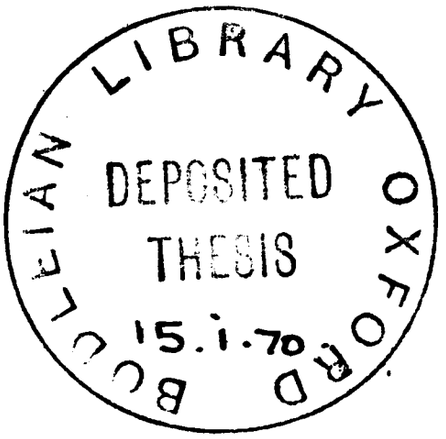
# AN INVESTIGATION OF THE MECHANISM OF ACTION OF PAPAIN

A Thesis submitted for
the Degree of Doctor of Philosophy
in the University of Oxford

by

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October 1969

## ACKNOWLEDGEMENTS

The author would like to thank his supervisor, Dr. G. Lowe, for his helpful advice and encouragement during the research. He is grateful to Dr. S. S. Husain and Dr. A. W. Lake for their assistance in the experimental work. Thanks are also due to Dr. J. M. Lawlor and G. Allen for fruitful discussions.

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ABSTRACT i

# CHAPTER 1

## INTRODUCTION[1]

Papain (EC 3.4.4.10) is an enzyme found in the fruits, the leaves and the stem of the tropical papaw tree (Carica papaya). The dried papaya latex is used in many practical ways, such as the treatment of dyspepsia, the tenderisation of meat and for removing scar tissue, which suggest that it has proteolytic properties. The enzymatic nature of papain was studied by Wurtz, who in 1880 showed that in the course of digestion of fibrin, the active principle in papaya latex was bound to the substrate.[2] Papain was the first proteinase shown to be active towards synthetic peptides,[3] and to depend for its activity on a thiol group.[4,5] The action of papain on synthetic peptides was studied extensively by Bergmann and his coworkers,[1a] who concluded that the specificity of papain was similar to, but broader than, that of trypsin. It is now placed in the same group of thiol proteinases as ficin and bromelain, the behaviour of all three being similar in many respects.

In 1937 Balls et al.[6] crystallised papain from fresh papaya latex. Crystalline papain was later obtained from

dried papaya latex by Kimmel and Smith,[7] and was shown to be homogeneous by electrophoresis and sedimentation at pH 4,[8] by ion-exchange chromatography,[9] by immunochemical studies,[1c] and by the presence of a single N-terminal isoleucine.[10] A molecular weight of 20,700 was found from sedimentation-diffusion measurements.[8] Osmotic pressure studies[11] gave a larger value of 27,000 ± 2,000. A value of 23,000 can be calculated from the now known amino acid sequence.

Papain has a wide range of temperature and pH stability. At 75°, only about 5% of its activity was lost in 3 minutes at neutral pH.[12] At pH 10.7 and 29°, about 10% inactivation resulted in 10 minutes.[13] Irreversible inactivation is rapid at acid pH values, however, especially at high temperatures.

The tertiary structure is little affected by small amounts of organic solvents, or in 8M urea, as studied from optical rotatory dispersion and viscosity measurements.[14] In guanidine hydrochloride of concentrations above 3M, however, the molecule is maximally unfolded and irreversible inactivation occurs.[15]

## The Structure of Papain

Investigation of the amino acid sequence of papain by Light *et al.*[16] indicated that it is a single-chain protein of about 200 amino acid residues. A three-dimensional X-ray study at 2.8 Å resolution made by Drenth *et al.*[17,18] has revealed that the enzyme molecule contains 212 residues. Alterations have to be made in Light's sequence: instead of Ileu-29 and Ileu-30, 13 new residues have to be inserted after Phe-28, and the peptide Thr-138 to Tyr-176 must be placed between these extra residues and Arg-31 (Light's nomenclature). The sequence of these 13 previously unknown residues has been worked out by Husain and Lowe.[19] Another alteration is the addition of a valine residue in position 130.[18] The revised, complete amino acid sequence is shown in Fig. 1-1, and will be referred to throughout this work.

There are seven half-cystine residues, of which only one, Cys-25, is in the reduced form. The others are engaged in disulphide bridges (residue 22 - 63, 56 - 95, 153 - 200). The folding of the polypeptide chain, shown in Fig. 1-2, is such that there is a large cleft in the molecule. The total helix content of the molecule is

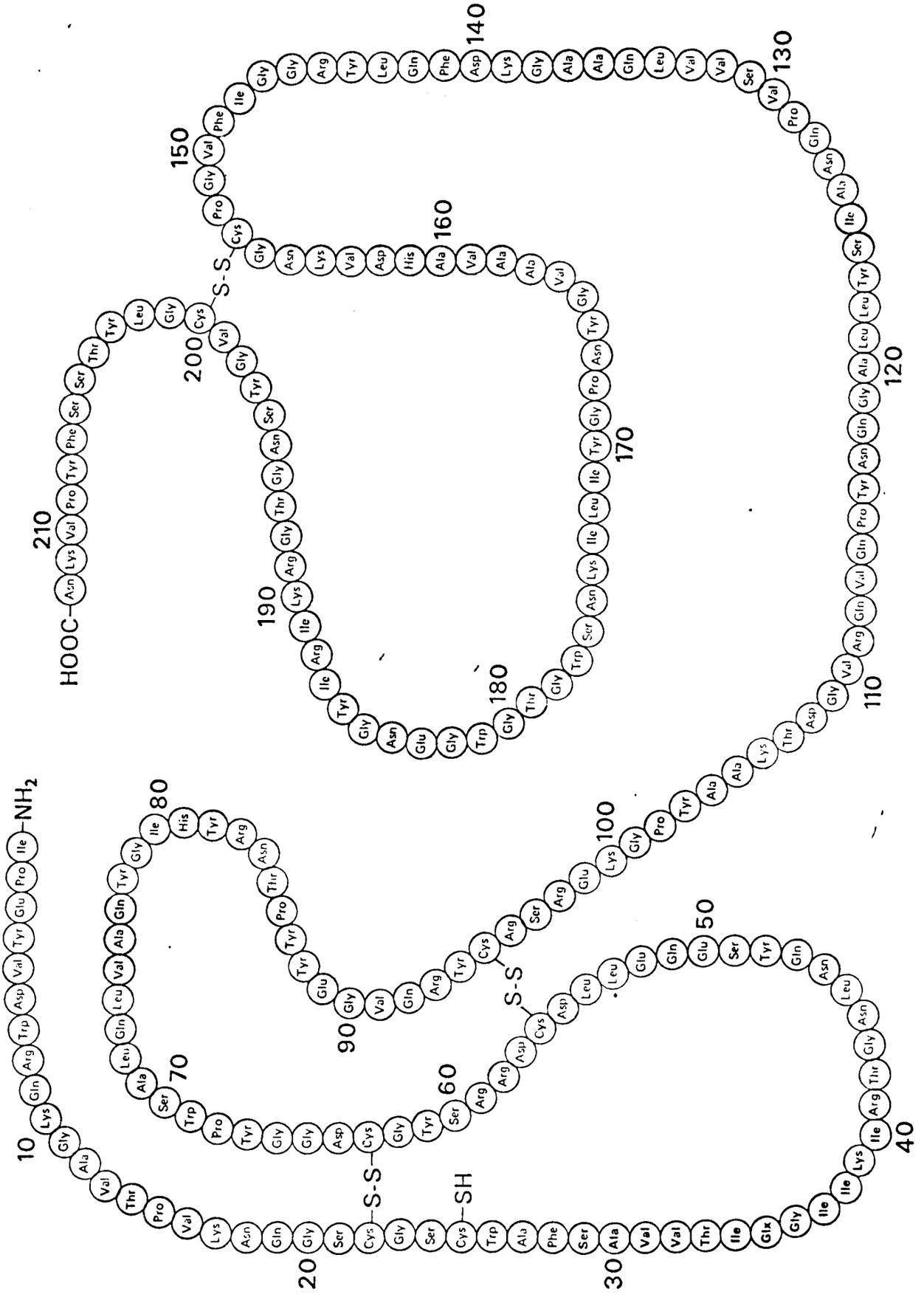



Fig. 1-1 Amino acid sequence of papain (ref. 19).

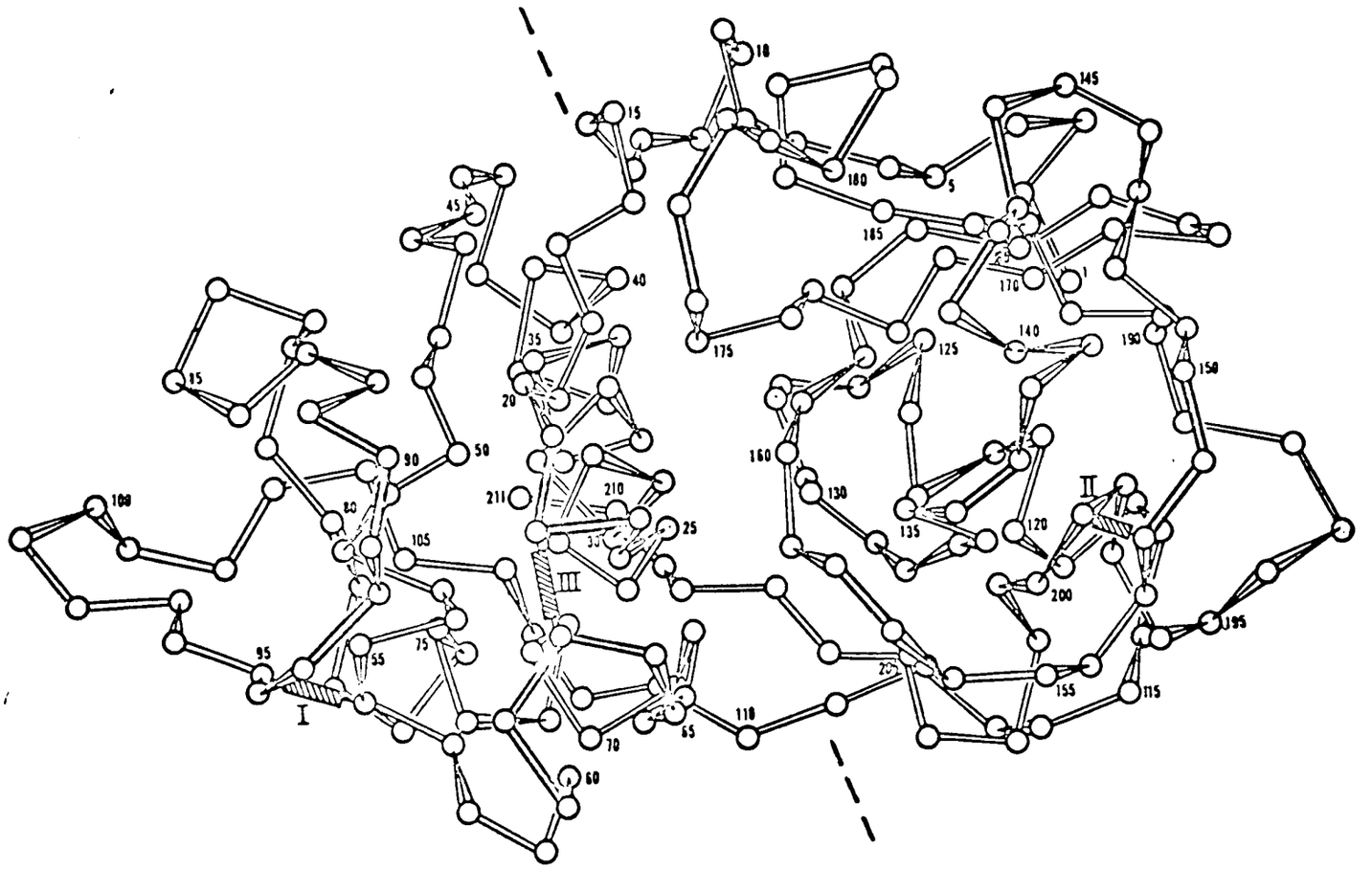



Fig. 1-2

The three-dimensional structure of papain (ref. 17). Subsequent work[18] has shown that an additional valine residue has to be inserted in position 130.

about 20%, in good agreement with the results obtained from optical rotatory dispersion studies.[14,20] There are four pieces of α-helix, three (residues 26 - 41, 50 - 56, 69 - 78) in the left-hand part as the molecule is shown in Fig. 1-2, and one (residues 116 - 126) in the right-hand part, which also contains a small portion of the β-structure (residues 163 - 172). The right-hand part is much more hydrophobic than the left-hand part.

