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KINETICS AND MECHANISM OF α-CHYMOTRYPSIN CATALYZED REACTIONS

by

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PREFACE

This thesis is divided into five chapters.

Chapter I deals with a discussion of the principle of microscopic reversibility. The principle as first enunciated by Tolman in 1924, applies to systems at equilibrium. Since that time it has found wide application to chemical reactions and many erroneous applications and conclusions have been made. Although there is no sound theoretical justification, the view has long been held that a reaction must occur in one direction by a mechanism that is the exact reverse of that in the reverse direction. The principle has been re-examined the object being to determine the conditions for which this view holds. A satisfactory solution to the problem was found by considering several different reaction schemes.

Chapters II, III and V deal with the pH dependence and mechanism of α-chymotrypsin catalyzed hydrolysis. Early workers considered the reaction to follow the classical Michaelis-Menten scheme in which a bimolecular complex between enzyme and substrate is formed. The next reaction was postulated to be the direct breakdown of this complex into free enzyme and products. This view was held for many years largely on the fact that all systems studied obeyed the kinetic

laws derived on the basis of this scheme. A new perspective was thrown on the problem of catalysis by hydrolytic enzymes when Hartley in 1954 postulated that the α-chymotrypsin catalyzed hydrolysis of p-nitrophenyl acetate proceeds through two intermediates. This hypothesis was shortly afterwards confirmed by stopped-flow studies and by the isolation of a new intermediate, the so-called acyl enzyme, in which the acyl moiety of an ester substrate has become esterified to a serine residue in the enzyme. This aspect became an area of intensive research with the result that several other hydrolytic enzymes, cholinesterase, trypsin, elastase and thrombin, were shown to have an acylated serine intermediate.

The inference that hydrolytic enzymes have a similar mechanism of action is inescapable and for this reason in the past five years prodigious efforts have been put forth in several laboratories to elucidate the mechanism of α-chymotrypsin catalyzed hydrolysis. The high interest in this particular enzyme stems from the fact that it is readily available in high purity, hydrolyses a wide variety of substrates, requires no activators or co-enzymes for activity and is stable in solution at ordinary temperatures. In spite of the many approaches used to study this enzyme, and the voluminous

amount of information accumulated, no firm decisions as to its mechanism of action have come forth, but many controversies have arisen. However, so much progress has been made that with no other enzyme is the complete understanding of the mechanism so close at hand. The present research was undertaken with a view to elucidating the nature and function of the chemical components of the active center in order to arrive at a more satisfactory over-all mechanism.

Chapter III deals with the inhibition of α-chymotrypsin catalyzed reactions. In 1960 Krupka and Laidler proposed novel kinetic schemes to explain inhibition phenomena for enzymes involving two intermediates. In particular, they demonstrated how mixed and non-competitive inhibition could arise without involving the classical explanation of ternary complexes between enzyme, substrate and inhibitor. As a further test of the generality of these schemes, the inhibition of α-chymotrypsin catalyzed hydrolysis was studied.

ACKNOWLEDGMENTS

This research was carried out under the supervision of Prof. K. J. Laidler without whose guidance this research could not have come to a successful completion. The author is especially grateful to him for the many hours he patiently devoted to discussions and advice during the preparation of this manuscript.

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ABSTRACT

The Kinetic Consequences of the Principle of Microscopic Reversibility

The principle of microscopic reversibility was formulated for elementary reactions at equilibrium. It follows from the principle that for any elementary reaction the favored reaction path in one direction must be the reverse of that in the opposite reaction, and that the ratio of rate constants is the equilibrium constant. The same is true for non-chain reactions occurring under steady-state conditions provided that the alternative paths are equivalent as far as kinetic order is concerned. For reactions occurring under non-steady-state conditions, for chain reactions even in the steady state, and for non-chain reactions in which the alternative paths are not kinetically equivalent, the preferred reaction path in one direction may be different from that in the other; in such cases the ratio of the over-all rate constants is not equal to the equilibrium constant. The principle of microscopic reversibility can never be used to prove the mechanisms of a reaction when that for the reverse reaction has been established; it can only be used to eliminate kinetically equivalent mechanisms which are not the exact reverse of that for the reaction in the opposite direction.

The Influence of pH on the Kinetics of Enzyme Reactions involving Two Intermediates

Enzyme reactions which occur according to the scheme $E + S \rightleftharpoons ES \longrightarrow P_1 + ES' \longrightarrow E + P_2$ follow the Michaelis law; this study deals with the effect of pH on the kinetic parameters which appear in the Michaelis equation. A general equation for pH effects is given. It is shown that the rate constant $\tilde{k}_c/\tilde{K}_m$, which is the second-order constant obtained by extrapolation to zero substrate concentration, can only give pK values for groups involved in the breakdown of the Michaelis complex. The various patterns of pH behavior are classified and discussed.

Studies in Mixed solvents; the Nature of the Ionizing Groups

The α-chymotrypsin-catalyzed hydrolysis of N-benzoyl-L-alanine methyl ester has been studied at low substrate concentrations over a range of pH, and in various dioxane-water mixtures. The results provide pK values for the groups that ionize in the free enzyme, and from the variation of these pKs with dielectric constant it is concluded that when protonated one group is cationic, the other neutral. The cationic group (pK $\sim$ 6.9 in water) is probably the imidazole group, which therefore is catalytically active in its neutral form. The neutral group (pK $\sim$ 9.2 in

pure water) is concluded to be probably a serine hydroxyl group, catalytically active in its neutral form.

Influence of pH, and the Mechanism of Reaction

An investigation has been made of the influence of pH on the kinetics of the α-chymotrypsin-catalyzed hydrolysis of N-benzoyl-D and L-alanine methyl esters, N-acetyl-L-tyrosine ethyl ester and p-nitrophenyl acetate. From studies over a range of substrate concentrations the variations with pH of $\tilde{k}_c$, $\tilde{K}_m$ and $\tilde{k}_c/\tilde{K}_m$ have been deduced. The results show that in the free enzyme there are ionizing groups of pK 6.9 and 9.2 and that these are involved in the subsequent breakdown of the enzyme-substrate complex. In the next stage, usually regarded as deacylation, only a group of pK 6.9 (presumably an imidazole group) is revealed. A number of possible reaction mechanisms, consistent with the evidence, are discussed. It is concluded that two imidazole groups probably play a role in the reaction. In complex formation one of these interacts with the carbonyl function of the substrate; in acylation the other imidazole group abstracts a proton from the serine hydroxyl and transfers it to the leaving alcohol moiety. The deacylation process is the reverse of this process, with water as the nucleophile.

The Inhibition of Enzyme Reactions Involving Two Intermediates

General steady-state equations are worked out for the case in which an inhibitor can combine with the free enzyme, the enzyme-substrate complex and also a second intermediate (e.g. an acyl enzyme). The equations are given in a form that is convenient for analyzing the experimental results, and a number of special cases are considered. It is shown how the type of inhibition depends not only on the nature of the inhibitor but on that of the substrate, an important factor being the rate-determining step of the reaction. A number of examples are given.

Mechanisms of Inhibition

An experimental study was made of the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tyrosine ethyl ester, inhibited by indole and phenol. Indole inhibits non-competitively, and analysis of the behavior shows that it binds to the enzyme and the acyl enzyme but not to the Michaelis complex; by binding to the acyl enzyme it blocks deacylation. Phenol exhibits competitive behavior, two molecules of phenol being bound to the free enzyme in a forced-order sequence. It is concluded from the kinetics that there is either no binding of phenol to the acyl enzyme,

or that there is binding which does not affect the rate of deacylation. A general mechanism of inhibition is shown to explain in a quantitative manner these and other inhibition results.

The Hydrolysis of D and L Esters

A temperature-dependence study was carried out on the α-chymotrypsin-catalyzed hydrolyses of N-benzoyl-D and L-alanine methyl esters. At 25°C there is a sharp break in the plot of log k against 1/T, the activation energy being lower at the higher temperature. This break is attributed to a rapid reversible denaturation of the enzyme at the higher temperatures. In the low temperature region the activation energies are $E_L^{\neq}$ = 16.2± 0.3 kcal. per mole and $E_D^{\neq}$ = 16.5± 0.6 kcal. per mole. The differences in the rates of deacylation are therefore due to the differences in entropies of activation, which at 298°K are $S_L^{\neq}$ = - 9.6 e.u. and $S_D^{\neq}$ = - 14.1 e.u. It is concluded that in the activated complex the enzyme is coiled differently around the two substrates.

CHAPTER I

GENERAL INTRODUCTION

Chymotrypsin is one of several enzymes found in the alimentary canal during the process of digestion. It is biosynthesized by the acinous cells of the pancreas in the form of an inactive precursor, chymotrypsinogen, and is carried as such by the pancreatic juice into the duodenum where it is activated to form chymotrypsin. The main role of this enzyme in the body is to catalyze the hydrolysis of peptide bonds during the intestinal digestion of proteins.

Activation of Chymotrypsinogen

The constituent proteins of pancreatic cells must be protected against autodestruction during the biosynthesis of proteolytic enzymes. The protective device adopted by the digestive organs is to manufacture such enzymes in the form of inactive zymogen precursors. Representative examples of zymogen formation in addition to chymotrypsinogen are trypsinogen, pepsinogen and procarboxypeptidase.

The first enzyme involved in the activation of pancreatic zymogens is enterokinase, a proteolytic enzyme

secreted by the mucous membranes of the intestine. Its prime function is the activation of trypsinogen to trypsin, which it accomplishes by cleaving the peptide bond between the sixth and seventh amino acids in the polypeptide chain of trypsinogen. Trypsin now becomes the key enzyme in the activation of chymotrypsinogen. It cleaves the peptide bond in the chymotrypsinogen molecule between residues fifteen and sixteen to yield an active chymotrypsin molecule. Unlike trypsin, chymotrypsin can act on itself, cleaving three additional bonds. The resulting fragments are enzymatically active only if the bond between the fifteenth and sixteenth residues is split by trypsin.

The activation process and the steps leading to the formation of α-chymotrypsin have been elucidated through the combined efforts of Jacobson, Neurath, Desnuelle and their co-workers(1-4). The steps which occur are listed below and are schematically illustrated in figs. 1 and 2.

Step I Trypsinogen $\xrightarrow{\text{enterokinase}}$ Trypsin

Step II Chymotrypsinogen $\xrightarrow{\text{trypsin}}$ δ-Chymotrypsin

One bond is opened between arginine_{15} and isoleucine_{16}. (The subscript denotes the position of the amino acid from the N-terminal residue in chymotrypsinogen).

Step III δ-Chymotrypsin $\xrightarrow{\text{chymotrypsin}}$ π-Chymotrypsin

$\text{seryl}_{14}\text{arginine}_{15}$

Figure 1

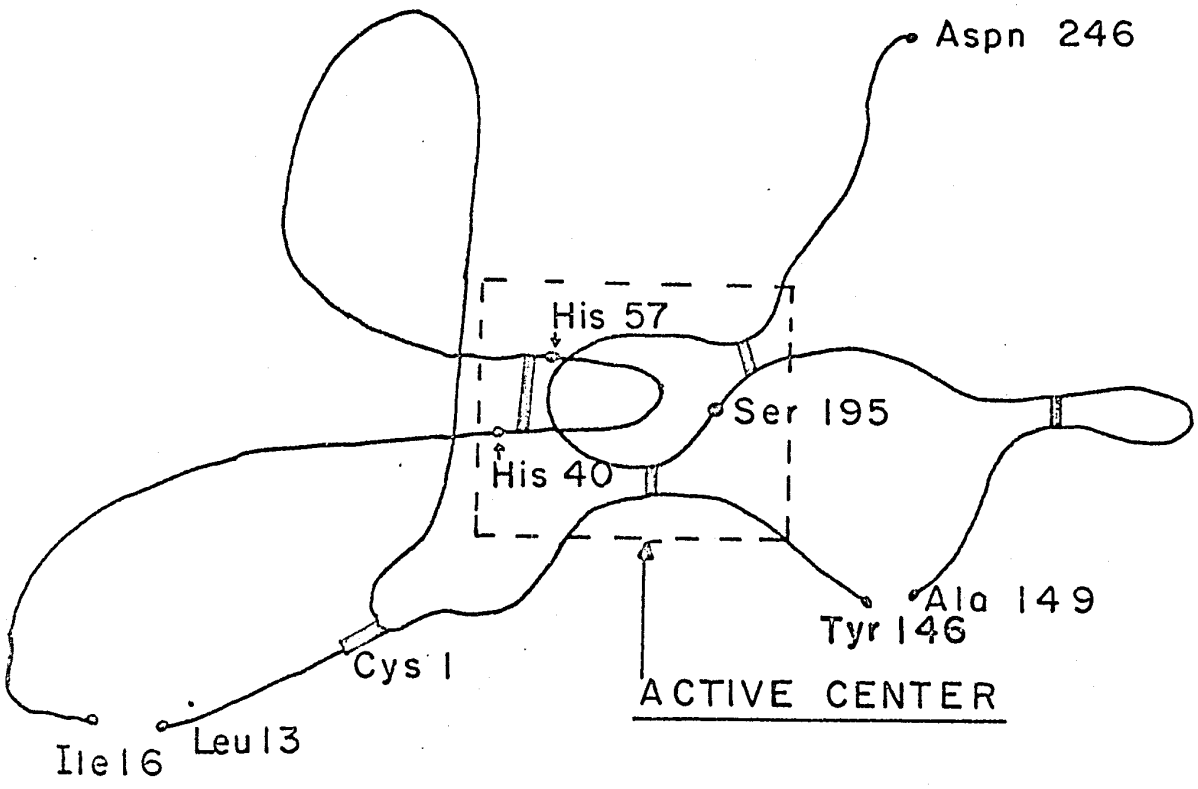
The amino acid sequences of chymotrypsinogen and trypsinogen (22). (↓ denotes a bond broken during the activation process.)

THE STRUCTURAL SIMILARITY OF TRYPSINOGEN AND CHYMOTRYPSINOGEN

```
                                1  2  3  4  5  6  7  8  9 10 11 12 13↓14 15↓16 17 18
Chymotrypsinogen                cys-gly-val-pro-ala-ile-gln-pro-val-leu-ser-gly-leu-ser-arg-ILE-VAL-GLY-
Trypsinogen                                                   val-asp-asp-asp-asp-lys-ILE-VAL-GLY-
                                                               1  2  3  4  5  6 ↑7  8  9

19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41
asp-glu-glu-ala-val-pro-gly-ser-trp-PRO-trp-GLN-VAL-SER-LEU-gln-asp-lys-thr-GLY-phe-HIS-PHE-
gly-tyr-thr-cys-gly-ala-asn-thr-val-PRO-tyr-GLN-VAL-SER-LEU-asn-        -ser-GLY-tyr-HIS-PHE-
10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25          26 27 28 29 30

42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64
CYS-GLY-GLY-SER-LEU-ILE-ASN-glu-asn-TRP-VAL-VAL-thr-ALA-ALA-HIS-CYS-gly-val-thr-thr-ser-asp-
CYS-GLY-GLY-SER-LEU-ILE-ASN-ser-gln-TRP-VAL-VAL-ser-ALA-ALA-HIS-CYS-tyr-lys-ser-gly-ile-gln-
31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53

65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87
VAL-val-val-ala-gly-glu-phe-asp-gln-gly-ser-ser-ser-glu-lys-ile-gln-lys-leu-lys-ile-ala-lys-
VAL-arg-leu-gly-glu-asp-asn-ile-asn-val-val-glu-gly-asp-glu-gln-phe-ile-ser-ala-ser-lys-ser-
54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76

88 89 90 91 92 93 94 95 96 97 98 99 100 101 102 103 104 105 106 107 108 109 110
val-phe-lys-asn-SER-lys-TYR-ASN-ser-leu-thr-ile-ASN-ASN-asn-ILE-thr-LEU-leu-LYS-LEU-ser-thr-
ile-val-his-pro-SER-    -TYR-ASN(pro,leu,thr,asn)ASN-ASN-asp-ILE-met-LEU-ile-LYS-LEU-lys-ser-
77 78 79 80 81      82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98

111 112 113 114 115 116 117 118 119 120 121 122 123 124 125 126 127 128 129 130 131 132 133
ALA-ALA-SER-phe-ser-gln-thr-VAL-ser-ala-val-cys-LEU-PRO-ser-ala-ser-asp-asp-phe-ala-ALA-GLY-
ALA-ALA-SER-leu-asn-ser-arg-VAL-ala-ser-ile-ser-LEU-PRO-thr-ser-cys-        -ala-ser-ALA-GLY-
99 100 101 102 103 104 105 106 107 108 109 110 111 112 113 114 115          116 117 118 119

134 135 136 137 138 139 140 141 142 143 144 145 146↓147 148↓149 150 151 152 153 154 155 156
THR-thr-CYS-val-thr-thr-GLY-TRP-GLY-leu-THR-arg-tyr-thr-asn-ala-asn-thr-PRO-ASP-arg-LEU-gln-
THR-glu-CYS-leu-ile-ser-GLY-TRP-GLY-asn-THR-lys-ser-ser-gly-thr-ser-tyr-PRO-ASP-val-LEU-lys-
120 121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136 137 138 139 140 141 142

157 158 159 160 161 162 163 164 165 166 167 168 169 170 171 172 173 174 175 176 177 178 179
gln-ala-ser-leu-PRO-leu-LEU-SER-asn-thr-asn-CYS-LYS-lys-tyr-trp-gly-thr-lys-ILE-lys-asp-ala-
cys-leu-lys-ala-PRO-ile-LEU-SER-asp-ser-ser-CYS-LYS-ser-ala-tyr-pro-gly-gln-ILE-thr-ser-asn-
143 144 145 146 147 148 149 150 151 152 153 154 155 156 157 158 159 160 161 162 163 164 165

180 181 182 183 184 185 186 187 188 189          190 191 192 193 194 195 196 197 198 199 200
MET-ile-CYS-ALA-GLY-ala-ser-gly-val-ser-        -SER-CYS-met-GLY-ASP-SER-GLY-GLY-PRO-leu-VAL-
MET-phe-CYS-ALA-GLY-tyr-leu-glu-gly-gly-lys-asn-SER-CYS-gln-GLY-ASP-SER-GLY-GLY-PRO-val-VAL-
166 167 168 169 170 171 172 173 174 175 176 177 178 179 180 181 182 183 184 185 186 187 188

201 202 203 204 205 206 207 208 209 210 211 212 213 214 215 216 217 218 219 220 221 222 223
CYS-lys-lys-asn-gly-ala-trp-thr-leu-val-GLY-ILE-VAL-ser-SER-TRP-GLY-SER-ser-thr-cys-ser-thr-
CYS-ser-gly-lys-leu-gln-              -GLY-ILE-VAL-   -SER-TRP-GLY-SER-gly-cys-ala-gln-lys-
189 190 191 192 193 194                195 196 197     198 199 200 201 202 203 204 205 206

224 225 226 227 228 229 230 231 232 233 234 235 236 237 238 239 240 241 242 243 244 245 246
ser-thr-PRO-GLY-VAL-TYR-ala-arg-VAL-thr-ala-leu-VAL-asn-TRP-val-gln-GLN-THR-leu-ALA-ala-ASN
asn-lys-PRO-GLY-VAL-TYR-thr-lys-VAL-cys-asn-tyr-VAL-ser-TRP-ile-lys-GLN-THR-ile-ALA-ser-ASN
207 208 209 210 211 212 213 214 215 216 217 218 219 220 221 222 223 224 225 226 227 228 229
```

Figure 2

A schematic diagram of α-chymotrypsin.


Aspn 246
His 57
Ser 195
His 40
Ala 149
Tyr 146
Cys 1
ACTIVE CENTER
Ile 16
Leu 13

Step IV π-Chymotrypsin $\xrightarrow{\text{chymotrypsin}}$ α-Chymotrypsin + threonyl$_{147}$asparagine$_{148}$

α-Chymotrypsin crystallizes more readily than the other chymotrypsins and thus is the most readily available form. Practically all kinetic studies on this enzyme have been carried out on α-chymotrypsin.

Active Center of α-Chymotrypsin

The term "active center" refers to those sites on an enzyme which are directly involved in the binding and activation of the substrate. Since substrates for enzymes are usually small compared to the entire enzyme, the active center is probably only a small fraction of the total enzyme. This view is supported by the fact that several hydrolytic enzymes have been reduced to smaller units with little or no loss of enzymic activity(5-7).

In the case of α-chymotrypsin, it has been possible to identify some components of the active center by the use of specific reagents which selectively attack functional groups. Diisopropyl phosphofluoridate reacts with α-chymotrypsin in a 1:1 stoichiometric reaction to give a product which contains one gram atom of phosphorus per mole of enzyme, and which is completely inactive enzymatically(8). When the inactive phosphorylated enzyme is degraded the phosphorus atom is found to be on the hydroxyl group of the amino acid serine(9). When

α-chymotrypsin is treated with p-nitrophenyl acetate at low pH, it is possible to isolate acetyl-α-chymotrypsin (10,11). Paralleling the finding with the phosphoryl-enzyme, the acetyl group is found to be bound to the hydroxyl group of a serine residue(12). Both diisopropyl phosphofluoridate and p-nitrophenyl acetate have been shown to inhibit the hydrolysis of N-acetyl-L-tyrosine ethyl ester, one of the best substrates of α-chymotrypsin(13). The evidence from these inhibition studies, coupled with that from degradative studies, indicates that α-chymotrypsin contains one active center per molecule and that this active center involves the amino acid serine.

The kinetic importance of an ionizable group of $pK_a \sim 7$ was first reported by Gutfreund and Sturtevant(14) and has later been substantiated by many workers(15). This group has been identified as the imidazole group of a histidine moiety, since imidazole is the only group in the enzyme with a pK_a near neutrality. There is much additional evidence which suggests that an imidazole group is catalytically active. For example, α-chymotrypsin is inactivated by the destruction of one histidine residue by either photooxidation(16) or by dinitrophenylation(17,18). A more recent study has shown that L-1-tosylamido-2-phenyl ethyl chloromethyl ketone inactivated α-chymotrypsin by alkylating a histidine residue(19). In summary, the evidence

for the participation of an imidazole group is well established experimentally.

Unfortunately, there is very little concrete information available concerning the binding loci which are an essential component of the active center. The best information available is derived from inhibition studies carried out with aromatic compounds by Niemann and co-workers(20). They have concluded that there are at least two binding sites, the primary binding site being a large planar surface surrounded by unsymmetrically disposed substituents which interact with the combining molecule. Other features are that there is a negative charge near one of the binding loci, and that the active center is bifunctional in the sense that one region is electron rich and the other electron deficient.

Amino Acid Sequence

Recently, the amino acid sequences of chymotrypsinogen and trypsinogen have been elucidated(21-24), and are shown in fig. 1. As a result of this work, it is possible to pin-point some of the active sites of α-chymotrypsin and trypsin. The reactive serine is located at position 195 in chymotrypsinogen and 183 in trypsinogen. The fact that there is a striking homology between the two enzymes in the regions extending from $serine_{190}$ to $half\text{-}cystine_{201}$ in chymotrypsinogen, and from $serine_{178}$ to

half-cystine$_{189}$ in trypsinogen, suggests that the entire region is of functional importance.

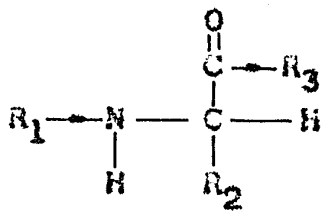
Chymotrypsinogen contains only two histidine residues (nos. 40 and 58) which are held very close together by means of a disulphide bridge. This fact alone would not be very significant, except for the fact that trypsinogen has an almost identical nineteen-amino-acid sequence containing two histidine residues. In view of the many similarities between chymotrypsin and trypsin it has been suggested that the finding of two histidines in close proximity on both enzymes is of mechanistic importance, and that two imidazole groups are catalytically active(25). Fig. 2 is a schematic diagram of α-chymotrypsin showing the position of the active sites.

Specificity

α-Chymotrypsin catalyses the hydrolysis of substrates whose structures and molecular dimensions vary over wide limits ranging from proteins to simple esters such as p-nitrophenyl acetate. For the natural protein and polypeptide substrates a marked specificity is shown for bonds connecting the carboxyl group of an aromatic residue to the amino group of another residue. From experiments carried out on a wide variety of substrates(26) it has been demonstrated that α-chymotrypsin rapidly hydrolyses tyrosyl, tryptophanyl and phenylalanyl peptides,

amides and esters. However, the specificity for aromatic bonds is not clear-cut since α-chymotrypsin will also split leucyl, methionyl, asparaginyl and glutaminyl bonds (27).

Hein and Niemann(28-30) have examined the specificity of reactions catalyzed by α-chymotrypsin, using acylated α-amino acid derivatives as model substrates. The binding of such a substrate as



proceeds with R_1, R_2 and COR_3 interacting with the active center of the enzyme. Specificity can therefore be studied by examining the effect of varying R_1, R_2 and R_3 on the Michaelis parameters $K_m(app)$ and $k_c(app)$. Two limiting types of substrates have been noted:

1) $S_{R_1\ R_3}$. R_1 and R_3 determine the magnitude of the binding constant of the substrate while R_2 is important only in the orientation of the substrate.

2) $S_{R_2\ R_3}$. R_2 and R_3 determine the binding constant and R_1 functions only to orientate the substrate.

In the transition $S_{R_1\ R_3} \longrightarrow S_{R_1\ R_2\ R_3} \longrightarrow S_{R_2\ R_3}$, increasing participation of R_2 in the binding process leads to decreasing values of $K_m(app)$ and increasing values of $k_c(app)$. Substrates of the limit type $S_{R_2\ R_3}$ are those for which R_2 = p-OH(C_6H_4) CH_2(tyrosyl), (C_6H_5) CH_2(phenylalanyl) or β-(C_8H_6N) CH_2(tryptophanyl) and R_3 = OCH_3 or OC_2H_5. The limit type $S_{R_2\ R_3}$ substrates are among the best substrates of α-chymotrypsin. The limit type $S_{R_1\ R_3}$ is approximated when R_2 = H or CH_3 or when R_3 = NH_2 or NH_2CH. $S_{R_1\ R_3}$ substrates are much poorer substrates than the limit type $S_{R_2\ R_3}$.

Removal of the acyl amino group or replacement of the α-amido group by a CH_2 leads to a marked decrease in activity(28). It therefore appears that the NH group in R_1' CONH- is an essential structural element with respect to the effectiveness of the R_1 group in orientation and binding of the substrate at the active site.

More recently Bender and Kézdy(31) have proposed that the specificity of α-chymotrypsin can be interpreted according to the equation

$$\log \frac{k_{cat}/K_m(app)\quad R_1R_2R_3}{k_{cat}/K_m(app)\quad R_{10}R_{20}R_{30}} = \sigma_{R_3}\,\rho_{R_3} + S_{R_1} + S_{R_2} \qquad (1)$$

in which R_{10}, R_{20} and R_{30} are arbitrarily chosen reference groups.

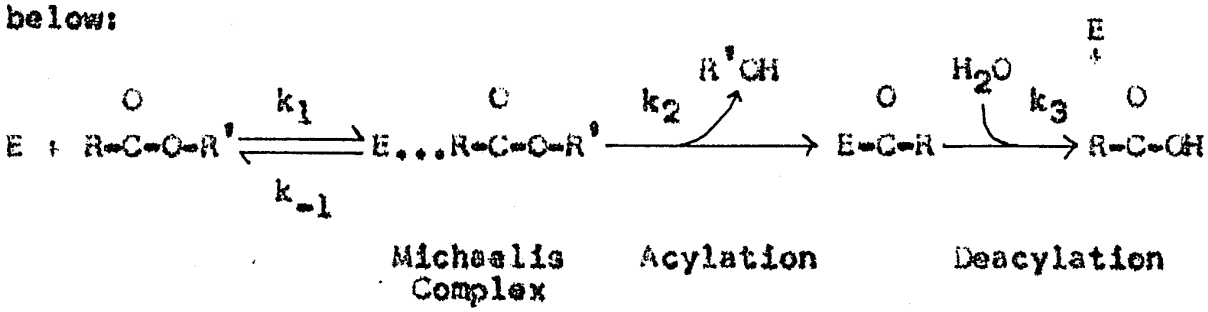
The first term arises from the fact that the rate constant $k_{cat}/K_m(app)$ shows Hammett-type linear-free-energy relationships for the leaving group R_3. The second and third terms are specificity factors for R_1 and R_2. The experimental results indicate that the relative specificities of R_1 and R_2 are independent of the nature of the other groups. Therefore either R_1 or R_2 can be assigned a specificity factor S_{R_1} or S_{R_2} relative to some arbitrary group R_{10} or R_{20}.

Stereospecificity

α-Chymotrypsin shows in most cases an absolute specificity for L-α-amino acids. However, if either R_1 or R_2 is small enough, the D-antipode can take up an orientation which places the R group in a position normally occupied by the α-hydrogen atom of the L-antipode, and will be hydrolyzed provided that COR_3 interacts at the appropriate locus. Examples(29) of loss of absolute stereospecificity occur when R_1 = H, CHO or R_2 = CH_3. In the case of D and L-3-carbomethoxy-dihydroisocarbostyril, there is an inversion of optical specificity, the D-antipode being hydrolyzed more rapidly than the L. In this case the loss of stereospecificity has been attributed to an exchange of binding loci for R_1 and R_2(28,30).

Mechanism

It is now well established, primarily through the efforts of Hartley and Kilby(22,33) and Gutfreund and Sturtevant(14), that the α-chymotrypsin-catalyzed hydrolysis of esters proceeds by a three-step mechanism as outlined below:



As mentioned previously, two catalytic entities have been identified as a serine hydroxyl and an imidazole group. At first it was thought that the imidazole group functions as a nucleophile, forming an acyl imidazole intermediate. This postulate was based largely on the fact that an acyl imidazole intermediate is formed in the imidazole catalysis of esters(34-36). However, the inactive monoacetyl derivative of α-chymotrypsin did not show any spectral evidence for acetyl histidine(37,38). More recently Bender and co-workers(39) failed to detect any observable build-up of an unstable acyl-imidazole in the deacylation of trans-cinnamoyl-α-chymotrypsin. Negative results do not, however, prove conclusively that an acyl imidazole intermediate does not exist, since it may just be present

in very small concentration. On the other hand Dixon and Neurath(40) reported that the deacetylation of acetyl-α-chymotrypsin is accompanied by a distinct increase in the absorption at 245 mμ, typical of the absorption of acetylimidazole, indicating that acetylimidazole makes a transitory appearance.

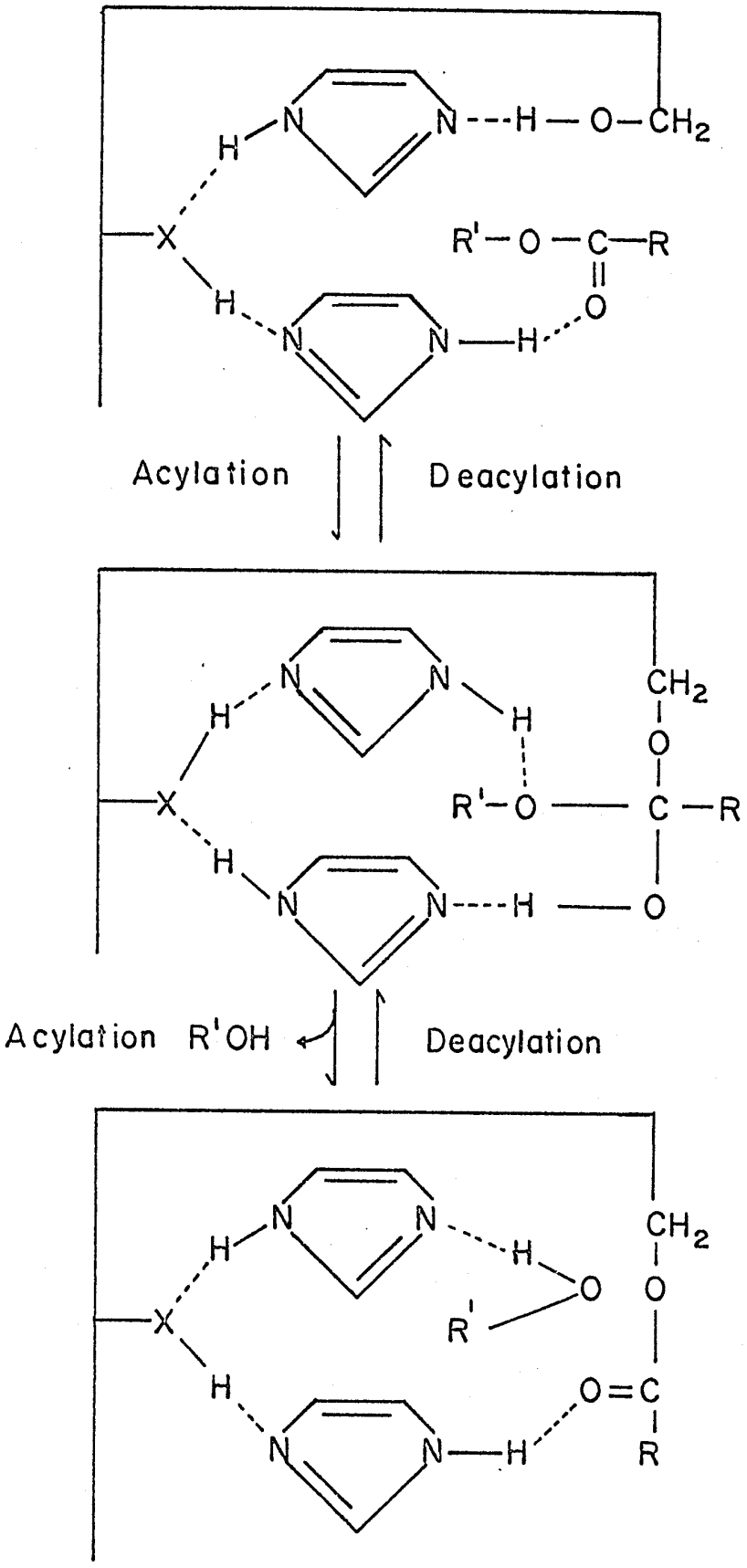
In order to reconcile the evidence for the participation of a serine and an imidazole group, Cunningham(41) and Neurath and Dixon(40) suggested that the imidazole group of histidine acts as a general base catalyst by abstracting a proton from the serine hydroxyl group, thereby aiding the nucleophilic attack by the serine hydroxyl. This hypothesis has recently been substantiated by Bender and co-workers(42), who have reported that deacylation in the presence of deuterium oxide produces an isotope effect (k_{H_2O}/k_{D_2O}) of 2-3. It therefore seems probable that in acylation and deacylation one imidazole functions as a general base catalyst carrying out a rate-determining proton transfer. In view of the recent finding that two imidazole groups may be involved, it seems reasonable to postulate that one functions as a general base and the other as a nucleophile. This hypothesis would reconcile the evidence for imidazole acting as a nucleophile and as a general base catalyst.

The efficiency of ∝-chymotrypsin action has been attributed to a concerted reaction with two or three catalytic centers participating simultaneously. The catalysis of the mutarotation of tetramethylglucose by o-hydroxypyridine, discovered by Swain and Brown(43), is an excellent example of such a concerted reaction. In the case of ∝-chymotrypsin the groups involved in a concerted reaction are a serine hydroxyl group and at least one imidazole group. Bender and co-workers(25), who have made the most significant contributions to the understanding of the ∝-chymotrypsin mechanism, have proposed a concerted reaction mechanism involving two imidazole groups and a serine hydroxyl. This mechanism is illustrated in fig. 3. In this mechanism two imidazole groups are postulated to form a π complex. In a cyclic process a proton is abstracted from the nucleophile by one imidazole group, and a proton added to the carbonyl oxygen atom by the other.

There are however, two difficulties with this mechanism. The first is that an unknown group -X-H must be postulated to take part. At present there is no evidence as to the participation of groups other than serine or imidazole. Second, it does not account for the observed pH dependencies; on the acid side of the pH optimum there is a strict pH dependence on only one imidazole

Figure 3

A mechanism of α-chymotrypsin catalyzed hydrolysis proposed by Bender and co-workers (25).


Acylation
Deacylation
Acylation R'OH
Deacylation

group. In view of these difficulties this mechanism must be considered as tentative.

There are as yet several aspects of the mechanism of α-chymotrypsin hydrolysis which require clarification. The problem of mixed and non-competitive inhibition observed with this enzyme has not been adequately investigated. Also, a dilemma has arisen in the explanation of the bell-shaped pH profile in acylation and the sigmoid profile in deacylation. The principle of microscopic reversibility has been employed in an attempt to explain the experimental evidence on this point(25). However, the question of the applicability and limitations of this principle arises. It is the objective of this thesis to critically examine these problems and to reconcile the available experimental evidence in the form of a concerted reaction mechanism.

CHAPTER II

THE KINETIC CONSEQUENCES OF THE PRINCIPLE OF MICROSCOPIC REVERSIBILITY

INTRODUCTION

It is commonly argued, on the basis of the principle of microscopic reversibility(44,45), that the favored kinetic pathway for a reaction occurring in one direction must be the same as that for the reverse reaction. This conclusion is certainly correct for all systems at equilibrium, and for all elementary processes whether at equilibrium or not. For many complex reaction systems, however, the conclusion is wrong even under steady-state conditions. In the present chapter we examine the conditions under which it is correct to conclude that forward and reverse paths must be identical, and that the ratio of rate constants is equal to the equilibrium constant.

