47, 317 (1969).
89. H. Kaplan and K.J. Laidler, Can. J. Chem., 45, 547 (1967).
90. G.E. Hein and C. Niemann, J. Am. Chem. Soc., 84, 4487 (1962).
91. L.W. Cunningham, Jr., J. Biol. Chem., 211, 13 (1954).
92. V. Kostka and F.H. Carpenter, J. Biol. Chem., 239, 1799 (1964).
93. N. Kobamoto, G. Löfroth, P. Camp, G. Van Amburg and L. Augenstein, Biochem. Biophys. Res. Commun., 24, 622 (1966).
94. G. Hein and C. Niemann, J. Am. Chem. Soc., 84, 4495 (1962).
95. H.R. Almond, Jr. and C. Niemann, Biochim. Biophys. Acta, 44, 143 (1960).

96. D.W. Ingles and J.R. Knowles, *Biochem. J.*, 99, 275 (1966).
97. K.R. Hanson, R. Ling and E. Havir, *Biochem. Biophys. Res. Commun.*, 29, 194 (1967).
98. C.I. Bliss and A.T. James, *Biometrics*, 22, 573 (1966).
99. M. Dixon, *Biochem. J.*, 55, 161, 170 (1953).
100. J. Labouesse and M. Gervais, *European J. Biochem.*, 2, 215 (1967).
101. J. Chevallier and J. Yon, *Biochim. Biophys. Acta*, 112, 116 (1966).
102. R. Shukuya and K. Watanabe, *J. Biochem. (Japan)*, 44, 481 (1957).
103. S.-S. Wang and F.H. Carpenter, *J. Biol. Chem.*, 243, 3702 (1968).
104. G. Gutfreund and J.M. Sturtevant, *Proc. Natl. Acad. Sci. U.S.*, 42, 719 (1956).
105. A. D'Albis and J.-J. B  chet, *Biochim. Biophys. Acta*, 140, 435 (1967).
106. H. Neurath, J.A. Rupley and W.J. Dreyer, *Arch. Biochem. Biophys.*, 65, 243 (1956).
107. H.L. Oppenheimer, B. Labouesse and G.P. Hess, *J. Biol. Chem.*, 241, 2720 (1966).

108. M. Mares-Guia, Arch. Biochem. Biophys., 127, 317 (1968).
109. T. Inagami, J. Biol. Chem., 239, 787 (1964).
110. J.T. Edsall and J. Wyman, "Biophysical Chemistry, Vol. I, Academic Press Inc., New York, 1958, p. 459, 470.
111. S.T. Scrimger and T. Hofmann, J. Biol. Chem., 242, 2528 (1967).
112. T.R. Hopkins and J.D. Spikes, Biochem. Biophys. Res. Commun., 28, 480 (1967) and 30, 540 (1968).
113. J. Chevallier, J. Yon, G. Spotorno and J. Labouesse, European J. Biochem., 5, 480 (1968).
114. B.S. Hartley and B.A. Kilby, Biochem. J., 50, 672 (1952).
115. B.S. Hartley and B.A. Kilby, Biochem. J., 56, 288 (1954).
116. L. Michaelis and M.L. Menten, Biochem. Z., 49, 333 (1913).
117. A.K. Balls and F.L. Aldrich, Proc. Natl. Acad. Sci. U.S., 41, 190 (1955).
118. H. Gutfreund and J.M. Sturtevant, Biochem. J., 63, 656 (1956).
119. G.H. Dixon and H. Neurath, J. Biol. Chem., 225, 1049 (1957).