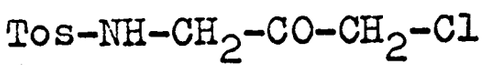
On the left-hand side of the cleft is Cys-25; on the right-hand side, His-159. The distance between the sulphur atom and the imidazole ring is only 3.4 Å. The indole ring of Trp-177 lies above this imidazole and is capable of interacting with it. Gln-19 and Asp-64 on the left-hand side, and Asp-158 on the right-hand side of the cleft are also in the active-site region. Residue 64, originally identified as aspartic acid, has recently been found to be asparagine.[21] Asp-158 is about 10 Å from the thiol group, and may take part in the binding of substrates and inhibitors.

## The Essential Functional Groups in Papain

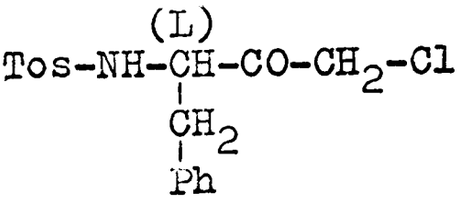
Early observations[4,5,11] showed that papain required

activation, and all the known activators like cyanide and L-cysteine were capable of reducing disulphide bonds. Mild oxidising agents reversibly inactivate papain, and so do mercuric compounds such as mercuric chloride and phenylmercuric chloride. Krebs[22] demonstrated that heavy metal ions were potent inhibitors of papain, and that their effect could be eliminated by pyrophosphate or citrate, which are metal-binding agents. These results suggest that a thiol group may be essential for catalysis.

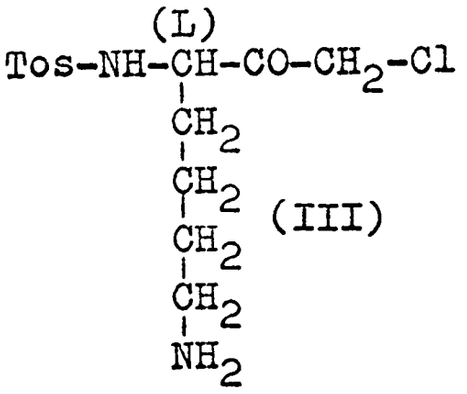
One molar equivalent of iodoacetate irreversibly inhibits active papain.[23] Alkylation by iodoacetamide at pH 5, and by iodoacetate and bromoacetate at pH 5 and 8, was shown to occur at the thiol group of Cys-25.[9,24] Irreversible inhibitors which bear resemblance to specific substrates are much more powerful than haloacetates. 1-Chloro-3-tosylamidopropan-2-one ('tosylglycyl chloromethyl ketone', TGCK, I),[25] L-1-chloro-4-phenyl-3-tosylamidobutan-2-one ('tosylphenylalanyl chloromethyl ketone', TPCK, II),[26,27,28] and L-7-amino-1-chloro-3-tosylamidoheptan-2-one ('tosyllysyl chloromethyl ketone', TLCK, III) are excellent irreversible inhibitors of papain. The rate of inhibition by TGCK is retarded by specific



(I)



(II)



(III)

substrates, suggesting attack at the active site.[25] Inhibition by TPCK[27] depends on a basic group of $pK_a$ 8.28, which could be that of the thiol group. The site of attachment was confirmed as Cys-25 for TGCK,[29] TPCK[28] and TLCK[28].

From photo-oxidation and alkylation experiments, Sun and Tsou[30] suggested that a histidine residue was involved in the activity of papain. Although the alleged observation that a histidine was alkylated at pH 5 has now been challenged,[24] there are many other indications for participation of the residue. The similarity of amino acid sequences around His-159 of

papain and the active-site histidine of bovine trypsin suggests that the former may also be in the active-site region.[31] Papain is irreversibly inhibited by 1,3-dibromoacetone,[32] which links the thiol group of Cys-25 with the imidazole N-1 of His-159. This result is in excellent agreement with those from X-ray studies,[17,18] and suggests that this imidazole may be involved in the mechanism of action. Husain and Lowe[32] proposed that in the active enzyme the thiol group is hydrogen-bonded to N-3 of the imidazole (Fig. 1-3). The $pK_a$ for protonation of this system, giving a free thiol and an imidazolium ion, would be expected to be lower than the $pK_a$ of a normal imidazole. A hydrogen bond also probably exists in α-chymotrypsin between the active-site serine and histidine,[33] and yet the value of the $pK_a$ of protonation, approximately 7.0, is normal. A difference between the two systems is that while a hydrogen-bonded hydroxyl-imidazolium can be formed, an analogous thiol-

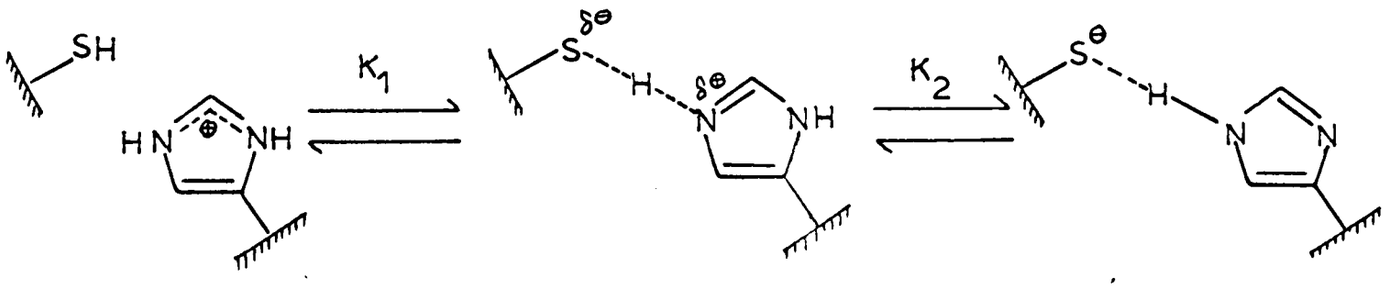



Fig. 1-3

imidazolium system cannot, sulphur being unable to accept a hydrogen bond. The $pK_a$ for deprotonation, giving a negatively charged hydrogen-bonded system, would in papain be expected to be between that of a free thiol and an imidazolium ion. These predictions account for the fact that papain activity for specific substrates depends on two groups in the free enzyme with apparent $pK_a$ values ca. 4 and 8.5.

Wallenfels and Eisele[34] observed a bell-shaped pH dependence for alkylation of papain by L(-)-α-iodopropionate, the apparent $pK_a$ values being 4.0 and 7.8. Inhibition by L(-)-α-iodopropionamide, however, shows a sigmoid pH dependence with an apparent $pK_a$ of 8.0. The limiting second-order rate constant for the L(-) acid is 25 times that for the L(-) amide, indicating that the ionised L(-)-α-iodopropionate must be more strongly attracted to the active site. These authors suggested that the L(-) acid was attracted by an imidazolium ion with $pK_a$ 7.8. The group with $pK_a$ 4.0 was considered to be a carboxylate of an aspartyl or glutamyl residue, which could be hydrogen-bonded to the active thiol, resulting in high nucleophilicity of the latter. In

view of more recent results from X-ray studies regarding the positions of the nearest carboxyl groups,[17,18] this interpretation is unlikely to be correct. A different explanation of these inhibition results was given by Husain and Lowe[32] that a polarised hydrogen-bonded thiol-imidazole system was required for alkylation by L(-)-α-iodopropionate. The negatively charged thiolate-imidazole formed at high pH values would repel the inhibitor carrying a like charge, and the free thiol existing at low pH values would lack sufficient nucleophilicity. The uncharged L(-) amide is not very strongly attracted to the active site and is subject only to attack by the more nucleophilic thiolate-imidazole.

From kinetic evidence many authors have suggested that a carboxylate anion is involved in the catalytic steps as well as in the binding of substrates and inhibitors. The X-ray results show that while the nearest aspartyl residues (residues 64 and 158) could take part in binding, they are probably too far, about 10 Å, from the active thiol to be actively involved in the catalytic steps, unless their positions in aqueous solutions are different from those in crystalline

forms. Furthermore, recent evidence from this laboratory has shown that residue 64 is asparagine and not aspartic acid.[21]

Blow et al.[33] reported the presence of a buried aspartyl residue (residue 102) in α-chymotrypsin, which is hydrogen-bonded to N-1 of the active-site histidine (residue 57) and is likely to play a crucial role in the catalytic mechanism. By analogy, since there are many similar features of the two enzymes, it may be expected that the active-site histidine in papain is subject to the same type of interaction. Residue 175 in papain, originally identified[16] and recently confirmed[21] as asparagine is indeed from X-ray evidence found to be hydrogen-bonded to N-1 of the histidine. Whether this interaction would affect the $pK_a$ of the imidazole, and/or be relevant to the mechanism of papain action, is still uncertain.

Gln-19 in the active-site region may take part in the binding of substrates and inhibitors through interactions of the γ-amide group, which is about 5 Å from the active-site thiol.

Participation of a tryptophan residue in catalysis has been suggested by Morihara and Nagami.[35] Their evidence was that papain was found to be inhibited by carbonyl reagents such as sodium bisulphite, by tetranitromethane and by Koshland's reagent (2-hydroxy-5-nitrobenzyl bromide). These results cannot be regarded as conclusive since the reagents used are not specific for tryptophan.

In contrast with the behaviour of N-terminal isoleucine of α-chymotrypsin, that of papain is not essential for enzyme activity.[36] Treatment of papain crystals with 1-fluoro-2,4-dinitrobenzene resulted in modification of the α-amino group, as shown by X-ray diffraction and acid hydrolysis, but did not affect the catalytic activity.

## The Mechanism of Activation of Papain

Murray,[37] and later Kimmel and Smith,[7] demonstrated that both a reducing agent and a metal-binding agent were necessary in order to produce maximal activity of papain. Once papain has been activated, the continued

presence of the activator is not necessary, as shown by Smith et al.,[38] who obtained active papain by passage through a series of ion-exchange resins containing a thioglycollate bed, and other beds which served to remove electrolytes. An early theory that activators of papain function as co-enzymes[39] cannot therefore be correct in the light of these results.

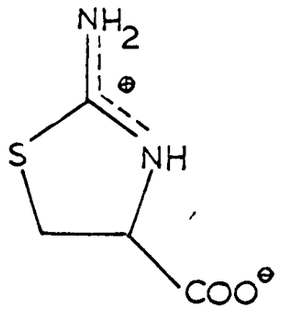
The observation that papain could be reversibly inactivated by iodine, oxygen or hydrogen peroxide led Bersin[40] to suggest that inactive papain is a dimer with the two molecules linked by a disulphide bridge, which is reduced by an activator. Since Glazer and Smith[41] found no significant difference between sedimentation coefficients of active and inactive papain, the dimer hypothesis is untenable.

From thermodynamic and kinetic considerations, and in order to account for the fact that the nitroprusside and the porphyrindine test on papain is negative, Smith[42] proposed that a high-energy thiolester bond existed in the active enzyme and was responsible for catalysis. Sanner and Pihl[43] have presented various criticisms of

this formulation, including the evidence obtained by Wieland[44] that thiolesters normally react with thiols. If a high-energy thiolester were the essential catalytic agent in papain, the enzyme should be inhibited, rather than activated, by added thiols. An activation mechanism has been proposed by Neumann et al.[45] implying the existence in the unactivated enzyme of a high-energy bond, by which the active-site cysteine is bound to an unknown group. It was claimed that in the course of activation of papain by phosphorothioate one molecule of the activator was reversibly incorporated into the protein molecule, resulting in the formation of one free thiol group which is directly related to the enzyme activity. The observation that the activator combines with papain is contradictory to that of Smith et al.[38] Since phosphorothioate may break disulphide bridges in papain and subsequently combine with the new thiol groups formed, the experiments of Neumann et al. cannot be regarded as conclusive.