For an elementary reaction the conclusion is certainly correct. Such a process can be represented by the flow of representative points over a potential-energy surface. Consider any molecular configuration, having certain bond angles and distances which define it. The

motion of such a system over a potential-energy surface can be expressed in terms of the vectorial velocities of the individual atoms. The probability of the existence of a species depends only on its energy, or on the squares of the individual velocities. Therefore, at equilibrium every molecular species has an exact counterpart which is moving over the potential-energy surface in the opposite direction and which is present at the same concentration. The favored reaction path over the surface must therefore be the same in both directions. For a system that can be represented simply as a motion over a surface the reaction path in one direction does not depend on the flow in the other direction. The favored reaction paths are therefore the same even if the system is not at equilibrium.

Reactions Occurring by Kinetically Equivalent Paths

The simplest type of non-elementary process is represented by the equation

$$A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} B \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} C$$

If a steady concentration of B is established the net rate of disappearance of A is

$$-\frac{d[A]}{dt} = \frac{k_1 k_2}{k_{-1} + k_2}[A] - \frac{k_{-1} k_{-2}}{k_{-1} + k_2}[C] \qquad (1)$$

If a kinetic experiment is done with pure A its rate of disappearance is given by the first term; with pure C its initial rate of disappearance is the second term. Suppose that there exists another intermediate B',

$$A \underset{k'_{-1}}{\overset{k'_1}{\rightleftharpoons}} B' \underset{k'_{-2}}{\overset{k'_2}{\rightleftharpoons}} C$$

Let the rate of reaction from left to right via B be x times that from left to right via B', and let the rate from right to left via B be y times that from right to left via B'. That is,

$$\frac{k_1 k_2}{k_{-1} + k_2} = x \frac{k'_1 k'_2}{k'_{-1} + k'_2} \quad (2) \qquad \text{and} \qquad \frac{k_{-1} k_{-2}}{k_{-1} + k_2} = y \frac{k'_{-1} k'_{-2}}{k'_{-1} + k'_2} \quad (3)$$

These equations reduce to

$$\frac{k_1 k_2}{k_{-1} k_{-2}} = \frac{x}{y} \frac{k'_1 k'_2}{k'_{-1} k'_{-2}} \quad (4)$$

However, $k_1k_2/k_{-1}k_{-2}$ and $k'_1k'_2/k'_{-1}k'_{-2}$ are equal, being equal to the over-all equilibrium constant; x and y are therefore equal. It follows that in this case a given path is favored to the same extent in the two directions.

This conclusion is not, however, true if the steady state is not established. Thus, at the very beginning of the reaction the forward and reverse rates via B are

$$-\frac{d[A]}{dt} = k_1 [A] \qquad (5) \qquad -\frac{d[C]}{dt} = k_{-2} [C] \qquad (6)$$

and via B'

$$-\frac{d[A]}{dt} = k_1' [A] \qquad (7) \qquad -\frac{d[C]}{dt} = k_{-2}' [C] \qquad (8)$$

There is no reason why k_1 should not be greater than k_1' and at the same time for k_{-2}' to be greater than k_{-2}. Prior to establishment of the steady state, therefore, the favored paths in the two directions need not be the same. Under these circumstances the ratio of the rate constants in the two directions is not equal to the equilibrium constant. The point is that under these conditions one is concerned with the rates of <u>different reactions</u> in the two directions.

The conclusions for the $A \rightleftharpoons B \rightleftharpoons C$ scheme can readily be extended to more complicated systems in which the alternative paths are equivalent as far as kinetic order is concerned; the conclusion is always that in the steady state the paths must be the same in the two directions, whereas if the steady state is not established this need not be the case.

$$-\frac{d[A]}{dt} = k_1[A] \qquad (5) \qquad -\frac{d[C]}{dt} = k_{-2}[C] \qquad (6)$$

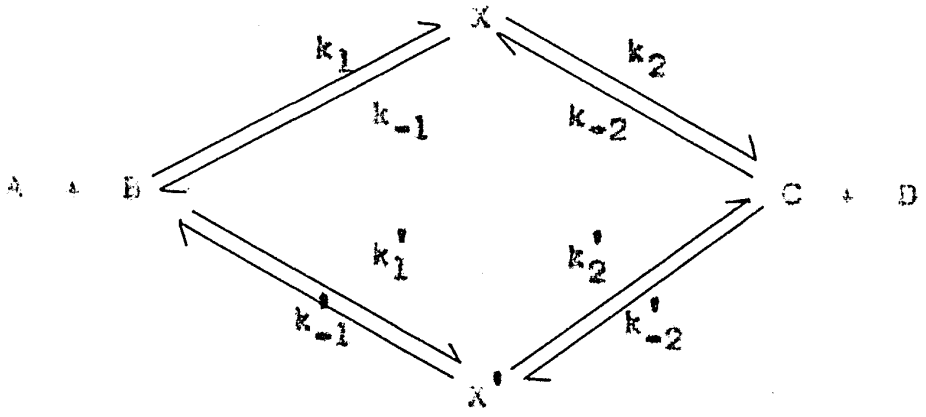
and via B'

$$-\frac{d[A]}{dt} = k_1'[A] \qquad (7) \qquad -\frac{d[C]}{dt} = k_{-2}'[C] \qquad (8)$$

There is no reason why k_1 should not be greater than k_1' and at the same time for k_{-2}' to be greater than k_{-2}. Prior to establishment of the steady state, therefore, the favored paths in the two directions need not be the same. Under these circumstances the ratio of the rate constants in the two directions is not equal to the equilibrium constant. The point is that under these conditions one is concerned with the rates of <u>different reactions</u> in the two directions.

The conclusions for the A B C scheme can readily be extended to more complicated systems in which the alternative paths are equivalent as far as kinetic order is concerned; the conclusion is always that in the steady state the paths must be the same in the two directions, whereas if the steady state is not established this need not be the case.

Special interest attaches to the scheme



in which B and D are supposed to be present at much larger concentrations than A and C respectively; A and C may therefore become saturated. This type of scheme applies to certain surface-catalyzed and enzyme-catalyzed reactions. If we start with pure A and B, at concentrations $[A]_o$ and $[B]_o$, the rates immediately after the establishment of the steady state are, for reaction via X,

$$v_f = \frac{k_1 k_2 [A]_o [B]_o}{1 + \frac{k_1}{k_{-1} + k_2} + \frac{k_1'}{k_{-1}' + k_2'} [B]_o} \qquad (9)$$

and for reaction via X'

$$v_f' = \frac{k_1' k_2' [A]_o [B]_o}{1 + \frac{k_1}{k_{-1} + k_2} + \frac{k_1'}{k_{-1}' + k_2'} [B]_o} \qquad (10)$$

It is to be noted that the ratio v_f/v_f' is independent of the concentrations of A and B. Similarly, for the reverse

reaction the initial steady-state rates are

$$v_r = \frac{k_{-1}\, k_{-2}\, [C]_o\, [D]_o}{1 + \frac{k_{-2}}{k_{-1} + k_2} + \frac{k_2'}{k_{-1}' + k_2'}\, [D]_o} \qquad (11)$$

$$v_r' = \frac{k_{-1}'\, k_{-2}'\, [C]_o\, [D]_o}{1 + \frac{k_{-2}}{k_{-1} + k_2} + \frac{k_{-2}'}{k_{-1} + k_2}\, [C]_o} \qquad (12)$$

Again it is found, making use of the relationship that $k_1k_2/k_{-1}k_{-2} = k_1'k_2'/k_{-1}'k_{-2}'$, that the ratio v_f/v_f' is always equal to v_r/v_r'; the favored path is therefore the same in the two directions.

The above conclusions are valid for non-chain mechanisms in which the alternative paths are equivalent as far as kinetic order is concerned. In such systems the concentrations of any alternative intermediates X and X' are affected <u>in the same way</u> by the reactant concentrations; the steady-state rates by alternative paths are therefore affected in the same way, so that one cannot favor different paths in opposite directions by adjusting reactant concentrations.

For unimolecular decompositions, and the reverse bimolecular processes (such as radical combinations), the Lindemann scheme applies approximately:

$$A + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} A^* + A$$

$$A^* \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} 2R$$

Using the same algebraic arguments as previously it is found that, under particular pressure conditions, the paths must be the same in the two directions. It follows that the change of kinetic order with change in pressure will occur over the same pressure range for the reaction in the two directions.

Reactions Occurring by Alternative Paths that are not Kinetically Equivalent

The situation is quite different if there are two or more alternative reaction paths that are not kinetically equivalent, in the sense that the reaction steps in the different paths are of different kinetic orders. A reaction between A and B might, for example, occur by the path

$$A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} X + P$$

followed by

$$X + B \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} Q$$

Here X is an intermediate and P and Q are the products. An alternative reaction path is

$$A + B \underset{k'_{-1}}{\overset{k'_1}{\rightleftharpoons}} Y \underset{k'_{-2}}{\overset{k'_2}{\rightleftharpoons}} P + Q$$

where Y is another intermediate. These two mechanisms are not kinetically equivalent. A special case of the first mechanism is an S_N1 reaction; an example of the second is an S_N2 reaction.

Application of the steady-state treatment leads to the conclusion that for the first mechanism the rate from left to right is

$$v_f = \frac{k_1 k_2 [A] [B]}{k_{-1}[P] + k_2[B]} \tag{13}$$

while that from right to left is

$$v_r = \frac{k_{-1} k_{-2} [P][Q]}{k_2[B] + k_{-1}[P]} \tag{14}$$

Similarly for the second mechanism

$$v'_f = \frac{k'_1 k'_2 [A] [B]}{k'_{-1} + k'_2} \tag{15}$$

$$v'_r = \frac{k'_{-1} k'_{-2} [P][Q]}{k'_{-1} + k'_2} \tag{16}$$

It is easy to show, for a given reaction mixture, using the relationship that $k_1k_2/k_{-1}k_{-2} = k'_1k'_2/k'_{-1}k'_{-2}$, that the ratio v_f/v'_f must be equal to v_r/v'_r. In other words, in a given reaction system a given path is equally favored in forward and reverse directions.

This, however, is not necessarily true if we first study the reaction in one direction and then in the other direction; the favored paths may now be different in the two directions. Suppose, for example, that when the reaction is studied from left to right $[B]_o$, the total concentration of B, is much greater than $[A]_o$, the total concentration of A. For reaction in the forward direction the total concentration of A is given by

$$[A]_o = [A] + [X] + [Y] \tag{17}$$

if reaction is going by both mechanisms. In the absence of P and Q the steady-state equations for [X] and [Y] are

$$k_1 [A] = k_2 [X] [B] \tag{18}$$

and

$$k'_1 [A][B]_o = (k'_{-1} + k'_2) [Y] \tag{19}$$

Elimination of [X] and [Y] between (17), (18) and (19) yields

$$[A]_o = [A]\left[1 + \frac{k_1}{k_2[B]_o} + \frac{k_1'[B]_o}{k_{-1}' + k_2'}\right] \qquad (20)$$

Elimination of [A] between (13) and (20) gives, for the initial rate by the first mechanism,

$$v_f = \frac{k_1[A]_o}{1 + \frac{k_1}{k_2[B]_o} + \frac{k_1'[B]_o}{k_{-1}' + k_2'}} \qquad (21)$$

Similarly for the initial rate by the second mechanism

$$v_f' = \frac{k_1' k_2' [A]_o [B]_o}{(k_{-1}' + k_2')\left(1 + \frac{k_1}{k_2[B]_o} + \frac{k_1'[B]_o}{k_{-1}' + k_2}\right)} \qquad (22)$$

The ratio of the rates in the forward direction by the two mechanisms is thus

$$\frac{v_f}{v_f'} = \frac{k_1(k_{-1}' + k_2')}{k_1' k_2' [B]_o} \qquad (23)$$

In exactly the same way we find for the ratio of rates in the reverse direction, assuming P to be in excess,

$$\frac{v_r}{v_r'} = \frac{k_{-2}(k_{-1}' + k_2')}{k_{-2}' k_{-1}' [P]_o} \qquad (24)$$

The ratio of these ratios is

$$\frac{v_f}{v_f'} \times \frac{v_r'}{v_r} = \frac{k_1 k_{-1}' k_{-2}' [P]_o}{k_{-2} k_1' k_2' [B]_o} \qquad (25)$$

which is not necessarily equal to unity. It follows that

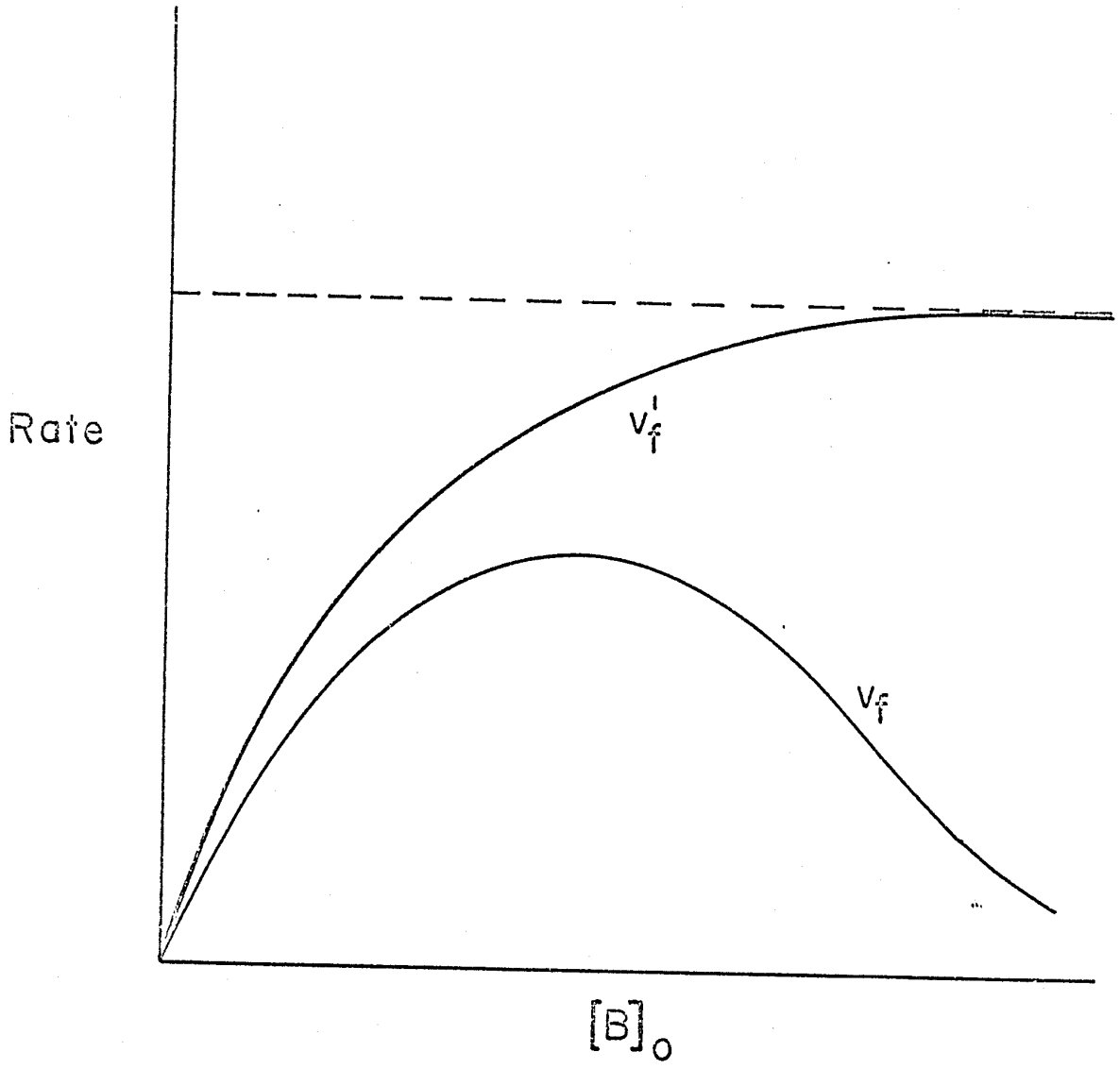
under these circumstances the favored paths may be different in the two directions.

It follows from equation (25) that the relative importance of the alternative paths in the two directions depends upon the ratio of the reactant concentrations - or the value of $[B]_o$, chosen for the study in the forward direction, divided by the concentration $[P]_o$ used in the reverse direction. The paths will be the same in the two directions only in the event that $[B]_o/[P]_o$ happens to equal $k_1k'_{-1}k'_{-2}/k_{-2}k'_1k'_2$. It is evident from equations (21) and (22) that the relative importance of the two paths in the forward direction depends upon the value of $[B]_o$. Figure 4 shows a schematic plot of the rates by the two mechanisms against $[B]_o$. Initially v_f varies with $[B]_o$, but at high $[B]_o$ v_f is inversely proportional to $[B]_o$; v'_f on the other hand initially varies with $[B]_o^2$ but reaches a limiting value at high $[B]_o$. Because v_f and v'_f vary <u>differently</u> with $[B]_o$ the relative importance of the two paths depends on $[B]_o$. Similar arguments apply to the dependence of v_r and v'_r on $[P]_o$.

Whenever the alternative reaction paths are not kinetically equivalent, in the sense that they do not consist of corresponding steps with the same kinetic orders, the relative importance of the paths will depend on concentrations. Therefore, in general the reaction will occur

Figure 4

A plot showing the dependence of initial rate on $[B]_o$.


Rate
v_f'
v_f
$[B]_o$

by different paths in the two directions, and the ratio of rate constants will not necessarily be the equilibrium constant.

Chain Reactions

A reaction proceeding by a chain mechanism need not, and generally does not, occur by the same path in opposite directions. In a chain reaction the intermediates (e.g. free radicals) are produced from the reactants in an initiation process, and the products are produced in the chain-propagating steps. The mechanism for the reverse reaction is obviously not obtained by writing all reactions in reverse; there must be a new initiation reaction which produces the radicals from the reactants. For example, in the decomposition of ethane (46-48) into ethylene and hydrogen the initiation reaction is

$$C_2H_6 \longrightarrow 2\ CH_3$$

and this is followed by

$$CH_3 + C_2H_6 \longrightarrow CH_4 + C_2H_5$$

The main products are produced in the chain-propagating steps

$$C_2H_5 \longrightarrow C_2H_4 + H$$

$$H + C_2H_6 \longrightarrow H_2 + C_2H_5.$$

If we start with pure ethylene and hydrogen a new initiation process occurs; the evidence (49) is that this is

$$C_2H_4 + C_2H_4 \longrightarrow C_2H_5 + C_2H_3$$

The chain-propagating steps are the reverse of one another in the forward and reverse reactions, but the initiation and termination processes are not the reverse of one another; the reaction paths are therefore different for the reactions in the two directions, and the ratio of rate constants is not the equilibrium constant. This may be shown in a more general way for a reaction in which a substance M is converted into P + Q. The following free-radical mechanisms lead to first-order kinetics in one direction and second-order in the other:

<u>Decomposition of M</u>

$$2M \xrightarrow{k_1} M + 2\beta$$

$$\beta + M \xrightarrow{k_2} \mu + P$$

$$\mu \xrightarrow{k_3} \beta + Q$$

$$2\mu \xrightarrow{k_4} P'$$

$$-\frac{d[M]}{dt} = k_3 \left(\frac{k_1}{k_4}\right)^{1/2} [M]$$

<u>Reaction between P and Q</u>

$$2Q \xrightarrow{k_1'} \mu + \mu'$$

$$\mu + P \xrightarrow{k_{-2}} \beta + M$$

$$\beta + Q \xrightarrow{k_{-3}} \mu$$

$$2\mu \xrightarrow{k_4} P'$$

$$-\frac{d[P]}{dt} = k_2 \left(\frac{k_1'}{k_4}\right)^{1/2} [P][Q]$$

In these schemes β represents a radical (e.g. H) which undergoes second-order reactions in the chain-propagating

steps, and μ is a radical which undergoes first-order reactions. P' is a minor product, and μ' a radical which is not involved in propagation. Although the kinetic laws in each direction are simple and correspond to the stoichiometry, the reaction paths are not the same in the two directions. The ratio of the rate constants, $(k_3/k_{-2})(k_1/k_1')^{1/2}$, is not equal to the equilibrium constant, which is $k_2k_3/k_{-2}k_{-3}$. After the system has reached equilibrium all of the elementary processes occur at equal rates in forward and reverse directions, and the ratio of over-all rate constants must then be equal to the equilibrium constant.

General Discussion

It follows from the discussion of the present chapter that great care must be exercised in deducing reaction mechanisms by use of the principle of microscopic reversibility. The principle may, in fact, only be used in a negative sense; if a reaction mechanism has been firmly established in one direction it is valid to eliminate certain mechanisms for the reaction in the reverse directions. Specifically, one may correctly discard mechanisms that are kinetically equivalent (i.e. involve corresponding steps of the same kinetic order) to that in the other direction, but which are not the exact reverse of the

reaction in the other direction. One must not, however, eliminate mechanisms that are not kinetically equivalent to one that has been established for the reverse direction. Moreover, contrary to what has recently been asserted by Burwell and Pearson(50), it is never correct to insist that a reaction must occur in one direction mainly by a mechanism that is the exact reverse of that in the reverse direction; one can never be certain that it does not occur mainly by a different mechanism that is not kinetically equivalent to that for the reverse reaction.

CHAPTER III

THE KINETICS AND MECHANISM OF α-CHYMOTRYPSIN CATALYZED HYDROLYSIS

A. The Influence of pH on the Kinetics of Enzyme Reactions Involving Two Intermediates

INTRODUCTION

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

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

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 in general pH-dependent quantities. At high substrate concentrations ($[S] \gg \tilde{K}_m$) the pH dependence of the over-all rate is that of $\tilde{k}_c$, while at low substrate concentrations ($[S] \ll \tilde{K}_m$) the pH dependence is that of $\tilde{k}_c/\tilde{K}_m$. The simplest mechanism consistent with equation (1) is

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

and for this case

$$\tilde{k}_c = \tilde{k}_2 \qquad (2)$$

and

$$\tilde{K}_m = \frac{\tilde{k}_{-1} + \tilde{k}_2}{\tilde{k}_1} \qquad (3)$$

The pH effects found in the case of this mechanism have been discussed in detail by Waley(51), Dixon and Webb(52), Laidler(53,54) and Peller and Alberty(55).

For many enzymes, however, such as the serine proteinases, chymotrypsin, trypsin, elastase and thrombin and the cysteine proteinases, papain, ficin and bromelain, the presence of a second intermediate has been demonstrated(31). Also, for the hydrolytic enzymes cholinesterase(56), ribonuclease(57) and α-amylase(58) a similar second intermediate has been demonstrated.

The mechanism is then

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} P_2 + E$$
$$\qquad\qquad\qquad\qquad\quad \downarrow P_1$$

where P_1 and P_2 are products of reaction. The Michaelis parameters are now

$$\tilde{k}_c = \frac{\tilde{k}_2 \tilde{k}_3}{\tilde{k}_2 + \tilde{k}_3} \qquad (4)$$

and

$$K_m = \frac{\tilde{k}_3}{\tilde{k}_2 + \tilde{k}_3} \cdot \frac{\tilde{k}_{-1} + \tilde{k}_2}{\tilde{k}_3} \qquad (5)$$

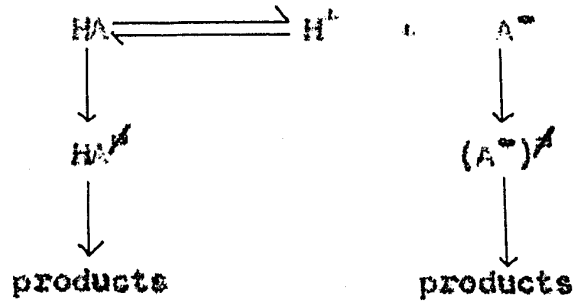
Some of the kinetic consequences of this scheme have been considered by Zerner and Bender(59). Krupka and Laidler(60) in particular have discussed the pH dependence of the over-all rates, on the basis of such a scheme, in terms of the nature of the ionizations of E, ES and ES', and taking into consideration the various possible rate-determining steps. More recently Krupka(61) and Webb(62) have dealt with cases in which the substrates and inhibitors are charged, and have considered the effects of the charged groups on the various ionizations.

The present treatment is concerned with the conditions under which an ionizing group is detected, and with the significance of the $\tilde{k}_c/\tilde{K}_m$ ratio. It deals also, for uncharged substrates, with the interpretation and classification of the various pH dependences of $\tilde{k}_2$, $\tilde{k}_3$ and $\tilde{k}_c/\tilde{K}_m$, with reference to the rate-determining step.

The Kinetic Detection of an Ionizing Group

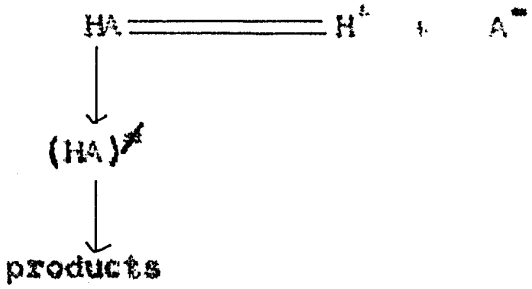
There are many ionizing groups on enzymes, and it is obvious that a kinetic study can only reveal the presence of those for which a change in state of ionization has some effect on the rate of reaction. For any elementary process $A \rightleftharpoons B$ we may consider three possibilities as far as pH dependence is concerned:

1) The reactant molecule A can exist in more than one state of ionization, but each one of these states can equally readily form an activated complex. For example, for two states of ionization,



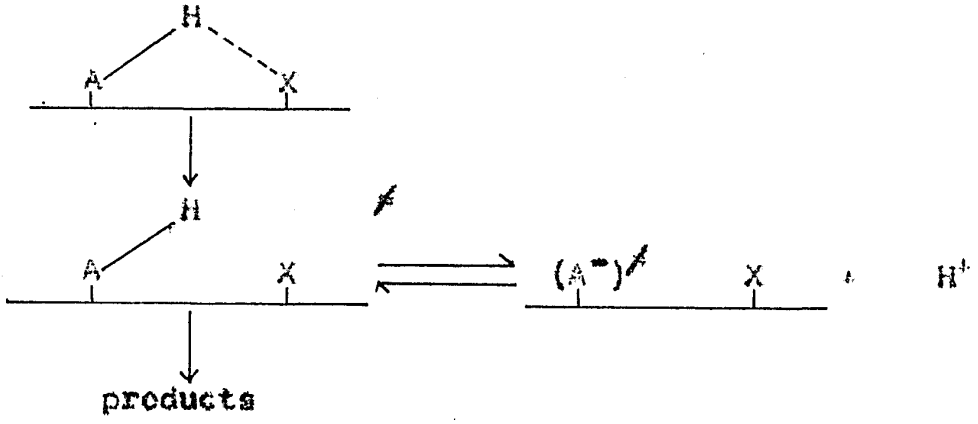
The rate is now unaffected by pH. This is the case of the ionizing group that is not essential to reaction.

2) If only one ionized state can give rise to an activated complex, the scheme is, for example,



Increase in pH decreases the concentration of HA and lowers the reaction rate.

3) The third possibility is that the reactant is not free to ionize, but the activated complex is. For example, ionization in the initial state might be inhibited by hydrogen bonding, but the hydrogen bonding might not exist in the activated state.



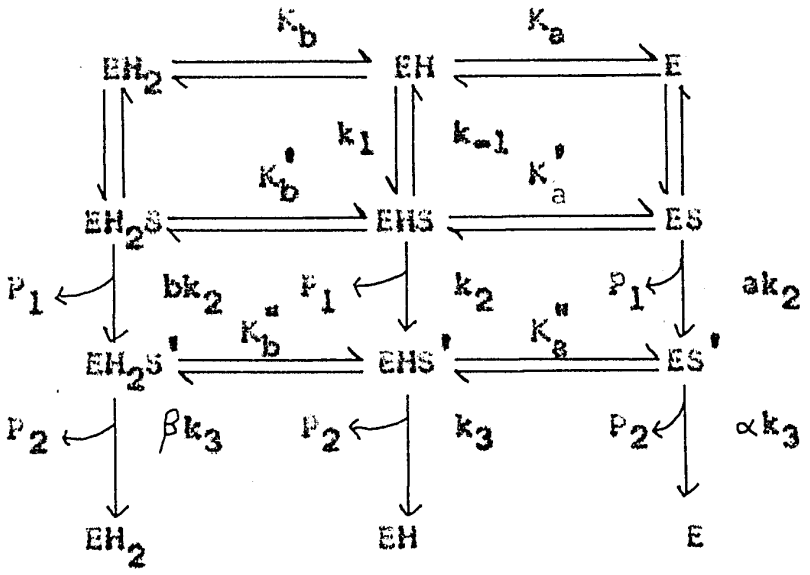
At first sight it might appear that raising the pH would remove activated complexes and reduce the rate. However, as recently emphasized (63-65), activated complexes are not in a state of true equilibrium, and their concentrations cannot be affected in this way; the rate of their decomposition is greater than their rate of ionization. The rate for this scheme is therefore uninfluenced by the pH.

It follows that a kinetic study only reveals the presence of an ionizing group when that group ionizes in the initial state, and when its ionization affects the

ease of formation of the activated state.

General Reaction Scheme

A general scheme involving two ionizing groups is:



K_a and K_b are both acid dissociation constants for the free enzymes, and representing the ionization constant of an acid and basic group, respectively. K_a', K_b' and K_a'' and K_b'' represent the ionization constants of the same groups in the Michaelis complex and acyl enzyme. The processes of breakdown of the Michaelis complex and of the second intermediate are shown to be irreversible, since under initial conditions the concentrations of P_1 and P_2 are negligible and no reverse reaction can take place. The scheme allows for the breakdown of different ionization states of the complexes. If, for example, a = 1 then that ionizing group is not essential for activity, but if a = o it is

essential. The steady-state solution for the above scheme leads to the following expressions for the Michaelis parameters:

$$\tilde{k}_c = \frac{k_2}{\left(\frac{1 + \frac{K_a'}{[H]} + \frac{[H]}{K_b'}}{1 + \frac{aK_a'}{[H]} + \frac{b[H]}{K_b'}}\right) + \frac{k_2}{k_3}\left(\frac{1 + \frac{K_a''}{[H]} + \frac{[H]}{K_b''}}{1 + \frac{K_a''}{[H]} + \frac{[H]}{K_b''}}\right)} \tag{6}$$

$$\tilde{K}_m = \frac{\frac{k_{-1} + k_2}{k_1}\left(\frac{1 + \frac{K_a}{[H]} + \frac{[H]}{K_b}}{1 + \frac{aK_a'}{[H]} + \frac{b[H]}{K_b'}}\right)}{\left(\frac{1 + \frac{K_a'}{[H]} + \frac{[H]}{K_b'}}{1 + \frac{aK_a'}{[H]} + \frac{b[H]}{K_b'}}\right) + \frac{k_2}{k_3}\left(\frac{1 + \frac{K_a''}{[H]} + \frac{[H]}{K_b''}}{1 + \frac{K_a''}{[H]} + \frac{[H]}{K_b''}}\right)} \tag{7}$$

$$\frac{\tilde{k}_c}{\tilde{K}_m} = \frac{k_1 k_2}{k_{-1} + k_2}\left(\frac{1 + \frac{aK_a'}{[H]} + \frac{b[H]}{K_b'}}{1 + \frac{K_a}{[H]} + \frac{[H]}{K_b}}\right) \tag{8}$$

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

The ratio $\tilde{k}_c/\tilde{K}_m$ is related to the kinetic behavior at low substrate concentrations. The way in which this ratio varies with the pH is shown by equation(8), and the following special cases are of particular interest

(1) Suppose that the enzyme-substrate complex ES ionizes in the same way as does the free enzyme (i.e. $K_a' = K_a$ and $K_b' = K_b$) and that a and b are unity. This is the case of non-essential ionizing groups. 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 the studies at low [S] reveal K_a and K_b for the ionization of the free enzyme. If a = o but b is not zero there will be pH dependence of $\tilde{k}_c/\tilde{K}_m$ on the basic side, and the results will reveal K_a; if b = o but a is not zero the pH dependence on the acid side will reveal K_b.

It is evident that when one obtains K_a and K_b values from studies at low [S], one can conclude that the corresponding ionizing groups are <u>essential to the subsequent reaction of the enzyme-substrate complex</u>; they may also be involved at a later stage, e.g. deacylation, but this must be investigated in other ways. It follows that even if one is using a substrate for which deacylation is

rate limiting one can still determine the ionizing groups involved in acylation by studying the pH variation of $\tilde{k}_c/\tilde{K}_m$.

The Patterns of pH Behavior

In the scheme under consideration, where there are two intermediates, there is ionization at three stages (E, ES and ES'), and there is the possibility of different rate-limiting steps for different substrates. There are therefore several different types of pH behavior, and these are classified in Table I. A positive sign (+) indicates that there is pH dependence of the Michaelis parameter indicated, and a zero (o) indicates no pH dependence. In the last three columns of the table are indicated the possible combinations that will give rise to the behavior observed, on the acid and basic sides. To simplify matters it has been assumed that if either K_a' or K_a'' is not equal to K_a it is very large (so that its ionization is outside the experimental range); similarly if K_b' or K_b'' is not equal to K_b it is very small. If these conditions do not hold the modifications are easily made. Table I will be useful in permitting preliminary conclusions to be drawn from experimental pH studies; a decision between alternative possibilities can then often be made on the basis of other evidence - for example, by using substrates for which different steps are rate-determining.

TABLE I

Classification of pH Behavior for the Case of Two Intermediates, for Uncharged Substrates

Case	$\tilde{k}_c$	$\tilde{K}_m$	$\frac{\tilde{k}_c}{\tilde{K}_m}$	$k_2 \ll k_3$		$k_2 \gg k_3$		$k_2 \sim k_3$	
				Acid	Base	Acid	Base	Acid	Base
1	+	+	+	N.P.	N.P.	N.P.	N.P.	(i) $b=0, \beta \neq 1$ (ii) $b=0, K''_b = \infty$	(i) $a=0, \alpha=1$ (ii) $a=0, K''_a=0$(a)
2	+	+	0	N.P.	N.P.	$b=1, \beta=0$	$a=1, \alpha=0$	$b=1, \beta=0$	$a=1, \alpha=0$
3	+	0	0	N.P.	N.P.	N.P.	N.P.	N.P.	N.P.
4	0	+	+	$K'_b = \infty$ (b)	$K'_a=0$(b)	(i) $b=0, \beta \neq 1$ (ii) $b=0, K''_b = \infty$ (ii)$K'_b = \infty, \beta=1$ (iv)$K'_b = \infty$, $K''_b = \infty$	(i) $a=0, \alpha=1$ (ii)$a=0, K''_a=0$(c) (iii)$K'_a=0, \alpha=1$ (iv) $K'_a=0$, $K''_a=0$	(i) $K'_b = \infty, \beta=1$ (ii)$K'_b = \infty, K''_b = \infty$	(i)$K'_a=0, \alpha=1$ (ii)$K'_a=0, K''_a=0$
5	0	0	+	N.P.	N.P.	N.P.	N.P.	N.P.	N.P.
6	+	0	+	b=0(d)	a=0(d)	(i) $b=0, \beta=0$(c) (ii)$K'_b = \infty, \beta=0$(e)	(i)$a=0, \alpha=1$ (ii)$K'_a=0$, $\alpha=0$(e)	(i)$K'_b = \infty, \beta=0$ (ii)$b=0, \beta=0$	(i)$K'_a=0, \alpha=0$ (ii)$a=0, \alpha=0$
7	0	+	0	N.P.	N.P.	N.P.	N.P.	N.P.	N.P.

Table I, continued on next page

Table I, Continued

(a) α-Chymotrypsin-catalysed hydrolysis of methyl hippurate (66,67).

(b) Cholinesterase-catalysed hydrolysis of N-methyl amino ethyl acetate (68).

(c) α-Chymotrypsin-catalysed hydrolysis of p-nitrophenyl acetate, N-acetyltyrosine ethyl ester, N-benzoyl-L-alanine methyl ester, N-benzoyl-D-alanine methyl ester (69);
trypsin-catalysed hydrolysis of p-toluene sulphonyl-L-arginine methyl ester (70).

(d) α-Chymotrypsin-catalysed hydrolysis of amides (42)
α-Chymotrypsin-catalysed hydrolysis of dihydromethyl cinnamate (71).

(e) Cholinesterase-catalysed hydrolysis of acetyl choline (68).

N.P. indicates that the behavior specified is not possible.

Some examples of the various types of behavior are listed at the foot of Table I. In some cases the case chosen is a matter of interpretation, and subsequent work might require a change of classification.

B. General Experimental

The apparatus and experimental procedure were very similar for the various experiments carried out; the general description given here thus applies to all the experimental work.