120. F.J. Kézdy and M.L. Bender, Biochemistry, 1, 1097 (1962).
121. T. Spencer and J.M. Sturtevant, J. Am. Chem. Soc., 81, 1874 (1959).
122. M.L. Bender, G.R. Schonbaum and B. Zerner, J. Am. Chem. Soc., 84, 2562 (1962).
123. M.L. Bender, F.J. Kézdy and J. Feder, J. Am. Chem. Soc., 87, 4953 (1965).
124. L. Faller and J.M. Sturtevant, J. Biol. Chem., 241, 4825 (1966).
125. M.L. Bender and T.H. Marshall, J. Am. Chem. Soc., 90, 201 (1967).
126. J.B. Milstien and T.H. Fife, Biochemistry, 8, 623 (1969).
127. Y. Pocker and J.T. Stone, Biochemistry, 7, 4139 (1968).
128. J.H. Park, B.P. Meriwether, P. Clodfelder and L.W. Cunningham, J. Biol. Chem., 236, 136 (1961).
129. C.G. Overberger, J.C. Salamone and S. Yaroslavsky, J. Am. Chem. Soc., 89, 6231 (1967).
130. J. Noguchi and H. Yamamoto, J. Biochem. (Japan), 65, 123 (1969).
131. G.E. Clement and M.L. Bender, Biochemistry, 2, 836 (1963).

132. M. Castañeda-Agulló and L.M. del Castillo, J. Gen. Physiol., 42, 617 (1959).
133. C.E. McDonald and A.K. Balls, J. Biol. Chem., 221, 993 (1956).
134. M.L. Bender, G.E. Clement, C.R. Gunter and F.J. Kézdy, J. Am. Chem. Soc., 86, 3697 (1964).
135. S.J. Singer, Advan. Protein Chem., 17, 1 (1962).
136. H. Hartridge and F.J.W. Roughton, Proc. Roy. Soc., A104, 376 (1923).
137. G.A. Millikan, Proc. Roy. Soc., A155, 277 (1936).
138. F.J.W. Roughton and B. Chance, "Technique of Organic Chemistry", S.L. Friess, E.S. Lewis and A. Weissberger (eds.), Vol. VIII, Part II, Interscience Publishers, New York, 1963, p. 703.
139. B. Chance, J. Franklin Inst., 229, 455, 613, 737 (1940).
140. Q.H. Gibson, Discuss. Faraday Soc., 17, 137 (1954).
141. M. Eigen and L. de Maeyer, "Technique of Organic Chemistry", S.L. Friess, E.S. Lewis and A. Weissberger (eds.), Vol. VIII, Part II, Interscience Publishers, New York, 1963, p. 895.
142. H. Gutfreund, "An Introduction to the Study of Enzymes", Blackwell Scientific Publications, Oxford 1965, p. 117..

143. K.J. Laidler, Can. J. Chem., 33, 1614 (1955).
144. L. Ouellet and K.J. Laidler, Can. J. Chem., 34, 146 (1956).
145. H. Gutfreund, Discuss. Faraday Soc., 20, 167 (1955).
146. L. Ouellet and J.A. Stewart, Can. J. Chem., 37, 737 (1959).
147. L. Peller and R.A. Alberty, Progress in Reaction Kinetics, 1, 236 (1961).
148. I.G. Darvey, J. Theoret. Biol., 19, 215 (1968).
149. H. Gutfreund, Discuss. Faraday Soc., 17, 220, (1954).
150. E.A. Guggenheim, Phil. Mag., 2, 538 (1926).
151. J.A. Stewart and H.S. Lee, J. Phys. Chem., 71, 3888 (1967).
152. W.E. Roseveare, J. Am. Chem. Soc., 53, 1651 (1931).
153. M. Goodman and K.C. Stueben, J. Am. Chem. Soc., 81, 3980 (1959).
154. G. Gomori, "Methods in Enzymology", S.P. Colowick and N.O. Kaplan (eds.), Vol. I, Academic Press Inc., New York, 1955, p. 138.
155. I.H. Hinberg, private communication.