Sluyterman[46] and Klein and Kirsch[47] provided conclusive results which led to formulation of an activation mechanism in which there is no incorporation

of the activator molecule. Sluyterman found that in the presence of air and low concentrations of L-($^{35}$S)-cysteine, active papain was reversibly inactivated with incorporation of radioactivity. The radioactive label was removed on reactivation. Klein and Kirsch treated unactivated papain prepared by the method of Kimmel and Smith[7] with potassium ($^{14}$C) cyanide and detected labelled 2-iminothiazolidine-4-carboxylic acid (IV) and a




(IV)

stoichiometric amount of free thiol in the activated enzyme, which contained no radioactivity. In a separate experiment, performic acid oxidation produced cysteic acid in an amount equivalent to that of (IV). It can be concluded that unactivated papain prepared by the method of Kimmel and Smith is a mixed disulphide formed between a thiol group of the enzyme and L-cysteine. The mechanism of activation proposed by Klein and Kirsch is shown in Fig. 1-4.

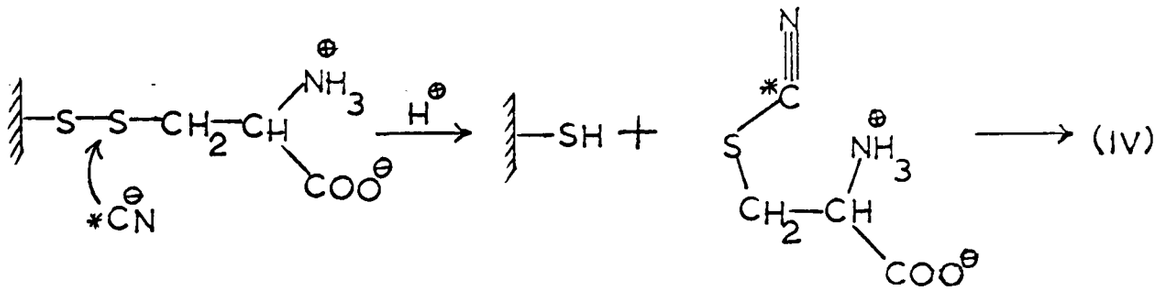


Fig. 1-4

## Interactions of the Substrate with the Active Site

The structural and stereospecificity of α-chymotrypsin has been explained by Niemann and his coworkers,[48] by assuming 'three-point interaction' between the substrate and the active site of the enzyme. An α-acylamino acid derivative consists of four groups attached to the α-carbon atom (Fig. 1-5). Three of these, $R_1CONH$, $R_2$ and $COR_3$ are assumed to interact most effectively with the corresponding sites $\rho_1$, $\rho_2$ and $\rho_3$ of the enzyme. $\rho_H$ is considered not to contribute substantially to binding. Other binding modes are possible, some indeed more favourable, but only orientations with $COR_3$ - $\rho_3$ interactions can lead to hydrolysis. Although papain has broader specificity than α-chymotrypsin, it is

useful to apply this concept of specificity to the former as well.

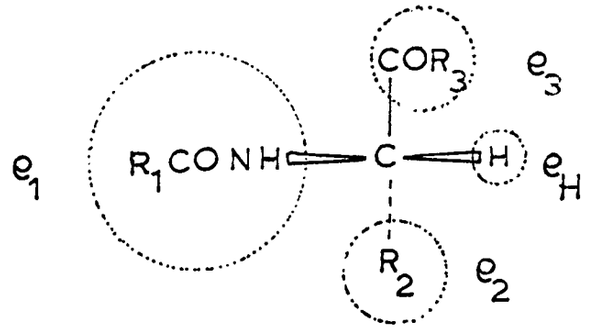



Fig. 1-5

Schechter and Berger,[49,50] using polypeptides containing D- and L-alanine, have shown that the size of the active site of papain is such that it is capable of interacting with a substrate of 25 Å length in extended conformation. Seven 'subsites', each interacting with an amino acid residue of the substrate, contribute significantly to binding (Fig. 1-6). The bond susceptible

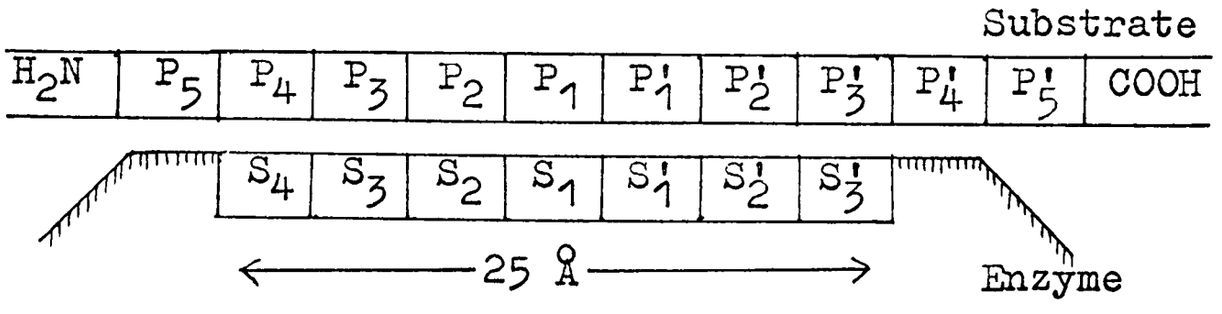



Fig. 1-6

A diagrammatic representation of the active site of papain

to hydrolysis is situated between subsites $S_1$ and $S_1'$, at which specificity for L-enantiomers is strongest. The $S_2$ subsite interacts most specifically with L-phenylalanine, the nature of interaction probably being mainly hydrophobic.[50] The size of the active site of papain may be compared with that of lysozyme,[51] which can accommodate six sugar units, and of carboxypeptidase-A[52] which can interact with a substrate with an extended conformation of 18 Å length.

Specificity of Papain

The broad specificity of papain extends to the L-form of polypeptides, α-N-acylamino acid amides, anilides, esters, thiolesters and thionesters. Amino acid derivatives with free α-amino groups are, however, normally poor substrates.

The nature of the acyl portion of α-N-acylamino acid derivatives is important for binding and kinetic specificity. Generally, the more hydrophobic, aromatic acyl groups such as benzoyl or benzyloxycarbonyl lead to better substrate activity than aliphatic acyl groups such as acetyl or methoxycarbonyl. More specifically,

the advantageous effect of placing an L-phenylalanyl residue in the $S_2$ subsite has been noted.[50] Lowe[1d] has pointed out the close analogy existing between the structure of the benzyloxycarbonyl and the phenylalanyl groups.

The optimum pH for neutral substrates is normally around pH 6. A substrate with a carboxylate group in the side chain such as α-N-benzyloxycarbonylisoglutamine is a poor substrate at this pH.[7] The rate of its hydrolysis is greater at pH 4, indicating that the negative charge is repelled by the $\rho_2$ site. α-N-Benzoyl-L-argininamide,[13,53] α-N-benzoyl-L-arginine ethyl ester (BAEE),[53,54] α-N-benzoyl-L-lysinamide,[1b] and α-N-benzyloxycarbonyl-L-lysine esters,[27] all of which have positively charged side chains, have been found to be very good substrates. These results suggest the existence in the $\rho_2$ site of a negative charge or a polar group capable of interacting with a charged or a polar side chain of the substrate. It is interesting to note, however, that α-N-benzoyl-L-citrulline methyl ester,[55] an analogue of BAEE with a δ-carbamyl in place of the guanidino group, and α-N-benzyloxycarbonyl-ε-N-formyl-L-lysine p-nitrophenyl ester,[53] both with no

net charge in the side chain, are almost as active as their positively charged parent compounds.

In contrast with α-chymotrypsin, papain does not depend critically for its activity on a hydrophobic side chain in the substrate. Acylglycine derivatives such as hippurylamide[56] or the corresponding esters[57,58] are specific substrates for papain, and the rates of hydrolysis of p-nitrophenyl esters of α-N-benzyloxycarbonyl-L-tryptophan, -L-phenylalanine, -L-leucine, -L-tyrosine and -glycine are all comparable.[59]

A peptide bond next to a free carboxyl terminal is not very susceptible to papain hydrolysis at neutral pH. As in the case of a carboxyl group in the side chain, it appears that progressive ionisation of that adjacent to the peptide bond decreases substrate binding. At pH 4 benzyloxycarbonylglycyl-L-tryptophan[7] and benzyloxycarbonylglycylglycine[56] are more quickly hydrolysed than at pH 8.

Papain has synthetic and transferase as well as hydrolytic activity. Bergmann and his coworkers[1a]

demonstrated that insoluble anilides and peptides could be synthesized by incubation with papain of acylamino acids and aniline or amino acid derivatives with free α-amino groups. The ability of many proteolytic enzymes such as α-chymotrypsin, cathepsin-C and trypsin to catalyse transamidation and transpeptidation, is also shared by papain. Fruton et al.[60] found, for example, that benzyloxycarbonylglycyl-L-phenylalaninamide could be synthesized at pH 7.5 from benzyloxycarbonylglycinamide and L-phenylalaninamide. These observations are not surprising, since hydrolytic, synthetic and transferase activity all belong to the same general type of reactions - substitution at a carbonyl carbon.

There is considerable evidence to indicate that the replacement nucleophiles, which include water, ammonia and amino acid derivatives, have to be bound on the enzyme surface, presumably at the $\rho_3$ site. For example, the L-form of amino acid derivatives are much better replacement agents than the D-form,[49,61] and methyl-L-tryptophanate enhances the deacylation rate of trans-cinnamoyl-papain more than the D-compound and simple amines.[62]

## A Brief Outline of the Mechanism of Action of Papain

A reaction mechanism of papain involving formation of an acyl-enzyme intermediate from the acyl portion of the substrate and a thiol group of the enzyme was suggested by Smith *et al.*,[38] mainly on the basis of the chemical nature of thiols and by analogy with other types of enzyme reactions[63]. Acyl-enzyme intermediates have been demonstrated in many hydrolytic[64] as well as group-transfer and electron-transport reactions[63]. The mechanistic scheme for papain was visualised by Smith as in eqn. (1-1). $k_{+1}$ was

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_0} E + P \qquad (1\text{-}1)$$

assumed to represent the constant for formation of the acyl-enzyme intermediate; $k_{-1}$, the back reaction. $k_0$, the constant for product formation, was considered to be possibly composite, representing more than one rate step. This conclusion was derived from the study of kinetics of papain-catalysed hydrolysis of α-N-benzoyl-L-argininamide (BAA)[13] and α-N-benzoyl-L-arginine ethyl ester (BAEE)[54], which was found to follow the Michaelis-Menten scheme. $(k_0/K_m)$ was interpreted as $k_{+1}$ from the effects of temperature,

dielectric constant, added alcohols and pH. This required that $k_0 \gg k_{-1}$, a condition which is now known not to be fulfilled.

Although many details of Smith's mechanism proposals are incorrect, the existence of the thiolester intermediate during the course of papain-catalysed hydrolysis has been demonstrated by other authors. Lowe and Williams[65] showed that hippuryl dithioester intermediates were formed during papain- and ficin-catalysed hydrolyses of the specific substrate methyl thionohippurate, $\lambda_{max}$ for the intermediates being 313 mμ (log ε 4.2) and 315 mμ (log ε 3.7) respectively. The variation with substrate concentration of ultraviolet extinction due to these intermediates was as expected from the Michaelis-Menten parameters found by an independent method. The suppression of extinction by methyl hippurate showed that both substrates were bound at the same site. Trans-cinnamoyl-imidazole, a non-specific substrate, was found by Brubacher and Bender[62] to give rise to trans-cinnamoyl-papain, with $\lambda_{max}$ 325 mμ (log ε 4.4), the rate of disappearance of which was identical to the rate of formation of trans-cinnamøic acid.

The existence of the acyl-enzyme intermediate was also shown indirectly [66] by the 'burst' of p-nitrophenol observed in the pre-steady phase of papain-catalysed hydrolysis of p-nitrophenyl α-N-benzyloxycarbonyl-L-tyrosinate and acetyl-DL-tryptophanate at pH 3.2.