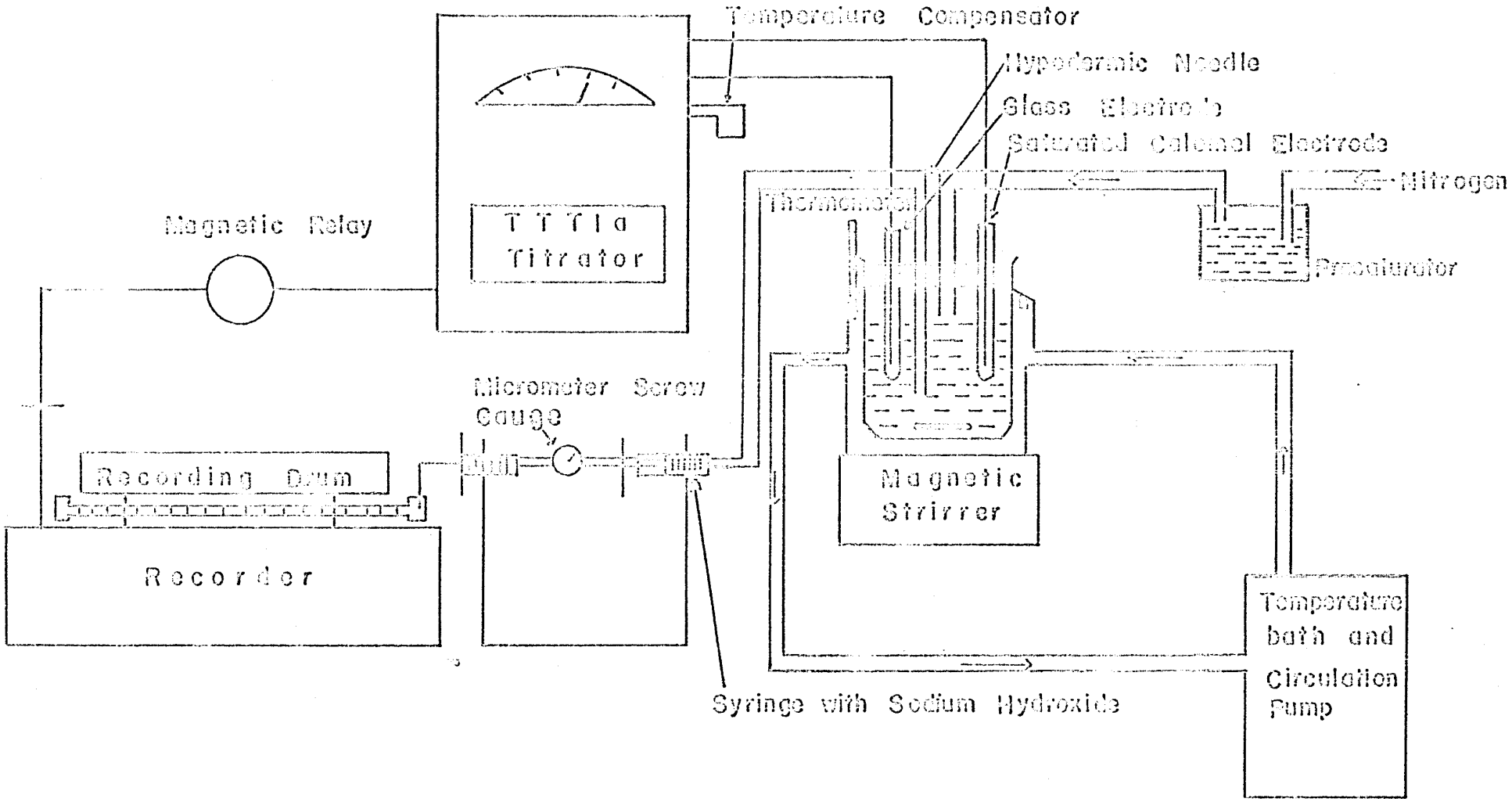
A pH-stat was employed for all kinetic measurements. Figure 5 shows schematically the titration assembly.

Three reaction vessels were used. Two were employed exclusively for enzyme experiments, and the other for buffer solutions used in the standardization of the pH meter. The commercially supplied assembly consists of one sealed glass vessel so that both buffer and enzyme solutions must be used in the same vessel. Hein and Niemann(72) reported that they could not obtain reproducible results with enzyme concentrations below 10^{-7}M. However, by conditioning the reaction vessels in the manner described above, reproducible results were obtained with enzyme concentrations as low as 10^{-9}M.

Two types of glass electrodes were employed. Work at 20°C or above was done with a Radiometer type G202B

Figure 9

A schematic diagram of the titration assembly.


Temperature Compensator
Hypodermic Needle
Glass Electrode
Saturated Calomel Electrode
Nitrogen
Thermometer
Magnetic Relay
TTTIa
Titrator
Presaturator
Micrometer Screw
Gauge
Recording Drum
Recorder
Magnetic
Stirrer
Temperature
bath and
Circulation
Pump
Syringe with Sodium Hydroxide

electrode, while for lower temperatures a type G202C was used. The reference electrode was a saturated calomel electrode with a porous pin junction.

The reaction vessel fitted into a thermostatted jacket connected to a temperature bath and circulation pump. The temperature in the reaction vessel was set by inserting a calibrated Beckmann thermometer into the reaction vessel and adjusting the temperature controls on the bath. The temperature variation in the cell was $\pm 0.05^{\circ}C$. A thermometer was inserted in the thermostatted jacket as a double check on the temperature control.

The delivery system for the titrant, 0.020N sodium hydroxide, consisted of a Yale B-D 1c.c. tuberculin syringe driven via a micrometer screw gauge by an armature on the recorder. The titrant entered the reaction medium through a hypodermic needle, the end of which was fitted with a platinum capillary wire. The rate at which the titrant was delivered could be controlled by replaceable cog-wheels in the driving system of the recorder. Calibration of the delivery system was carried out using water. By means of replaceable gears on the armature, either 10.8 or 21.6 microlitres of titrant could be delivered per centimeter deflection of the recording needle.

Procedure

In a typical run substrate and sodium chloride were mixed to 14.0 ml. The pH was adjusted to the desired value with 2N base delivered by means of a micrometer screw gauge. In this manner the volume change during pH adjustment was kept negligible. The system was allowed five minutes to come to temperature equilibrium, then 1.0 ml of enzyme was added to start the reaction. In the cases where there was non-enzymic hydrolysis of the substrate the reaction was started by injecting 1.0 ml of substrate. During the course of a run the reaction medium was kept free of carbon dioxide by passing nitrogen over the system. The reactions were in all cases followed to less than 5% completion and in most cases to approximately 1% completion.

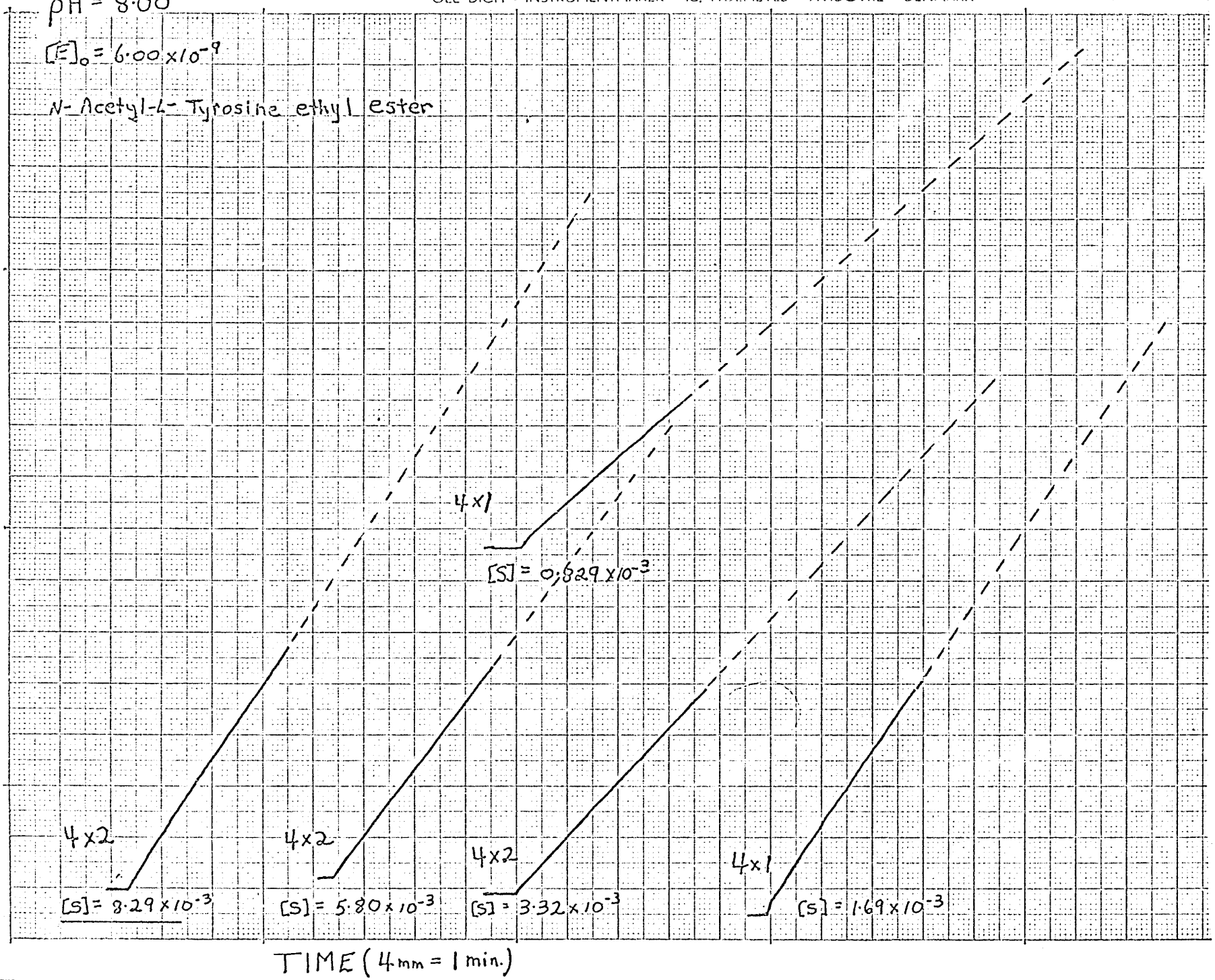
Analysis of Data

The rates of reaction were obtained by measuring the initial slopes of the ΔA versus time curves, where ΔA is the amount of acid produced. The plots were practically linear for the first fifteen minutes which, allowed an accurate initial slope to be taken. The gearing of the recorder was adjusted so that the initial slopes obtained varied between 0.7 and 2.0. Figure 6 shows some typical time course curves for the hydrolysis of N-acetyl-L-tyrosine ethyl ester by α-chymotrypsin at pH 8.00. The dotted line

Figure 6

Typical time course curves for the α-chymotrypsin catalyzed hydrolysis of N-acetyl-L-tyrosine ethyl ester. (Actual photograph of experimental data).

OLE DICH - INSTRUMENTMAKER - 18, HOLMEVEJ - HVIDOVRE - DENMARK



is an extrapolation of the initial slope.

The kinetic parameters of the Michaelis-Menten equation (cf. equation (1)) were obtained from Eadie plots(73) as shown in figure 7 using the method of least squares to evaluate the slope and intercepts.

The method of Dixon(74) was employed to obtain ionization constants from the variation of the kinetic parameters with pH. The method is schematically illustrated in figure 8.

Examples of applications of these methods will be given in the presentation of experimental results.

C. Studies in Mixed Solvents; the Nature of the Ionizing Groups

INTRODUCTION

Ionizing groups on an enzyme may be divided into two classes:

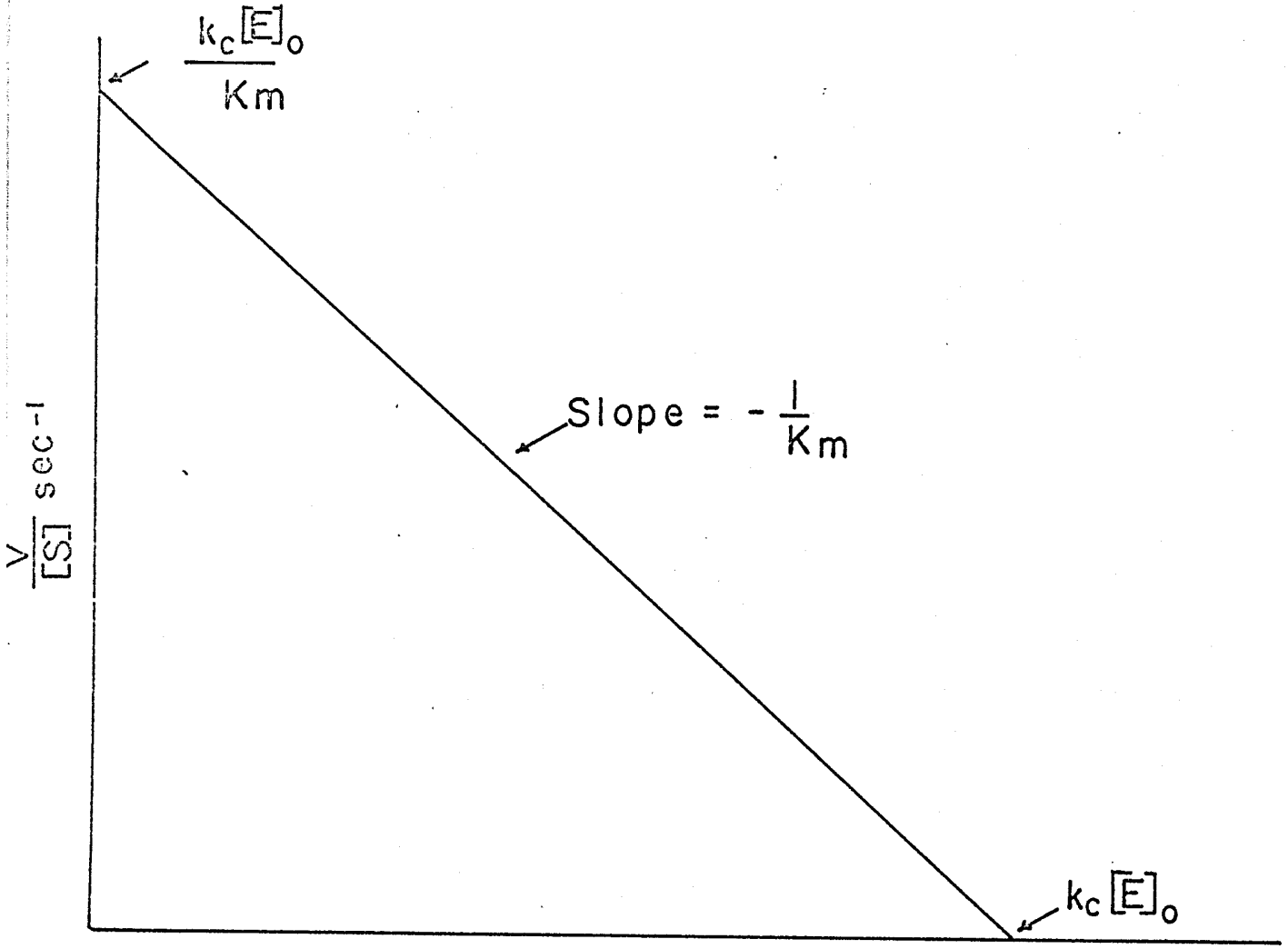
(a) Neutral groups, such as -COOH and -OH, which dissociate into positive and negative species on ionization,

$$-A-H \rightleftharpoons H^{+} + -A^{-}$$

(b) Cationic groups, such as $-NH_3^+$, which dissociate into a proton and a neutral group,

Figure 7

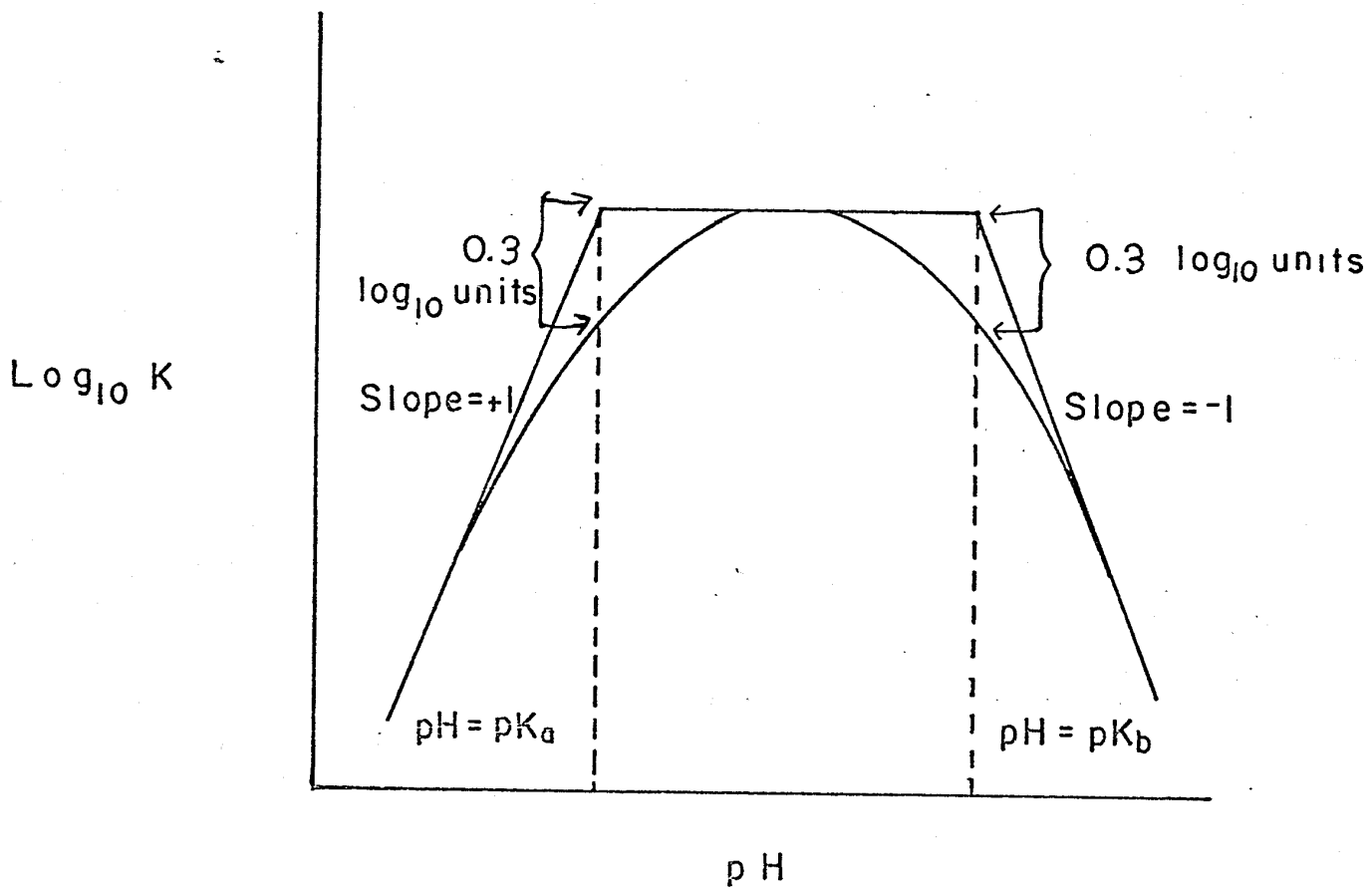
Eadie Plot (73).


$\frac{k_c[E]_o}{Km}$
Slope = $-\frac{1}{Km}$
$k_c[E]_o$
$\frac{V}{[S]}$ sec^{-1}
V moles litre^{-1} sec^{-1}

EADIE PLOT

Figure 8

A schematic illustration of Dixon's method for obtaining pK values (74).


Log10 K
0.3 log10 units
0.3 log10 units
Slope=+1
Slope=-1
pH = pKa
pH = pKb
p H

$$-BH^{+} \rightleftharpoons -B + H^{+}$$

The effect of changing the dielectric constant of the solvent is very different in the two cases. In (a), an increase in dielectric constant increases K_a (and decreases pK_a); in (b) it has very little effect on K_a, since cationic species are involved on both sides of the equation. This type of behavior is well established experimentally. For example, the pK of acetic acid is 4.76 in water and 10.14 in an 82% (w/w) dioxane-water mixture(75). On the other hand the pKs corresponding to the $-NH_3^+$ groups of amino acids are only very slightly affected by a change in the dielectric constant(76).

The present investigation is concerned with the variation of the pKs of the two ionizing groups in α-chymotrypsin with the dielectric constant of the solvent. The dielectric constant has been varied by using a series of dioxane-water mixtures. The question of whether there might be effects due to factors other than change in dielectric constant is considered in the Appendix, where it is concluded that the dielectric constant effect is by far the most important.

In the case of α-chymotrypsin, as with many other hydrolytic enzymes, the existence of bell-shaped rate-pH profiles at low substrate concentration reveals the presence

of two kinetically-significant ionizing groups in the free enzyme. Each of these may be either neutral or cationic, so that there are four possibilities,

(1) neutral - neutral

(2) neutral - cationic

(3) cationic - neutral

(4) cationic - cationic

The type of behavior expected if the dielectric constant is reduced, by the addition of inert solvent, is shown in figure 9. These curves have been normalized so as to make the maximum rates the same in all cases. By observing what pattern of behavior actually occurs it is possible to distinguish between the four possibilities.

This procedure resembles, but is simpler than, that employed by Findlay, Mathias and Rabin(77) for ribonuclease. They used buffers, and had to take into account the effect of the dielectric constant on the ionization of the buffer. In the present work, done with a pH-stat, no buffer is employed.

EXPERIMENTAL

Materials

All water used was doubly distilled, deionized and free of carbon dioxide. The dioxane was purified by the method of Fieser(78) and was stored under nitrogen.

Figure 9

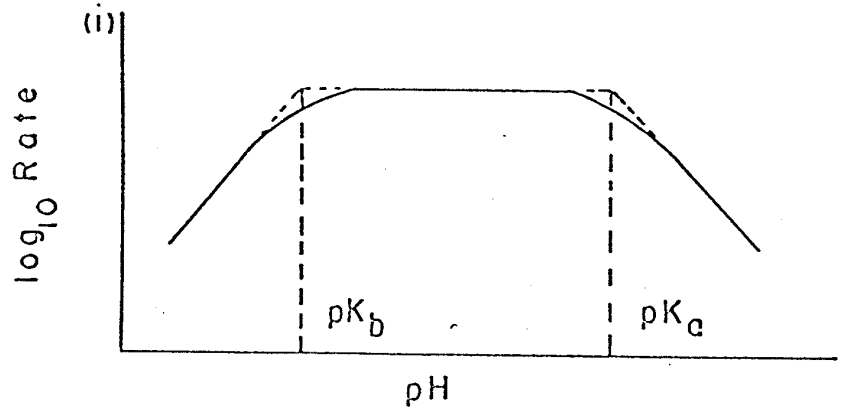
The expected effect of inert organic solvent on the pH profile for various ionizing pairs at the active centre.

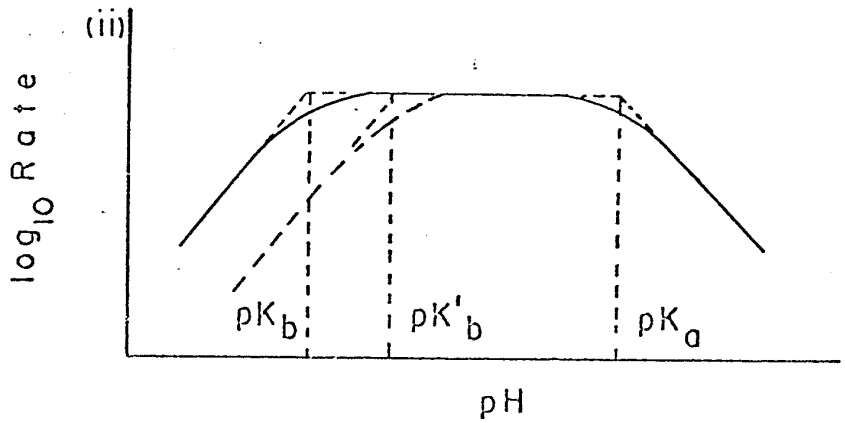
(i) neutral - neutral

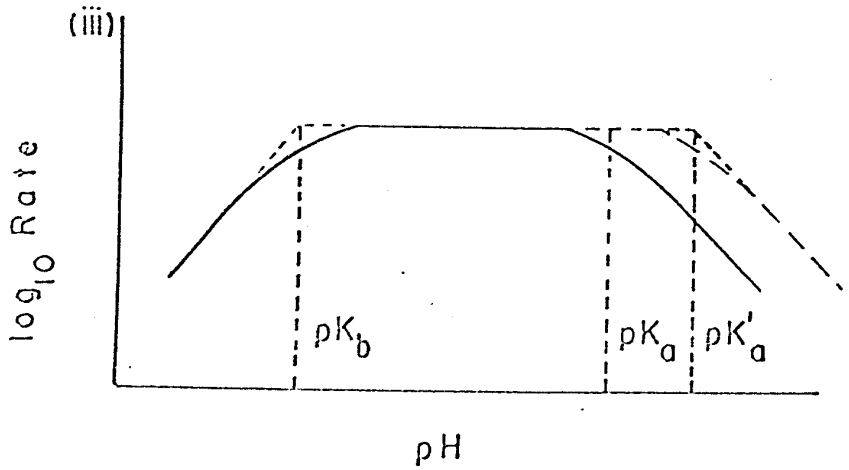
(ii) neutral - cationic

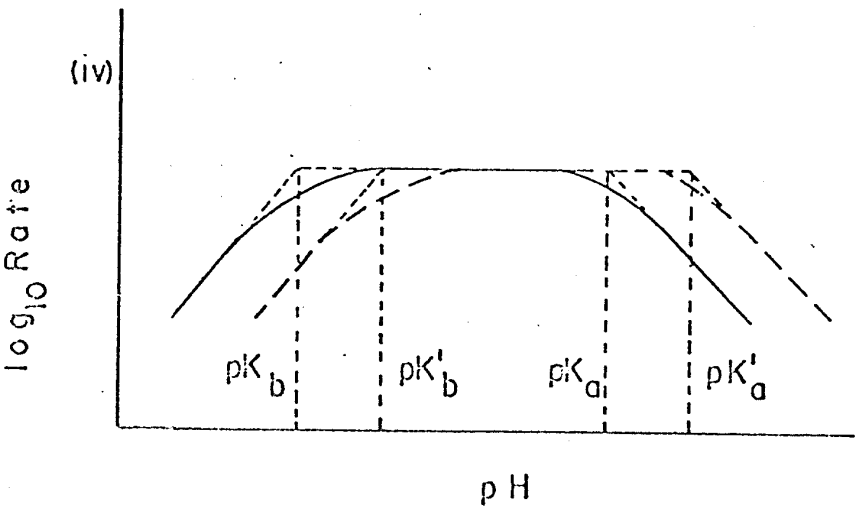
(iii) cationic - neutral

(iv) cationic - cationic


(i)
log10 Rate
pKb
pKa
pH


(ii)
log10 Rate
pKb
pK'b
pKa
pH


(iii)
log10 Rate
pKb
pKa
pK'a
pH


(iv)
log10 Rate
pKb
pK'b
pKa
pK'a
pH

Precautions were taken to prevent the solvent from coming into contact with the atmosphere, so as to avoid the formation of peroxide impurities.

N-Benzoyl-L-alanine methyl ester was used as substrate. It was prepared by essentially the method used by Hein and Niemann(29), with a modification to the benzoylation procedure which doubled the previously reported yield. Esterification of 5.0 gms of L-alanine was carried out with methanol and thionyl chloride according to the procedure of Brenner and Huber(79). Benzoylation of the unpurified ester hydrochloride was carried out using a pH-stat according to the following procedure. The ester hydrochloride was dissolved in water and the pH adjusted to 8.0 with base. One equivalent of benzoyl chloride was slowly added. The pH was kept constant at 8.0 by means of sodium hydroxide solution delivered by the pH-stat, and a milky white liquid settled to the bottom of the flask. The liquid was separated and recrystallized twice from high boiling petroleum ether to give 7.4 gms (63%) of N-benzoyl-L-alanine methyl ester, m.p. 57-58°C, $[\alpha]_D$ = +30.7 in 5% sym-tetrachloroethane.

The titrant used to follow the course of the reaction was 0.02N sodium hydroxide, prepared from the Fisher Certified reagent.

Kinetic Procedure

The apparatus used was that described in Section B.

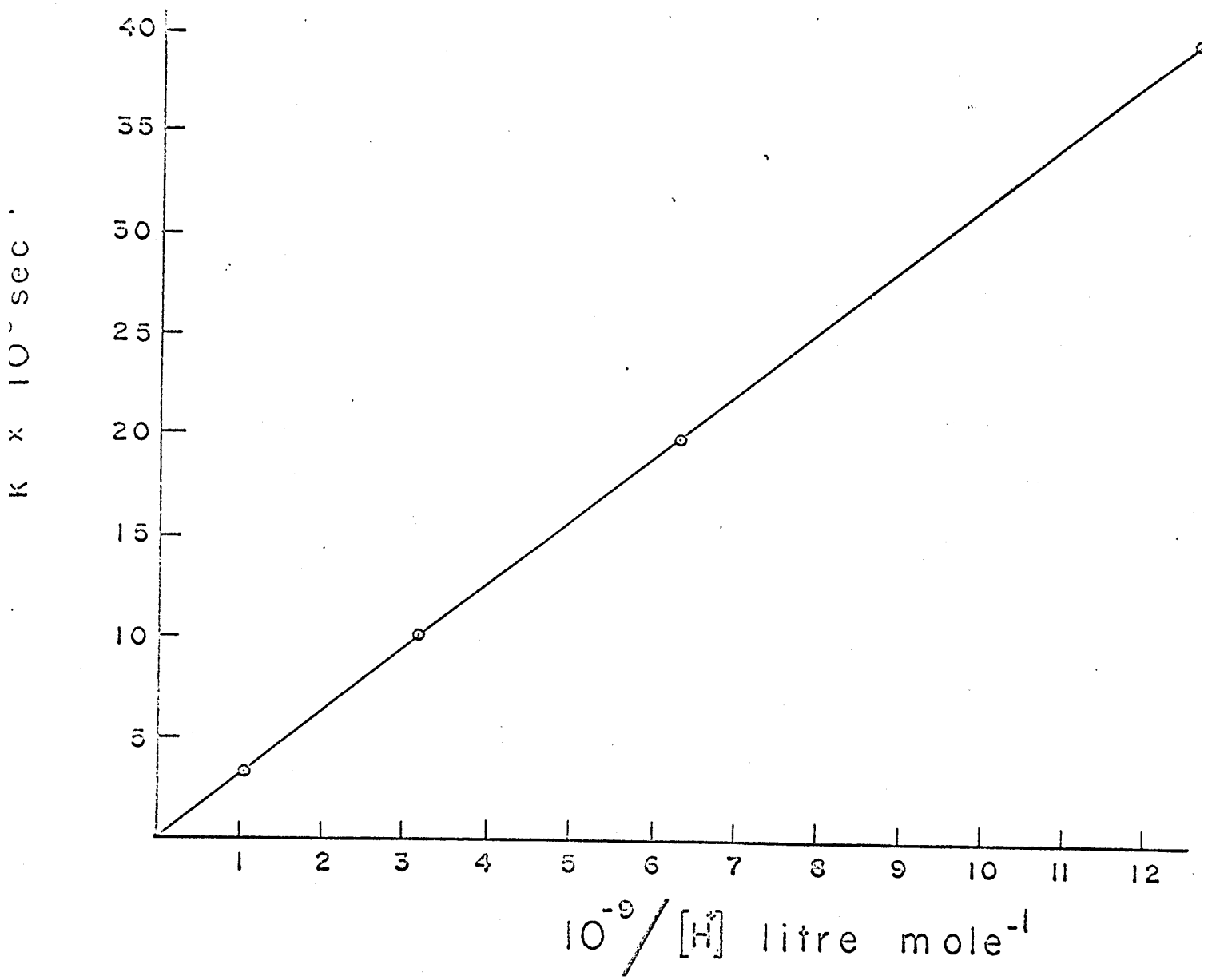
The reactions were carried out in 15.0 ml of solution containing 15 meq. of sodium chloride and maintained at 20.0 ± .05 in a thermostatically controlled bath. Stock solutions of enzyme and substrate were made up in water. Enzyme concentrations were in the region of 1 to 5 x 10^{-5}M, based on a molecular weight of 24,800(80,81), and substrate concentrations were from 5 x 10^{-4} to 3 x 10^{-3}M depending upon the solvent composition and pH.

In a typical run, substrate and sodium chloride solutions were mixed to 10.0 ml. Then dioxane or water or both were added to a total volume of 4.0 ml. The pH was adjusted to the desired value with 2N base delivered by means of a micrometer screw gauge. 1.0 ml of enzyme was added to start the reaction. The reactions were followed to less than 5% completion.

Since reactions were carried out up to high pH values, considerable basic hydrolysis was encountered. It was found that the best method for correction was to find the first-order constant $k = v/[S]$ at various pHs and then to plot k vs. $1/[H^+]$. These plots, of which an example is shown in Figure 10, were linear. The correction can then be directly calculated.

Figure 10

A plot of k against $1/[H^+]$.


40
35
30
25
20
15
10
5
K x 10⁻ sec⁻
1
2
3
4
5
6
7
8
9
10
11
12
10⁻⁹ / [H⁺] litre mole⁻¹

RESULTS AND DISCUSSION

Since the rates measured were at low substrate concentrations they relate to the second-order constant $\tilde{k}_c/\tilde{K}_m$(cf. Section A), and the pKs obtained are for the free enzyme. A plot of rate against [S] should be a straight line going through the origin; figure 11 shows that this is the case.

Figure 12 shows the effect of pH in pure water, and figure 13 is for 6.67%, 13.3% and 26.6% (v/v) dioxane-water mixtures. Figure 13 also shows a superposition of the curves, with the maximum rates normalized to the same value. Tables 3, 4, 5 and 6 summarize the data for the various solvent mixtures. The data clearly correspond to Case (iii), in which the ionizing groups are cationic-neutral. The lower pK remains constant at about 6.9, but the upper one is increased markedly as dioxane is added. The pK values in the various mixtures are summarized in Table 2.

The group of lower pK has previously been postulated to be an imidazole group, on the basis of the studies using a specific imidazole inhibitor (19,82), and more recently from a comparison of the amino acid sequences in chymotrypsinogen and trypsinogen(21-24,83,84). The present results are in agreement with this conclusion, the

Figure 11

Plots of rates against [S] for the hydrolysis of N-benzoyl-L-alanine in 13.3%(v/v) dioxane-water.

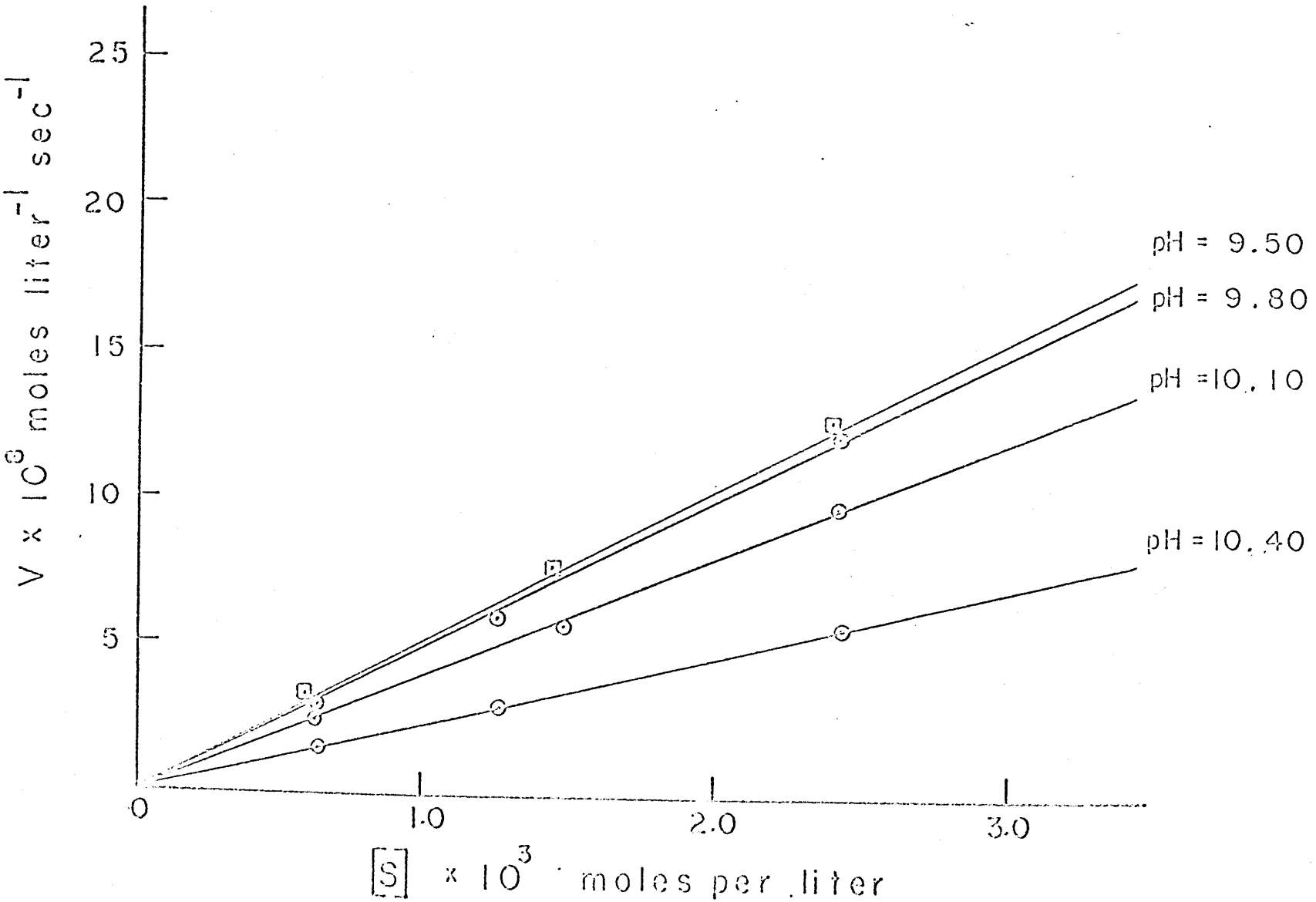
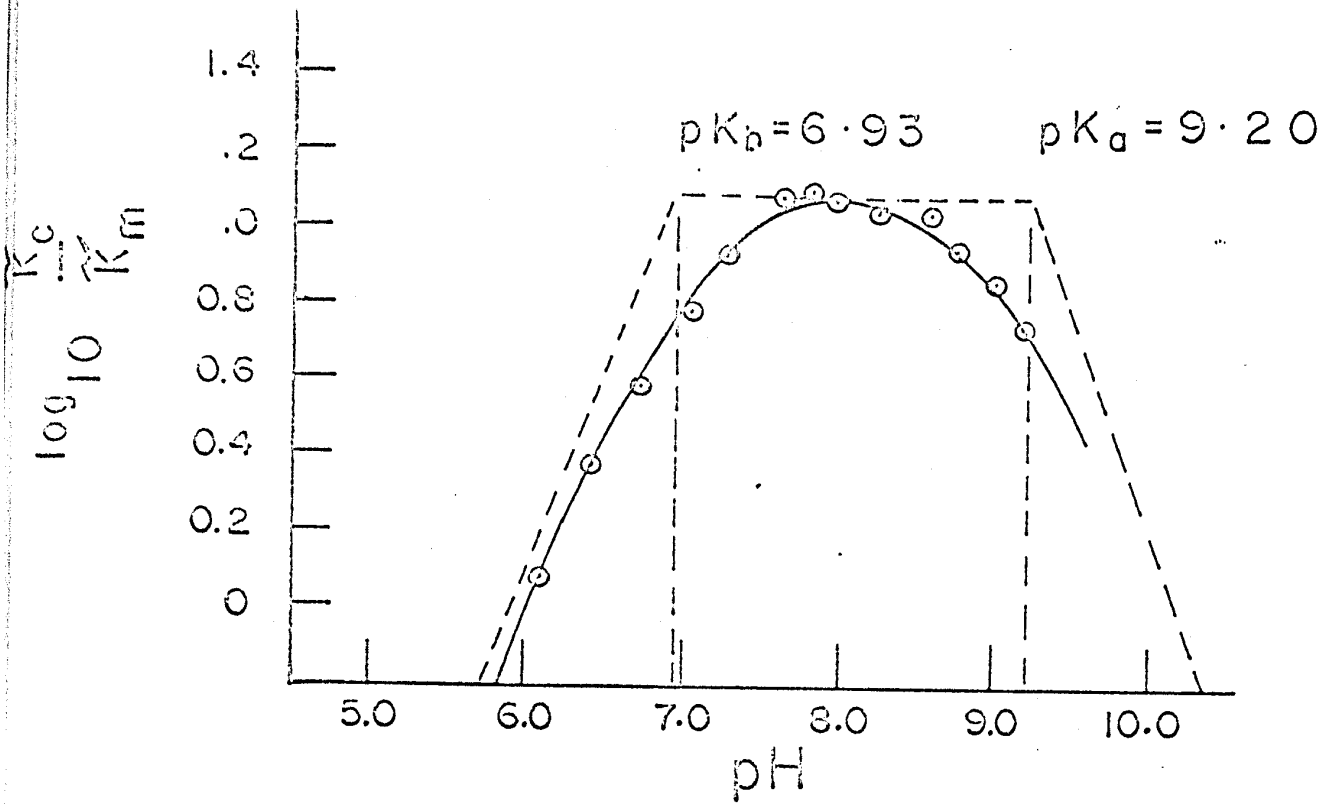

V x 10^8 moles liter^-1 sec^-1
25
20
15
10
5
0
1.0
2.0
3.0
[S] x 10^3 moles per liter
pH = 9.50
pH = 9.80
pH = 10.10
pH = 10.40

Figure 12

Logarithms of $\tilde{k}_c$ and $\tilde{k}_c/\tilde{K}_m$ in water plotted against pH for the α-chymotrypsin catalyzed hydrolysis of N-benzoyl-L-alanine methyl ester; T=20.0°C, 0.10N sodium chloride.