156. H. Lineweaver and D. Burk, J. Am. Chem. Soc., 56, 658 (1934).
157. G. Åkerlöf, J. Am. Chem. Soc., 54, 4125 (1932).
158. E.J. Cohn and J.T. Edsall, "Proteins, Amino Acids and Peptides", Reinhold Publishing Co., New York, 1943, p. 473.
159. M. Bier and F.F. Nord, Arch. Biochem. Biophys., 33, 320 (1951).
160. W. Kauzmann, Advan. Protein Chem., 14, 1 (1959).
161. I.M. Klotz, "Protein Structure and Function", Brookhaven Symposium in Biology, Brookhaven National Laboratory, New York, 1960, p. 25.
162. I.M. Klotz and S.B. Farnham, Biochemistry, 7, 3879 (1968).
163. C. Tanford, "Physical Chemistry of Macromolecules", Wiley and Sons Inc., New York, 1961, chap. 8.
164. F. Seydoux, J. Yon and G. Némethy, Biochim. Biophys. Acta, 171, 145 (1969).
165. J.A. Stewart, H.S. Lee and J.E. Dobson, J. Am. Chem. Soc., 85, 1537 (1963).
166. J.A. Stewart and J.E. Dobson, Biochemistry, 4, 1086 (1965).

167. M.L. Bender, M.J. Gibian and D.J. Whelan, Proc. Natl. Acad. Sci. U.S., 56, 833 (1966).
168. N.K. Schaffer, S.C. May and W.H. Summerson, J. Biol. Chem., 206, 201 (1954).
169. A.K. Balls and H.N. Wood, J. Biol. Chem., 219, 245 (1956).
170. R.A. Oosterbaan, P. Kunst, J. Van Rotterdam and J.A. Cohen, Biochim. Biophys. Acta, 27, 556 (1958).
171. M.L. Bender, G.E. Clement, F.J. Kézdy and H. d'A. Heck, J. Am. Chem. Soc., 86, 3680 (1964).
172. T. Inagami, S.S. York and A. Patchornik, J. Am. Chem. Soc., 87, 126 (1965).
173. M. Eigen and G.G. Hammes, Advan. Enzymol., 25, 1 (1963).
174. M. Eigen, G.G. Hammes and K.J. Kustin, J. Am. Chem. Soc., 82, 3482 (1960).
175. K. Hofmann, "Imidazole and its Derivatives", Part I, in the Chemistry of Heterocyclic Compounds, A. Weissberger (consult. ed.), Interscience Publishers, Inc., New York, 1953, p. 30.
176. H. Goldenberg and V. Goldenberg, Arch. Biochem. Biophys., 29, 154 (1950).
177. M. Castañeda-Agulló and L.M. del Castillo, J. Gen. Physiol., 44, 19 (1960).

178. M. Castañeda-Agulló and L.M. del Castillo, *Biochim. Biophys. Acta*, 139, 398 (1967).
179. R. Mason and C.A. Ghiron, *Biochim. Biophys. Acta*, 51, 377 (1961).
180. R. Lumry and H. Eyring, *J. Phys. Chem.*, 58, 110 (1954).
181. D.E. Koshland, Jr., *Proc. Natl. Acad. Sci. U.S.*, 44, 98 (1958).
182. A. Yapel, R. Lumry and M.H. Han, *Fed. Proc.*, 25, 650 (1966).
183. W.P. Jencks, "Current Aspects of Biochemical Energetics", N.O. Kaplan and E. Kennedy (eds.), Academic Press Inc., New York, 1966, p. 273.
184. D. Karibian, C. Laurent, J. Labouesse and B. Labouesse, *European J. Biochem.*, 5, 260 (1968).
185. Y. Inada, M. Kamata, A. Matsushima and K. Shibata, *Biochim. Biophys. Acta*, 81, 323 (1964).
186. V. Holeyšovský and M. Lazdunski, *Biochim. Biophys. Acta*, 154, 457 (1968).
187. F.J. Kézdy, J. Feder and M.L. Bender, *J. Am. Chem. Soc.*, 89, 1009 (1967).
188. A.N. Glazer and F. Sanger, *Biochem. J.*, 90, 92 (1964).

189. J.F. Wootton and G.P. Hess, J. Am. Chem. Soc., 84, 440 (1962).
190. M.-A. Coletti-Previero, A. Previero and E. Zuckerkandl, J. Mol. Biol., 39, 493 (1969).