It has been observed[67] that papain catalyses the exchange of $^{18}O$ from water to the carboxyl group of α-N-benzyloxycarbonylamino acids and vice versa: these acids are 'virtual substrates' for papain, and the mechanism of exchange has been proposed, in which equilibria are set up between the free acid, the enzyme-substrate complex and the acyl-enzyme. Almost no $^{18}O$ exchange with the solvent occurs during papain-catalysed hydrolysis of ethyl (carbonyl-$^{18}O$) hippurate.[68] The rate of exchange in the hydroxide-ion-catalysed hydrolysis of the same substrate is 1/14 of the hydrolytic rate, indicating that a symmetrical tetrahedral intermediate is formed. In papain-catalysed hydrolysis no intermediate identical to those in hydroxide-ion-catalysed hydrolysis is present.

The observation that various aryl and alkyl esters of hippuric acid possess remarkably similar values for $k_0$,

about 3 $sec^{-1}$, led Lowe and Williams[57] to postulate that all these substrates have a common rate-determining step, most probably the breakdown of the acyl-enzyme. In order to account for anomalously low values of $k_0$ for hippuryl-amide (0.6 $sec^{-1}$), n-butyl (1.3 $sec^{-1}$) and isopropyl (0.4 $sec^{-1}$) hippurate, a three-step mechanism was postulated (eqn. (1-2)).

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} \underset{+P_1}{ES'} \underset{k_{-3}}{\overset{k_d}{\rightleftharpoons}} E + P_2 \qquad (1\text{-}2)$$

ES = the enzyme-substrate complex

ES'= the acyl-enzyme intermediate

$P_1$ = amine, ammonia, alcohol or other 'leaving' molecule

$P_2$ = acid

$k_{+1}$, $k_{-1}$ = rate constants for formation of ES and the back reaction respectively

$k_{+2}$, $k_{-2}$ = rate constants for formation of ES' and the back reaction respectively

$k_d$, $k_{-3}$ = rate constants for deacylation and the back reaction respectively

When initial rates are measured $k_{-2}$ and $k_{-3}$ can be ignored. The observed Michaelis-Menten parameters are composite, and eqn. (1-3) and (1-4) can be derived from

$$k_0 = \frac{k_{+2}k_d}{k_{+2}+k_d} \qquad (1\text{-}3)$$

$$K_m = \frac{k_{-1}+k_{+2}}{k_{+1}} \cdot \frac{k_d}{k_{+2}+k_d} \qquad (1\text{-}4)$$

the steady-state assumption. Kirsch and Igelström[69] also observed nearly identical $k_0$ values for various esters of α-N-benzyloxycarbonylglycine, and offerred the same interpretation, pointing out however that this evidence alone was not decisive since the common rate-determining step could be a conformational change among other possibilities. The constancy of $k_0$ for esters of α-N-benzyloxycarbonyl-L-lysine has also been reported.[27]

Previously, Smith and his coworkers[13,54] also suggested, from similarity of the observed $k_0$ values at 37°, that the deacylation step was rate-determining for the hydrolysis of BAA and BAEE. Whitaker and Bender,[53] however, reported that at 25° $k_0$ for BAA was only half of that for BAEE. They suggested that for the former substrate acylation was predominantly rate-determining, while for the latter deacylation was. The problem of identifying the rate-determining step was elegantly solved by Sluyterman, who showed that BAEE combined

with the active site of unactivated papain with a dissociation constant equal to $K_m$ of the active enzyme,[70] and that the rate of inactivation of the enzyme by chloroacetate was not affected by the presence of BAEE.[71] Presumably chloroacetate could attack the enzyme-substrate complex at the same rate as the free enzyme. The observations indicated that, for BAEE as well as for BAA, acylation of the enzyme is the rate-determining step. The same conclusion was made by Whitaker for the enzyme ficin from the observation that the inhibition constant for the D-form of BAEE was comparable with $K_m$ for the L-form, and that in the presence of saturating amounts of either D- or L-BAEE the rate of inactivation of ficin by chloroacetamide was seven times faster than in their absence.[72]

The rate of inactivation of papain by chloroacetate is decreased in the presence of ethyl hippurate.[71] The magnitude of the decrease agrees with calculation based on the assumption that the acyl-enzyme is protected from the activating agent, while the free enzyme and the enzyme-substrate complex are not. The result shows clearly that for this substrate deacylation is rate-determining.

A. Binding and acylation. The condition $k_{+2} \ll k_{-1}$ probably holds for papain-catalysed hydrolyses as it is known to do for those of α-chymotrypsin.[73] The lower limits of $k_{+1}$ for almost all enzymes so far studied[74] are $10^7$ - $10^9$ $M^{-1}sec^{-1}$, and it was suggested that $k_{+1}$ for papain is in this range.[27] The values of $(k_{-1}+k_{+2})/k_{+1}$ are typically $10^{-3}$ - $10^{-4}$ M, and therefore $(k_{-1}+k_{+2})$ = $10^4$ $sec^{-1}$. For substrates having rate-determining acylation rates, it is clear that $k_{+2} \ll k_{-1}$, and $K_m = K_s$ (substrate constant, $k_{-1}/k_{+1}$). For many ester substrates for which acylation is not rate-determining it may still be true that $k_{+2} \ll k_{-1}$.

A plot of log $(k_0/K_m)$ against σ, the Hammett substituent constants, for four p-substituted phenyl esters of hippuric acid[57] gives a good straight line with ρ=+1.2. Since $(k_0/K_m) = k_{+1}k_{+2}/(k_{-1}+k_{+2})$, the fact that a good correlation was obtained indicates that $k_{+2} \ll k_{-1}$, and $(k_0/K_m)$ is linearly related to the acylation rate. Thiolate-catalysed hydrolysis of aryl acetates was found to give a ρ value of +1.7. The lower ρ value for the acylation of the enzyme suggests that it is subject to acid as well as nucleophilic catalysis.

Stockell and Smith[13] had previously observed that $(k_0/K_m)$ for BAA showed an inverse deuterium oxide isotope effect, which is characteristic of many acid-catalysed reactions.[75]

In the papain-catalysed hydrolysis of hippuric acid derivatives, leaving agents such as ammonia and alcohols seem to have played little part in substrate binding. Log $(k_0/K_m)$ (=log $(k_{+2}/K_s)$) for the amide and various types of esters form a roughly linear plot with $k_{OH^-}$, the hydroxide-ion-catalysed rates.[76] The plot of log $(1/K_m)$ against log $k_{OH^-}$ for ethyl and substituted phenyl esters of α-N-benzyloxycarbonylglycine is also a good straight line.[69]

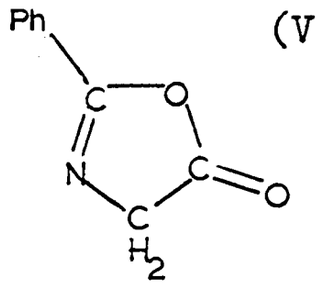
$(k_0/K_m)$ for all substrates of papain with no free carboxyl groups depend on a group with an apparent $pK_a$ $(pK_1)$ of about 4.3, and another group with an apparent $pK_a$ $(pK_2)$ of about 8.5. Provided that a substrate does not change its state of ionisation, the variation with pH of $(k_0/K_m)$ should, in principle, reveal the prototropic groups on the free enzyme, which are essential for its subsequent activity after the substrate has been bound.[77,78] $pK_1$ has been assigned to an ω-carboxyl group of aspartic

or glutamic acid by various authors.[1c,27,53,57] It was assigned by Husain and Lowe[32] to protonation of a hydrogen-bonded thiol-imidazole system. $pK_2$ of $(k_0/K_m)$ for BAEE shows a heat of ionisation of 5.1 kcal $M^{-1}$ at 0°.[54] Data from literature[79,80] indicate for the thiol group $pK_a$ 8.3 - 8.6, $\Delta H$ 6 - 7 kcal $M^{-1}$; the imidazole of histidine $pK_a$ 5.6 - 7.0, $\Delta H$ 6.9 - 7.5 kcal $M^{-1}$; the α-ammonium group $pK_a$ 7.6 - 8.4, $\Delta H$ 10.0 - 13.0 kcal $M^{-1}$. The α-ammonium group has been shown not to be essential for papain activity.[36] Sluyterman[58] and Lowe and Williams[57] considered it possible that $pK_2$ represents protonation of an imidazole. Many authors concluded that the essential group of $pK_2$ is the unionised active-site thiol group.[1c,62,69] Husain and Lowe [32] postulated deprotonation of the thiol-imidazole system to be responsible for the observed $pK_2$.

If the functional groups of the enzyme which are essential for catalysis interact with the substrate in the binding step, the $pK_a$ values of the free enzyme, as shown from the $(k_0/K_m)$ pH profile, should be different from the $pK_a$ values observed in subsequent steps in which enzyme-substrate combination has already occurred. It should be noted that the $pK_a$ values for acylation steps

of p-nitrophenyl, benzyl and methyl esters of α-N-benzyloxycarbonyl-L-lysine[27] are the same as $pK_a$ values of $(k_0/K_m)$, indicating lack of perturbation of the enzyme catalytic groups on binding.

B. Deacylation and other possible rate steps. It is conceivable that the acyl-enzyme intermediates which have been shown to exist during hydrolyses of methyl thionohippurate[65] and trans-cinnamoylimidazole[62] are not formed with more labile substrates. In the hydroxide-ion-catalysed hydrolysis of p-nitrophenyl hippurate, 2-phenyloxazolin-5-one (V) is formed as an intermediate, the breakdown of which is rate-determining at alkaline pH values; this intermediate is not formed to any appreciable extent during the hydroxide-ion-catalysed hydrolysis of methyl hippurate.[81] (V) has, however, recently been found to be a good substrate for papain, probably forming an acyl-enzyme intermediate during its reaction.[82] It appears reasonable to assume that the acyl-thiol intermediate is universal in papain catalysis, regardless of the lability of the substrate. Whether its



(V)

formation is a part of the direct hydrolytic pathway, or whether it has no active role apart from existing in equilibrium with a more reactive, as yet undetected intermediate - probably an acyl-imidazole in a small concentration - can be decided by studying the pH dependence and the types of catalysis in the acylation and the deacylation steps.

Lowe and Williams[57] proposed that in the deacylation step the acyl moiety is transferred from the active cysteine residue to an imidazole group of a histidine residue through rate-determining nucleophilic attack. The N-acyl intermediate is then hydrolysed rapidly in a kinetically unobservable step. Results in support of this mechanism were further obtained by Lake and Lowe[83] who reported that $k_0$ for p-nitrophenol production in papain- and ficin-catalysed hydrolyses of p-nitrophenyl hippurate is independent of added methanol concentration up to 1.5M, while $k_0$ for hippuric acid production in hydrolyses of methyl hippurate decreases with increasing methanol concentration. The interpretation is that the hydrolytic and the methanolytic steps are fast, and follow a rate-determining step which is not affected by added methanol (see scheme B of Appendix 2). It should be noted that the

proposed mechanism demands both the formation of the S-acyl and of the N-acyl intermediates as necessary parts of the hydrolytic pathway.

Henry and Kirsch[84] observed an increase in $k_0$, when p-nitrophenol production was followed, for the papain-catalysed hydrolysis of p-nitrophenyl hippurate in the presence of added methanol and ethanol, and a decrease in $k_0$ for hippuric acid production when methyl hippurate was the substrate. The experiments of Brubacher and Bender[62] on trans-cinnamoyl-papain also revealed that its deacylation rate was enhanced in the presence of added nucleophiles. In view of the different results obtained by Lake and Lowe and by Henry and Kirsch, it appears that more conclusive experiments on specific substrates are needed with nucleophiles other than alcohols. If the hydrolysis of the thiolester intermediate is rate-determining as the results of Henry and Kirsch appear to show, it is probably subject to general base catalysis for two main reasons. First, large deuterium oxide kinetic isotope effects have been observed for deacylation of α-N-benzoyl-L-arginyl-papain (2.75),[54] and trans-cinnamoyl-papain (3.35).[62] Secondly, the rates of reactions

of added nucleophiles with trans-cinnamoyl-papain do not have any simple correlation with their basicity.[62] If there is no general base catalysis, the efficiency of nucleophiles of the same type should roughly reflect their basicity. It has been pointed out,[62,83] however, that neither of these criteria is free from criticism: large secondary isotope effects are possible, and the efficiency of nucleophiles depend on other factors than basicity, such as their strength of binding on the enzyme surface.