$\log_{10} \tilde{K}_c : \tilde{K}_m$
1.4
.2
.0
0.8
0.6
0.4
0.2
0
$pK_b = 6 \cdot 93$
$pK_a = 9 \cdot 20$
5.0
6.0
7.0
8.0
9.0
10.0
pH

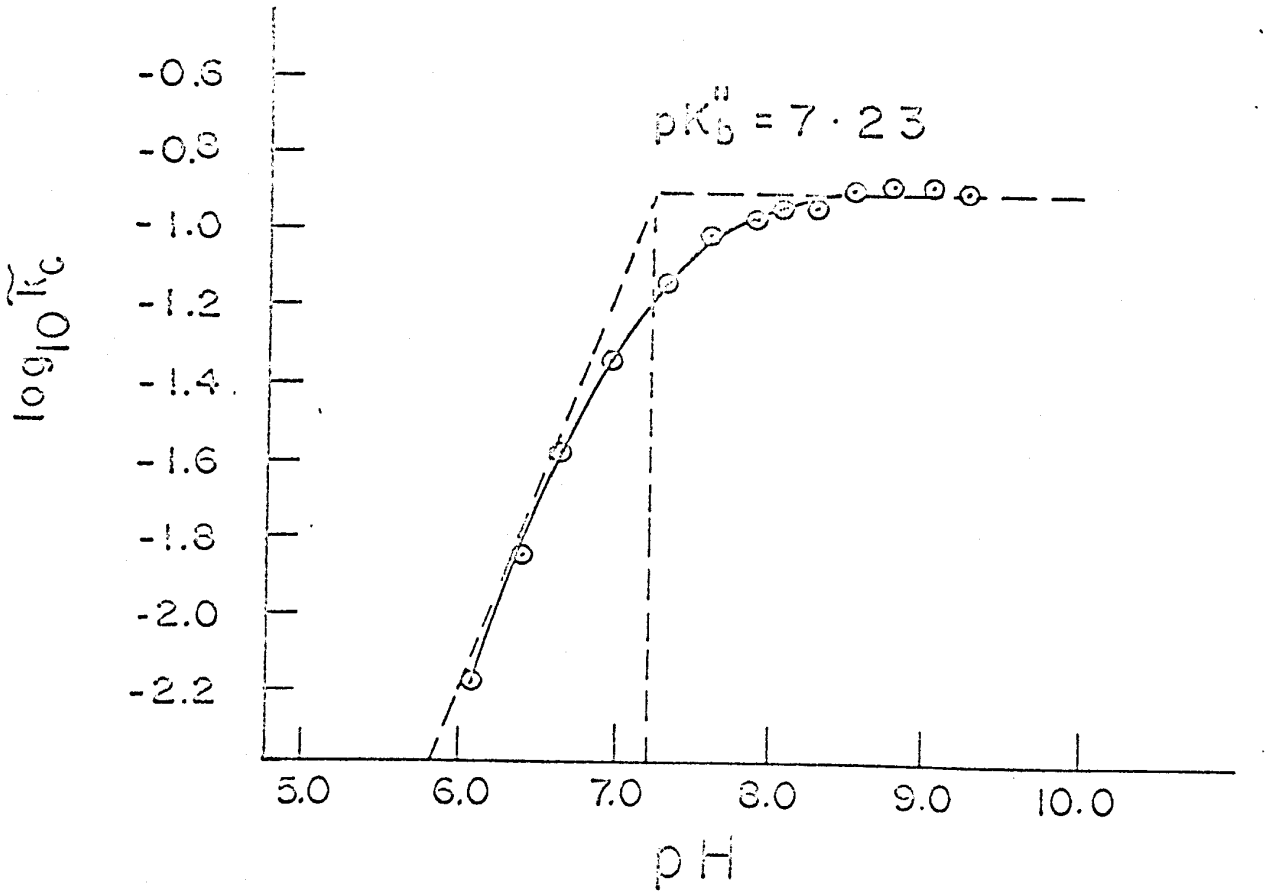
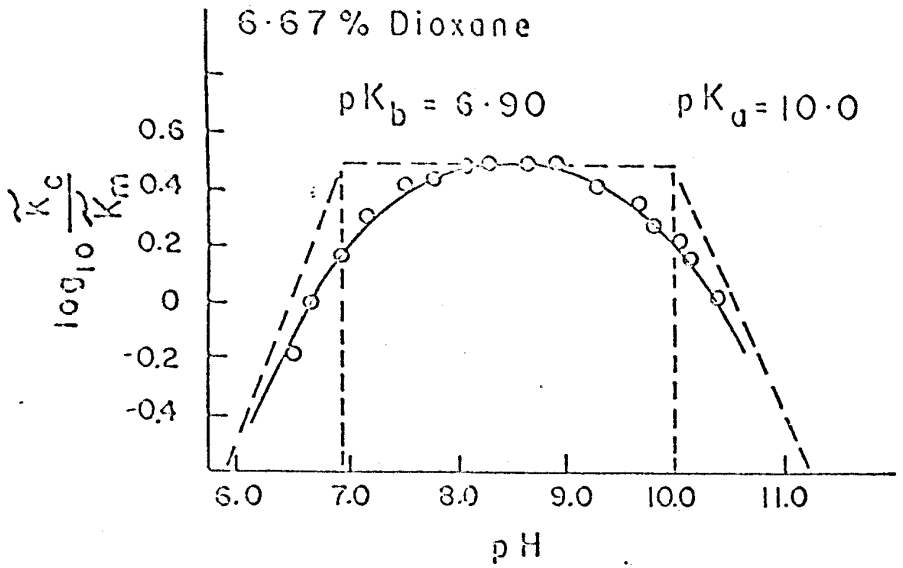
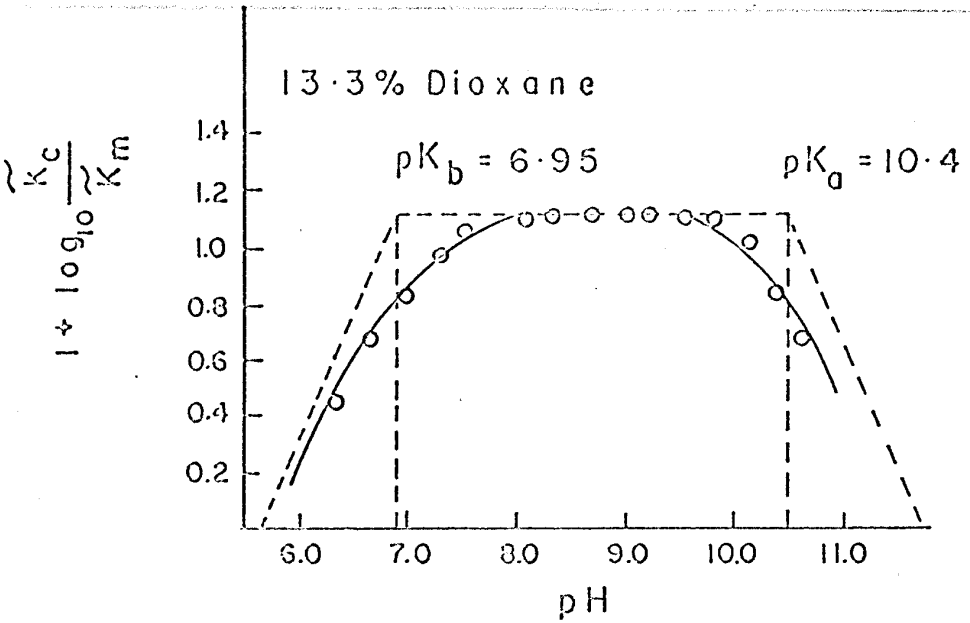
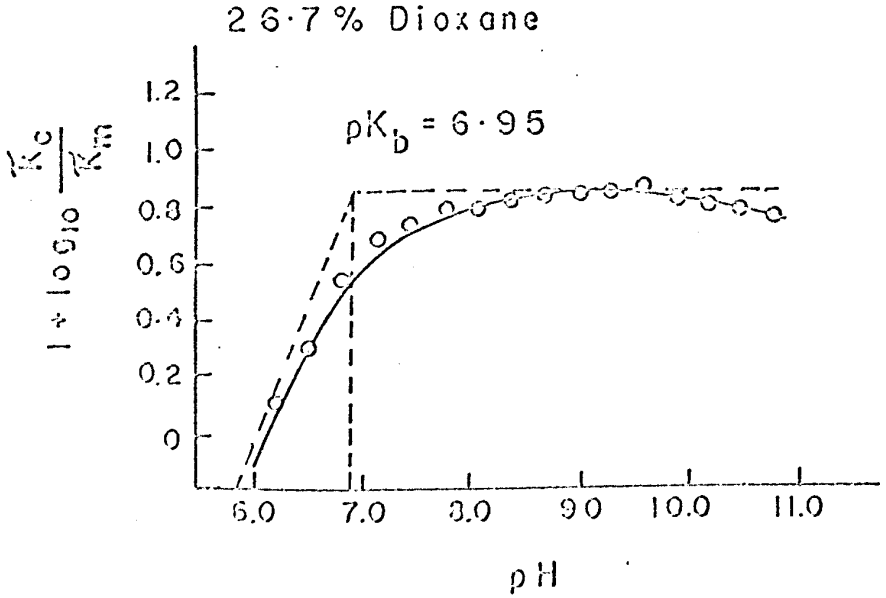

$\log_{10} \tilde{K}_c$
-0.6
-0.8
-1.0
-1.2
-1.4
-1.6
-1.8
-2.0
-2.2
$pK_b'' = 7 \cdot 23$
5.0
6.0
7.0
8.0
9.0
10.0
pH

Figure 13

Logarithm of $\tilde{k}_c/\tilde{K}_m$ plotted against pH for N-benzoyl-L-alanine methyl ester in 6.67%, 13.3%, and 26.7%(v/v) dioxane-water and the superposition of these curves; T=20.0°C, 0.10N sodium chloride.


6·67 % Dioxane
pK_b = 6·90
pK_a = 10·0
log10 kc/km
0.6
0.4
0.2
0
-0.2
-0.4
6.0
7.0
8.0
9.0
10.0
11.0
pH


13·3 % Dioxane
pK_b = 6·95
pK_a = 10·4
1 + log10 kc/km
1.4
1.2
1.0
0.8
0.6
0.4
0.2
6.0
7.0
8.0
9.0
10.0
11.0
pH


26·7 % Dioxane
pK_b = 6·95
1 + log10 kc/km
1.2
1.0
0.8
0.6
0.4
0.2
0
6.0
7.0
8.0
9.0
10.0
11.0
pH

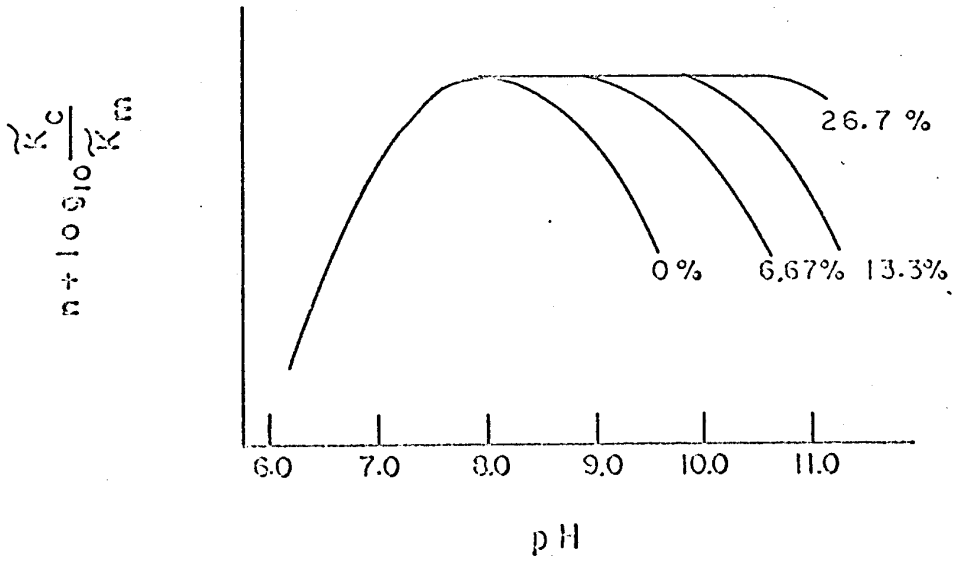

n + log10 kc/km
26.7 %
0 %
6.67%
13.3%
6.0
7.0
8.0
9.0
10.0
11.0
pH

Table 2

pK values in the various solvent mixtures

Volume % Dioxane	$D_{(100)}$	pK_b	pK_a
0	80.38	6.93	9.2
6.67	74.5	6.90	10.0
13.3	69.0	6.95	10.4
26.7	57.0	6.90	11.2

Table 3

$\tilde{k}_c/\tilde{K}_m$ Values in Water at 20°C

pH	$\tilde{k}_c/\tilde{K}_m$ litre $mole^{-1} sec^{-1}$	$\log_{10} \tilde{k}_c/\tilde{K}_m$
6.10	1.07	0.0067
6.40	2.28	0.359
6.70	3.78	0.578
7.00	6.23	0.795
7.30	8.82	0.945
7.60	12.1	1.081
7.80	12.3	1.089
8.00	11.7	1.071
8.25	11.2	1.052
8.50	10.8	1.034
8.75	8.79	0.944
9.00	7.08	0.848
9.25	5.66	0.753

Table 4

$\tilde{k}_c/\tilde{K}_m$ Values in 6.67%(v/v) Dioxane at 20°C

pH	$\tilde{k}_c/\tilde{K}_m$ litre $mole^{-1}sec^{-1}$	$\log_{10} \tilde{k}_c/\tilde{K}_m$
6.40	0.645	-0.190
6.60	0.972	-0.022
6.90	1.45	0.163
7.20	1.95	0.290
7.50	2.51	0.400
7.75	2.76	0.442
8.00	3.13	0.496
8.30	3.16	0.500
8.60	3.18	0.502
8.90	3.13	0.496
9.20	2.31	0.364
9.50	2.05	0.312
9.70	2.31	0.364
9.80	1.91	0.280
10.00	1.79	0.253
10.10	1.49	0.173
10.30	1.07	0.030

Table 5

$\tilde{k}_c/\tilde{K}_m$ Values in 13.3%(v/v) Dioxane at 20°C

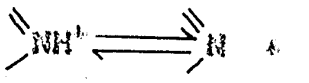
pH	$\tilde{k}_c/\tilde{K}_m$ x 10 litre $mole^{-1} sec^{-1}$	$1 + \log_{10} \tilde{k}_c/\tilde{K}_m$
6.30	2.89	0.461
6.60	4.88	0.688
6.90	7.37	0.868
7.20	9.54	0.980
7.50	11.3	1.05
8.00	12.7	1.10
8.30	13.8	1.14
8.60	13.9	1.14
8.90	14.0	1.15
9.20	14.1	1.15
9.50	13.6	1.13
9.80	12.9	1.11
10.10	10.5	1.02
10.40	6.54	0.816
10.63	2.60	0.445

Table 6

$\tilde{k}_c/\tilde{k}_m$ Values in 26.7%(v/v) Dioxane at 20°C

pH	$\tilde{k}_c/\tilde{k}_m$ x 10 litre mole^{-1}sec^{-1}	$1 + \log_{10} \tilde{k}_c/\tilde{k}_m$
6.20	1.29	0.111
6.50	2.05	0.312
6.80	3.34	0.524
7.10	5.08	0.706
7.40	5.58	0.747
7.70	6.25	0.796
8.00	6.29	0.799
8.30	6.79	0.832
8.60	6.70	0.826
8.90	7.06	0.849
9.20	7.17	0.856
9.50	7.69	0.886
9.80	6.80	0.832
10.1	6.39	0.806
10.4	6.85	0.836
10.7	6.30	0.800

ionization in question being of the type



The active species is the neutral-group  .

The group of pK ~ 9 (in water) has previously been postulated to be the α-ammonium group of the N-terminal isoleucine residue, the evidence being that when this group is acetylated, δ-chymotrypsin is inactivated (25,31,85,86). This result, however, does not prove that this group is involved directly in the bond breaking process; it might play merely a conformational role. The present results are not consistent with the conclusion that the group is an amino group; the group must be a neutral group, in view of the very considerable effect of dielectric constant on the ease of its ionization.

Possible groups to be considered are:

1) The -OH group of a serine residue. There is much evidence that a serine is involved in the acylation of the enzyme. However, the pK of an ordinary serine residue is greater than 13 (87). A downward shift of 4 pK units, while large, is possible if neighboring groups give rise to strong electrostatic effects or hydrogen bonding. On this hypothesis the serine hydroxyl group acts as a powerful nucleophile, and this requires that the bond between the oxygen and hydrogen atoms is weakened by interaction with neighboring groups.

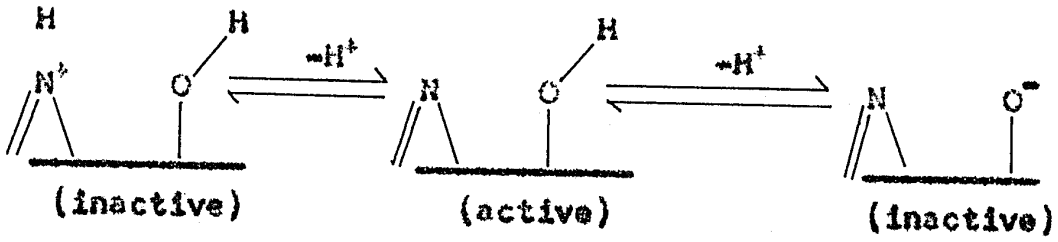
2) An -SH group. This ionizes in the right pH range, but in chymotrypsin all the -SH groups have been shown to be tied up in disulfide bridges(24). Furthermore, the enzyme is not inactivated by sulfhydryl inhibitors(88). This possibility therefore does not seem likely.

3) A tyrosine -OH group. This ionizes in the right range. Comparison of the amino acid sequence in trypsin and chymotrypsin(23) shows that there are two tyrosine residues, nos. 94 and 229, in analogous positions in the two enzymes. Filmer and Koshland(89) have concluded from iodination of tyrosine residues in chymotrypsin that none are essential for activity. However, it is possible that iodination has not changed the property of tyrosine responsible for its function, viz., the proton-transferring ability of the hydroxyl group. An indication that tyrosine may be essential comes from the observation of Dube and co-workers(90) that a tyrosine residue is protected by diisopropylfluorophosphate against iodination.

Possibilities (1) and (3) are the most reasonable and on the whole we favor (1), i.e., that the group that ionizes at a pK of about 9 is the serine hydroxyl group. The mechanistic implications of this are considered in the following section.

The conclusion from this work is therefore that as far as ionizing groups are concerned the active center

of the enzyme may be represented as follows:



It may be noted incidentally that the maximum k_c/K_m values decrease as the dielectric constant of the medium decreases (cf. Figures 12 and 13). This result is consistent with a previously observed (91-93) increase in K_m as the dielectric constant is lowered, and suggests that there is an increase in polarity as the complex is formed from enzyme and substrate.

Appendix

An important question is whether one is justified in interpreting the kinetic results in mixed solvents on the basis of a dielectric constant effect. This matter is now considered.

The addition of an organic solvent such as dioxane to an enzyme system may affect the behavior in several ways:

1) It may alter the conformation and give rise to a reversible or irreversible denaturation. If there were a rapid irreversible denaturation the system would not be suitable for study along the present lines; slow

irreversible denaturation could be allowed for. In the case of α-chymotrypsin there is no evidence for a significant amount of denaturation, since the value of $\tilde{k}_c$ is only slightly decreased by the addition of organic solvents (91,94). In any case, even if some reversible denaturation did occur it would make no difference in studies of the present kind, which are based on relative values of the kinetic parameters. What is being measured in a given solvent mixture is the variation with pH of the amounts of active enzyme, and on this basis the pK shifts are deduced.

2) The solvation effects will be different in the different solvent mixtures. This is equally true in non-enzymatic systems, for which a consideration of dielectric constant has proved satisfactory in interpreting pK values(95). To some extent solvation effects are included in dielectric constant effects.

3) The added organic substance may act as an enzyme inhibitor by competing for active sites on the enzyme. In the case of α-chymotrypsin the true K_m value is independent of pH(42,71), so that the substrate binding is independent of pH. Inhibitor binding would also be independent of pH, so that the present results are not affected by this factor. If there were a variation with pH of solvent binding this would have to be corrected for.

4) The concentration of water varies. This will not affect the present results for the same reasons as above.

It is concluded that it is valid to interpret the results of the present investigation in terms of dielectric constant effects.

Dioxane is a particularly satisfactory solvent for this work for the following reasons:

1) It is completely miscible with water.

2) It has been demonstrated that with a glass electrode one measures the true pH value in dioxane-water mixtures(96-98).

3) It cannot enter into reaction with the substrate (unlike methanol, which has been shown(99) to give rise to methanolysis).

4) Its low dielectric constant(100) allows the dielectric constant of the solvent to be lowered to a considerable extent by the addition of a small amount of dioxane.

D. Influence of pH, and the Mechanism of Reaction

INTRODUCTION

Numerous studies have been made of the influence of pH on chymotrypsin kinetics, and the results have recently been summarized and interpreted by Bender(42). As we have discussed earlier in Section A, measurements at low substrate concentrations reveal the pK values for groups in the free enzyme that are involved in the breakdown of the enzyme substrate complex. The present section describes the results of a study over a range of pH values and substrate concentrations, of the hydrolyses of N-benzoyl-D and L-alanine methyl esters, N-acetyl-L-tyrosine ethyl ester and p-nitrophenyl acetate. The results are compared with other pH results, and on the basis of them, and of other evidence, a conclusion is drawn as to the reaction mechanism.

EXPERIMENTAL

The experimental technique was exactly as described in Sections III B and III C. N-benzoyl-D-alanine methyl ester was prepared as described for the L-enantiomer; m.p., 57-58°C; $[\alpha]D = -30.6°$ in 5% sym-tetrachloroethane. N-acetyl-L-tyrosine ethyl ester was purchased in pure form

from Mann Biochemicals and used without further purification. p-Nitrophenyl acetate was prepared by the method of Chattaway(101) and was purified by three crystallizations from an alcohol-water mixture; m.p. 82-84°C.

In the study of the hydrolysis of p-nitrophenyl acetate a correction to the rate has to be made since p-nitrophenol ionizes in the pH range studied. A portion of the p-nitrophenol will be titrated and the apparent rate of acetate production will be faster than the true rate. The correction may be calculated as follows.

$$O_2N\text{-}\phi\text{-}O\text{-}Ac \xrightarrow{H_2O} O_2N\text{-}\phi\text{-}OH + HAc \qquad (1)$$

$$HAc \underset{}{\overset{K_1}{\rightleftharpoons}} Ac^- + H^+ \qquad (2)$$

$$O_2N\text{-}\phi\text{-}OH \underset{}{\overset{K_2}{\rightleftharpoons}} O_2N\text{-}\phi\text{-}O^- + H^+ \qquad (3)$$

Since acetic acid has a pK_a of 4.75, it will be entirely in the acetate form at alkaline pHs. From 1, 2, and 3 it follows that

$$[Ac^-]_{true} = [O_2N\text{-}\phi\text{-}OH] + [O_2N\text{-}\phi\text{-}O^-] \qquad (4)$$

$$[Ac^-]_{app} = [Ac^-]_{true} + [O_2N\text{-}\phi\text{-}O^-] \qquad (5)$$

and

$$[Ac^-]_{app} = [O_2N\text{-}\phi\text{-}OH] + 2[O_2N\text{-}\phi\text{-}O^-] \qquad (6)$$

From the Henderson-Hasselbalch equation

$$\frac{[O_2N\text{-}\phi\text{-}O^-]}{[O_2N\text{-}\phi\text{-}OH]} = \text{antilog } (pH\text{-}pK_2) \qquad (7)$$

From equations 4, 6 and 7 it follows that

$$\frac{[Ac^-]_{true}}{[Ac^-]_{app}} = \frac{1 + \text{antilog } (pH\text{-}pK_2)}{1 + 2 \text{ antilog } (pH\text{-}pK_2)} \qquad (8)$$

and

$$\frac{d[Ac^-]_{app}}{dt} = V_{app} = \frac{\tilde{k}_c \dfrac{[1 + 2 \text{ antilog } (pH\text{-}pK_2)]}{[1 + \text{antilog } (pH\text{-}pK_2)]} [E]_o[S]}{\tilde{K}_m + [S]} \qquad (9)$$

Therefore the value of $\tilde{K}_m$, obtained from an Eadie plot, will not change but the value of $\tilde{k}_c$ obtained must be multiplied by the factor $[1 + \text{antilog } (pH\text{-}pK_2)]/[1 + 2 \text{ antilog } (pH\text{-}pK_2)]$ to obtain the true value of $\tilde{k}_c$.

Results

Figures 14, 15 and 16 show the pH dependencies of $\tilde{k}_c$, $\tilde{K}_m$ and $\tilde{k}_c/\tilde{K}_m$ for the three substrates. Figure 12 of the preceding section shows similar results for N-benzoyl-L-alanine methyl ester. The parameter $\tilde{k}_c$ is in all cases independent of pH on the basic side of the pH optimum and dependent on pH on the acid side. $\tilde{K}_m$ shows an inverse behavior to that of $\tilde{k}_c$, being independent of pH on the

Figure 14

Logarithms of $\tilde{k}_c$, $\tilde{K}_m$ and $\tilde{k}_c/\tilde{K}_m$ plotted against pH for the α-chymotrypsin catalyzed hydrolysis of p-nitrophenyl acetate in 20%(v/v) isopropyl alcohol-water, T=20.0°C and 0.10N sodium chloride.

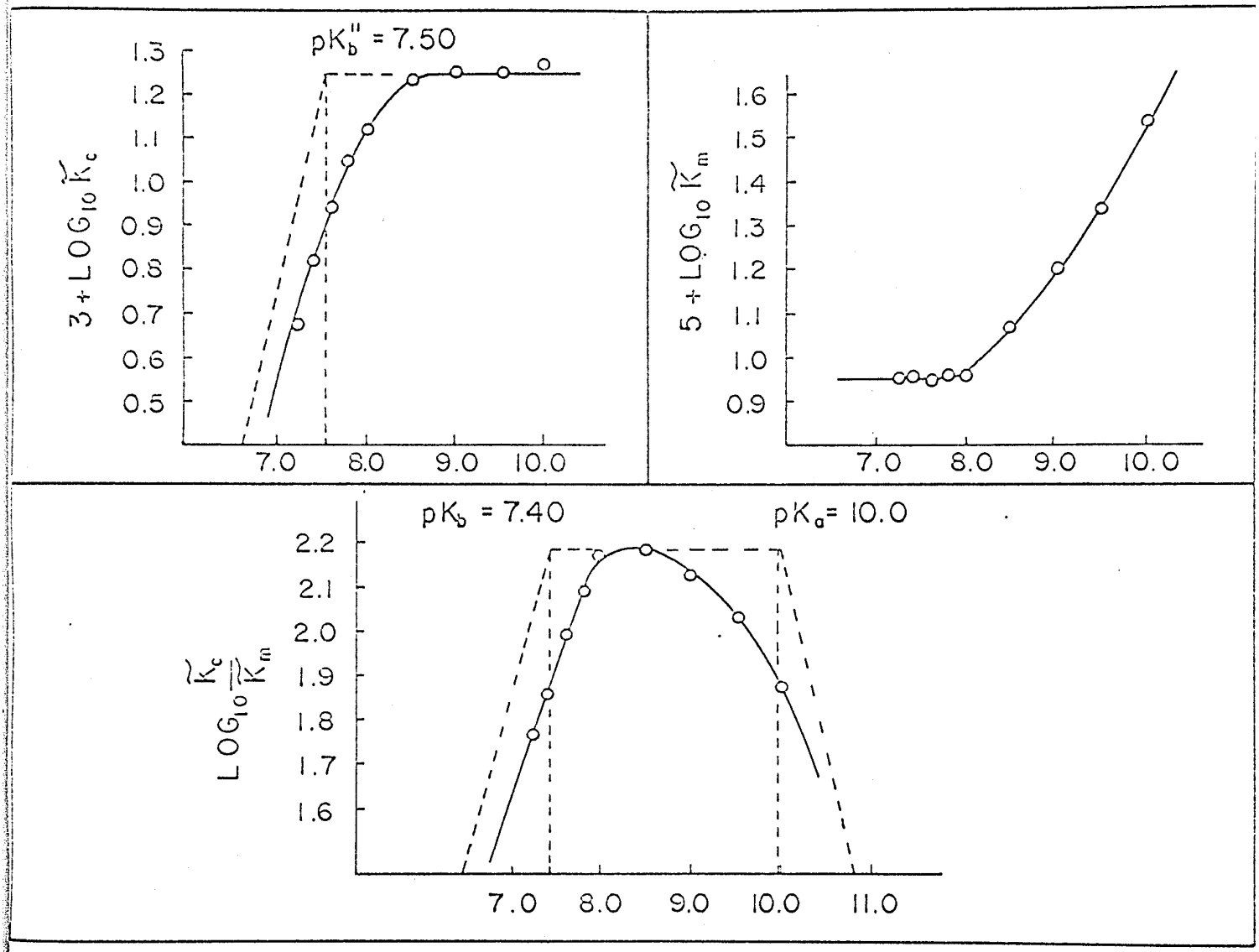
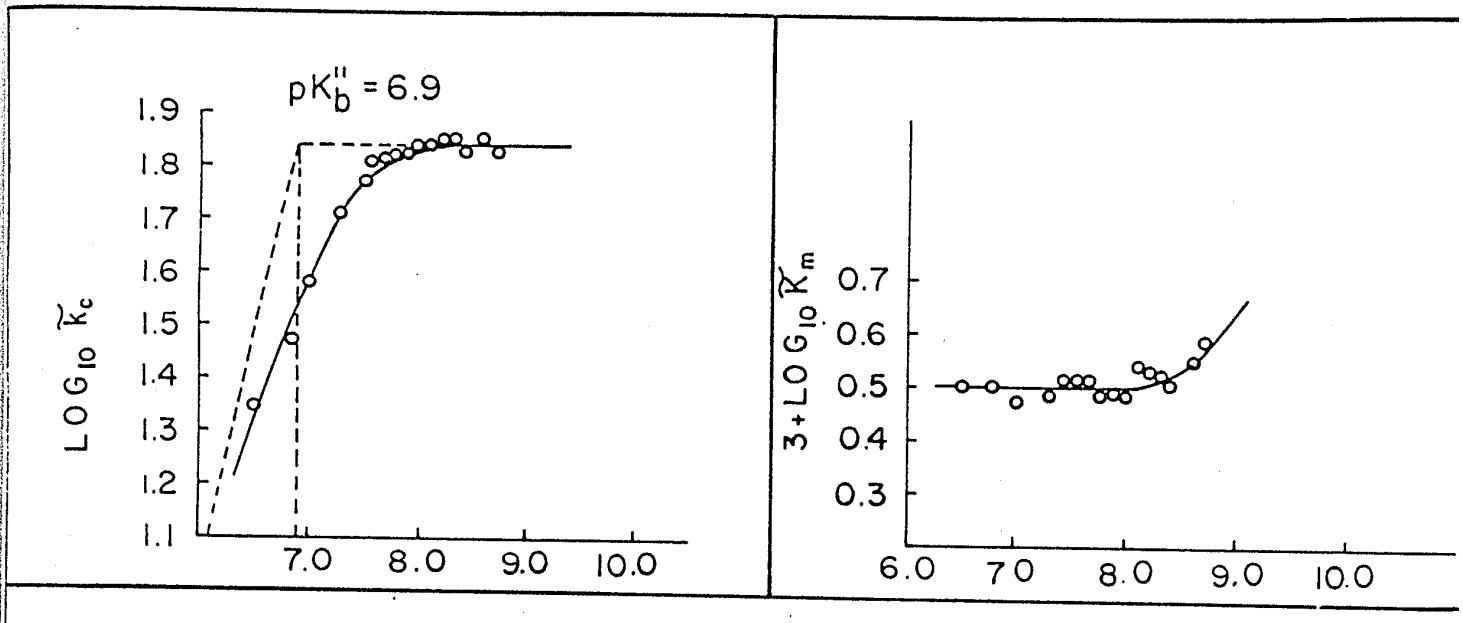
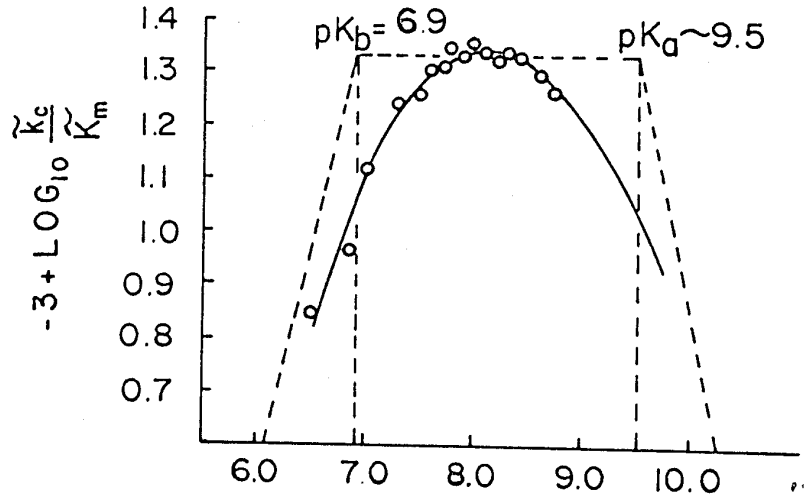

pK'b = 7.50
3 + LOG10 K̃c
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
7.0
8.0
9.0
10.0
5 + LOG10 K̃m
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
7.0
8.0
9.0
10.0
pKb = 7.40
pKa = 10.0
LOG10 K̃c / K̃m
2.2
2.1
2.0
1.9
1.8
1.7
1.6
7.0
8.0
9.0
10.0
11.0
pH

Figure 15

Logarithms of $\tilde{k}_c$, $\tilde{K}_m$ and $\tilde{k}_c/\tilde{K}_m$ plotted against pH for the α-chymotrypsin catalyzed hydrolysis of N-acetyl-L-tyrosine ethyl ester in 5.0%(v/v) dioxane-water, T=20.0°C and 0.10N sodium chloride.