The deacylation rate ($k_d$) depends only slightly on the acyl moiety of a specific substrate. For esters of α-N-benzyloxycarbonyl-ε-N-formyl-L-lysine[27] $k_d$= 30 - 50 $sec^{-1}$. For BAEE and BAA[71] $k_d$> 10 $sec^{-1}$. For other α-N-acylamino acid derivatives, such as those of glycine, L-leucine, L-phenylalanine, L-tryptophan and L-tyrosine,[59] $k_d$ is typically 1 - 10 $sec^{-1}$. Trans-cinnamoyl-papain ($k_d$= 0.00368 $sec^{-1}$)[62] and α-N-tosylsarcosyl-papain[85] are among the most stable acyl-papains studied; they are formed, however, from non-specific substrates. The similar values of $k_d$ for specific substrates raise the question whether a conformational change of the enzyme could be rate-determining. $k_d$ values of substrates of the same series

are not identical within limits of experimental errors, and this has been interpreted by Henry and Kirsch[84] as possibly indicating that the rates of departure of the leaving groups are partially rate-determining, possibly being accompanied by a conformational change. The results of Lake and Lowe can be explained in another way by postulating a rate-determining conformational change of the thiolester intermediate. Little can be said at present concerning kinetic significance of conformational changes in papain owing to lack of data.

Deacylation of α-N-benzyloxycarbonyl-L-lysyl-,[27] benzoyl-L-arginyl-,[58] hippuryl-,[58] benzyloxycarbonyl-glycyl,[86] and trans-cinnamoyl-papain[62] are independent of pH in the alkaline region. The decrease in $k_0$ above pH 8.5 for α-N-benzyloxycarbonyl-L-lysine esters have been attributed to the effect of $k_{+2}$. The observed values of $pK_2$ of acylation and its heat of ionisation, and its absence in deacylation, provide strong evidence that it represents deprotonation of the active-site thiol or the thiol-imidazole system. Consequently, the thiolester intermediate must have an active role in the hydrolytic pathway, and is not merely formed in equilibrium with a more reactive intermediate.

Unlike $pK_1$ for acylation, that for deacylation is not constant but varies according to the nature of the substrate, from 3.5 for α-N-benzyloxycarbonyl-L-lysine esters[27] to 4.7 for trans-cinnamoylimidazole.[62] The identity of the prototropic group, presumably the same as that involved in the acylation step but now not possibly interacting with the active-site cysteine, is again uncertain. Studies on thiolester hydrolyses as models for the deacylation step showed that carboxylate is a much poorer nucleophilic catalyst than imidazole.[87] The intramolecular-catalysed hydrolyses of S-hippurylthioglycollic acid and S-ethyl monothiolsuccinate at neutral pH are slower than deacylation of hippuryl-papain by a factor of about $10^5$, the difference being largely due to less favourable energies of activation for the former. In contrast, the energies of activation of intermolecular imidazole-catalysed hydrolyses of the two model systems are very similar to that for deacylation of hippuryl-papain.

C. The essential prototropic groups. While it is almost certain that the unionised, possibly hydrogen-bonded active-site thiol is essential for catalysis, and its ionisation constant is $pK_2$, the identity of the $pK_1$ group

is still a subject of considerable controversy. Evidence for and against assigning it to a carboxylate anion, and an imidazole group is summarised below.

The main evidence for participation of a carboxylate anion is that the $pK_1$ values of approximately 4 are typical of such a functional group. The observation that $pK_1$ values of $(k_0/K_m)$ for BAEE, α-N-benzyloxycarbonyl-L-histidinamide and hippurylamide are virtually independent of temperature has been interpreted[1c] as another supporting evidence, carboxyl groups normally having small heats of ionisation (± 1.5 kcal $M^{-1}$).[79] In view of the fact that $pK_1$ of $k_0$ for BAEE varies with temperature (3.5 at 25° and 3.7 at 37°)[1c] arguments from observed effects of temperature on $pK_1$ must be regarded with caution. Evidence against identification of the $pK_1$ group as a carboxylate ion includes the observations that 1) no carboxyl groups are within 5 Å of the active-site thiol in crystalline papain, which has been shown to be catalytically active,[88] and 2) a carboxylate ion is a poor catalyst, being approximately 5000-fold less effective than imidazole as a nucleophile and 100-fold less effective as a general base catalyst.[27]

Evidence suggesting that the $pK_1$ group is an imidazole is as follows. 1) Its presence within 4 Å of the active-site thiol suggests a possible catalytic function. 2) Bateman[89] observed that at 35° in 20% dioxan ($\varepsilon$=60) $pK_1$ of ($k_0/K_m$) for methyl hippurate is 4.3, the same as in water ($\varepsilon$=75), and Allen[90] observed that with a similar change of solvent and decrease in dielectric constant $pK_1$ for deacylation of <u>trans</u>-cinnamoyl-, acetylglycyl- and acetyl-L-tryptophanyl-papain are decreased by 0.6, 0.3 and 0.5 units respectively. These results are consistent with the interpretation that the protonated $pK_1$ group is a cationic species; the $pK_a$ of a neutral acid is expected to increase with decreasing dielectric constant. For a model compound 4-bromoimidazole at 22° $pK_a$ is decreased from 3.8 to 3.4, and for hippuric acid at 35° $pK_a$ is increased from 3.7 to 4.1 with a similar decrease in dielectric constant of the medium.[89]

Objections against assignment of the $pK_1$ group to an imidazole are as follows. 1) An imidazole usually has a $pK_a$ of 5.6 - 7.0, which is sensitive to temperature ($\Delta H$ = 6.9 - 7.5 kcal $M^{-1}$). Hydrogen bonding could depress $pK_a$, as the imidazole-serine hydroxyl hydrogen bonding[91]

probably does by about 0.5 units in α-chymotrypsin, but this would only be possible in the acylation step. Since the environment around the active site may be very different from normal aqueous environment it is conceivable however that the active-site imidazole is 'buried' and therefore has anomalous $pK_a$. 2) Studies on quenching of papain fluorescence[92] indicate dependence of quenching on protonation of a group with $pK_a$ 6.6. Papain fluorescence is presumably due to tryptophan residues and may therefore depend on interactions between the active-site imidazole and the active-site indole. It is not known, however, that the group with $pK_a$ 6.6 is the active-site imidazole or another group not involved in active-site interactions. None of these objections are therefore very serious, and it appears reasonable to identify the $pK_1$ group with the active-site thiol-imidazole system.

## The Object of this Thesis

The work described in this thesis concerns the binding, acylation and deacylation steps in papain-catalysed hydrolyses, which it is hoped will provide further insight into the mechanism of action of this proteolytic enzyme.

# CHAPTER 2

## INVESTIGATION OF BINDING AND KINETIC SPECIFICITY

Quantitative results have been obtained on the binding and subsequent kinetic steps for various substrates with the aim of studying the specificity of the $\rho_1$, $\rho_2$ and $\rho_3$ areas. By varying the acyl group in a series of N-acylglycine esters and anilides the nature of the $\rho_1$ area was studied. The importance of the amide linkage in the $\rho_1$ area, next to the susceptible bond, was tested by modification to analogous linkages. Specificity of the $\rho_2$ area was studied by comparing the activities of p-nitroanilides of hippuric acid and α-N-benzoyl-L-arginine. Specificity of the $\rho_3$ area is discussed from studies with some anilides, amides and thioamides as substrates and nitriles as inhibitors.

### Specificity of the $\rho_1$ Area

Schechter and Berger[49,50] showed clearly that specificity for peptide substrates includes at least seven residues, four on the carbonyl and three on the amino moiety of the peptide bond being cleaved. The

first four subsites $S_1$ - $S_4$, excluding an area in $S_1$ interacting with the side chain of the substrate residue $P_1$, might be regarded as the $\rho_1$ region (see p. 18). The advantageous effect of having an L-phenylalanyl residue at the $S_2$ subsite is here confirmed. Tables 2-1 and 2-2 give the kinetic parameters for esters and anilides of some α-N-acylglycine derivatives.

Since for the esters deacylation is rate-determining it is not possible to separate the parameters for binding and acylation. $(k_0/K_m)$ $(= k_2/K_s)$, a measure of both combined, increases in magnitude in the order α-N-acetyl-<benzoyl-<benzyloxycarbonyl-<acetyl-L-phenylalanyl-glycine esters. The same order of activity was found for p-nitroanilides, the differences between them being more pronounced. Lowe[1d] has pointed out the similarity in structure and hydrophobicity of the last three moieties. ( Fig. 2-1). The fact that acetyl-L-phenylalanylglycine (APG) esters and p-nitroanilide possess the greatest substrate activity appears to indicate that hydrophobicity is not the sole requirement. The polar bonds of the peptide linkages of the substrate, which interact with the $\rho_1$ area of the enzyme, must play a part in binding

## Table 2-1

Papain-catalysed hydrolyses of some α-N-acylglycine esters.

pH 6.0; 35°; μ 0.3M; $10^{-3}$M EDTA (ethylenediamine tetraacetate); p-thiocresol-activated papain.

| Substrate | Organic solvent | $k_0$ $sec^{-1}$ | $K_m$ mM | $k_0/K_m$ ($M^{-1}sec^{-1}$) |
|---|---|---|---|---|
| p-Nitrophenyl N-acetylglycinate | 2% DMF[a] | 2.04 ±0.07 | 0.68 ±0.02 | 3,000 ±200 |
| Methyl hippurate[b] | None | 2.72 ±0.06 | 20.5 ±2 | 133 ±3 |
| p-Nitrophenyl hippurate | 1.9% acetonitrile | 2.60 ±0.07 | 0.0117 ±0.0003 | 222,000 ±13,000 |
| Methyl N-benzyloxycarbonylglycinate[c] | None | 3.68 ±0.05 | 8.4 ±0.1 | 440 ±7 |
| p-Nitrophenyl N-benzyloxycarbonylglycinate | 1.9% ethanol | 5.71 ±0.17 | 0.0077 ±0.0004 | 743,000 ±20,000 |
| Methyl acetyl-L-phenylalanylglycinate | None | 5.43 ±0.18 | 0.032 ±0.001 | 170,000 ±15,000 |
| p-Nitrophenyl acetyl-L-phenylalanylglycinate | 2% DMF | 6.60 ±0.05 | $3.90\times10^{-4}$ $\pm0.07\times10^{-4}$ | $1.7\times10^{7}$ $\pm0.2\times10^{7}$ |

(a) DMF=dimethylformamide

(b) Ref. 57.

(c) pH 7.0

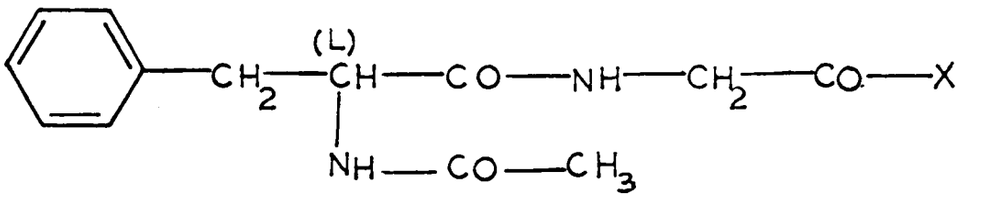
Table 2-2

Papain-catalysed hydrolyses of some α-N-acylglycine p-nitroanilides. Unless otherwise stated, the conditions are: pH 6.0, 35°, μ0.05M, $10^{-3}$M EDTA; L-cysteine (0.01M) activated papain.

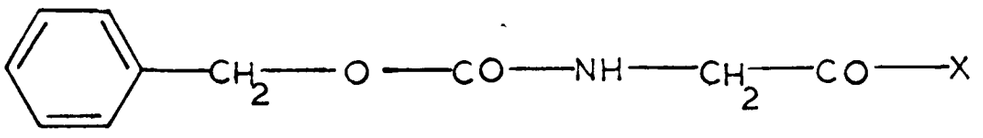
| Substrate | Organic solvent | $k_0$ $sec^{-1}$ | $K_m$ mM | $k_0/K_m$ $M^{-1}sec^{-1}$ |
|---|---|---|---|---|
| Acetylglycine p-nitroanilide | 20% DMF | $>5x10^{-7}$ | >5 | $8.7x10^{-5}$ $\pm1.0x10^{-5}$ |
| Hippuryl p-nitroanilide | 20% DMF | $2.6x10^{-3}$ $\pm1.9x10^{-3}$ | 7.2 ±5.1 | 0.36 ±0.03 |
| Acetyl-L-phenylalanyl glycyl p-nitroanilide [a] | 10% DMF | 1.30[b] ±0.18 | 0.88[b] ±0.13 | 1,500 ±200 |

(a) μ 0.3M

(b) assuming $k_d$= 6.6 $sec^{-1}$ (see Chapter 3), it can be calculated that $k_{+2}$= 1.6 $sec^{-1}$ and $K_s$= 1.07mM. $k_0$ and $K_m$ for the other two substrates are $k_{+2}$ and $K_s$ respectively.

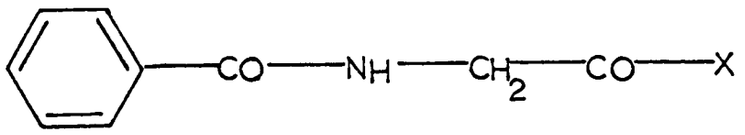


Acetyl-L-phenylalanylglycyl,



benzyloxycarbonylglycyl,

Fig. 2-1



and hippuryl derivatives.

and subsequent steps as well.

A study of anilide substrates is illustrative, the binding and acylation steps being separable since the latter are wholly or partially rate-determining. Upon changing the acyl moiety from hippuryl to APG, binding is improved ($K_s$ decreased) by only about 4 - 7 times, whereas the acylation rates become approximately 1000-fold faster (see Tables 3-1 and 3-2 in Chapter 3). These results show that difference in specificity for the two groups of substrates originates mainly from the acylation step and not from binding.

The effect of modification of the substrate structure at the amide linkage in the $\rho_1$ area next to the susceptible bond has been studied. Analogues of hippuric esters

show decreasing substrate activity, as measured by ($k_0/K_m$), in the order -CO-NH-> -CS-NH-> -CO-$CH_2$-> -CO-O-> -CO-S-> -$CH_2$-$NH_2^+$-, -$CH_2$-$CH_2$- (Table 2-3). The experimental conditions were not identical for all the substrates, since in some cases low ionic strengths and high concentrations of organic solvents were required in order to increase substrate solubility. Data from Lowe and Williams[65] indicated that in aqueous 30% acetone ($k_0/K_m$) for methyl hippurate was decreased by about six times from the value in pure water. Effects of organic solvents having been allowed for, data from Table 2-3 still show that various analogues have significantly different substrate properties. Differences in catalytic activities may partly stem from the inherent chemical nature of the acyl portions of the substrates. In order to estimate the latter, the rates of hydroxide-ion-catalysed hydrolyses ($k_{OH^-}$) of some p-nitrophenyl esters were determined (Table 2-4). Since the mechanism of such hydrolysis of p-nitrophenyl hippurate is anomalous in involving rate-determining breakdown of an oxazolone intermediate[81], $k_{OH^-}$ for p-nitrophenyl α-N-benzyloxycarbonylglycinate is used instead as a rough measure of inherent chemical activity of the hippuryl moiety. It is clear that variation of ($k_0/K_m$) is not

Table 2-3

Papain-catalysed hydrolyses of analogues of hippuric esters.

Unless otherwise stated, the conditions are as in Table 2-1.

| Substrate | Organic solvent | $k_o$ $sec^{-1}$ | $K_m$ mM | $k_o/K_m$ $M^{-1}sec^{-1}$ |
|---|---|---|---|---|
| Methyl hippurate[a] | None | 2.72 ±0.06 | 20.5 ±2 | 133 ±3 |
| p-Nitrophenyl hippurate | 1.9% acetonitrile | 2.60 ±0.07 | 0.0117 ±0.0003 | 222,000 ±13,000 |
| Methyl thiobenzoylglycinate[b] | 2.5% acetone | 1.43 ±0.31 | 32.7 ±7.1 | 43.8 ±2.1 |
| p-Nitrophenyl 3-benzoylpropionate[b] | 10% DMF | 2.38 ±0.54 | 0.531 ±0.120 | 4,500 ±220 |
| Methyl O-benzoylglycollate | 3.6% acetonitrile | - | - | 12.2 |
| p-Nitrophenyl O-benzoylglycollate[b] | 25% acetone | 1.99 ±0.19 | 1.43 ±0.14 | 1390 ±20 |
| p-Nitrophenyl S-benzoyl-2-thiolacetate[c] | 25% acetone | - | - | 570 |
| Methyl benzylglycinate | None | - | - | Not detectable |
| p-Nitrophenyl 4-phenylbutyrate[d] | 10% DMF | - | - | 7 |

(a) ref. 57

(b) $\mu$ 0.05M

(c) $\mu$ 0.1M

(d) pH 6.3

Table 2-4

Hydroxide-ion-catalysed hydrolyses of p-nitrophenyl esters of hippuric acid analogues at pH 6 - 8.5, 35°, 0.1 - 0.3M ionic strength (phosphate-citrate or borate buffers).

| Ester | Organic solvent | $k_{OH^-}$ $M^{-1}sec^{-1}$ |
|---|---|---|
| p-Nitrophenyl O-benzoyl-glycollate | 25% acetone | 900 |
| p-Nitrophenyl N-benzyloxy-carbonylglycinate | 2.3% ethanol | 330[a] |
| p-Nitrophenyl 3-benzoyl-propionate | 10% DMF | 165 |
| p-Nitrophenyl 4-phenyl-butyrate | 10% DMF | 77 |

(a) At 25° and with different buffers, a value of 115 $M^{-1}sec^{-1}$ was reported in ref. 69, while a value of 510 $M^{-1}sec^{-1}$ was reported in ref. 93. The difference may spring from specific buffer catalysis, which was shown to be absent in ref. 69.

entirely determined by inherent chemical activity of the acyl moieties. Interactions of the $\rho_1$ site of the enzyme with the acylamino or analogous groups must also be important for catalytic activity. It appears that the -NH- portion of the amide linkage next to the susceptible bond plays only a subsidiary, although not negligible, part in binding and catalysis, whereas the presence of a carbonyl or a thioamide function is necessary.

The result with the $-CH_2-NH_2^+-$ analogue presents a

problem for interpretation, since the positive charge may influence the binding constant by altering the partition equilibrium between the solvent and the enzyme active site. The poor activity of p-nitrophenyl 4-phenylbutyrate shows clearly that hydrophobic bonding between the enzyme and the substrate alone is not sufficient to produce efficient catalysis.

All the determined values of $k_0$ for analogues of hippuric esters are near 2 $sec^{-1}$. Presumably, as for the parent esters, deacylation is rate-determining, and it is probably little affected by modification of the amide linkage in the $\rho_1$ area. The anomalously slow deacylation rate for N-tosylsarcosyl-papain, reported by Wilson,[85] is possibly due to steric effects.

Kinetic data for analogues of hippuryl p-anisidide, for which acylation is rate-determining, are given in Table 2-5. Modification of -CO-NH- to -CO-O- and -CO-$CH_2$- are seen clearly not to affect binding but acylation. Hippuryl, O-benzoylglycollyl and 3-benzoylpropionyl p-anisidide all have very similar binding constants. The first two also have similar acylation rates, but the last

Table 2-5

Papain-catalysed hydrolyses of p-anisidides of some hippuric acid analogues at pH 6.0, 35°, $\mu$ 0.05M; phosphate-citrate buffer; 20% DMF; $10^{-3}$M EDTA; L-cysteine (0.01M) activated enzyme.

| p-Anisidide | $k_0$ $msec^{-1}$ | $K_m$ mM | $k_0/K_m$ $M^{-1}sec^{-1}$ |
|---|---|---|---|
| Hippuryl | 24.0 ± 6.6 | 15.9 ±4.3 | 1.51 ± 0.06 |
| O-Benzoylglycollyl | 38.5 ± 9.9 | 26.3 ±6.8 | 1.46 ± 0.30 |
| 3-Benzoylpropionyl | 0 | 19.5[a] | 0 |
| 4-Phenylbutyryl | 0 | - | 0 |

(a) From competitive inhibition of methyl hippurate hydrolysis (pH 6.0, 35°, $\mu$ 0.1M (sodium chloride), 20% DMF)

shows no activity at all, being merely a competitive inhibitor. The binding constant for this compound was obtained from measurement of the effect of its presence on papain-catalysed hydrolysis of methyl hippurate. Finally, the $-CH_2-CH_2-$ analogue, 4-phenylbutyryl p-anisidide, is neither a substrate nor a competitive inhibitor.

The importance of polar interactions of the α-acylamino group with the $\rho_1$ site should not be exaggerated. Lucas[94] found no simple relationship between inhibition constants of N-trihaloacetylethanolamines ($CX_3CONHCH_2CH_2OH$) and $pK_a$

values of the corresponding trihaloacetamides. It is likely that the strength of binding and efficiency of catalysis depend on both hydrophobic and polar interactions. Other factors may no doubt be involved.

## A Possible Role of Gln-19

Inspection of a model of papain and its substrates show that interactions in the $\rho_1$ area may include those at Gln-19. This residue is on the same side of the molecular cleft as Cys-25, and the γ-amide group lies at a distance of some 5 Å from the active thiol group. A carbonyl function of an amide or an analogous linkage of the substrate, next to the susceptible bond on the acyl side, can interact through hydrogen bonding with the amino portion of this γ-amide. The observation that such a carbonyl function is vital to binding lends support to this suggestion. The fact that methyl thiobenzoylglycinate is a good substrate is consistent with the interpretation, since a thioamide function can also form hydrogen bonding.[95] Until detailed studies by X-ray diffraction of papain-inhibitor complexes have been made, however, no definite conclusion can be drawn on the nature

of interactions at the $\rho_1$ site.

## Specificity of the $\rho_2$ area

New data confirm the known generalisation that the side chain of L-arginine in the $\rho_2$ site produces an accelerating effect on catalytic rates (Table 2-6). α-N-benzoyl-DL-arginine p-nitroanilide is more than 150 times as active a substrate as hippuryl p-nitroanilide, as measured by $k_0/K_m$. Insufficient solubility of the former prevents determination of individual constants, but it can be estimated that the acylation rate is >100 times that of the latter. Hydrolyses were performed at pH 6.0, 35° and $\mu$ 0.05 - 0.1M, with phosphate-citrate buffer.

Table 2-6

| p-Nitroanilide | Organic solvent | $k_0$ $sec^{-1}$ | $K_m$ mM | $k_0/K_m$ $M^{-1}sec^{-1}$ |
|---|---|---|---|---|
| α-N-Benzoyl-DL-arginine[a] | 1.6% acetonitrile | $\gg 0.3$ | $\gg 2$ | 150 |
| Hippuryl[b] | 20% DMF | $2.6\times10^{-3}$ $\pm 1.9\times10^{-3}$ | 7.2 $\pm 5.1$ | 0.37 $\pm 0.03$ |
| Hippuryl[c] | 8% acetonitrile | - | - | 0.66 |

(a) $\mu$ 0.07M; p-thiocresol-activated enzyme. (b) $\mu$ 0.05M; L-cysteine (0.01M) activated enzyme. (c) $\mu$ 0.1M; p-thiocresol-activated enzyme.

## Specificity of the $\rho_3$ Area

Papain-catalysed hydrolyses include those of esters, thiolesters, thionesters, amides and anilides. When acylation is rate-determining, as is the case for some esters, amides and anilides, the binding constant is measured directly by $K_m$. Substrate constants for substituted anilides of hippuric acid are in the range of 2 - 60 mM (Table 3-1), and those of acetyl-L-phenylalanyl-glycine are 1 - 4 mM (Table 3-2).