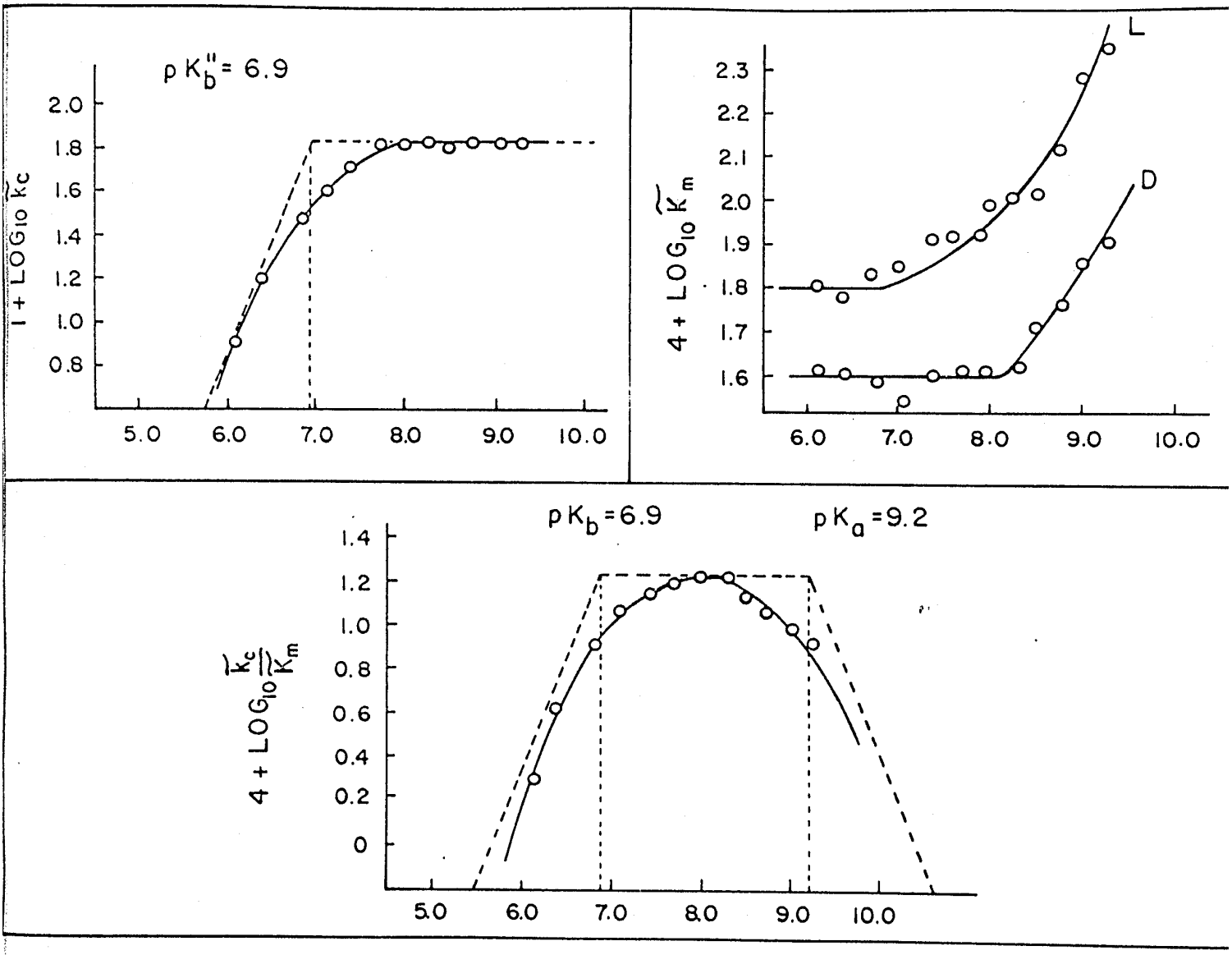

pK'b = 6.9
LOG10 k̃c
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
7.0
8.0
9.0
10.0
3+LOG10 K̃m
0.7
0.6
0.5
0.4
0.3
6.0
7.0
8.0
9.0
10.0


pKb = 6.9
pKa ~ 9.5
-3+LOG10 k̃c/K̃m
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
6.0
7.0
8.0
9.0
10.0

pH

Figure 16

Logarithms of $\tilde{k}_c$, $\tilde{K}_m$ and $\tilde{k}_c/\tilde{K}_m$ plotted against pH for the α-chymotrypsin catalyzed hydrolysis of N-benzoyl-D-alanine methyl ester in water, T=20.0°C and 0.10N sodium chloride.


pK''b = 6.9
1 + LOG10 k̃c
2.0
1.8
1.6
1.4
1.2
1.0
0.8
5.0
6.0
7.0
8.0
9.0
10.0
L
D
4 + LOG10 K̃m
2.3
2.2
2.1
2.0
1.9
1.8
1.7
1.6
6.0
7.0
8.0
9.0
10.0
pKb = 6.9
pKa = 9.2
4 + LOG10 k̃c / K̃m
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
5.0
6.0
7.0
8.0
9.0
10.0
pH

acid side of the pH optimum and dependent on pH on the basic side. The ratio $\tilde{k}_c/K_m$ shows a pH dependence on both sides of the pH optimum.

Figures 17 to 20 show some typical Eadie plots for the substrates studied, while Tables 7 to 10 contain the data for the Eadie plots at pH 8.00 in order to illustrate the magnitude of the rates measured. The data for the pH dependence of the kinetic parameters are summarized in Tables 11 to 14.

DISCUSSION

The pH dependence of the hydrolysis of p-nitrophenyl acetate has previously been investigated at least four times (42,102-104). In all but one study the pH dependence was not studied above pH 7.8. Bender and co-workers(42), have studied the reaction up to pH 9.98 using the stopped-flow technique in the second-order kinetic region in which $[E]_o \simeq [S]_o \ll K_s$. The results leave little doubt that p-nitrophenyl acetate is hydrolysed by a three-step mechanism, the first step being the binding of the substrate, the second acylation and the third deacylation. In the present work, we have studied to pH 10.0 the overall steady-state kinetics. It should be noted that while the four substrates studied have considerable structural differences, and different kinetic specificities for the enzyme, they all show almost identical pH depen-

Figure 17

Eadie plots for p-nitrophenyl acetate on the acid side of the pH optimum.

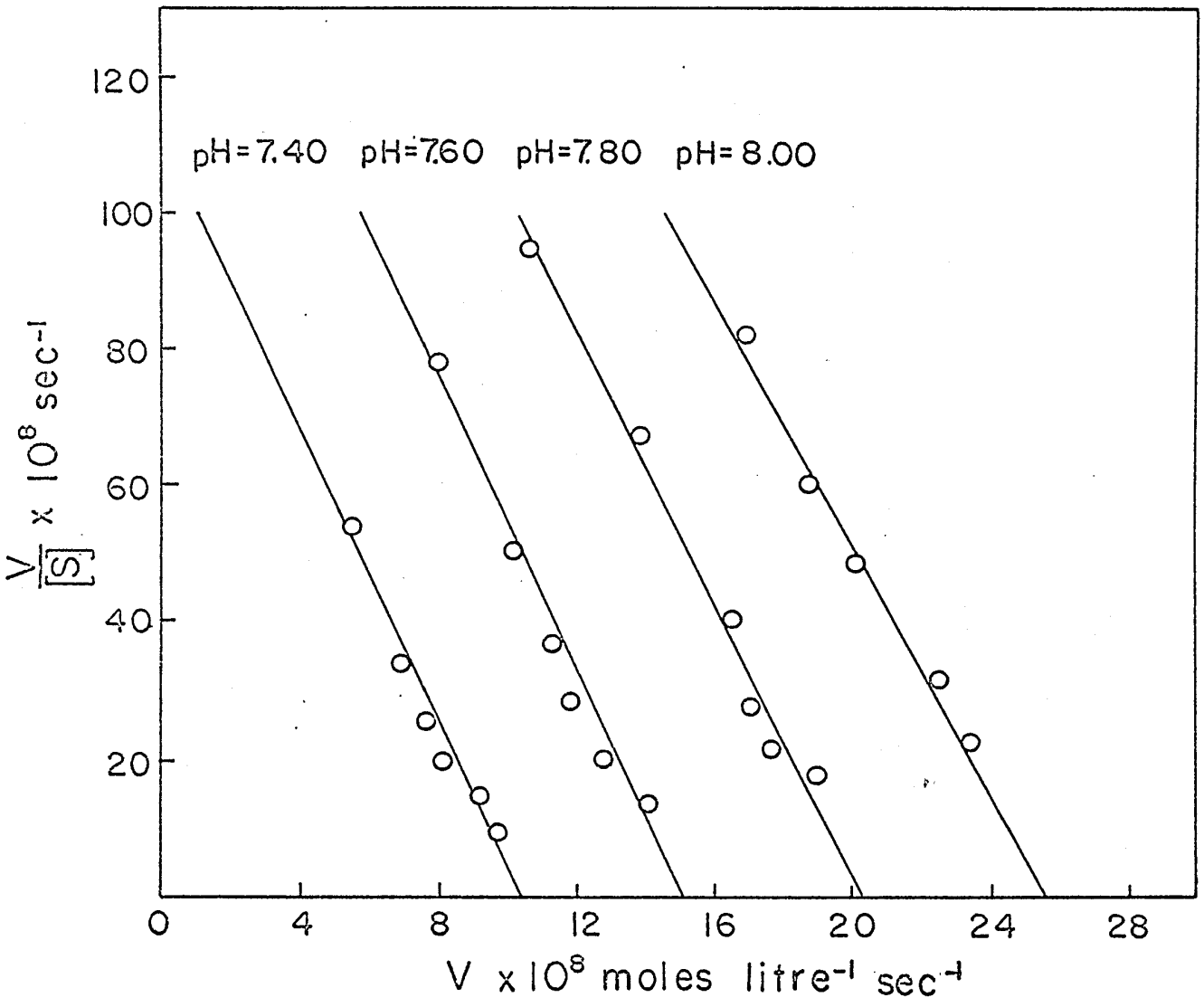

pH=7.40
pH=7.60
pH=7.80
pH=8.00
120
100
80
60
40
20
0
4
8
12
16
20
24
28
$\frac{V}{[S]} \times 10^8 \text{ sec}^{-1}$
V x 10^8 moles litre^{-1} sec^{-1}

Figure 18

Eadie plots for N-acetyl-L-tyrosine ethyl ester on the acid side of the pH optimum.

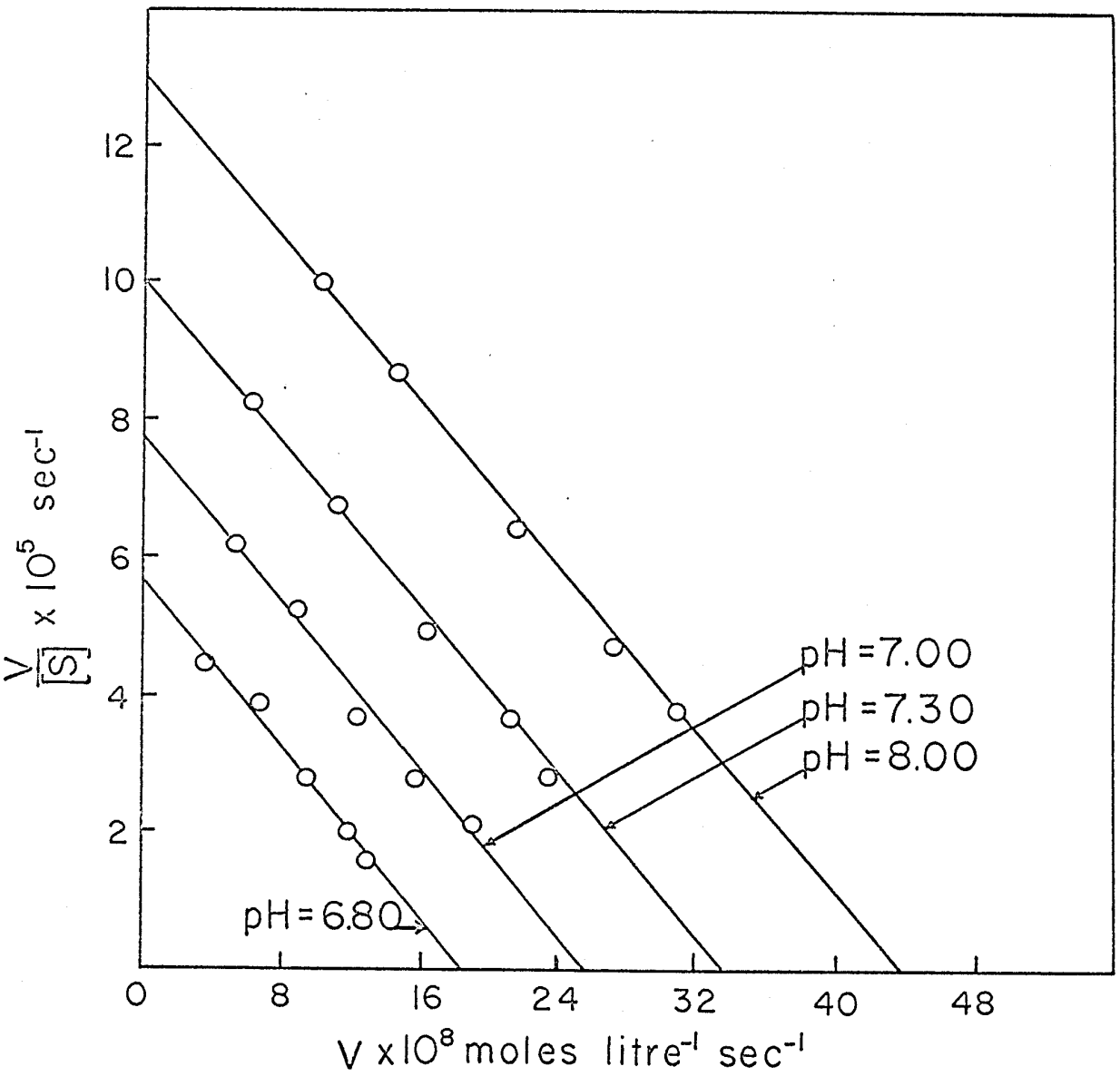

12
10
8
6
4
2
0
8
16
24
32
40
48
$\frac{V}{[S]} \times 10^5$ sec^{-1}
V x10^8 moles litre^{-1} sec^{-1}
pH=7.00
pH=7.30
pH=8.00
pH=6.80

Figure 19

Eadie plots for N-benzoyl-L-alanine methyl ester on the basic side of the pH optimum.

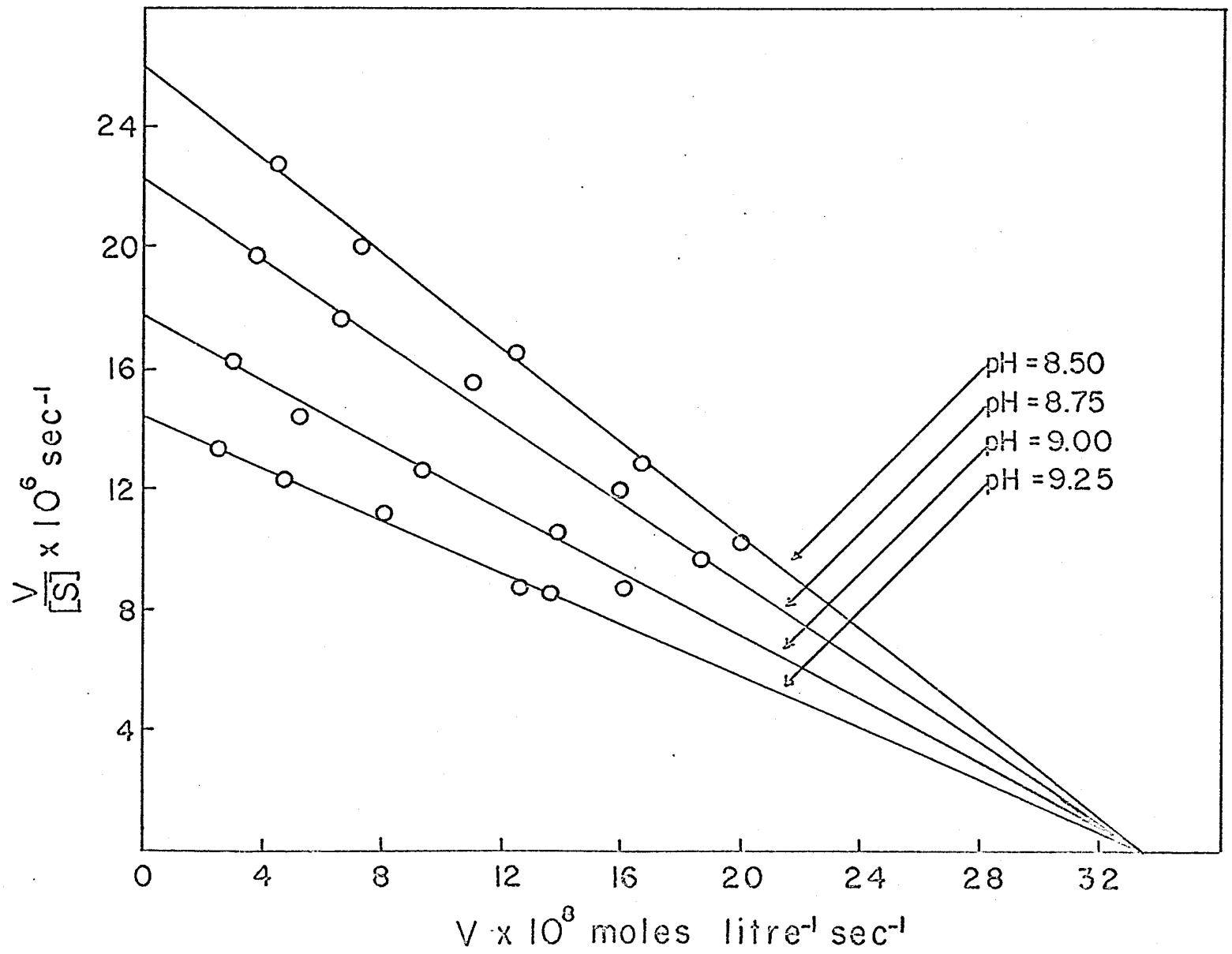

24
20
16
12
8
4
0
4
8
12
16
20
24
28
32
V/[S] x 10^6 sec^-1
V x 10^8 moles litre^-1 sec^-1
pH = 8.50
pH = 8.75
pH = 9.00
pH = 9.25

Figure 20

Eadie plots for N-benzoyl-D-alanine methyl ester on the basic side of the pH optimum.

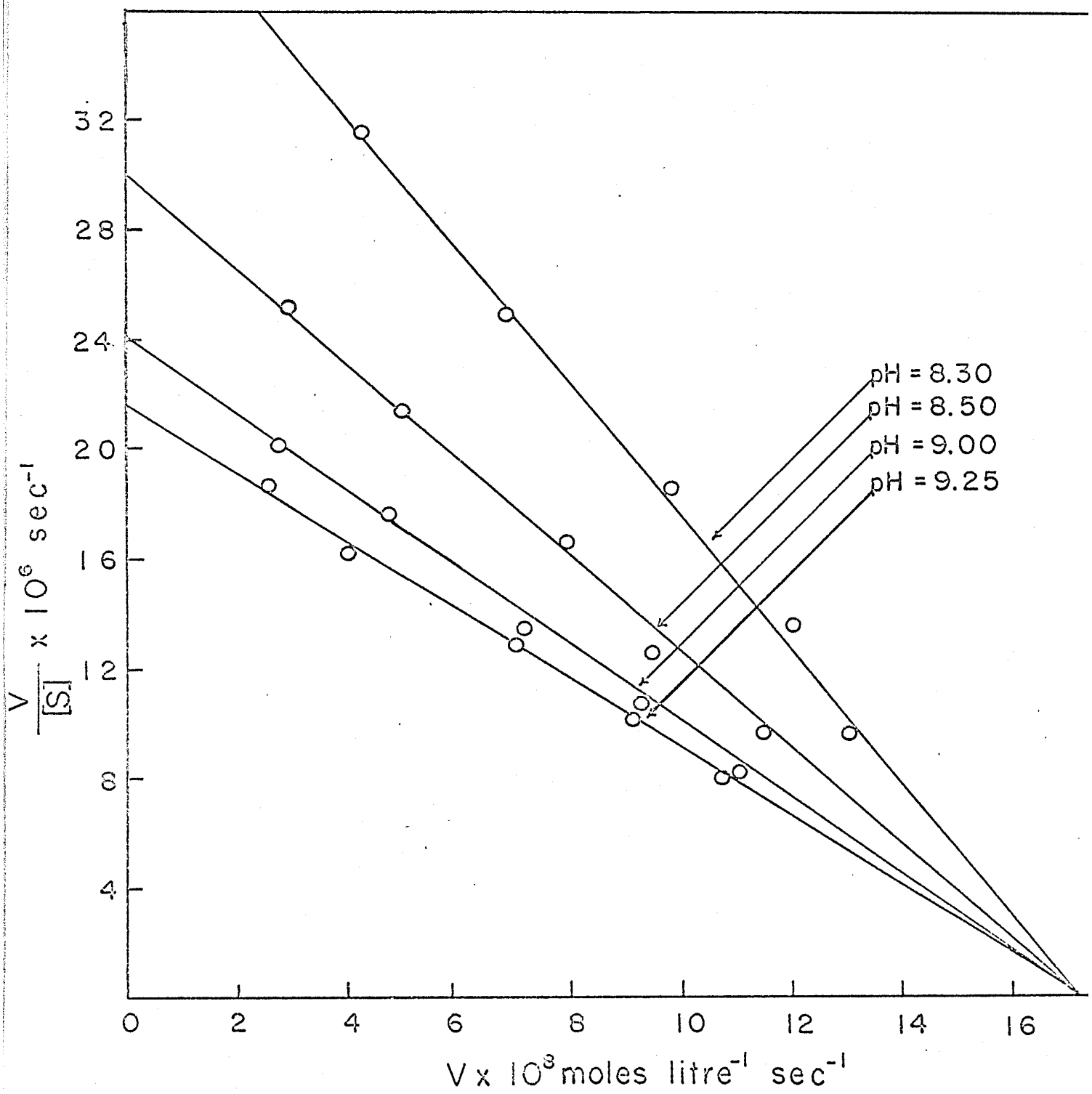

pH = 8.30
pH = 8.50
pH = 9.00
pH = 9.25
$\frac{V}{[S]} \times 10^6$ sec^{-1}
V x 10^3 moles litre^{-1} sec^{-1}
0
2
4
6
8
10
12
14
16
4
8
12
16
20
24
28
32

Table 7

Hydrolysis of p-Nitrophenyl Acetate at pH 8.00 in 20%(v/v) Isopropyl Alcohol and 0.10N Sodium Chloride at 20.0°C.
$[E]_o = 1 \times 10^{-5}$M

$[S] \times 10^4$M	$V \times 10^8$ moles litre^{-1}sec^{-1}	$v/[S] \times 10^5$ sec^{-1}
10.2	23.2	22.7
7.15	22.3	31.2
4.08	20.0	49.1
3.06	18.5	60.5
2.04	16.9	82.6
1.02	13.6	133.3

Table 8

Hydrolysis of N-Acetyl-L-Tyrosine Ethyl Ester at pH 8.00 in 5%(v/v) Dioxane and 0.10N Sodium Chloride at 20.0°C.
$[E]_o = 6.00 \times 10^{-9}$M

$[S] \times 10^3$M	$V \times 10^8$ moles litre^{-1}sec^{-1}	$v/[S] \times 10^5$ sec^{-1}
8.32	31.0	3.73
5.82	27.3	4.69
3.33	21.1	6.35
1.66	14.6	8.80
0.832	9.15	11.00

Table 9

Hydrolysis of N-Benzoyl-L-Alanine Methyl Ester at pH 8.00 in Water and 0.10N Sodium Chloride at 20.0°C.

$[E]_o = 2.50 \times 10^{-6}$M

$[S] \times 10^3$M	$V \times 10^8$ moles litre^{-1}sec^{-1}	$V/[S] \times 10^6$ sec^{-1}
17.9	18.6	10.4
12.6	16.1	12.8
7.18	12.3	17.2
3.59	7.78	21.7
1.79	4.42	24.6

Table 10

Hydrolysis of N-Benzoyl-D-Alanine Methyl Ester at pH 8.00 in Water and 0.10N Sodium Chloride at 20.0°C.

$[E]_o = 2.50 \times 10^{-5}$M

$[S] \times 10^3$M	$V \times 10^8$ moles litre^{-1}sec^{-1}	$V/[S] \times 10^6$ sec^{-1}
13.9	13.4	9.61
9.07	11.3	12.4
5.58	9.80	17.6
2.79	6.74	24.1
1.39	4.38	31.4

Table 11

pH Dependence of $\tilde{k}_c$, $\tilde{K}_m$ and $\tilde{k}_c/\tilde{K}_m$ for p-Nitrophenyl Acetate in 20%(v/v) Isopropyl Alcohol and 0.10N Sodium Chloride at 20°C

pH	$\tilde{k}_c \times 10^3$ sec^{-1}	$\tilde{K}_m \times 10^4$ moles litre^{-1}	$\tilde{k}_c/\tilde{K}_m$ litre moles^{-1} sec^{-1}
7.20	4.79	9.00	5.32
7.40	6.49	9.10	7.13
7.60	8.79	9.01	9.75
7.80	11.2	8.98	12.4
8.00	13.4	8.99	14.9
8.50	17.7	11.8	15.0
9.00	17.8	16.0	11.1
9.50	17.6	21.8	8.07
10.00	18.2	34.8	5.24

Table 12

pH Dependence of $\tilde{k}_c$, $\tilde{K}_m$ and k_c/K_m for N-Acetyl-L-Tyrosine Ethyl Ester in 5%(v/v) Dioxane and 0.10N Sodium Chloride at 20°C

pH	$\tilde{k}_c$ sec^{-1}	$\tilde{K}_m \times 10^3$ moles litre^{-1}	$\tilde{k}_c/\tilde{K}_m \times 10^{-3}$ litre mole^{-1} sec^{-1}
6.50	22.4	3.16	7.08
6.80	29.4	3.16	9.33
7.00	38.8	2.93	13.3
7.30	51.9	3.01	17.2
7.50	59.4	3.27	18.2
7.60	64.7	3.24	20.0
7.70	65.6	3.22	20.4
7.80	66.4	3.02	22.0
7.90	66.8	3.14	21.3
8.00	68.0	3.01	22.6
8.10	69.9	3.46	20.2
8.20	70.3	3.36	20.9
8.40	68.0	3.21	21.8
8.50	73.2	3.66	20.0
8.60	71.1	3.61	19.7
8.70	67.6	3.92	17.3

Table 13

pH Dependence of $\tilde{k}_c$, $\tilde{K}_m$ and $\tilde{k}_c/\tilde{K}_m$ for N-Benzoyl-L-Alanine Methyl Ester in Water and 0.10N Sodium Chloride at 20°C

pH	$\tilde{k}_c \times 10^2$ sec^{-1}	$\tilde{K}_m \times 10^3$ moles litre^{-1}	$\tilde{k}_c/\tilde{K}_m$ litre moles^{-1} sec^{-1}
6.10	0.644	6.33	1.02
6.40	1.36	5.97	2.28
6.70	2.53	6.69	3.78
7.00	4.34	6.96	6.23
7.30	7.20	8.16	8.82
7.60	10.0	8.29	12.1
7.80	10.4	8.47	12.3
8.00	11.5	9.81	11.8
8.25	11.6	10.3	11.3
8.50	12.5	11.5	10.8
8.75	13.5	15.4	8.79
9.00	13.3	18.9	7.08
9.25	12.6	22.3	5.66

Table 14

pH Dependence of $\tilde{k}_c$, $\tilde{K}_m$ and $\tilde{k}_c/\tilde{K}_m$ for N-Benzoyl-D-Alanine Methyl Ester in Water and 0.10N NaCl at 20°C

pH	$\tilde{k}_c \times 10^4$ sec^{-1}	$\tilde{K}_m \times 10^3$ moles litre^{-1}	$\tilde{k}_c/\tilde{K}_m$ litre moles^{-1} sec^{-1}
6.10	8.32	4.21	0.198
6.40	16.4	3.95	0.416
6.80	30.9	3.87	0.799
7.10	40.5	3.50	1.16
7.40	55.0	3.94	1.39
7.70	65.4	4.11	1.59
8.00	67.7	4.09	1.66
8.30	69.8	4.23	1.65
8.50	65.2	5.26	1.24
8.75	68.2	5.92	1.15
9.00	67.7	7.09	0.954
9.25	68.9	7.93	0.867

dencies for the kinetic parameters $\tilde{k}_c$, $\tilde{K}_m(app)$ and $\tilde{k}_c/\tilde{K}_m(app)$. This observation suggests that there is a similar mechanism of action of α-chymotrypsin on all these substrates.

Recently evidence has been presented that the closely related enzyme trypsin does not proceed through an acyl intermediate (105,106). However, Bender and co-workers have presented evidence to the contrary (107-109). It might be suggested that specific substrates of α-chymotrypsin do not proceed through an acyl intermediate; p-nitrophenyl acetate might be a special case, acting as an acylating agent by virtue of its chemical composition. Such a conclusion is difficult to reconcile with the pH dependencies presented here.

The present results show that the free enzyme contains two ionizing groups, of pK 7 and 9, which are involved in the acylation reaction. This conclusion is consistent with that arrived at on the basis of studies using substrates for which the acylation processes is slower than the deacylation one; examples are methyl hydrocinnamate (71) and the amides of aromatic amino acids (42). For these substrates the rate constant at high substrate concentrations, $\tilde{k}_c$, can be identified with $\tilde{k}_2$, so that the pH dependence reveals the pK values for the Michaelis complex. The pH dependence for these substrates is bell-shaped, and indicates pK values of about 7 and 9, in

agreement with the present results.

On the other hand, for substrates such as the ethyl esters of N-acetyl-L-tryptophan, N-acetyl-L-phenylalanine, and N-acetyl-L-tyrosine (42), N-benzoyl-D and L-alanine methyl esters and p-nitrophenyl acetate (cf. figures 14-16) the slow process is deacylation, and $\tilde{k}_c$ is equal to $\tilde{k}_3$. The pH dependence is sigmoid, there being no falling off of $\tilde{k}_3$ at high pH. These results indicate that the group of pK 7 is still catalytically active in the deacylation process. The group of pK 9 is not revealed in $\tilde{k}_3$, and two different types of explanation are possible for this:

(1) The group of pK 9 may not be free in the acyl enzyme. It might have undergone covalent bonding, or be interacting with other groups (e.g. by hydrogen bonding) in such a way that it is unable to lose its proton at high pH values.

(2) The group of pK 9 may simply not be involved in the deacylation process. Of these two possibilities Bender and Kézdy(25) favor the second. Our own preference is for the first type of explanation, for reasons discussed below; it is still, however, not possible to arrive at a firm decision.

Mechanistic Information

In arriving at a conclusion about the mechanism of the action of chymotrypsin it is necessary to take into

account a considerable number of experimental results. We have listed below the most important mechanistic information, classifying it according to its relevance to (1) the formation of the enzyme-substrate addition complex (the Michaelis complex), (2) the acylation process, and (3) the deacylation process. Some of the specific conclusions drawn are also indicated; the more general conclusions are discussed in a later section.

(1) Complex formation

(a) The pH work shows that two ionizing groups, of pK 7 and 9, are free in the free enzyme, and are also free in the complex (present work).

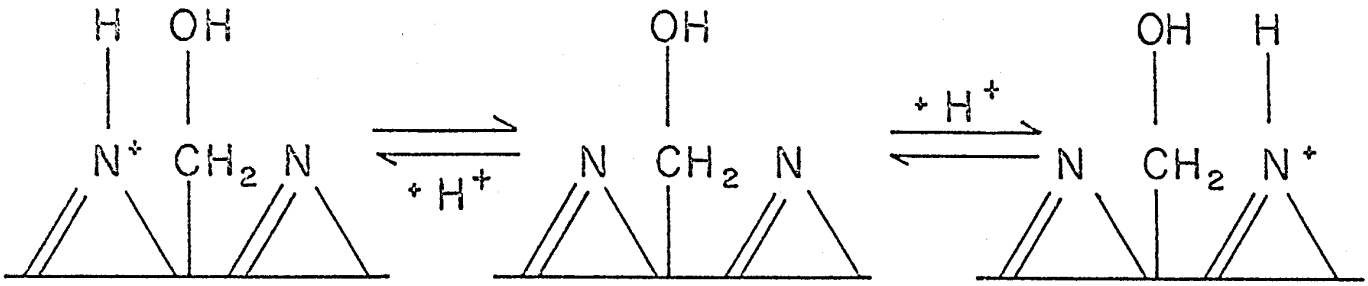
(b) Studies of complex formation in mixed solvents have shown in a number of instances that the complex is more polar than the separate enzyme and substrate (91-94).

(c) The fact that two histidine residues are very close in space to one another in chymotrypsin, and also in trypsin which is kinetically very similar, suggests that both are involved in the catalytic action.

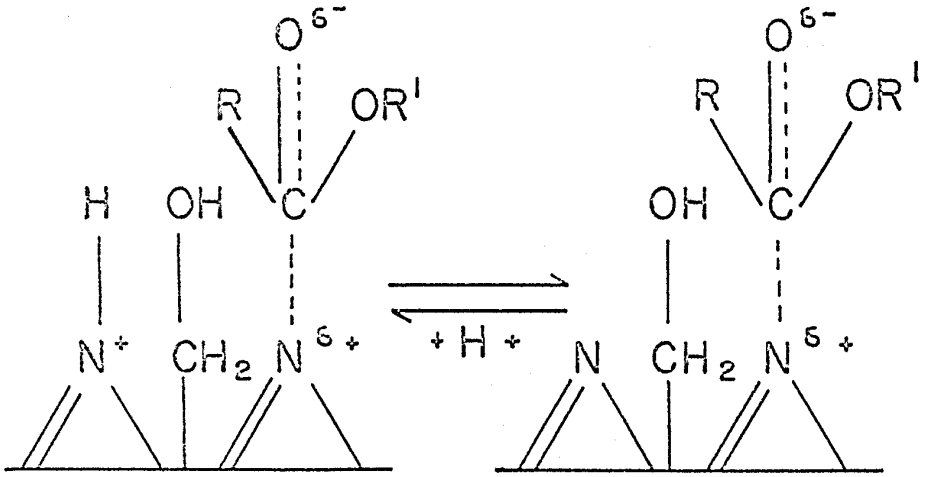
To interpret these facts we suggest that the process of complex formation is as represented in figure 21. One of the histidine residues is directly involved in complex formation by donating an electron pair to the carbonyl carbon atom; the formation of a charged

Figure 21

A mechanism of binding for substrates of α-chymotrypsin.



FREE ENZYME



MICHAELIS COMPLEX

intermediate explains the strong solvent effect that is observed. The other histidine group and the group of pK 9 play no role in complex formation. If a proton adds on to this second imidazole group, electrostatic effects prevent the addition of a proton to the group involved in complex formation.

(2) Acylation

(a) The pH work indicates that both ionizing groups are free in the complex and are involved in the acylation process.

(b) Studies in mixed solvents indicate that the group pK 7 is a cationic acid and the group of pK 9 a neutral acid (Section III C).

(c) There is much evidence that in the acyl enzyme the acyl group is attached to a serine residue (9, 12). One cannot, however, exclude the possibility that the appearance of the acyl group on the serine residue is an artefact, and that in the enzymatic process the group is attached to some other residue, perhaps a tyrosine hydroxyl group of pK 9. This possibility is discussed below.

Further consideration of the deductions from the mechanistic information regarding acylation is deferred to a later section.

(3) Deacylation

(a) The pH work shows sigmoid behavior, the group of pK 9 not being revealed. This means either that the group of pK 9 is tied up in the acyl enzyme, or that it is not involved in reaction.

(b) The deacylation process is first order in water or in any other nucleophile (such as methanol) that is present (99).

(c) No intermediate can be detected in the deacylation process.

(d) The deuterium-isotope effect in the deacylation process indicates that a proton transfer is involved (42).

Bender and Kézdy (31) have pointed out that facts (b) and (c) together lead to the conclusion that the imidazole group on the enzyme undergoes a general basic (proton-abstracting), rather than a nucleophilic (electron-donating), action in the deacylation process. Thus, if the action were nucleophilic it would have to be followed by the addition of a water molecule; since the reaction is first-order in water the nucleophilic attack would have to be fast and the water addition slow. An acyl-imidazole intermediate should therefore be detectable; since it is not detected, it is unlikely that there is a nucleophilic attack. This is also the conclusion from (d).

Function of the Imidazole Group

The evidence is that one of the imidazole groups is free to ionize in the free enzyme, in the enzyme-substrate complex and in the acyl enzyme, and that it plays a role in both the acylation and deacylation processes. In the deacylation process this imidazole group plays a general basic and not a nucleophilic role. The second imidazole group, if it plays any part at all, is probably involved in substrate binding, as indicated in figure 21.