Substrate constants for hippurylamide,[56] isopropyl[57] and n-butyl hippurate[57] have been found to be 202, 48 and 10.2 mM respectively. Bender and Brubacher[27] have calculated the values of substrate constants for some esters of α-N-benzyloxycarbonyl-L-lysine to be in the range of 1 - 10 mM. Therefore for a wide range of substrates the substrate constants are similar.

There is a linear correlation between log $(k_O/K_m)$ and σ of p-substituted phenyl hippurates,[57] between log $(k_O/K_m)$ and log $k_{OH^-}$ of the amide and esters of hippuric acid,[76] and between log $(1/K_m)$ and log $k_{OH^-}$ of

some N-benzyloxycarbonylglycine esters.[69] The results imply that derivatives of the same acylamino acid do not normally have widely different values for binding constants. That the $\rho_3$ site must have some stereo-selectivity, however, is shown by the fact that the L-form of amino acid derivatives are much better nucleophiles than the D-form.[27,49]

## Activity of Thioamides

Papain is found to be inactive towards thioamide bonds. Methoxycarbonyl-L-phenylalanylglycine thioamide is hydrolysed at a very slow rate in the presence of the enzyme ($k_0 \sim 10^{-5} sec^{-1}$), in spite of the known specificity for esters and anilides of the closely related acetyl-L-phenylalanylglycine (APG). The compound is, however, a good competitive inhibitor with an inhibition constant of 0.61mM (pH 6.0, 35°), which is in the same range as substrate constants for APG substituted anilides. The inhibition constant was determined from the effect on papain-catalysed hydrolysis of methyl hippurate (Table 2-7). Sinclair[96] has previously found that hippuryl thioamide is also not a substrate for papain, but a competitive inhibitor with an inhibition constant of

## Table 2-7

Competitive inhibition of papain-catalysed hydrolysis of methyl hippurate by methoxycarbonyl-L-phenylalanylglycine thioamide. pH 6.0; 35°; μ 0.3M (sodium chloride); 2% acetonitrile; $10^{-3}$M EDTA; p-thiocresol activated papain. 5 - 6 points were used for each Lineweaver-Burk plot. The inhibition constant $K_i$ was calculated from the equation $K_m(app)=K_m^0+(K_m^0/K_i)(i)$, where (i)=inhibitor concentration, and $K_m^0$ and $K_m(app)$ are the Michaelis constants in the absence and presence of (i) respectively. Calculated $K_i$=0.61mM

| (i)(mM) | $k_0(sec^{-1})$ | $K_m$(mM) | (i)(mM) | $k_0(sec^{-1})$ | $K_m$(mM) |
|---|---|---|---|---|---|
| 0 | 2.72 | 20.0 | 0.348 | 3.0 | 29.9 |
| 0.116 | 2.7 | 23.8 | 0.472 | 3.2 | 39.5 |
| 0.236 | 2.8 | 25.8 | 0.665 | 3.0 | 39.2 |

## Table 2-8

Competitive inhibition of papain-catalysed hydrolysis of methyl hippurate by hippuronitrile. pH 6.0; 35°; μ 0.3M; 2% acetonitrile; $10^{-3}$M EDTA; p-thiocresol-activated papain. A Lineweaver-Burk plot (4 points) for 0.6mM of the nitrile confirms that $k_0$ is unaffected by the inhibitor, and this is assumed for determination of $K_m$ at other inhibitor concentrations, for each of which 1 - 4 points were used. $K_i$(0.46mM) was calculated as described in Table 2-7.

| (i)(mM) | $K_m$(mM) | (i)(mM) | $K_m$(mM) |
|---|---|---|---|
| 0 | 20.0 | 0.955 | 58.8 |
| 0.358 | 34.5 | 1.195 | 72 |
| 0.598 | 47.6 | 2.39[a] | 125 |

(a) 4% acetonitrile.

2.3mM (pH6.0, 35°). It is notable that hippuryl thioamide is approximately 100 times more favourably bound than hippurylamide, a specific amide substrate.

One reason for poor activity of the thioamides may be their weaker basicity than the corresponding amides. At room temperature, $pK_a$ for acetamide is -0.7, while for thioacetamide, -1.7.[97] Since acylation of papain by amides and thioamides is likely to depend on electrophilic catalysis as is the case for anilides (see Chapter 3), it may be expected that thioamides are less reactive than amides on this ground alone. It is possible, however, that inactivity of the thioamides is due to the nature of specificity of the $\rho_3$ site.

A possible use resulting from inactivity of thioamide substrates is for X-ray studies of the nature of binding.

Competitive Inhibition by Nitriles

Williams has found that hippuronitrile is a good competitive inhibitor of papain at neutral and acidic pH.[98] The observation has been confirmed in this laboratory, and

Table 2-9

Competitive inhibition of papain-catalysed hydrolysis of methyl hippurate by methoxycarbonyl-L-phenylalanylaminoacetonitrile. Experimental conditions and procedure were the same as in Table 2-8. A Lineweaver-Burk plot (4 points) confirms that $k_0$ is not affected by the inhibitor (0.006mM), and this is assumed for determination of $K_m$ at all inhibitor concentrations, for each of which 1 point was used. $K_i$ (0.0018mM) was calculated as described in Table 2-7.

| (i)(mM) | $K_m$(mM) | (i)(mM) | $K_m$(mM) |
|---|---|---|---|
| 0 | 20.0 | 0.00634 | 93.5 |
| 0.00317 | 52.7 | 0.00793 | 109 |
| 0.00476 | 71.5 | | |

an inhibition constant of 0.46mM (pH 6.0, 35°) has been determined from the effect on papain-catalysed hydrolysis of methyl hippurate (Table 2-8). Methoxycarbonyl-L-phenylalanylaminoacetonitrile has also been found to be an excellent competitive inhibitor. The inhibition constant (0.0018mM at pH 6.0 and 35°; Table 2-9), determined from the effect on papain-catalysed hydrolysis of methyl hippurate, is smaller than that of any known competitive inhibitor for the enzyme at neutral pH. The binding is over 500 times more favourable than that of APG anilides (Table 3-2), although the anilide moieties may inherently be capable of more interactions with the enzyme than the nitrile group. Apparently a considerable amount of 'strain' has been introduced into the enzyme-anilide complexes, which is smaller or absent in the enzyme-nitrile complex.

It appears that the nitrile inhibitors are potentially useful for X-ray studies of the nature of binding. The enzyme-nitrile complexes are formed at neutral pH and are probably stable, unlike enzyme-acid inhibitor complexes which are formed in significant amounts only at acid pH, and exist in equilibrium with the corresponding acyl-enzymes[67].

Binding versus Kinetic Specificity

It follows from the discussion above that the magnitude of the substrate constant gives no reliable clue to prediction of substrate activity. Specificity for substrates of papain is more profitably measured in terms of rates than binding. ($k_o/K_m$) is a measure of both and is the criterion for overall specificity. In terms of individual steps, acylation appears to be a better guide than either binding or deacylation. A comparison of kinetic behaviour between two substrates may be of value. Acetyl-L-phenylalanylglycine p-chloroanilide has a 1,300-fold faster acylation rate than hippuryl p-chloroanilide, while the substrate constant for the former is only four times smaller (Tables 3-1, 3-2). The deacylation rate of acetyl-L-phenylalanylglycyl-papain is about twice that of hippuryl papain (Table 2-1).

# CHAPTER 3

## INVESTIGATION OF THE ACYLATION STEP

Steady-state kinetic studies have been made on papain-catalysed hydrolyses of substituted anilides of hippuric acid and acetyl-L-phenylalanylglycine (APG). In the first series of compounds, acylation is rate-determining. In the second series, acylation and deacylation rates are comparable in magnitude. The nature of the acylation step is discussed in the light of available results on the electronic effect of the anilide substituents, the Arrhenius parameters and pH dependence.

### A Scheme for the Mechanism of Acylation

A plausible mechanism of acylation of papain at Cys-25 involves formation of a tetrahedral intermediate:

$$R\text{-}CO\text{-}X + EH \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} (RCOX\ldots EH) \underset{k'_{-2}}{\overset{k'_{+2}}{\rightleftharpoons}} R\text{-}\overset{\overset{O^-}{|}}{\underset{\underset{EH_+}{|}}{C}}\text{-}X \xrightarrow{k''_{+2}} R\text{-}CO\text{-}E + XH \qquad (3\text{-}1)$$

The mechanism is in essence the same as that proposed by Bender[99] on non-enzymatic substitution at a carbonyl carbon. A steady-state treatment of scheme (3-1) gives

the overall initial acylation rate $k_{+2}$ as a composite constant,

$$k_{+2} = \frac{k''_{+2}}{\frac{k'_{-2} + k''_{+2}}{k'_{+2}} + 1}. \qquad (3\text{-}2)$$

Two limiting cases are possible. For substrates with good leaving groups, such as esters, it is proposed that $k''_{+2} \gg k'_{+2}$, $k'_{-2}$ and therefore

$$k_{+2} = k'_{+2}. \qquad (3\text{-}3)$$

For substrates with poor leaving groups, such as anilides and amides, it is proposed that $k''_{+2} \ll k'_{-2}$ and

$$k_{+2} = k''_{+2}.K/(K+1) \qquad (3\text{-}4)$$

where $K = k'_{+2}/k'_{-2}$, the equilibrium constant for formation of the tetrahedral intermediate from the Michaelis complex.

## Esters and Anilides

The rates of papain-catalysed hydrolyses depend partly on the chemical nature of the bond being hydrolysed. For anilides and amides, in which anilines and amines are relatively poor leaving groups, the rate-determining step is likely to be the acylation rate, although this is not true for some specific anilide substrates. A decrease in electron-attracting power of the leaving group tends to

reduce $k'_{+2}$ and increase $k''_{+2}/k'_{-2}$, both leading to a decrease in $k_{+2}$.[99] Although it is not possible to determine $k_{+2}$ for ester substrates, deacylation being rate-determining, the relative difference between $k_{+2}$ of alkyl and p-nitrophenyl esters is reflected in $K_m$ ($=K_s.k_d/k_{+2}$), which are larger for alkyl esters. $k_{+2}$ for alkyl esters are likely to be smaller because of smaller electron-attracting power compared to p-nitrophenyl esters.

Esters and anilides show difference in sensitivity, regarding acylation rates, to modification of the amide linkage in the acyl moiety nearest to the susceptible bond (see Tables 2-3 and 2-5). The difference is understandable if it is assumed that acylation rates for the former are $k'_{+2}$, while for the latter, $k''_{+2}.K/(K+1)$. Replacement of such an amide linkage by any other function affects electronic distribution in the susceptible bond, which in turn may affect $k'_{+2}$ and $k''_{+2}.K/(K+1)$ differently. Apart from an influence on the substrate molecule, such modification must also have an influence on enzyme-substrate interactions, which may again produce different results on $k'_{+2}$ and $k''_{+2}.K/(K+1)$.

## Electronic Requirements for Acylation

Lowe and Williams[57] found that acylation by p-substituted phenyl hippurates is subject to nucleophilic catalysis the Hammett plot of log ($k_o/K_m$) versus σ having a slope (ρ value) of +1.2. Since for thiolate-ion-catalysed hydrolysis of aryl acetates ρ is +1.7, it was concluded that in the enzymic acylation there is some acid catalysis as well as nucleophilic catalysis. In the investigation now reported, it has been found that acylation of papain by anilides is subject to electrophilic catalysis. A Hammett plot of log $k_{+2}$ versus σ for various p-substituted anilides of hippuric acid gives a ρ value of -1.2 (Table 3-1, Fig. 3-1). The anilides used for the plot were p-nitroanilide, p-bromoanilide, p-chloroanilide, the parent anilide, p-anisidide and p-hydroxyanilide. $k_o$ values at 35° are only 3 - 50 $msec^{-1}$ and hence can be safely assumed to represent acylation, since the deacylation constant for hippuryl-papain is approximately 3 $sec^{-1}$.