Microscopic Reversibility

Arriving at a conclusion about the role of the acidic group of pK 9 is a matter of considerably greater difficulty. A complication arises from the different pH dependencies of acylation and deacylation. Bender and co-workers(25,110) argue that acylation and deacylation are essentially the reverse of one another; if a methyl ester is hydrolyzed the methanolysis is indeed the exact reverse of the acylation reaction. They then argue on the basis of microscopic reversibility that acylation and deacylation must follow the same reaction paths. Since the pH results suggest that the acidic group does not play a kinetic role in deacylation, they conclude that it does not do so in acylation. To explain the pH results for acylation they suggest that the acidic group (which

they believe to be $-NH_3^+$) plays a conformational role. Since this conformational effect has a direct bearing on the kinetics, it is not clear that this suggestion avoids the alleged difficulty.

We have elsewhere shown (Chapter II) that from this principle it is not always valid to assume that a reaction and its reverse follow the same path away from equilibrium. In such cases, however, the paths available must have a different kinetic order under steady-state conditions. In the acylation-deacylation system, methanolysis of the acyl enzyme will almost certainly be the reverse of acylation under steady-state conditions, since no reasonable pathway of a different kinetic order can be postulated.

Since the principle of microscopic reversibility only applies to a single reaction at equilibrium, no absolutely firm conclusions may be drawn for hydrolysis under steady-state conditions. However, in view of the conclusions reached for the case of methanolysis, it seems reasonable to postulate that the hydrolysis of the acyl enzyme will follow a similar pathway to that followed by the reverse of acylation.

Bender's Mechanism

The mechanism at which Bender and his co-workers

arrive is shown in modified form in figure 22. Their mechanism has to be amended because they thought the acidic group was $-NH_3^+$, which could exert a conformational role in the Michaelis complex by attracting an anionic group; in basic solution, when the $-NH_3^+$ becomes $-NH_2$, there would be a change of conformation leading to a species incapable of forming the acyl enzyme.

It is now very likely (Section III C) that the acidic group is a neutral acid group. This could play a conformational role by hydrogen bonding with an unknown group X; in basic solution this hydrogen bond is broken. An essential feature of the scheme is that the -O-H group is not directly involved in deacylation, so that the two ionized forms of the acyl enzyme are deacylated at the same speed.

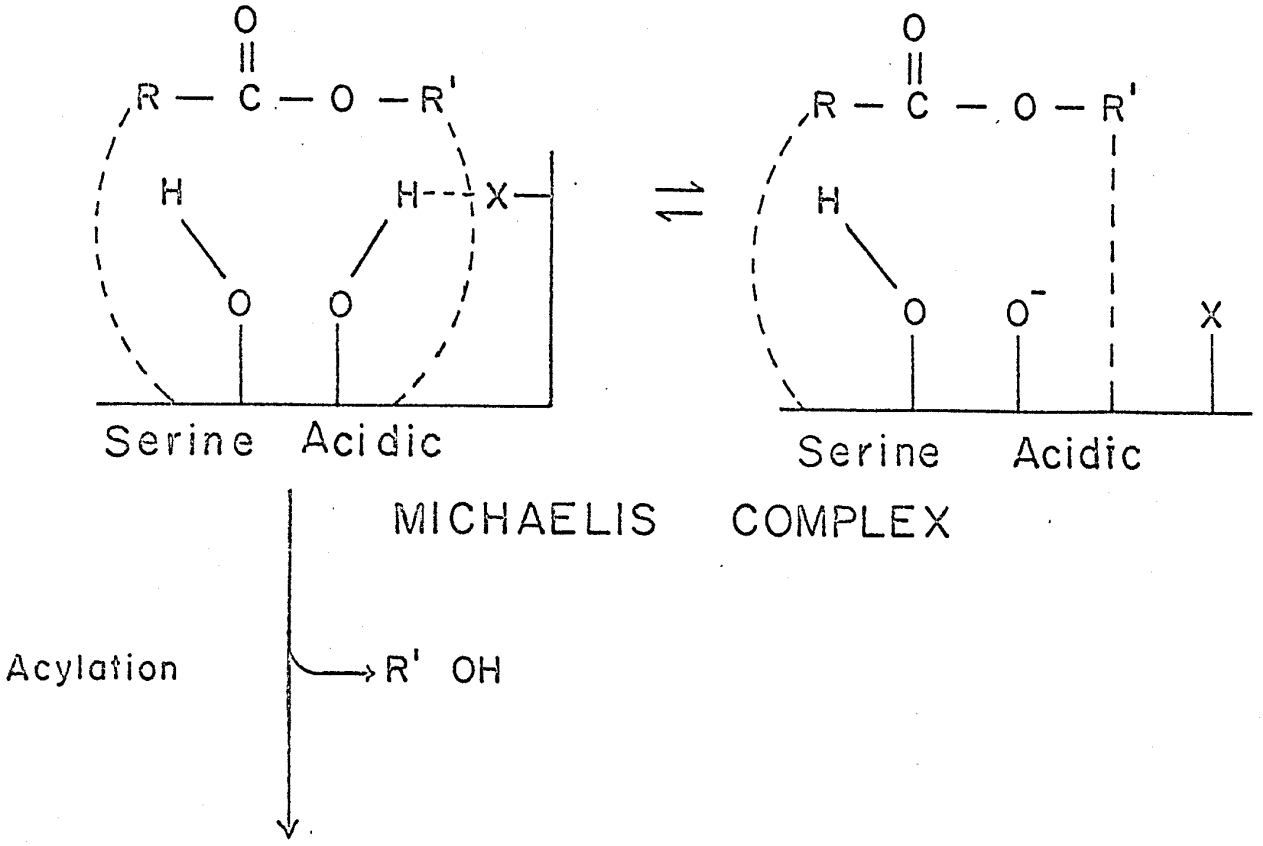
The main weakness of this mechanism relates to the fact that, as shown in the present work, the Michaelis complex ionizes on the basic side with the same pK as the free enzyme. If the hydroxyl groups were involved in hydrogen bonding in the Michaelis complex it would be more difficult for the proton to leave, i.e., the pK would be higher than in the free enzyme.

The Acidic Hydroxyl Group Aids Deacylation

A mechanism in which the acidic group functions as a general acid catalyst is shown in figure 23. In the

Figure 22

A mechanism in which the acidic group has a conformational role.


R — C — O — R'
H
H--X
Serine
Acidic
MICHAELIS COMPLEX
Acylation
R' OH

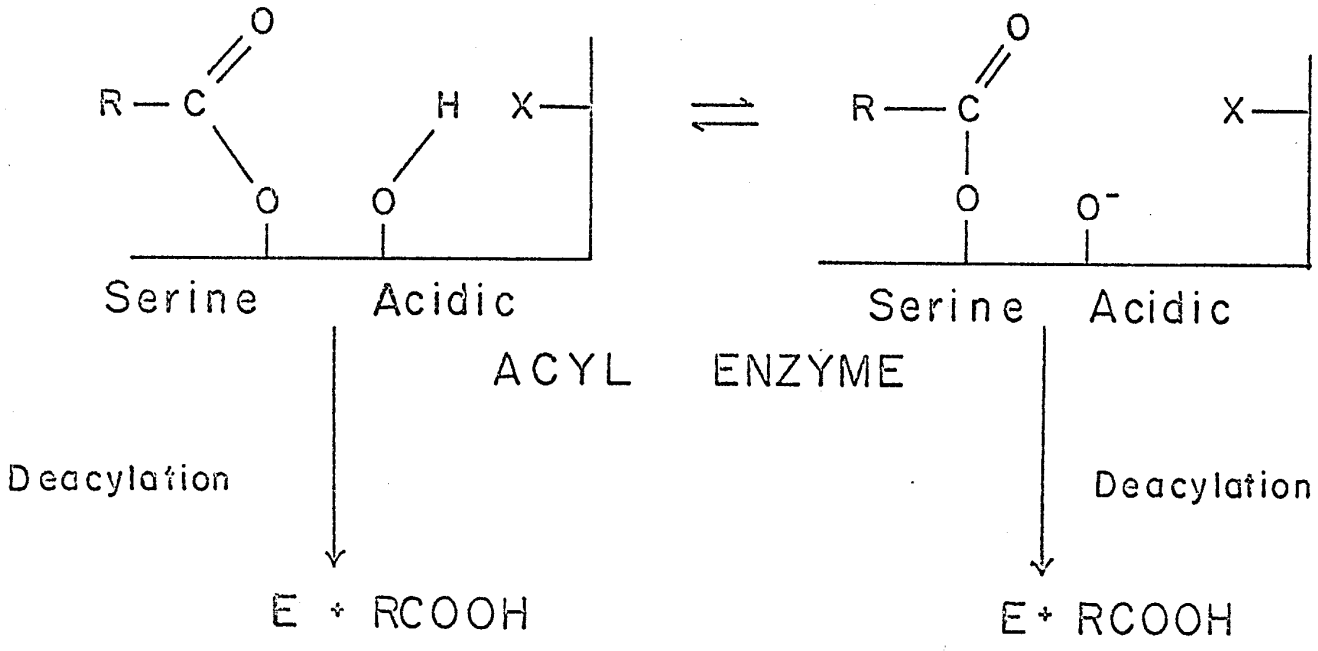
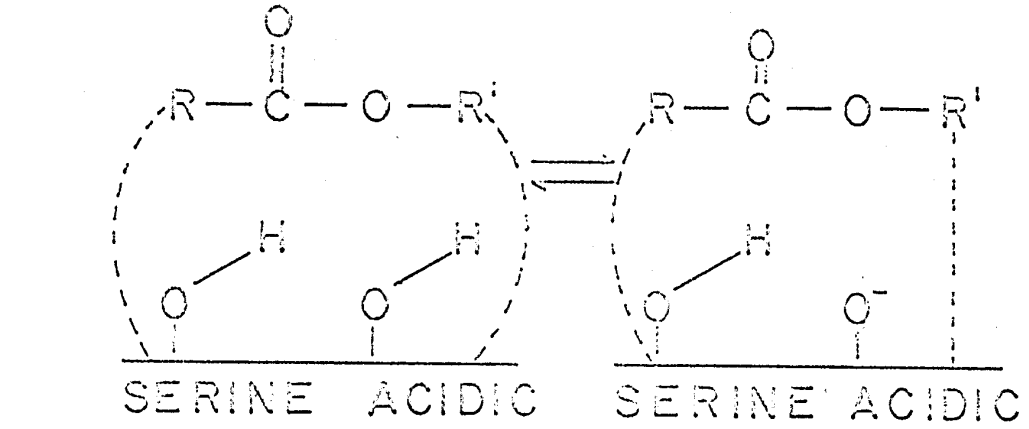
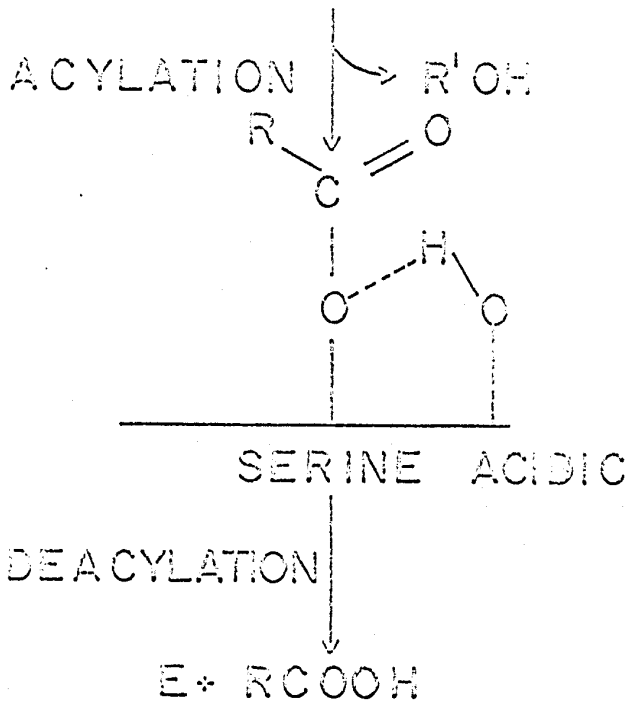

R — C
H
X
Serine
Acidic
ACYL ENZYME
Deacylation
E + RCOOH

Figure 23

A mechanism in which the acidic group functions as a general acid catalyst.

MICHAELIS COMPLEX





DEACYLATION

E + RCOOH

Michaelis complex the hydrogen atom of the acidic -OH group is free to ionize, but is hydrogen bonded in the acyl enzyme to the serine oxygen atom, and is therefore not free to split off at high pH values. This hydrogen atom is involved in the deacylation process by being transferred to the serine oxygen atom, so aiding the acyl group to leave.

This mechanism explains satisfactorily the observed pH dependencies on the basic side of the pH optimum. However, while the deacylation process has the same character as the reverse of acylation, it is not mechanistically the reverse; this mechanism must therefore be considered unlikely.

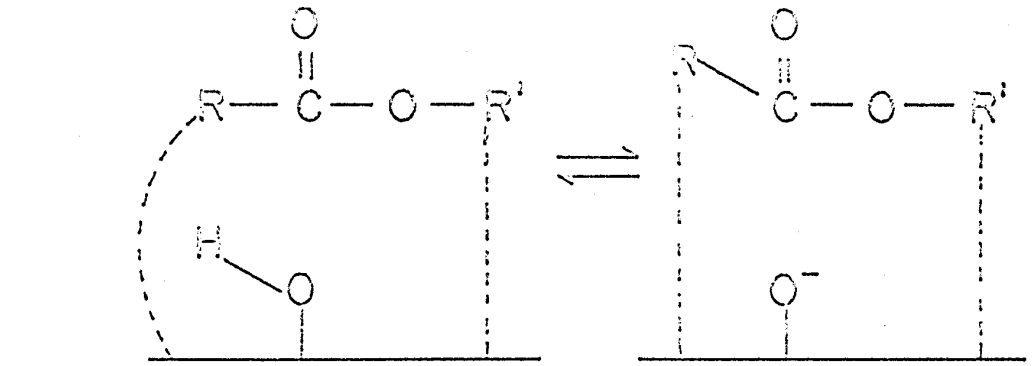
Acylation of the Acidic Hydroxyl Group

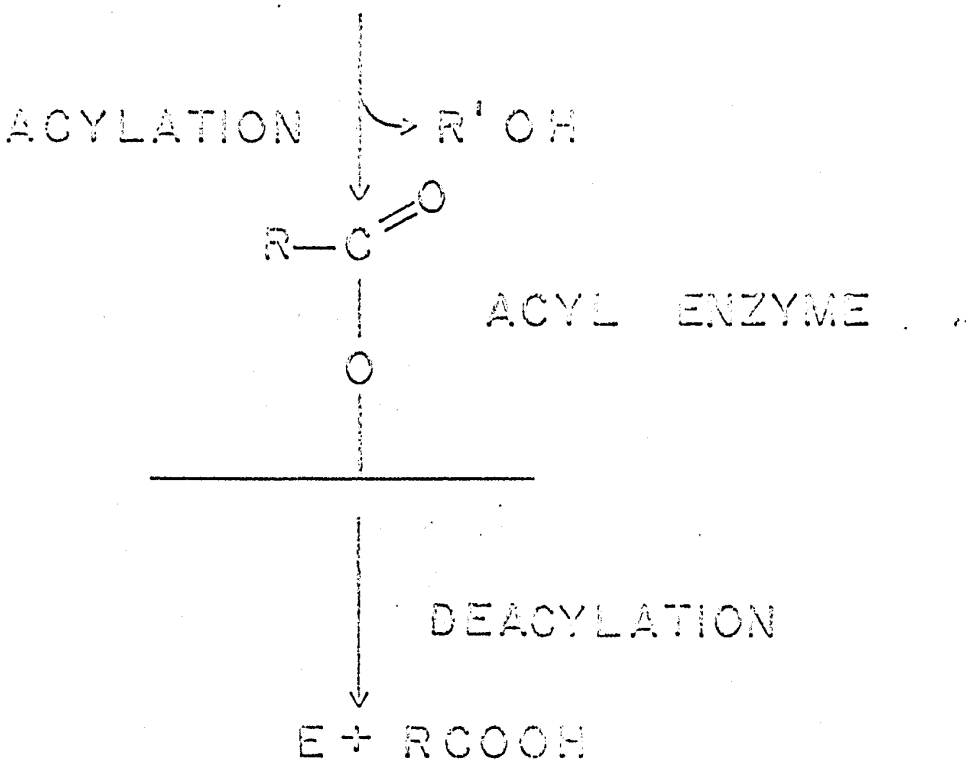
The most straightforward explanation of the bell-shaped pH dependence of acylation, and the sigmoid pH dependence of deacylation, is that it is the acidic group that is acylated. This mechanism is represented in figure 24. There are, however, some difficulties:
1) The chemical evidence, referred to above, strongly suggests that it is a serine hydroxyl group that is acylated. The pK of a normal serine hydroxyl group, however, is above 13, much higher than the measured value of 9.2 for the acidic group. It is possible, however, that

Figure 24

A mechanism in which the acidic group is acylated.

MICHAELIS COMPLEX





neighboring group effects could reduce the pK of the serine hydroxyl group to 9.2.

(2) There remains the possibility that it is not really the serine group that is initially acylated, but that a tyrosine hydroxyl group of normal pK is acylated. The spectrum and behavior of cinnamoyl-chymotrypsin are consistent with this possibility (39). According to this, the finding of acylated serine in the breakdown products from the acyl enzyme must be an artefact, involving transfer from tyrosine to serine. This does not seem particularly likely in view of the abundant evidence of the involvement of serine for other hydrolytic enzymes.

The view we favor is that the group of pK 9.2 in acylation is the serine hydroxyl which is acylated, since this seems to involve the least difficulties in formulating a satisfactory mechanism.

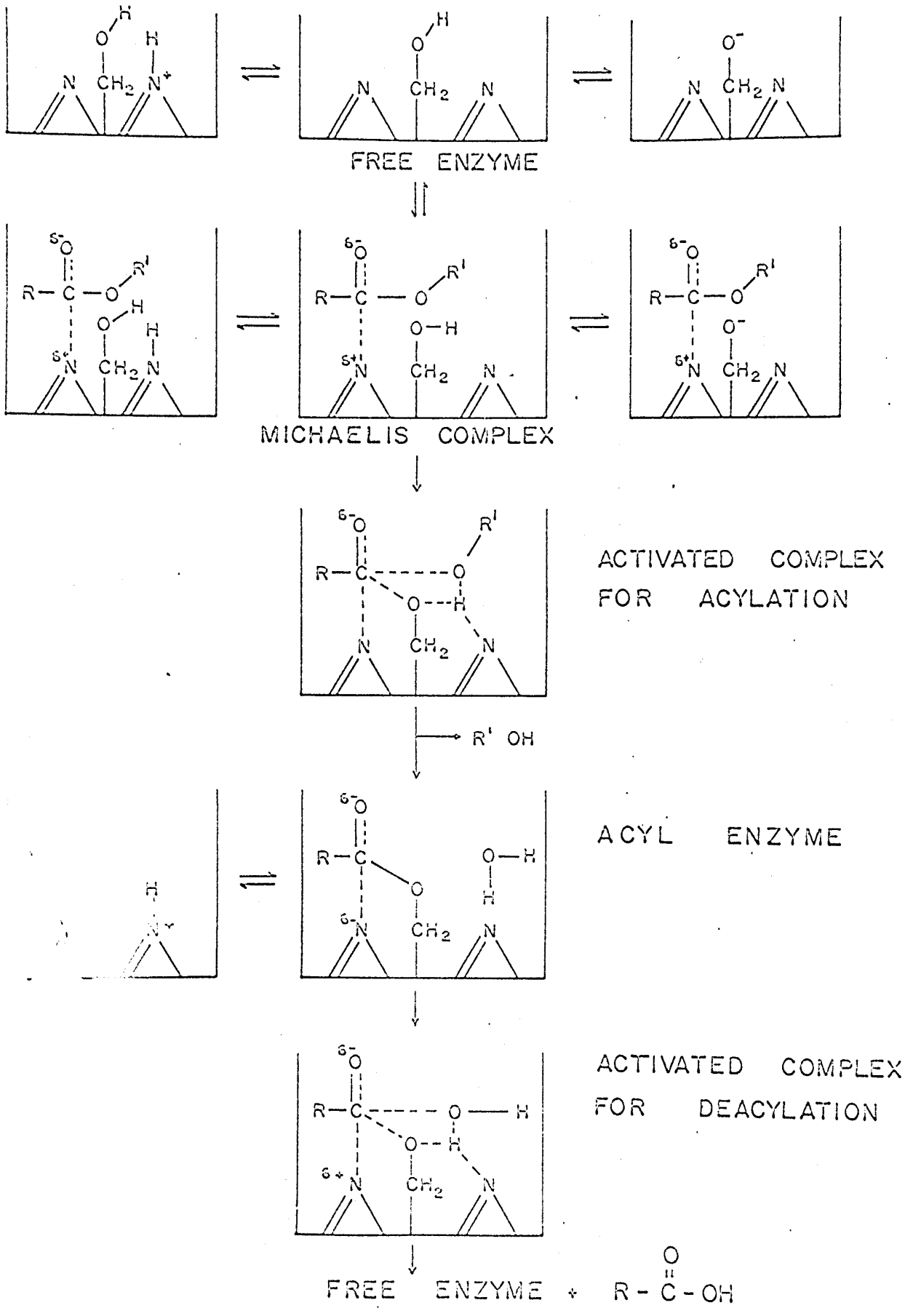
The Over-all Mechanism

In figure 25 the various conclusions are pieced together into a consolidated mechanism. In the case of acylation and deacylation the suggested activated complexes are shown, so that the various interactions can be clearly seen. The most important features of the mechanism are as follows:

1) Acylation occurs by a concerted mechanism in which one imidazole group donates electrons to the carbonyl function,

Figure 25

Proposed mechanism for α-chymotrypsin catalyzed hydrolysis.


FREE ENZYME
MICHAELIS COMPLEX
ACTIVATED COMPLEX FOR ACYLATION
R' OH
ACYL ENZYME
ACTIVATED COMPLEX FOR DEACYLATION
FREE ENZYME + R - C - OH

thereby making for a more facile attack by the serine hydroxyl group. The other imidazole group functions as a general base, general acid catalyst, helping the proton off the serine hydroxyl and onto the departing alcohol.
2) Deacylation is the reverse of acylation. The second imidazole group helps the proton off the water molecule and onto the departing serine hydroxyl group.

It has been emphasized in the discussion above that one cannot yet arrive at a firm decision as to mechanism for this enzyme system. The mechanism proposed in figure 25 appears to be entirely plausible, and to be the simplest and most straightforward explanation consistent with all of the facts. It should be useful in planning further experimental work.

Appendix

An interesting consequence of the mechanism outlined in figure 25 is that the ionizations of the imidazole groups may not be random; the ionization of one group may prevent the ionization of the other. The evidence is that two imidazole groups are brought into close proximity by means of a disulphide bridge, so that the protonation of one will prevent the protonation of the other. It can easily be shown that

$$\frac{1}{K_{b(app)}} = \frac{1}{K_{b1}} + \frac{1}{K_{b2}} \qquad (1)$$

If $K_{b1} = K_{b2}$ then

$$K_{b(app)} = \frac{K_b}{2} \qquad (2)$$

The measured pK value is thus 0.3 units higher than the true value. In the case of α-chymotrypsin the values determined are in the range of 6.7 to 7.0 for the free enzyme so that the true values are 6.4 to 6.7, which is closer to the true value of 6.1 for the imidazole group of histidine.

CHAPTER IV

INHIBITION

A. The Inhibition of Enzyme Reactions Involving Two Intermediates

INTRODUCTION

The simplest types of kinetic behavior of inhibitors in enzyme systems are those corresponding to rate equations of the form

$$v = \frac{k[S]}{1 + K[S] + K_i[I]} \qquad (1)$$

and

$$v = \frac{k[S]}{(1 + K[S])(1 + K_i[I])} \qquad (2)$$

where k, K and K_i are constants and [S] and [I] are the substrate and inhibitor concentrations. Behavior that corresponds to equation (1) is described as <u>competitive</u>, the degree of inhibition being dependent on [S]; that corresponding to (2) is <u>non-competitive</u>, the degree of inhibition being independent of [S].

In terms of the conventional Michaelis-Menten equation

$$v = \frac{V[S]}{K_m + [S]} = \frac{k_c[E]_o[S]}{K_m + [S]} \qquad (3)$$

it is to be seen that with competitive inhibition the Michaelis parameters V and K_m are of the form

$$V = \frac{k}{K} \qquad (4) \qquad K_m = \frac{1 + K_i[I]}{K} \qquad (5)$$

that is, V is independent of [I] but K_m varies with [I]. For non-competitive inhibition

$$V = \frac{k}{K\,(1 + K_i[I])} \qquad (6) \qquad K_m = \frac{1}{K} \qquad (7)$$

so that now K_m is independent of [I] and V varies with [I]. When both V and K_m vary with [I] the inhibition may be referred to as <u>mixed</u>.

In terms of the classical Michaelis-Menten scheme, involving a single intermediate, the enzyme-substrate complex, the classes of behavior are simply explained as follows:

(1) In competitive inhibition the inhibitor can bind to the free-enzyme but cannot bind to the enzyme-substrate complex.

(2) In non-competitive inhibition the substrate binds to the complex as readily as to the free enzyme.

(3) In mixed inhibition the inhibitor has a different affinity for the complex than for the free enzyme.

There is now abundant evidence (31,56-58,111) that two intermediates are of kinetic significance in many enzyme systems, in particular with hydrolytic enzymes such as α-chymotrypsin and cholinesterase. For such systems inhibition schemes that neglect the second intermediates are obviously incomplete. Krupka and Laidler(112) have developed inhibition equations based on the two-intermediate mechanism, and have applied them quantitatively to reactions catalyzed by cholinesterase. Their experimental results with this enzyme could not be reconciled with a scheme based on a single intermediate, but could be interpreted satisfactorily in terms of a two-intermediate mechanism.

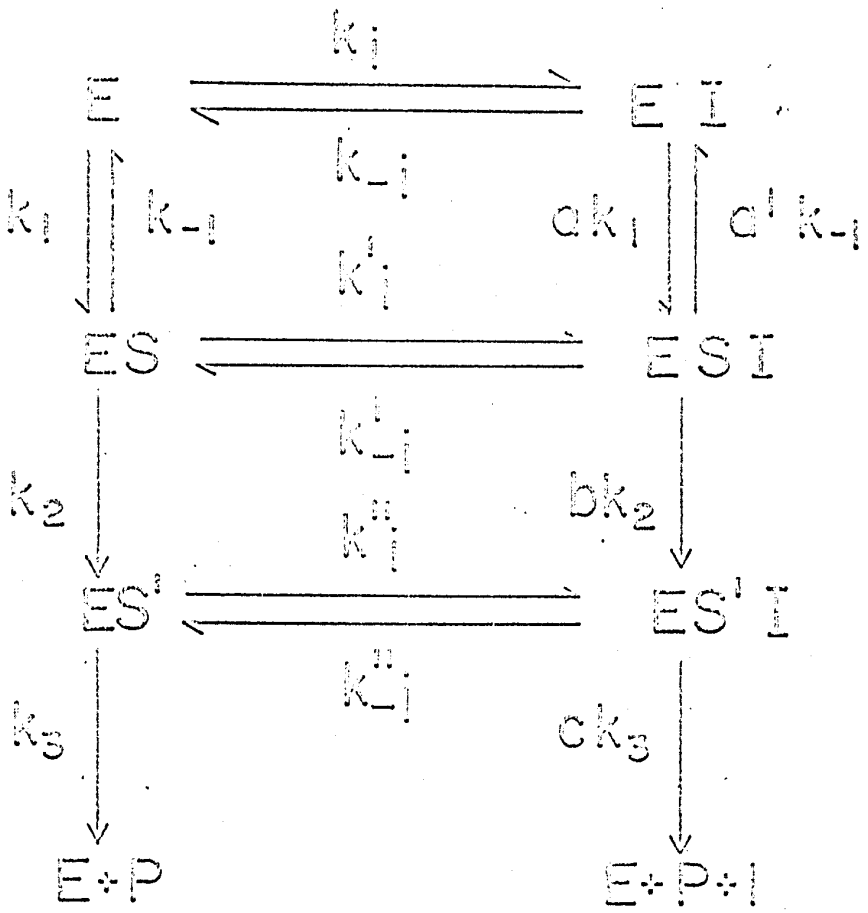
In the present section we develop the steady-state equations for inhibited systems in a somewhat more general form than was done by Krupka and Laidler, and we classify the special cases in a way that makes it easy to apply them to the experimental results. A few examples will show the applicability of this treatment; a later section will describe a quantitative application of the treatment to the α-chymotrypsin system.

The General Inhibition Equations

The scheme for which the steady-state equations have been worked out is shown in figure 26. As discussed

Figure 26

A general inhibition scheme.


E
I
EI
ES
ESI
ES'
ES'I
E+P
E+P+I
k1
k-1
k'1
k'-1
k''1
k''-1
k2
k3
ak1
a'k-1
bk2
ck3

in a previous treatment of steady-state equations in enzyme kinetics (113,114), reaction schemes involving cycles lead to exceedingly complicated rate equations which are not useful for the analysis of experimental data. A satisfactory approximation in cases of this kind is to treat the horizontal reactions as rapid and therefore in equilibrium, but to apply the steady-state to the vertical reactions. This procedure is justified by the fact that the horizontal reactions are simply binding and dissociation processes, whereas the vertical reactions involve chemical change.

The equilibrium equations corresponding to the three horizontal reactions are

$$[EI] = K_1 [E] [I] \tag{8}$$

$$[ESI] = K_1' [ES] [I] \tag{9}$$

$$[ES'I] = K_1'' [ES'] [I] \tag{10}$$

where $K_1 = k_1/k_{-1}$, $K_1' = k_1'/k_{-1}'$ and $K_1'' = k_1''/k_{-1}''$. The sum of the steady-state equations for ES and ESI is

$$k_1[E][S] + a\, k_1[EI][S] = (k_{-1} + k_2)[ES] + (b\, k_2 + a' k_{-1})[ESI] \tag{11}$$

whence, using (8) and (9),

$$k_1[E][S]\,(1 + a\, K_1[I]) = \bar{k}[ES]\,(1 + \bar{a}\, K_1'[I]) \tag{12}$$

where $\bar{k} = k_{-1} + k_2$ and $\bar{a} = (b\, k_2 + a' k_{-1})/\bar{k}$. Similarly, the sum of the steady-state equations for ES' and ES'I is

$$k_2[ES]\,(1 + b\,K_i'\,[I]) = k_3[ES']\,(1 + c\,K_i''\,[I]) \qquad (13)$$

The total enzyme concentration, $[E]_o$, is

$$[E]_o = [E] + [EI] + [ES] + [ESI] + [ES'] + [ES'I] \qquad (14)$$

and the rate is

$$v = k_2\,[ES] + b\,k_2\,[ESI] \qquad (15)$$

Elimination of [E], [EI], [ES], [ESI], [ES'] and [ES'I] using equations (8) to (14) leads finally to the rate equation

$$v = \frac{k_c(app)[E]_o[S]}{K_m(app)} \qquad (16)$$

where

$$k_c(app) = \frac{k_2}{\dfrac{1 + K_i'[I]}{1 + b\,K_i'[I]} + \dfrac{k_2\,(1 + K_i''[I]}{k_3\,(1 + c\,K_i''[I]}} \qquad (17)$$

and

$$\frac{1}{K_m(app)} = \frac{k_1(1 + a\,K_i[I])(1 + K_i'[I])}{\bar{k}(1 + K_i[I])(1 + \bar{a}\,K_i'[I])} + \frac{k_2(1 + b\,K_i'[I])(1+K_i''[I])}{k_3(1 + K_i'[I])(1+c\,K_i''[I])} \quad (1$$

Special Cases

Equations (17) and (18) are in a particularly useful form for arriving at the relationships that apply in special cases, and in deriving the conditions that apply to systems that have been studied experimentally. Two extreme

cases are represented by $k_2 \ll k_3$ (acylation rate-determining) and $k_3 \ll k_2$ (deacylation rate-determining). In addition, it is possible that the inhibitor cannot bind to the enzyme-substrate complex, in which case $K_1' = 0$ and $a = 0$; in this case, the complex $ES'I$ may be inactive ($c = 0$), or may undergo deacylation ($c \ll 1$). Alternatively, the enzyme-substrate complex may bind the inhibitor, but the acyl enzyme may not do so ($K_1'' = 0$).

Table 15 summarizes the most interesting special cases that may arise, and indicates in each case whether the inhibition is competitive, non-competitive or mixed. Figure 27 shows the mechanisms corresponding to each of the three main cases.

Some clear-cut examples of the behavior are as follows:

Case I (blocking of deacylation)

(a) $k_2 \ll k_3$: Cholinesterase-catalyzed hydrolysis of methylaminoethyl acetate, competitively inhibited by cis-2-dimethylaminocyclohexanol (112); α-chymotrypsin-catalyzed hydrolysis of nicotinyl-L-tryptophanamide, competitively inhibited by indole (115, Chapter IV B).

(b) $k_3 \ll k_2$: Cholinesterase-catalyzed hydrolysis of acetylcholine, non-competitively inhibited by cis-2-dimethylaminocyclohexanol (112); α-chymotrypsin-

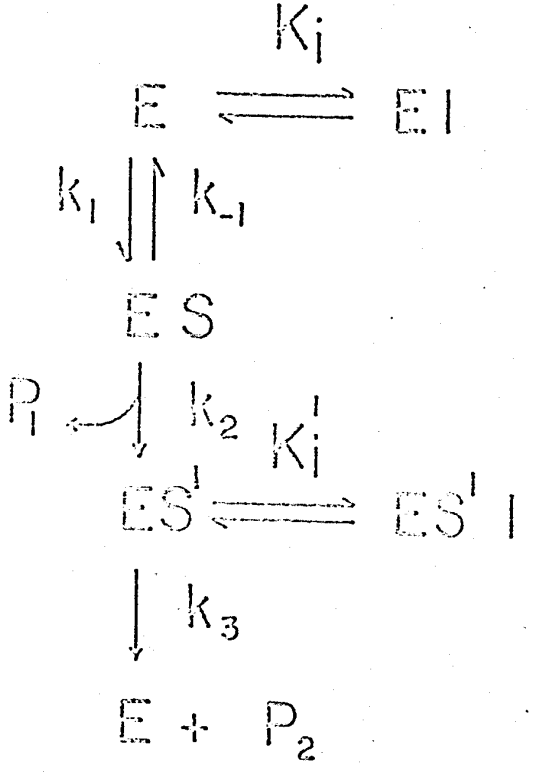
Table 15

Inhibition Equations: Special Cases

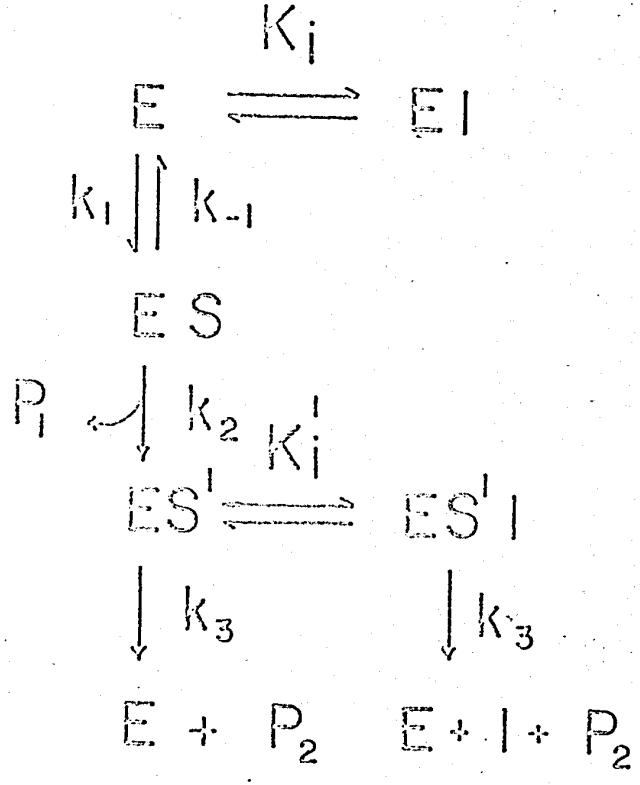
Case	Conditions	$k_2 \ll k_3$		$k_2 \simeq k_3$		$k_3 \ll k_2$	
		k_c	K_m	k_c	K_m	k_c	K_m
I	$K_1'=0; c=0$ $K_1''=K_1$	k_2	$\dfrac{1+K_1[I]}{\bar{K}}$	$\dfrac{k_2}{1+\frac{k_2}{k_3}(1+K_1[I])}$	$\dfrac{1+K_1[I]}{\bar{K}+\frac{k_2}{k_3}\bar{K}(1+K_1[I])}$	$\dfrac{k_3}{1+K_1[I]}$	$\dfrac{k_3}{k_2\bar{K}}$
		COMPETITIVE		MIXED		NON-COMPETITIVE	
II	$K_1'=0$ $K_1''=K_1$ $c=1$	k_2	$\bar{K}(1+K_1[I])$	$\dfrac{k_2}{1+\frac{k_2}{k_3}}$	$\dfrac{\bar{K}(1+K_1[I])}{1+\frac{k_2}{k_3}}$	k_3	$\dfrac{k_3\bar{K}(1+K_1[I])}{k_2}$
		COMPETITIVE		COMPETITIVE		COMPETITIVE	
III	$K_1'' = 0$ $K_1' = K_1$	$\dfrac{k_2}{1+K_1[I]}$	$\dfrac{1}{\bar{K}}$	$\dfrac{k_2}{1+K_1[I]+\frac{k_2}{k_3}}$	$\dfrac{1+K_1[I]}{\bar{K}(1+K_1[I]+\frac{k_2}{k_3}}$	k_3	$\dfrac{k_3(1+K_1[I])}{k_2\bar{K}}$
		NON-COMPETITIVE		MIXED		COMPETITIVE	

Figure 27

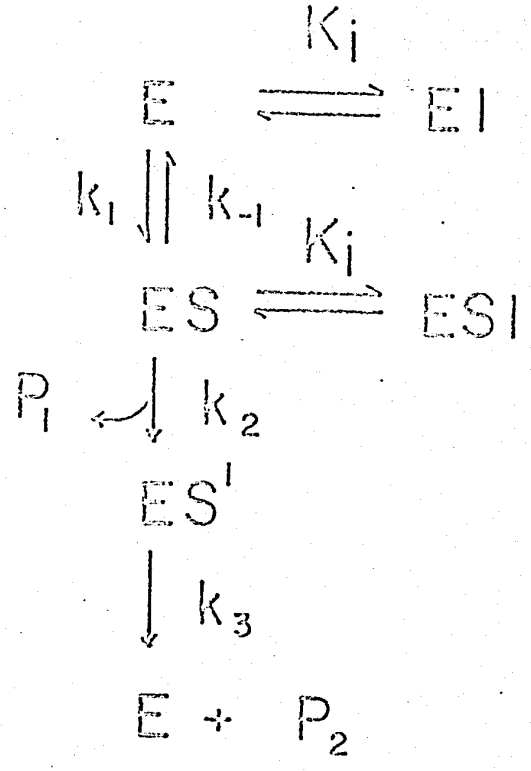
Mechanisms of inhibition.



CASE 1



CASE 2



CASE 3

catalyzed hydrolysis of N-acetyl-L-tyrosine ethyl ester, non-competitively inhibited by indole (Chapter IV B).

(c) $k_2 \simeq k_3$: α-chymotrypsin-catalyzed hydrolysis of methyl hippurate, mixed inhibition by indole (116, Chapter IV B).

Case II (no blocking of deacylation)

(a) $k_2 \ll k_3$: Cholinesterase-catalyzed hydrolysis of methylaminoethyl acetate, competitively inhibited by choline, carbachol and eserine (117).

(b) $k_3 \ll k_2$: Cholinesterase-catalyzed hydrolysis of acetylcholine, competitively inhibited by choline, carbachol and eserine (117).

B. Mechanisms of Inhibition

INTRODUCTION

A considerable number of inhibition studies on α-chymotrypsin-catalyzed reactions have been carried out, particularly by Niemann and his co-workers (115,118,119), and the results have been interpreted in terms of the simple Michaelis-Menten scheme involving a single intermediate. Krupka and Laidler(112) developed inhibition equations for the two-intermediate mechanism, which certainly applies to α-chymotrypsin-catalyzed reactions, and we have recently extended and generalized their treatment (Chapter IV A). The present investigation is concerned with making a quantitative test of these equations with reference to results for α-chymotrypsin-catalyzed reactions.

In the present work we have chosen systems which were expected, on the basis of the theory and certain information about the substrates and inhibitors, to give rise to well-defined behavior. The inhibitor indole presented itself as an obvious choice, since with it three types of inhibition had already been observed - competitive, non-competitive and mixed. Phenol has also been investigated in the present work. What was needed to test the

theory was a substrate for which the deacylation process was rate-limiting, since in this way one can deduce the effect of the inhibitor on the acyl enzyme and on the deacylation reaction. The pH data show (Chapter III D) that for N-acetyl-L-tyrosine ethyl ester the deacylation is slower than acylation, and this was the substrate used in the present work. Conclusions are also drawn from the results of investigations with other substrates; with nicotinyl-L-tryptophanamide, for which acylation is rate limiting, and with methyl hippurate, for which the acylation and deacylation rates are similar to one another.

EXPERIMENTAL

Materials

The work was all carried out in water, which was doubly distilled and deionized, and was free from carbon dioxide. The pH was maintained at 8.0 by addition of 0.02 N sodium hydroxide solution, prepared from Fisher Certified Reagent.

The substrate, N-acetyl-L-tyrosine ethyl ester, was a Mann Assayed Reagent, and was used without further purification. Indole was purchased in pure form from Nutritional Biochemicals Corp. and phenol was purified by distillation. Three-times recrystallized α-chymotrypsin was obtained from Nutritional Biochemicals Corp.

Kinetic Procedure

The general procedure was exactly as described

previously, use being made of the pH-stat technique. The reactions were carried out in 15.0 ml. of solution containing 15 meq of sodium chloride and maintained at 20.0°C in a thermostatically controlled bath. 14.0 ml. of solution containing substrate and inhibitor were first held in the reaction cell until temperature equilibration was achieved; the reaction was then started by the injection of 1.0 ml. of enzyme solution.

RESULTS AND DISCUSSION

Inhibition by Indole

Figure 28 shows a plot of v_o/v_i against [I]; v_o is the rate in the absence of inhibitor, and v_i that in its presence. In this type of plot non-competitive behavior is indicated if the points fall on a single straight line. Figure 29 is a plot of $1/v_i$ against [I]; non-competitive behavior is now indicated if the lines meet on the negative [I] axis. The inhibition by indole of the N-acetyl-L-tyrosine ethyl ester hydrolysis is thus clearly non-competitive. The (association) inhibition constant calculated from these results is 1.17×10^3 M^{-1}. Using the substrate nicotinyl-L-tryptophanamide, for which the inhibition is competitive, Foster and Niemann(115) obtained an inhibition constant of $1/8.0 \times 10^{-4} = 1.25 \times 10^3$ M^{-1}, in satisfactory agreement.

We may now draw conclusions from the behavior

Figure 28

A plot of v_o/v_i against [I] for the α-chymotrypsin catalyzed hydrolysis of N-acetyl-L-tyrosine ethyl ester inhibited by indole.

△ - [S] = 3.00×10^{-3}M

○ - [S] = 1.50×10^{-3}M

■ - [S] = 1.05×10^{-3}M

□ - [S] = 0.600×10^{-3}M

⬠ - [S] = 0.450×10^{-3}M

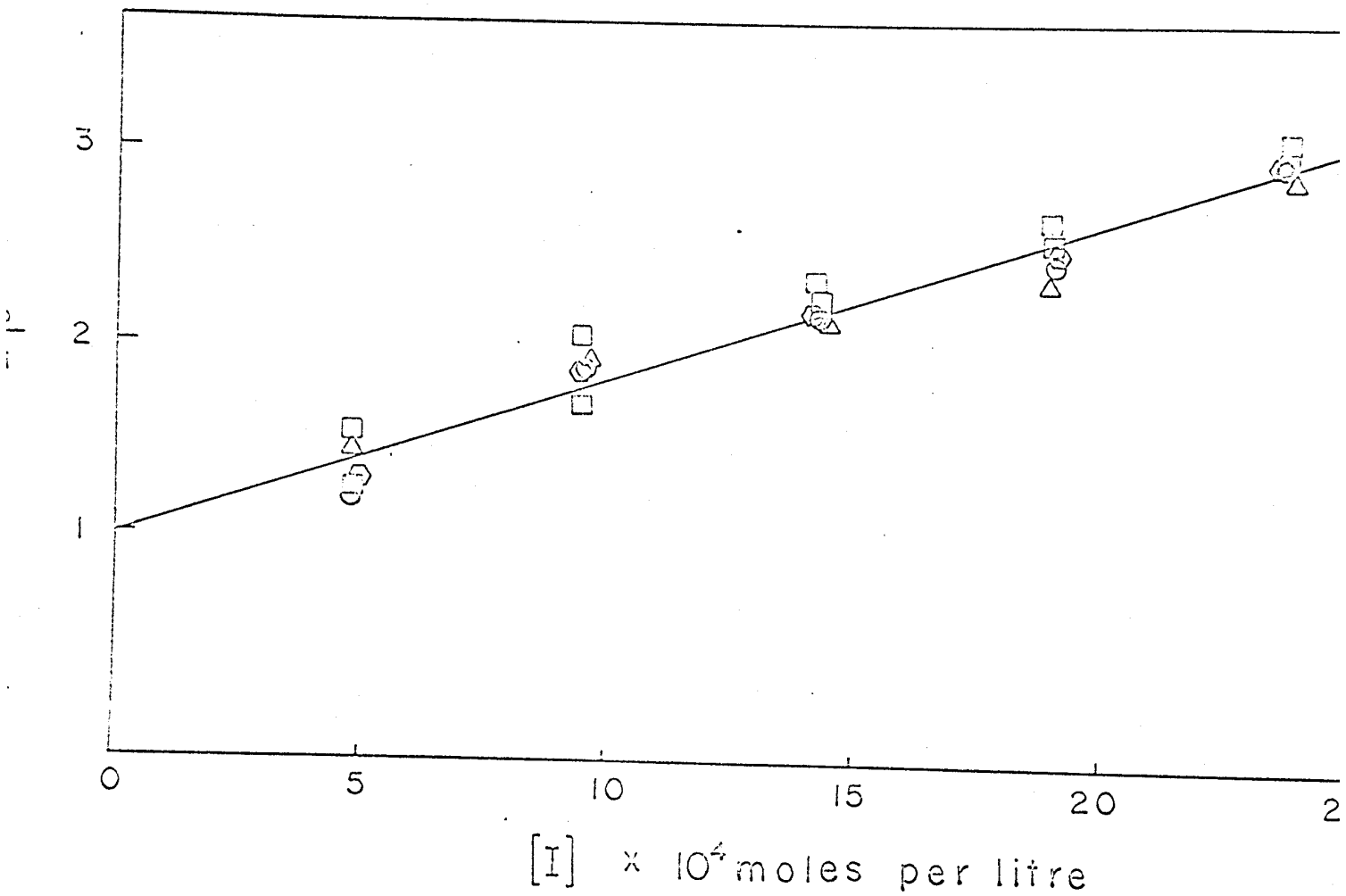
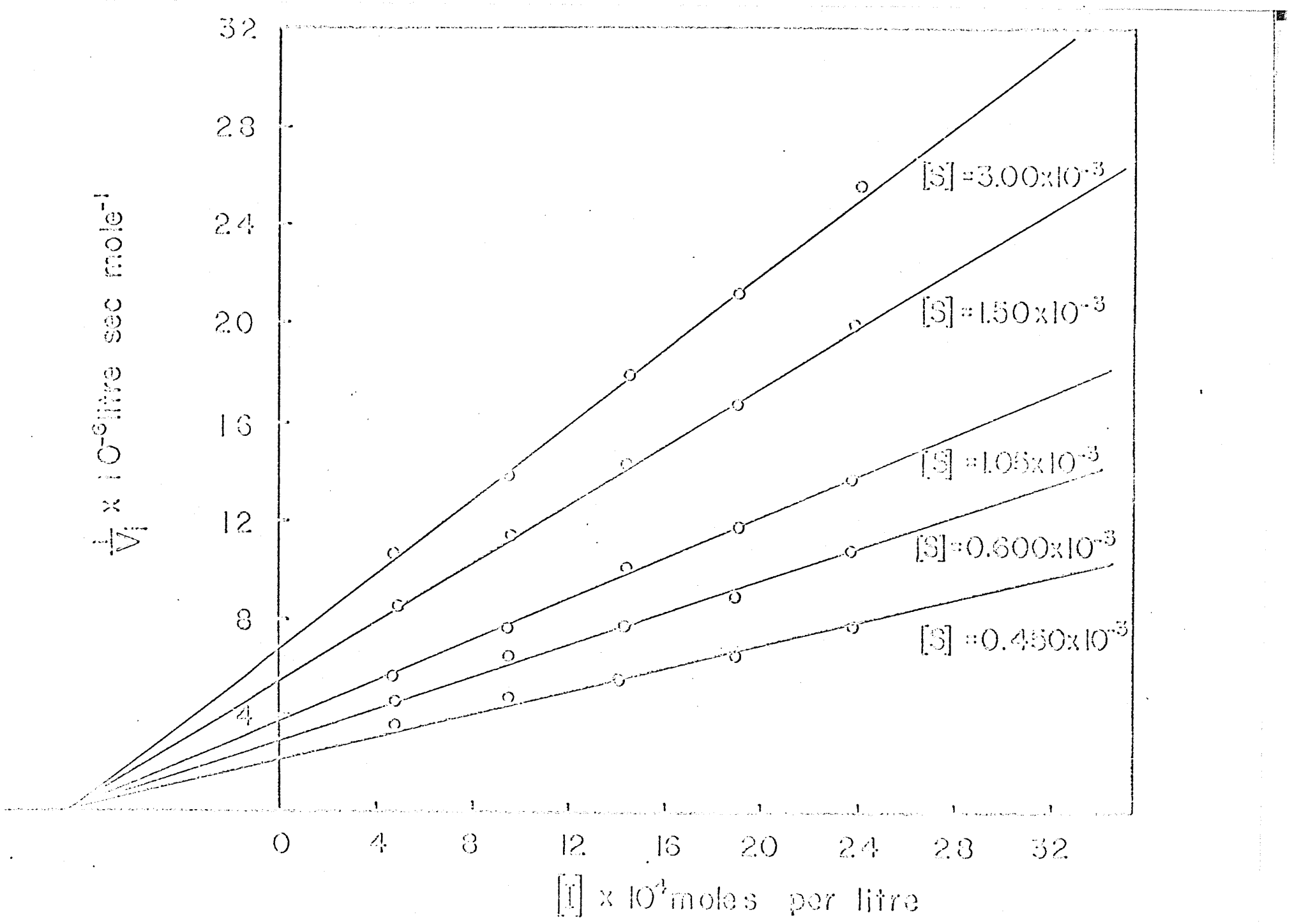

3
2
1
0
5
10
15
20
2
[I] × 10⁴ moles per litre

Figure 29

A plot of $1/v_i$ against [I] for the α-chymotrypsin catalyzed hydrolysis of N-acetyl-L-tyrosine ethyl ester inhibited by indole.


32
28
24
20
16
12
8
4
0
4
8
12
16
20
24
28
32
$\frac{1}{V_i} \times 10^{-6}$ litre sec mole^{-1}
[I] x 10^{-3} moles per litre
[S] =3.00x10^{-3}
[S] =1.50x10^{-3}
[S] =1.05x10^{-3}
[S] =0.600x10^{-3}
[S] =0.450x10^{-3}

shown by indole with three substrates: nicotinyl-L-tryptophanamide ($k_2 \ll k_3$) for which the inhibition is strictly competitive (115); N-acetyl-L-tyrosine ethyl ester ($k_3 \ll k_2$), for which the inhibition is non-competitive (present results) and methyl hippurate ($k_2 \simeq k_3$), for which the inhibition is mixed (116). The above conclusions about the relative magnitudes of k_2 and k_3 are clearly indicated by the pH data (15, Chapter III D).

For the case of $k_2 \ll k_3$ (nicotinyl-L-tryptophanamide) the Michaelis parameters, obtained from equations (17) and (18) of Chapter IV A are

$$k_c = \frac{k_1 (1 + b K_i' [I])}{1 + K_i' [I]} \qquad (1)$$

$$K_m = \frac{\bar{k} (1 + K_i [I])(1 + \bar{a} K_i [I])}{k_2 (1 + a K_i [I])(1 + K_i' [I])} \qquad (2)$$

Competitive inhibition requires that k_c is independent of [I] and that K_m varies linearly with [I]; this can only occur if $a = 0$ (which requires that $a' = 0$ and that $K_i' = 0$, since $K_i' = a K_i / a'$) and if $\bar{a} = 0$ (which means that $b = 0$). The conclusion is therefore that the inhibitor is not bound to the enzyme-substrate complex.

When $k_3 \ll k_2$ (N-acetyl-L-tyrosine ethyl ester) the Michaelis parameters become, with $K_i' = 0$ and $a = 0$,

$$k_c = \frac{k_3 (1 + c K_i'' [I])}{1 + K_i'' [I]} \qquad (3)$$

$$K_m = \frac{\bar{k} k_3 (1 + K_i [I])(1 + c K_i'' [I])}{k_1 k_2 (1 + K_i'' [I])} \qquad (4)$$

The inhibition is now non-competitive, which means that V is inversely proportional to $1 + K_i[I]$ and K_m is independent of [I]. From equations (3) and (4) it follows that $c = 0$ and $K_i'' = K_i$; that is, the inhibitor is bound to the acyl enzyme just as strongly as it is to the free enzyme, and it blocks the deacylation process.

Another way of analyzing the results with indole and N-acetyl-L-tyrosine ethyl ester is as follows. The rate equation corresponding to equations (3) and (4), with c equal to zero, can be written as

$$\frac{1}{v} = \frac{\bar{k} k_3}{k_1 k_2 [E]_o [S]} + \frac{1}{k_3 [E]_o} + [I]\left[\frac{\bar{k} k_3 K_i}{k_1 k_2 [E]_o [S]} + \frac{K_i''}{k_3 [E]_o}\right] \qquad (5)$$

A plot of 1/v against the inhibitor concentration for various substrate concentrations will therefore give straight lines with intercepts and slopes that are functions of the substrate concentration. A plot of the slopes of such lines against the reciprocal of the substrate concentration will give a straight line having an intercept on the 1/[S] axis equal to

$$\frac{K_i''}{k_3[E]_o}$$

A zero value for the intercept indicates that ES'I is not formed, and a non-zero value that it is formed. Figure 30 shows a plot of the slopes of the lines in figure 29 against 1/[S]. The intercept is positive, which indicates that indole does bind to the acyl enzyme. The value of K_i'' calculated from this intercept is 1.0×10^3, in satisfactory agreement with the value of 1.17×10^3 for K_i, calculated by the conventional procedure.

The inhibition by indole of the hydrolysis of methyl hippurate is a mixture of competitive and non-competitive inhibition (116). There is evidence which indicates that for this substrate k_2 and k_3 may have comparable values (66), so that mixed inhibition is expected (cf. Table 15). It was established above that $b = 0$, $K_i' = 0$ and $K_i'' = K_i$; equations (17) and (18) of Chapter IV A then become

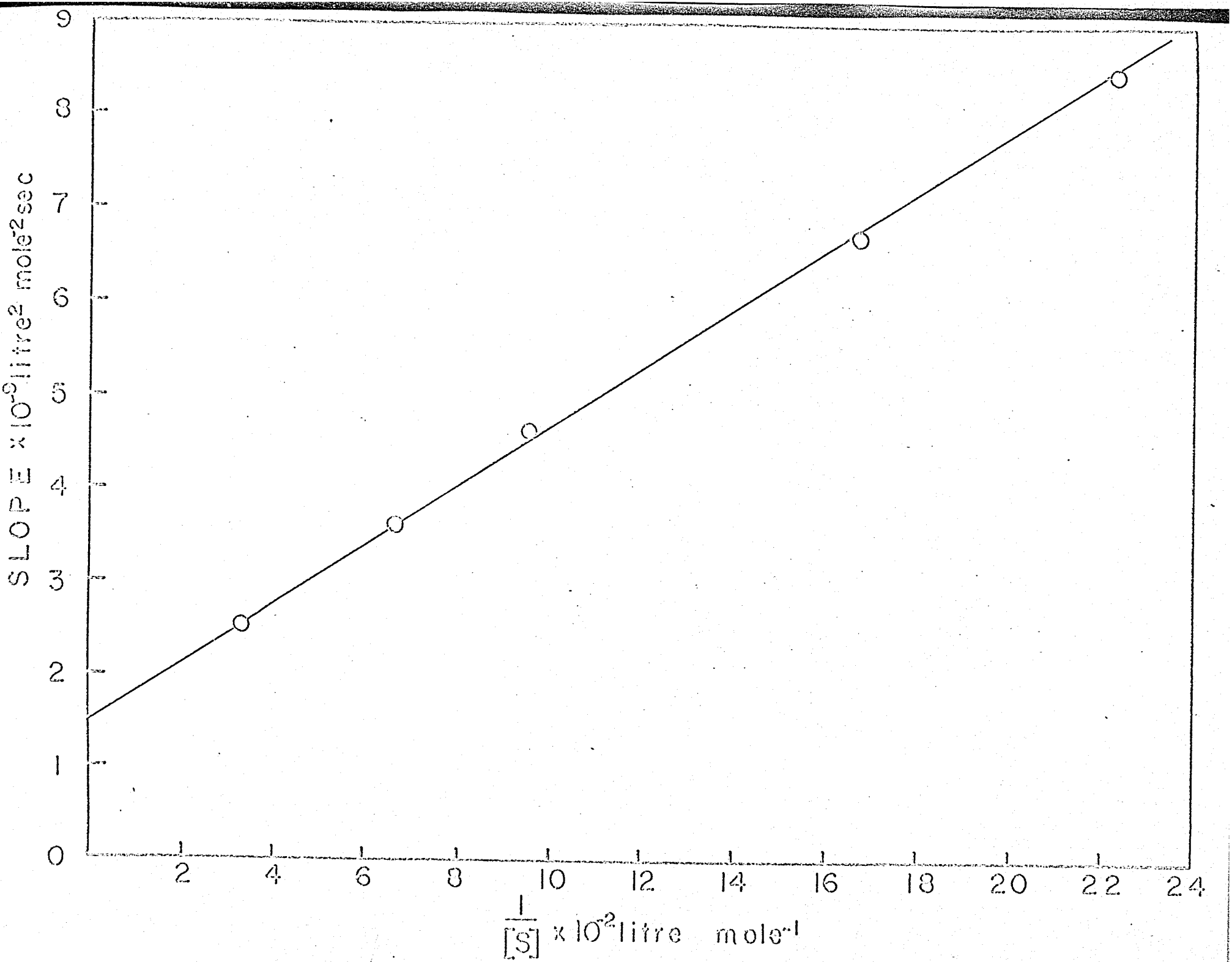
$$k_c = \frac{k_2}{1 + \frac{k_2}{k_3}(1 + K_i[I])} \qquad (6)$$

$$K_m = \frac{K_m(1 + K_i[I])}{1 + \frac{k_2}{k_3}(1 + K_i[I])} \qquad (7)$$

Plots of $1/k_c$ against $1 + K_i[I]$ and of $1/K_m$ against

Figure 30

A plot of the slopes of the lines in figure 29 against 1/[S].


SLOPE x 10^{-9} litre^2 mole^{-2} sec
0
1
2
3
4
5
6
7
8
9
2
4
6
8
10
12
14
16
18
20
22
24
$\frac{1}{[S]}$ x 10^{-2} litre mole^{-1}

$1/(1 + K_i[I])$ should therefore be linear; such plots are shown in figures 31 and 32. From the plot shown in figure 31 the following values are calculated:

$$k_2 = 3.3 \times 10^{-3} \text{ sec}^{-1}$$

$$k_3 = 27 \times 10^{-3} \text{ sec}^{-1}$$

The above explanation of the mixed inhibition brought about by indole with this substrate seems to us to be preferable to much more complicated mechanisms that have previously been put forward; Hein and Niemann(28,30) postulated three binding centers on the enzyme and 12 different interactions between substrate and enzyme, while Bender and Kézdy(31) postulated two different enzyme-substrate complexes (involving different modes of interaction) and two acyl enzymes. The experimental results do not require any such complicated explanations.

Inhibition by Phenol

Figure 33a shows a plot of v_o/v_i against [I] for the phenol inhibition of the hydrolysis of N-acetyl-L-tyrosine ethyl ester. The plots are displaced upwards as the substrate concentrations are lowered, indicating the behavior to be competitive. There is a pronounced curvature, which shows that the dependence on the inhibitor concentration is to a power greater than unity.

Figure 33b shows a plot of $1/v_i$ against $1/[S]$

Figure 31

A plot of $1/k_c$ against $(1 + K_I[I])$ for the α-chymotrypsin catalyzed hydrolysis of methyl hippurate inhibited by indole.

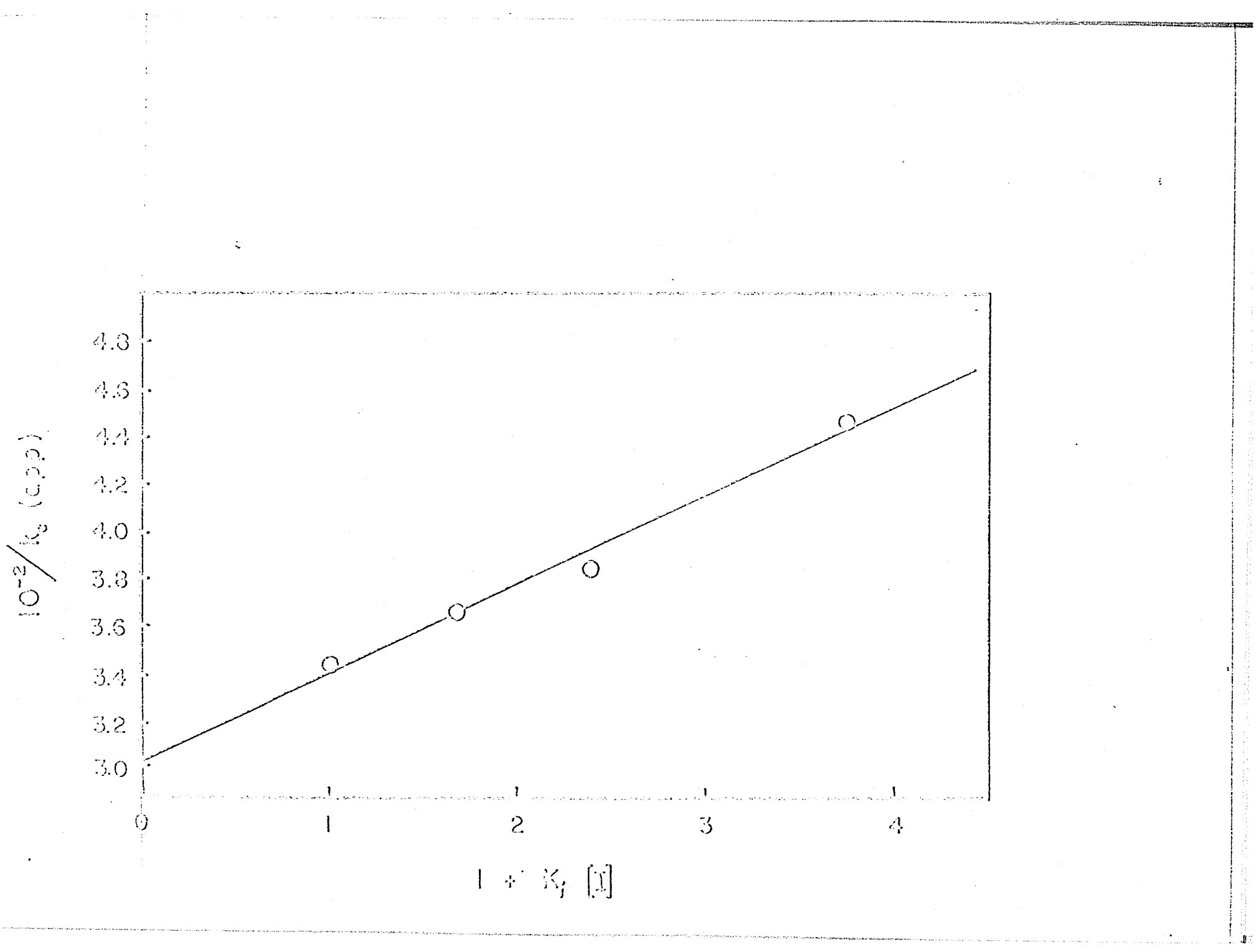

4.8
4.6
4.4
4.2
4.0
3.8
3.6
3.4
3.2
3.0
0
1
2
3
4
1 + K_I [I]

Figure 32

Plot of $1/K_m$ against $1/(1 + K_I[I])$ for the α-chymotrypsin catalyzed hydrolysis of methyl hippurate inhibited by indole.

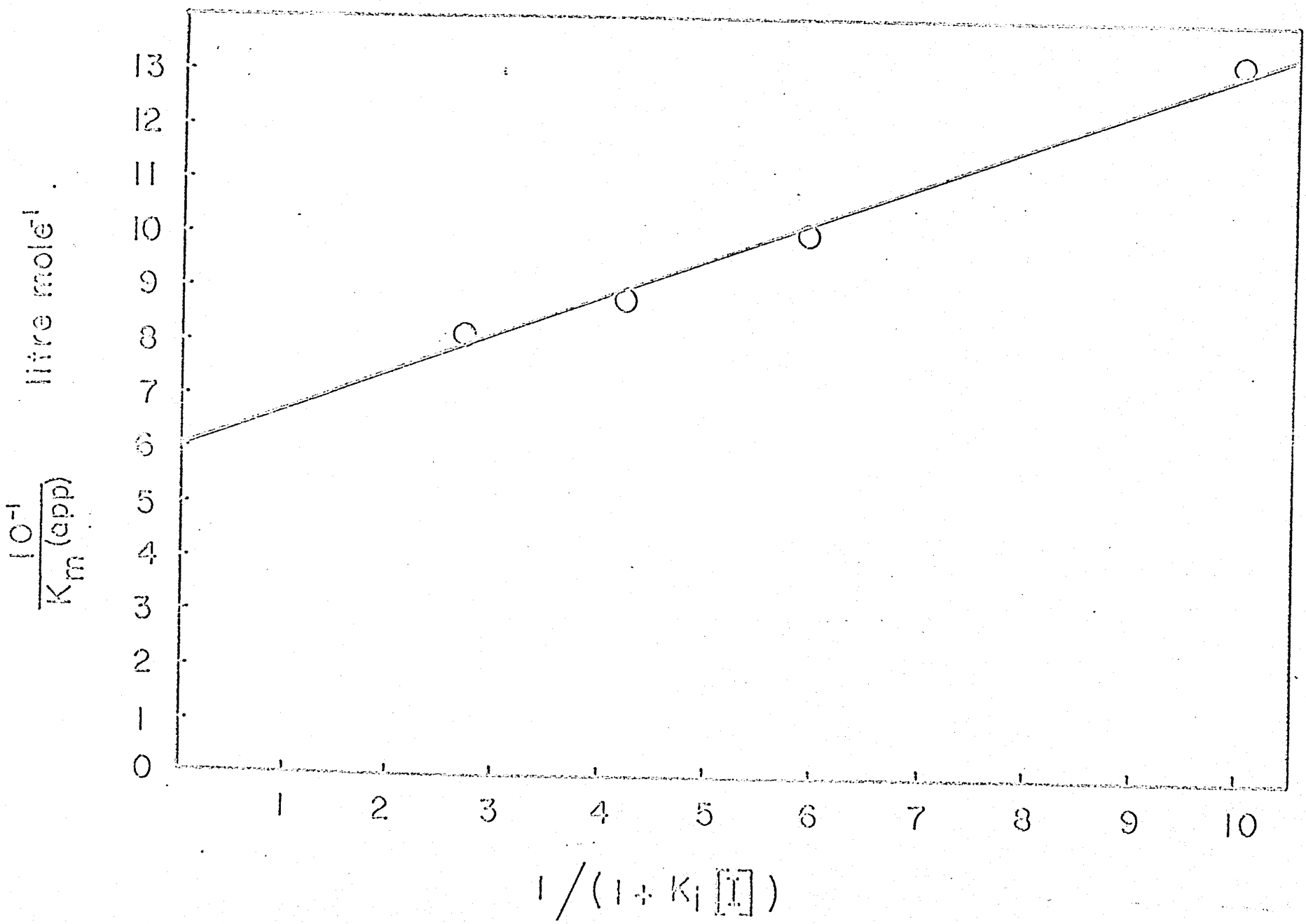
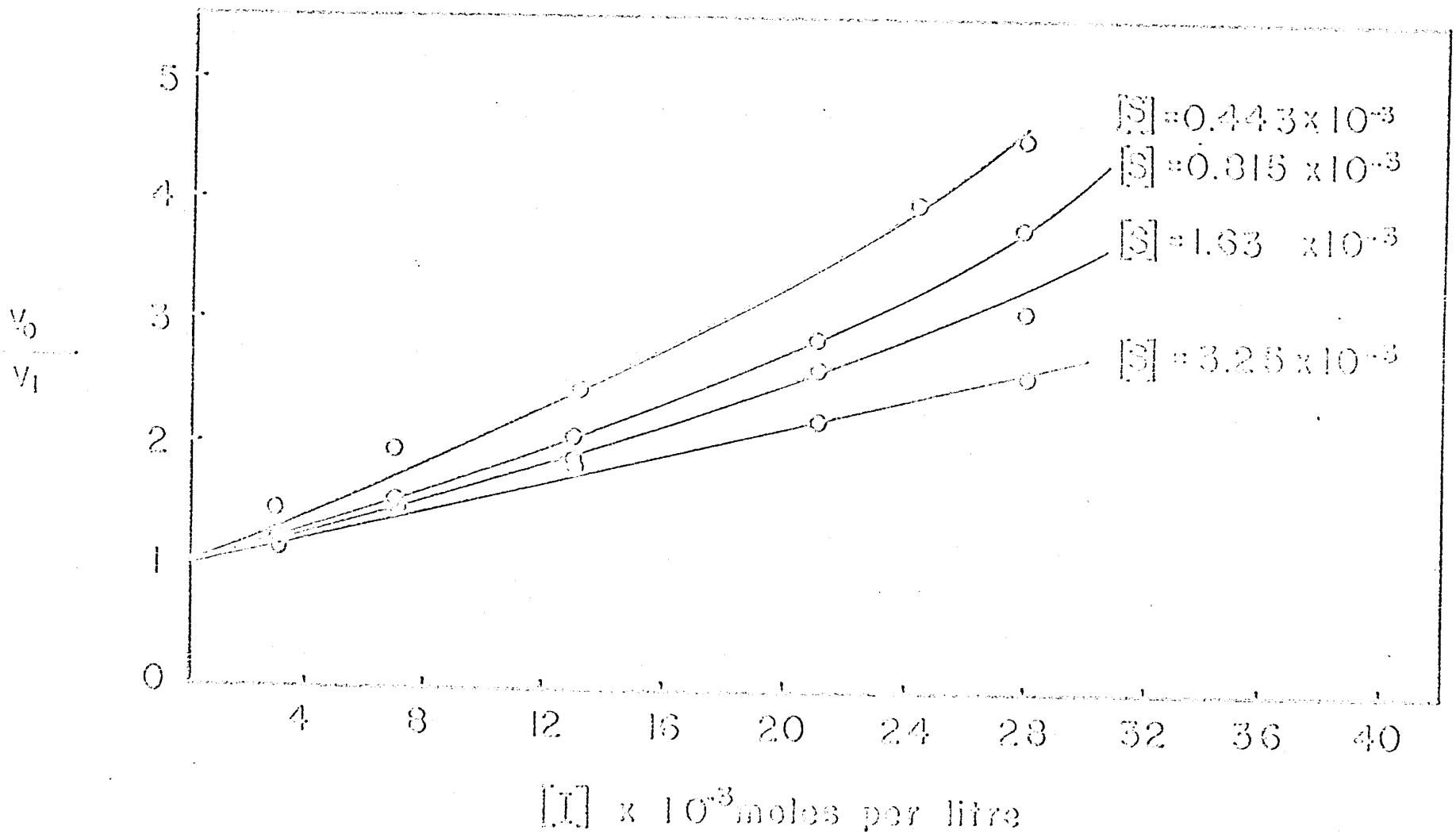

$\frac{10^{-1}}{K_m(app)}$ litre mole^{-1}
0
1
2
3
4
5
6
7
8
9
10
11
12
13
1
2
3
4
5
6
7
8
9
10
$1/(1+K_i[I])$

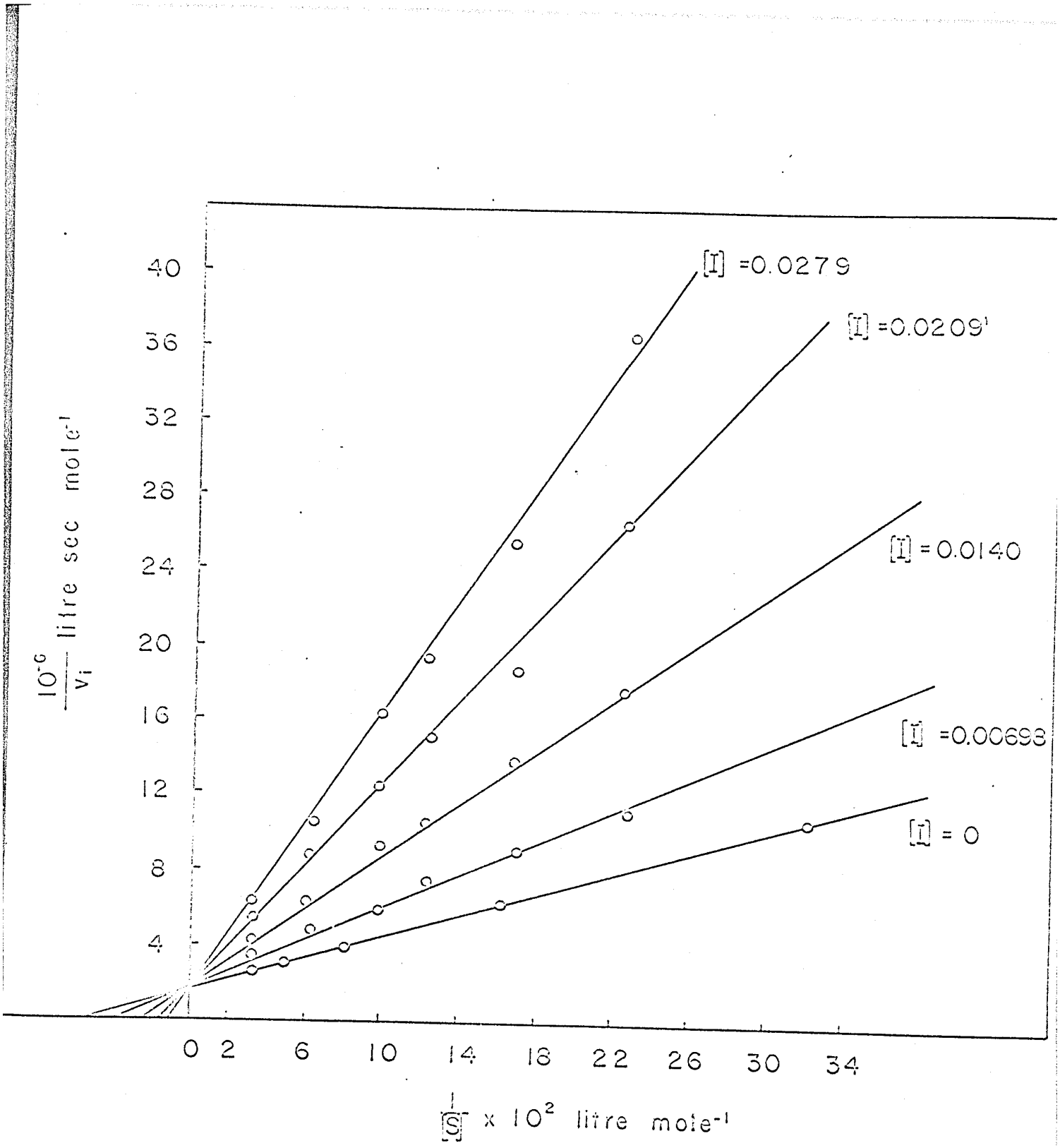
Figure 33a

A plot of v_o/v_i against [I] for the phenol inhibition of the α-chymotrypsin catalyzed hydrolysis of N-acetyl-L-tyrosine ethyl ester.

Figure 33b

Plot of $1/v_i$ against $1/[S]$ for the phenol inhibition of the α-chymotrypsin catalyzed hydrolysis of N-acetyl-L-tyrosine ethyl ester.


V_0/V_1
5
4
3
2
1
0
4
8
12
16
20
24
28
32
36
40
[S] =0.443x10⁻³
[S] =0.815 x10⁻³
[S] =1.63 x10⁻³
[S] =3.25x10⁻³
[I] x 10⁻³moles per litre


40
36
32
28
24
20
16
12
8
4
0 2 6 10 14 18 22 26 30 34
$\frac{10^{-6}}{v_i}$ litre sec mole^{-1}
$\frac{1}{[S]} \times 10^2$ litre mole^{-1}
[I] =0.0279
[I] =0.0209
[I] =0.0140
[I] =0.00698
[I] = 0

for the same data. The plots are linear and meet on the $1/v_i$ axis corresponding to a non-zero value; this also shows that the behavior is competitive. If the dependence were to the first power of the inhibitor concentration the K_i values calculated from each of the lines in figure 34 should be the same; in fact, as shown in Table 16, K_i is a function of the inhibitor concentration.

The simplest explanation of the behavior is that two inhibitor molecules can bind to the enzyme, either in a random or forced-order sequence as shown in figure 34. The apparent K_i values corresponding to these schemes are, for the random order

$$K_i(app) = (K_1 + K_2) + K_1K_2[I] \qquad (8)$$

and for forced order

$$K_i(app) = K_1 + K_1K_2[I] \qquad (9)$$

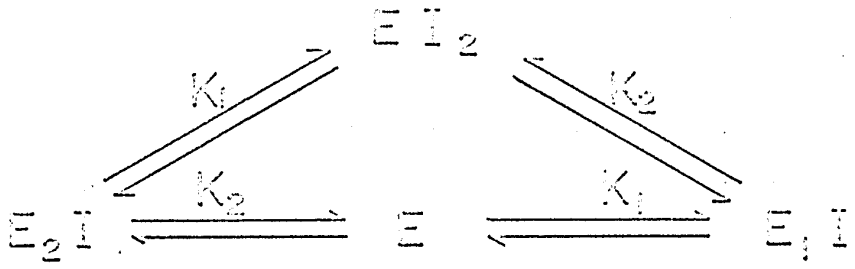
Each of these schemes predicts that a plot of K_i against [I] should be linear, and this is verified in figure 35. In both cases the slope is equal to K_1K_2 (equations (8) and (9)); for random order the intercept is $K_1 + K_2$, while for forced order it is K_1. Calculations of K_1 and K_2 from the data led, on the basis of the random-order scheme, to imaginary values, and it is concluded that forced-order binding applies; the values calculated on this basis are

K_1 = 42 liters per mole

Figure 34

Random and forced order binding schemes.

RANDOM ORDER



FORCED ORDER

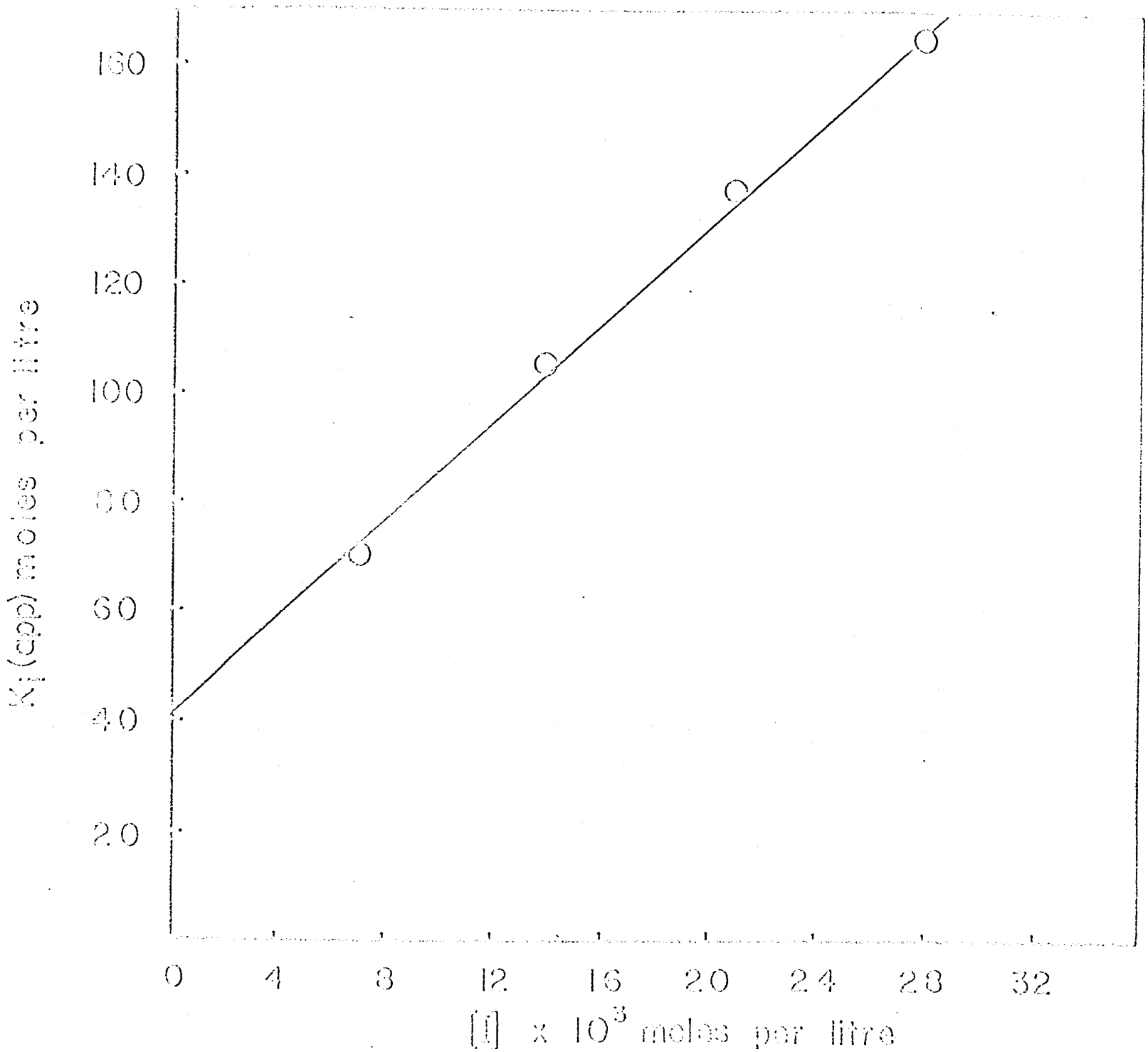
Table 16

Values of Association Inhibition Constants for Inhibition by Phenol

Phenol Concentration x 10^3 moles per liter	Apparent Inhibition Constant liter per mole
6.98	70.4
13.97	105
20.95	136
27.93	167

Figure 35

A plot of $K_I(app)$ against [I] for phenol inhibition.


160
140
120
100
80
60
40
20
K_I (app) moles per litre
0
4
8
12
16
20
24
28
32
[I] x 10^3 moles per litre

K_2 = 110 liters per mole.

Previously K_1 values of 160 and 140 M^{-1} have been deduced (119,120), on the assumption that only one molecule of inhibitor is involved.

The fact that the inhibition by phenol is competitive means one of two things:

1) Phenol does not become bound to the acyl enzyme, or

2) It does become bound, but the binding does not affect the rate of deacylation.

The second of these possibilities seems more likely; since the larger molecule indole can become bound to the acyl enzyme, it is probable that the phenol molecule can also be bound.

General Discussion

The inhibition results with indole are of special interest in that they conform exactly to the pattern of behavior predicted on the basis of the two-intermediate mechanism, when consideration is given to the different rate-determining steps found with different substrates. It is evident that the type of inhibition that is observed depends not only on the nature of the inhibitor but on the nature of the substrate, as was previously emphasized with acetylcholinesterase (112,121).

A point of interest is the binding site of indole and phenol. Indole probably binds at the same site as does the phenolic group of a tyrosine substrate or the indole group of a tryptophan substrate. Because this site is covered in the Michaelis complex there can be no binding of indole. In the acyl enzyme the aromatic moiety of the substrate probably interacts at the same site as in the Michaelis complex; that this interaction is important to the kinetic specificity of deacylation is suggested by the relative rate constants for the hydrolysis of various acyl enzymes (99). When the aromatic residue is dissociated from its binding site in the acyl enzyme, the acyl group cannot escape since it is covalently bonded, but the deacylation processes will be blocked. Therefore, if indole competes for this site, or if the binding of phenol interferes with the interaction at this site, deacylation will be blocked. That indole does not block deacylation by preventing the water molecule from approaching, or by interfering with the ionizing groups at the active site, is demonstrated by the fact that the inhibition is competitive with N-benzoyl-D and L-alanine methyl esters (29) for which deacylation is rate limiting (Chapter III D).

The fact that p-nitrophenyl acetate and aromatic

esters such as N-acetyl-L-tyrosine ethyl ester are substrates which compete for the same binding site on chymotrypsin (13) suggests why two phenol groups can become attached to the enzyme; there must be one site X that binds the aromatic ring in p-nitrophenyl acetate, and another Y that binds the aromatic ring in the aromatic esters. It is likely that binding at site X would not interfere with deacylation, since it does not interfere with acylation. Site Y, which is large enough to accommodate an indole group, is probably large enough to accommodate two phenolic residues; binding by phenol at this site in the acyl enzyme will therefore not interfere with deacylation.

CHAPTER V

HYDROLYSIS OF D AND L ESTERS

INTRODUCTION

Hein and Niemann(29) were the first to demonstrate the stereospecificity of α-chymotrypsin towards N-benzoyl-D and L-alanine methyl esters. From the pH dependence exhibited by these substrates (Chapter III) it has been concluded that the rate-limiting step is deacylation, with the L-antipode being hydrolysed approximately seventeen times faster than the D-antipode, in spite of the fact that there is no chemical difference between the two forms of acyl enzyme. It was therefore considered to be of interest to determine whether this difference is associated with different energies or entropies of activation.

Experimental

N-Benzoyl-D-alanine methyl ester and N-benzoyl-L-alanine methyl ester were prepared as previously described.

All reactions were carried out in 0.10M sodium chloride at pH 8.0, the kinetic procedure being the same as previously described.

RESULTS AND DISCUSSION

Figure 36 shows a plot of $\log_{10} k_c$ against 1/T, and the data relating to the temperature dependence of the kinetic parameters is summarized in Tables 17 and 18. There is seen to be a break at 25°C, with an apparent lowering of the activation energy at higher temperatures. This type of behavior has often been observed in enzyme reactions, and may be attributed to several factors (122). The possibilities relevant to the present case are: (1) the reaction might involve two intermediates, with each reaction step showing a different temperature coefficient; (2) the reaction might be accompanied by the rapid and reversible denaturation of the enzyme.

Two intermediates are known to be involved, the reaction scheme being

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow[P_1]{k_2} E' \xrightarrow{k_3} E + P_2$$

The kinetic parameters are given by

$$k_c(app) = \frac{k_2 + k_3}{k_2 + k_3} \qquad (1)$$

and

$$K_m(app) = \frac{k_3}{k_2 + k_3} \; K_m \qquad (2)$$

The rate constants k_2 and k_3 may have different activation

Figure 36

A plot of $\log_{10}k_3$ against 1/T for N-benzoyl-D and L-alanine methyl esters.

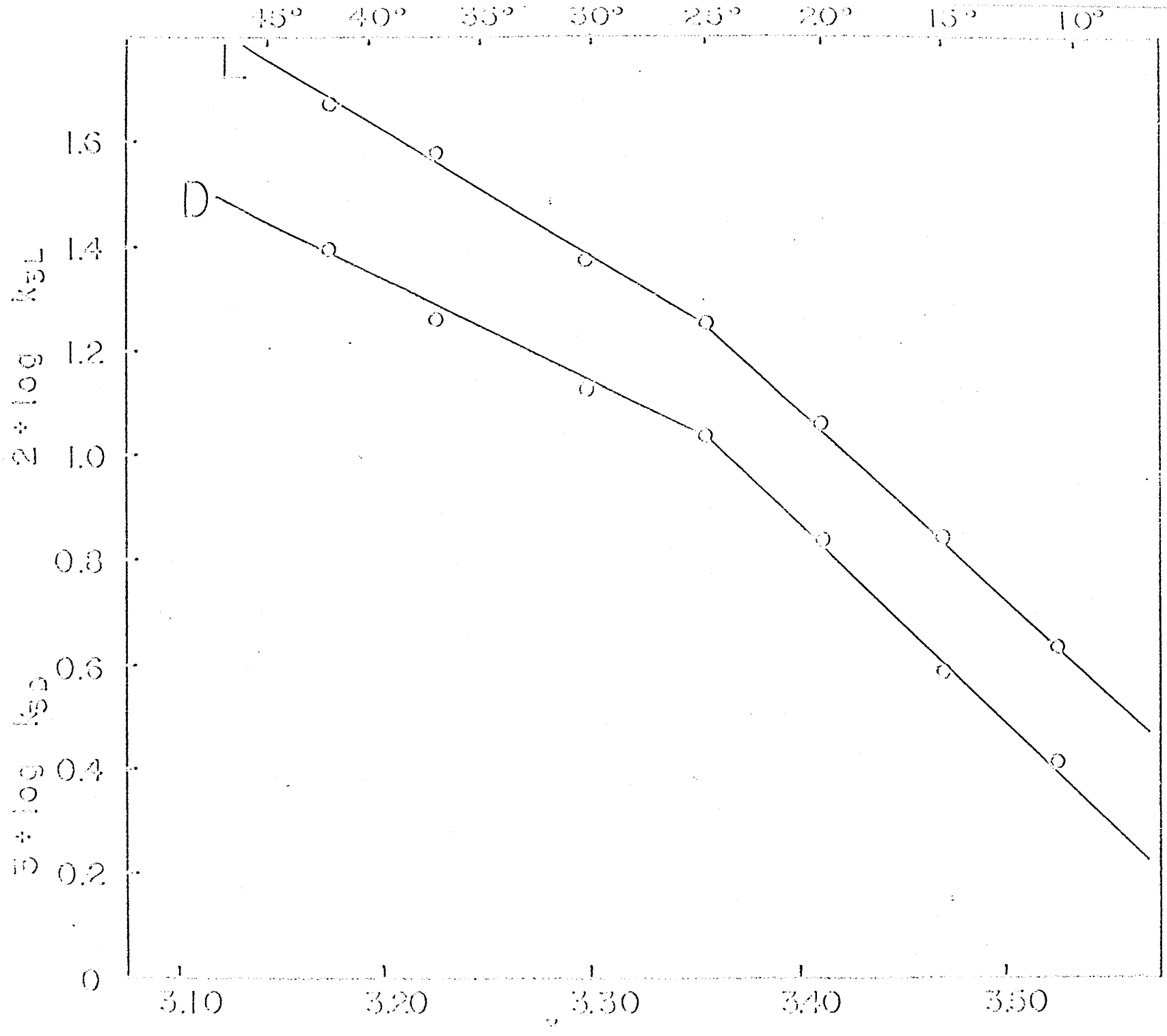

45°
40°
35°
30°
25°
20°
15°
10°
L
D
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
2 + log kEL
2 + log kD
3.10
3.20
3.30
3.40
3.50

Table 17

Temperature Dependence of the Hydrolysis of N-Benzoyl-L-Alanine Methyl Ester

T°C	$k_3 \times 10^2 sec^{-1}$	$K_m \times 10^3$ moles litre^{-1}
10.5	4.27	6.99
15.0	6.91	8.12
20.0	11.5	8.47
25.0	17.6	8.42
30.0	24.3	8.32
37.0	37.1	8.75
42.0	46.9	8.54

Table 18

Temperature Dependence of the Hydrolysis of N-Benzoyl-D-Alanine Methyl Ester

T°C	$k_3 \times 10^3 sec^{-1}$	$K_m \times 10^3$ moles litre^{-1}
10.5	2.62	3.14
15.0	3.74	2.77
20.0	6.54	4.01
25.0	10.8	3.71
30.0	13.4	3.28
37.0	19.1	3.48
42.0	24.7	3.49

energies, so that one reaction becomes rate limiting in the low-temperature region and the other in the high-temperature region. If such were the case the experimental curve at the inflection point is expected to lie 0.3 log units below the intersection point of the individual Arrhenius lines. Figure 36 shows that the change in slope at high temperatures is inconsistent with this explanation, the change of slope being much too sharp. Furthermore, if this explanation were applied a similar inflection would be found in $K_m(app)$. Figure 37 shows that such is not the case; $K_m(app)$ is independent of temperature over the entire temperature range studied for both the D and L compounds.

The second possibility, that the reaction is accompanied by a rapid reversible denaturation of the enzyme molecule, is represented by the scheme shown in figure 38. Bender and co-workers(42) have studied the irreversible denaturation of α-chymotrypsin and found it to be first order in enzyme concentration.

For the case in which $k_2 \gg k_3$,

$$k_c(app) = \frac{k_3}{1 + K} \qquad (3)$$

where K equal to $[E_{inactive}]/[E_{active}]$, is the constant for the equilibrium between the active and inactive enzyme.

Figure 37

A plot of $\log_{10}K_m$ against 1/T for N-benzoyl-D and L-alanine methyl esters.

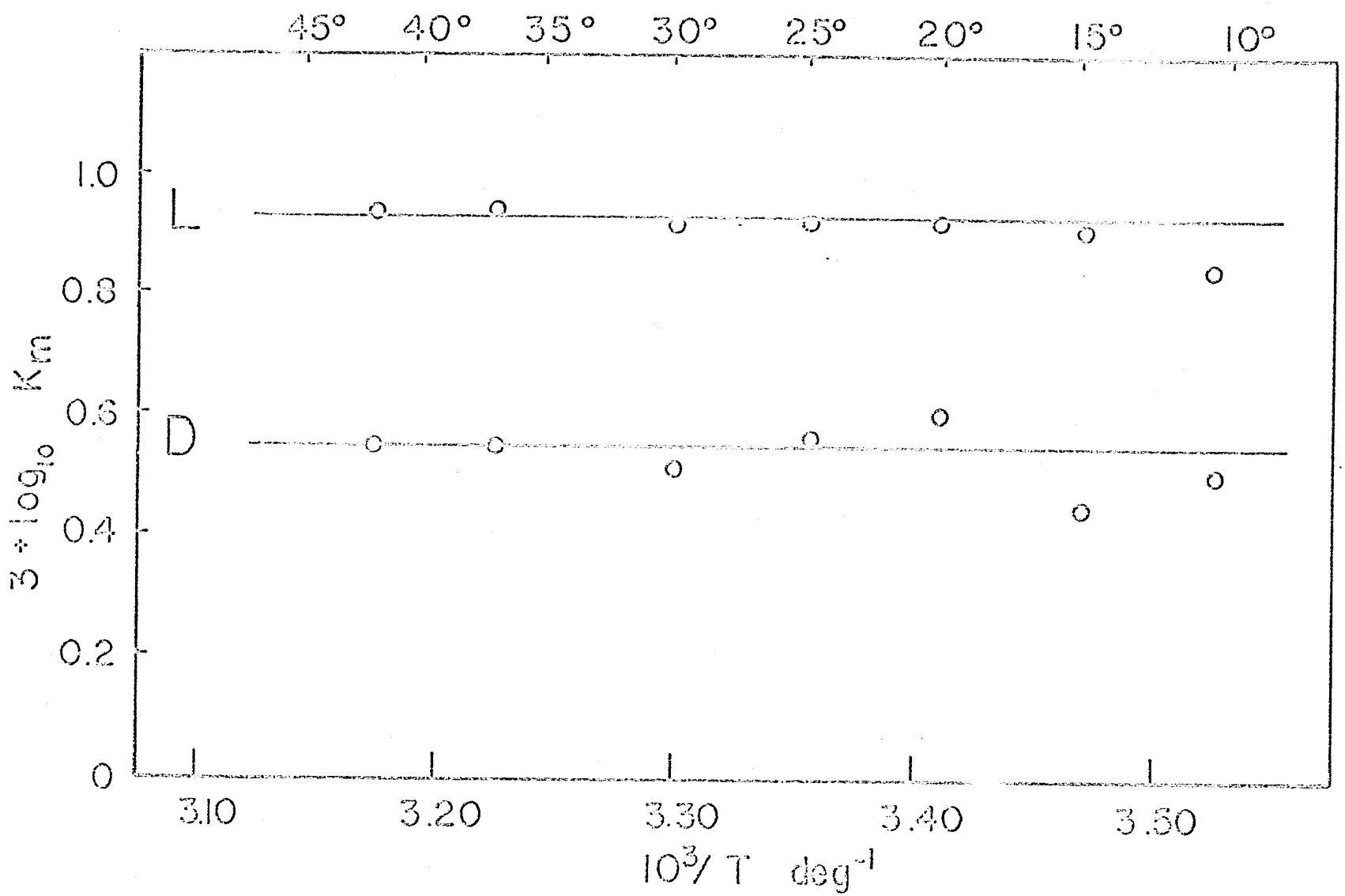
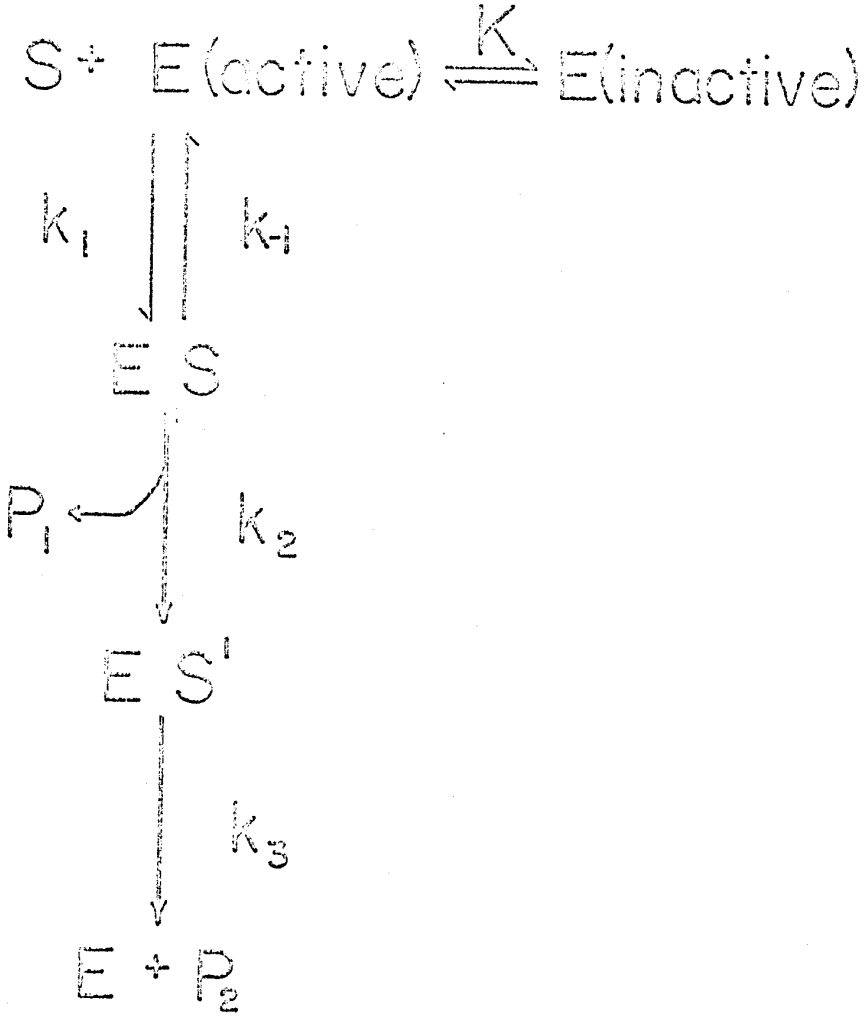

45°
40°
35°
30°
25°
20°
15°
10°
1.0
0.8
0.6
0.4
0.2
0
L
D
3 + log10 Km
3.10
3.20
3.30
3.40
3.50
10³/ T deg⁻¹

Figure 38

A scheme for reversible denaturation of α-chymotrypsin.


S + E (active) K E (inactive)
k1
k-1
E S
P1
k2
E S'
k3
E + P2

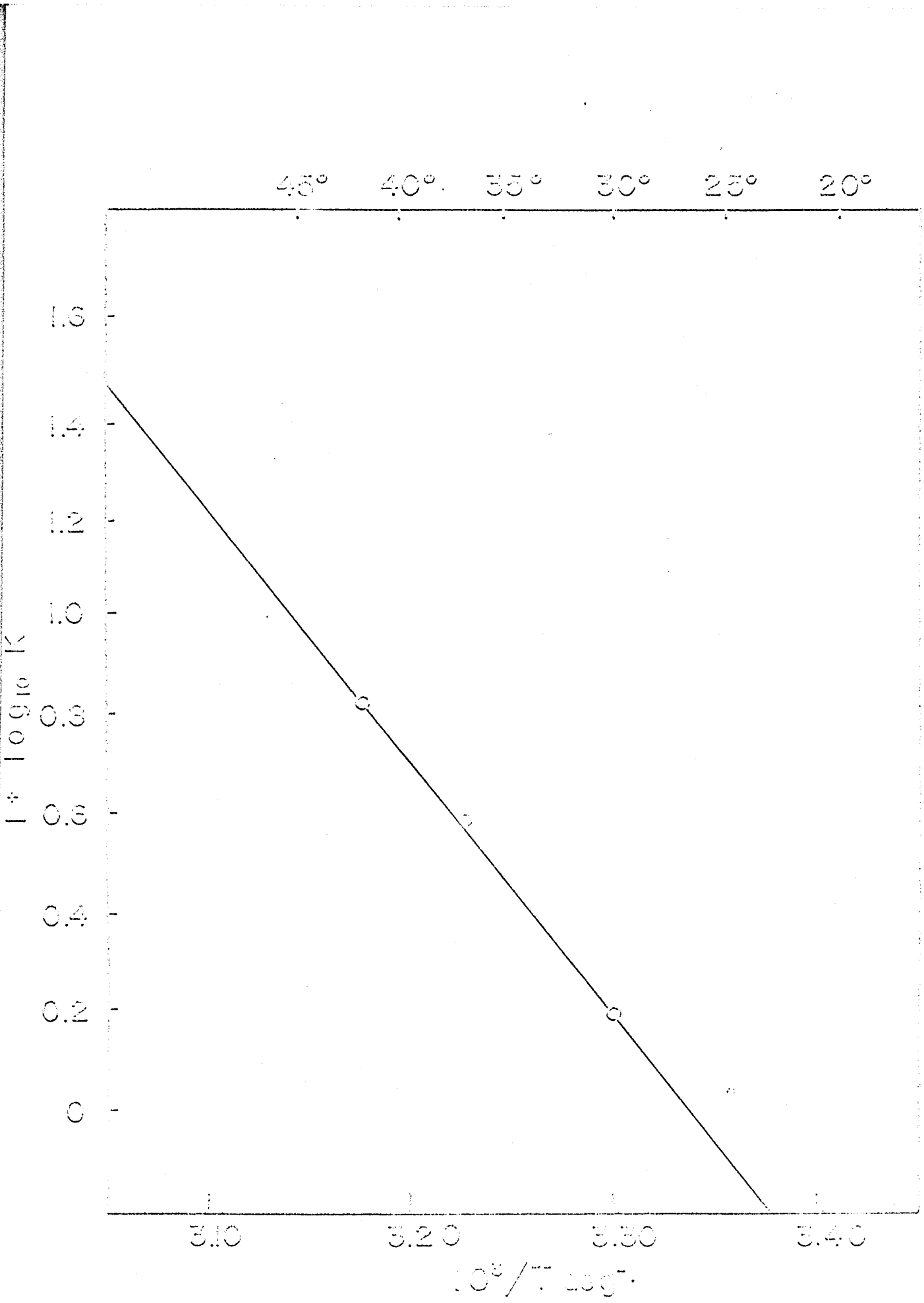
It is possible to calculate K from the amount the observed value lies below the expected value. A plot of log K against 1/T (figure 39) gives $\Delta H=25$ kcal and $\Delta S=80$ e.u., which are reasonable values for denaturation phenomena. It is therefore reasonable to conclude in this case that the break in the plot of log k against 1/T is due to a rapid reversible denaturation.

Of greater interest is the fact that in the low temperature region the plots are linear and give activation energies of 16.2 ± 0.3 kcal and 16.5 ± 0.6 kcal for the D and L antipodes respectively. This is a rather surprising result, in that it might have been expected that groups on the D-antipode may cause steric interference; there would then be a more highly strained activated complex, and a higher activation energy. The difference in the rates of deacylation must therefore be due to a less favorable entropy of activation for the D-antipode. The calculated entropies of activation are - 9.6 e.u. and - 14.1 e.u. for the L and D antipodes respectively.

Since there is no chemical difference between the two substrates the entropies of the ground states are the same in both reactions. The difference in entropy of activation must therefore arise from the different conformations of the enzyme when it is interacting with the

Figure 39

Plot of $\log_{10}K$ against 1/T.


45°
40°
35°
30°
25°
20°
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
I + log10 K
3.10
3.20
3.30
3.40
10³/T deg⁻¹

D and L substrates in the activated complexes. In the activated complex of the D-antipode the enzyme is probably in a more extended form.

Bender and co-workers(99) have postulated that the large differences in rates of deacylation are related to the entropy of activation, the poorer substrates having a less favorable entropy of activation. The higher entropies in the case of the best substrates has been attributed to a fixation of the acyl moiety, with the result that there is a smaller loss of rotational entropy during the activation process. On these grounds the entropies of activation for D and L-alanine methyl esters would be expected to be greater than that for N-acetyl-L-tyrosine ethyl ester, which has a $\Delta S^{\neq}$ = -13.4 e.u. (99). While the rotational entropy is undoubtedly a contributing factor it appears from these results that the entropy changes resulting from changes in the conformation of the enzyme are also important.

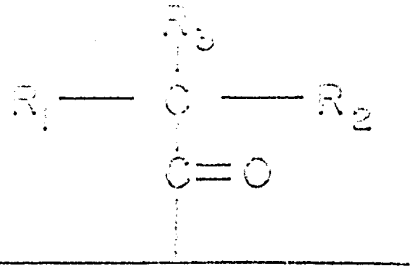
The various possible orientations of the groups in the acyl enzyme are shown in figure 40. Each group interacts with a specific locus on the enzyme, with 1 (a) being the natural configuration for the L-substrate. Structures 2 (a) and 3 (a) can be ruled out since bulky groups would be interacting at a locus normally occupied by a hydrogen, and this has been shown to lead to a lowering of the rate

Figure 40

The possible orientations of groups in the acyl enzyme.

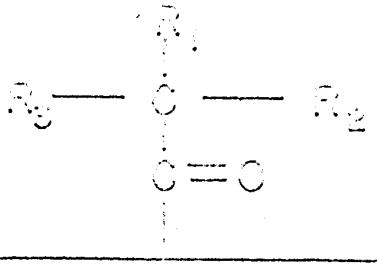
R_1=H , R_2= $-\overset{H}{\underset{|}{N}}-\overset{O}{\overset{\|}{C}}-\Phi$ R_3=CH_3

L

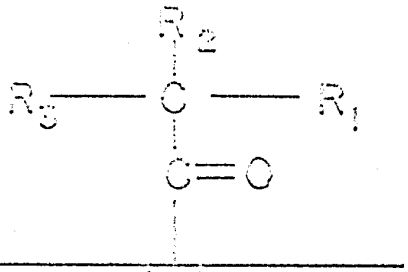
(1) 

(a)

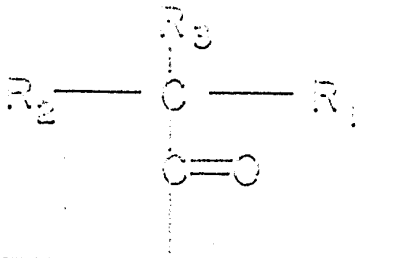
D



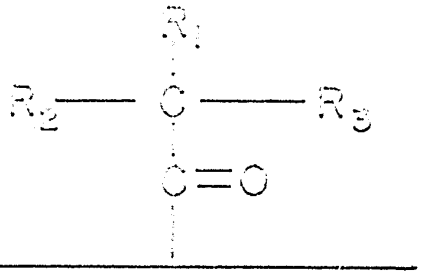
(a)

(2) 

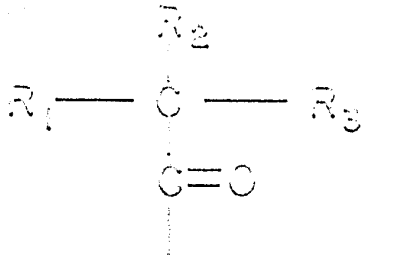
(c)



(b)

(3) 

(d)



(b)

(123). For the D antipode 1 (b) and 3 (b) are possible modes of interaction; 2 (b) can be ruled out since R_2 is too bulky a group to interact at the locus normally occupied by a hydrogen.

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CLAIMS TO ORIGINAL RESEARCH

1. The principle of microscopic reversibility was examined for reactions occurring under non-steady-state and steady-state conditions. By considering several different reaction schemes it was shown that the principle of microscopic reversibility can never be used to prove the mechanism of a reaction when that for the reverse reaction has been established; it can only be used to eliminate kinetically equivalent mechanisms which are not the exact reverse of that for the reaction in the opposite direction.

2. A general equation for pH effects was formulated and the various patterns of pH behavior were classified. It was shown that the pH dependence of the $\tilde{k}_c/\tilde{K}_m$ ratio can only give pK values for groups involved in the breakdown of the Michaelis complex.

3. The α-chymotrypsin catalyzed hydrolysis of N-benzoyl-L-alanine methyl ester was studied at low substrate concentrations over a range of pH, and in various dioxane-water mixtures. From the variation of the pKs of the ionizing groups it was concluded that one group is cationic, probably an imidazole group, the other neutral, probably a serine hydroxyl group.

4. An investigation has been made of the influence of pH on the kinetics of the α-chymotrypsin catalyzed hydrolysis of N-benzoyl-D and L-alanine methyl esters, N-acetyl-L-tyrosine ethyl ester and p-nitrophenyl acetate. The results show that these four substrates have a similar mechanism of hydrolysis with two ionizing groups of pK 6.9 and 9.2 involved in the breakdown of the enzyme substrate complex and a group of pK 6.9 involved in the breakdown of the acyl enzyme.

5. A new mechanism of action was proposed for α-chymotrypsin catalyzed hydrolysis. Two imidazole groups are postulated to play a role in the reaction. In complex formation one of these interacts with the carbonyl function of the substrate; in acylation the other imidazole group abstracts a proton from the serine hydroxyl and transfers it to the leaving alcohol moiety. The deacylation process is the reverse of this process, with water acting as the nucleophile.

6. General steady-state equations were formulated for the inhibition of enzyme reactions involving two intermediates. The various types of inhibition were classified and a number of special cases

considered. In particular, it was shown how the type of inhibition observed depends not only on the substrate and inhibitor, but also on the rate-determining step of the reaction.

7. A study was made of the inhibition of the α-chymotrypsin catalyzed hydrolysis of N-acetyl-L-tyrosine ethyl ester, inhibited by indole and phenol. Indole exhibits non-competitive behavior, and an analysis of the behavior showed that it binds to the free enzyme and acyl enzyme but not the Michaelis complex. Phenol exhibits competitive behavior, two molecules of phenol being bound to the free enzyme in a forced-order sequence.

8. A temperature study was carried out on N-benzoyl-D and L-alanine methyl esters. A sharp break in the log k_3 against 1/T plot was attributed to a rapid reversible denaturation of the enzyme at higher temperatures. In the low temperature region the activation energies are $E_L^{\ddagger} = 16.2 \pm 0.3$ Kcal. and $E_D = 16.5 \pm 0.6$ kcal. per mole. It was concluded that the difference in rates is due to the different ways the enzyme is coiled around the two substrates in the activated complex and is manifested in different entropies of activation.