Another Hammett plot of the same slope is obtained from acylation rates for p- and m-substituted anilides of APG (Table 3-2, Fig. 3-2). The anilides used for the plot

Table 3-1

Papain-catalysed hydrolyses of hippuryl p-substituted anilides at pH 6.0, 35° and $\mu$ 0.05M; phosphate-citrate buffer; 20% DMF; $10^{-3}$M EDTA; L-cysteine (0.01M) activated enzyme; (E)=0.5-1x$10^{-4}$M; (S)=0.1-1x$10^{-2}$M; initial rate method. 6-10 points were used for a Lineweaver-Burk plot. Each value is the average of 2 - 3 determinations.

| Substrate | $k_0$ $msec^{-1}$ | $K_m$ mM | $k_0/K_m$ $M^{-1}sec^{-1}$ | $\sigma^a$ |
|---|---|---|---|---|
| Hippuryl | | | | |
| p-hydroxyanilide | 45.3 ± 28.2 | 57.1 ± 35.5 | 0.79 ± 0.05 | -0.37 |
| p-anisidide | 24.0 ± 6.6 | 15.9 ± 4.3 | 1.51 ± 0.06 | -0.27 |
| anilide | 15.1 | 19.4 | 0.77 ± 0.06 | 0 |
| p-chloroanilide | 6.7 | 6.5 | 1.03 | +0.23 |
| p-bromoanilide | 3.1 ± 1.5 | 1.8 ± 0.9 | 1.70 ± 0.15 | +0.23 |
| p-nitroanilide | 2.6 ± 1.9 | 7.2 ± 5.1 | 0.36 ± 0.03 | +0.78 |

(a) ref.100.

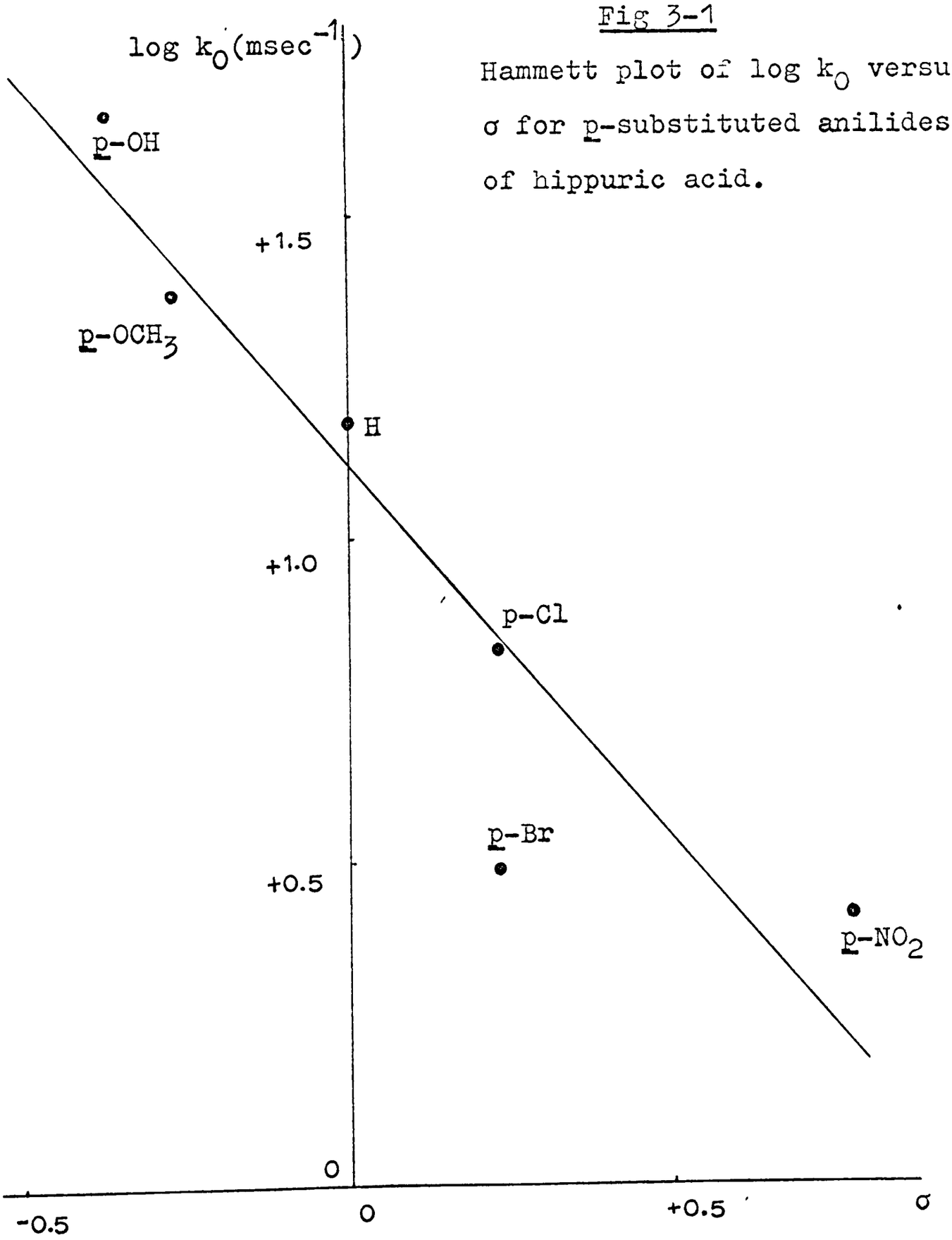



Fig 3-1

Hammett plot of log $k_0$ versus σ for p-substituted anilides of hippuric acid.

Table 3-2

Papain-catalysed hydrolyses of substituted anilides of acetyl-L-phenylalanylglycine at pH 6.0, 35° and μ 0.3M; phosphate-citrate buffer; 10% DMF; $10^{-3}$M EDTA; $5\times10^{-4}$M L-cysteine; (E)= $10^{-5}$M; (S)=0.1-1mM. The total progress method was used for all hydrolyses except that of the p-nitroanilide, for which the initial rate method was used. About 5 points were used for a Lineweaver-Burk plot. Each value is an average of 2-3 determinations.

| Substrate APG | $k_0$ $sec^{-1}$ | $K_m$ mM | $k_0/K_m$ $M^{-1}sec^{-1}$ |
|---|---|---|---|
| p-anisidide | 5.10 ± 0.26 | 0.96 ± 0.05 | 5,300 ± 100 |
| anilide | 4.32 ± 0.43 | 1.55 ± 0.16 | 2,800 ± 200 |
| p-chloroanilide | 3.72 ± 1 | 0.78 ± 0.2 | 4,800 ± 200 |
| sulphanilamide | 2.44 ± 0.4 | 0.79 ± 0.1 | 3,100 ± 300 |
| m-nitroanilide | 1.64 ± 0.18 | 0.78 ± 0.08 | 2,100 ± 100 |
| p-nitroanilide | 1.35 ± 0.18 | 1.00 ± 0.13 | 1,350 ± 200 |

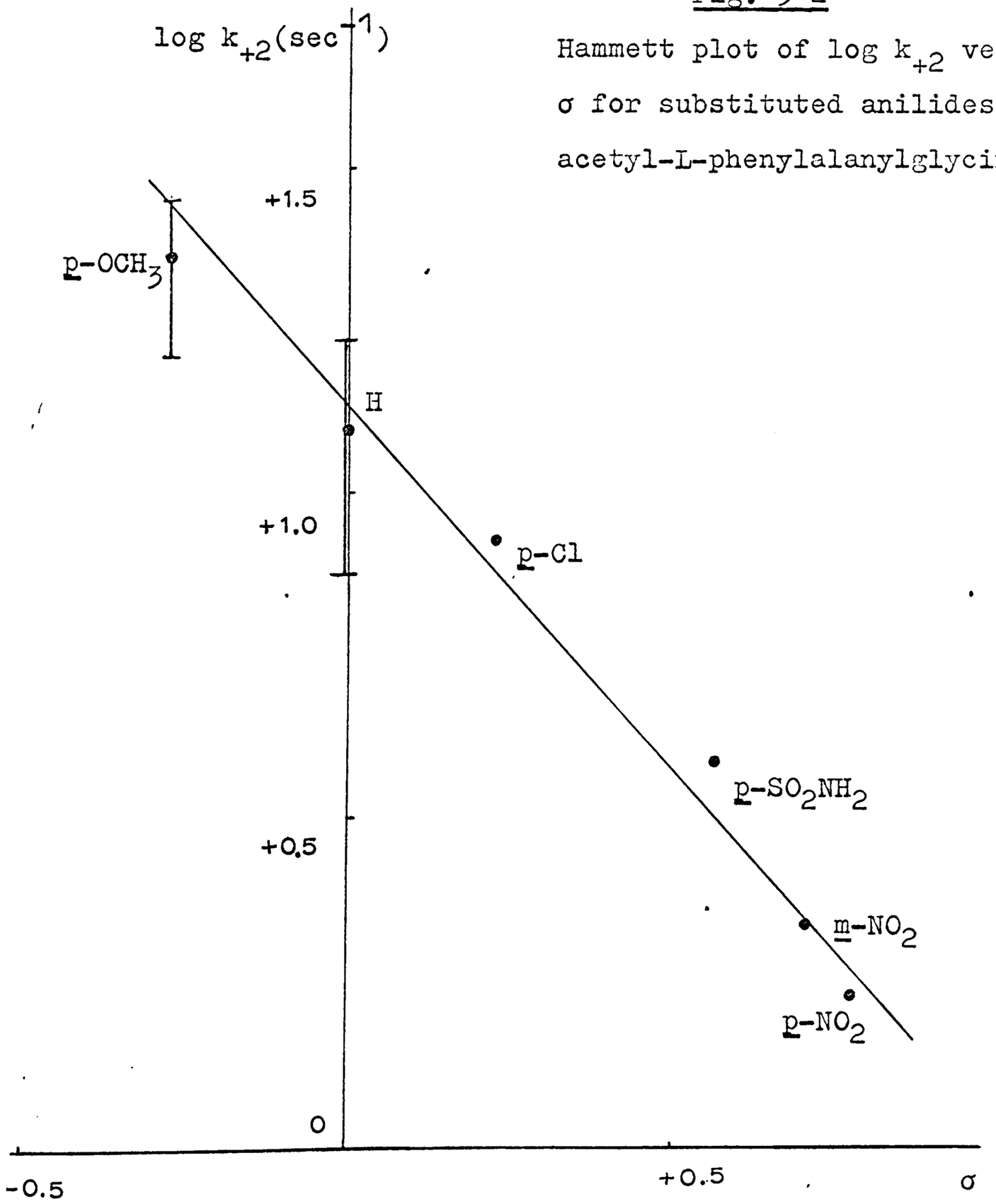
Calculation of acylation rates and binding constants for the anilides was made from the relationship $(1/k_0)=(1/k_{+2})+(1/k_d)$, assuming $k_d=6.6$ $sec^{-1}$, and the relationship $K_s=k_{+2}/(k_0/K_m)$.

| Substrate | $k_{+2}$ $sec^{-1}$ | $K_s$ mM | $\sigma$ a |
|---|---|---|---|
| p-Anisidide | 22.4 ± 6 | 4.2 | -0.27 |
| Anilide | 12.5 ± 5 | 4.3 | 0 |
| p-Chloroanilide | 8.5 | 1.78 | +0.23 |
| Sulphanilamide | 3.9 | 1.26 | +0.57 |
| m-Nitroanilide | 2.2 | 0.96 | +0.71 |
| p-Nitroanilide | 1.7 | 1.26 | +0.78 |

(a) ref. 100.

Fig. 3-2

Hammett plot of log $k_{+2}$ versus σ for substituted anilides of acetyl-L-phenylalanylglycine



were p-nitroanilide, m-nitroanilide, sulphanilamide, p-chloroanilide, the parent anilide and p-anisidide. Acylation rates for these anilides are nearly equal to or greater than the deacylation rate (the value for APG p-nitrophenyl ester is 6.6 sec at 35°), and were calculated from the relationship $k_0=k_{+2}k_d/(k_{+2}+k_d)$.

The electrophilic catalyst in the acylation step is probably the hydrogen-bonded thiol-imidazole system, which can donate the hydrogen-bonded proton to the leaving group while the thiolate anion attacks the carbonyl carbon of the substrate. In scheme (3-1) the nucleophilic attack from the thiolate of the hydrogen-bonded system occurs prior to electrophilic (general acid) catalysis (Fig. 3-3). The

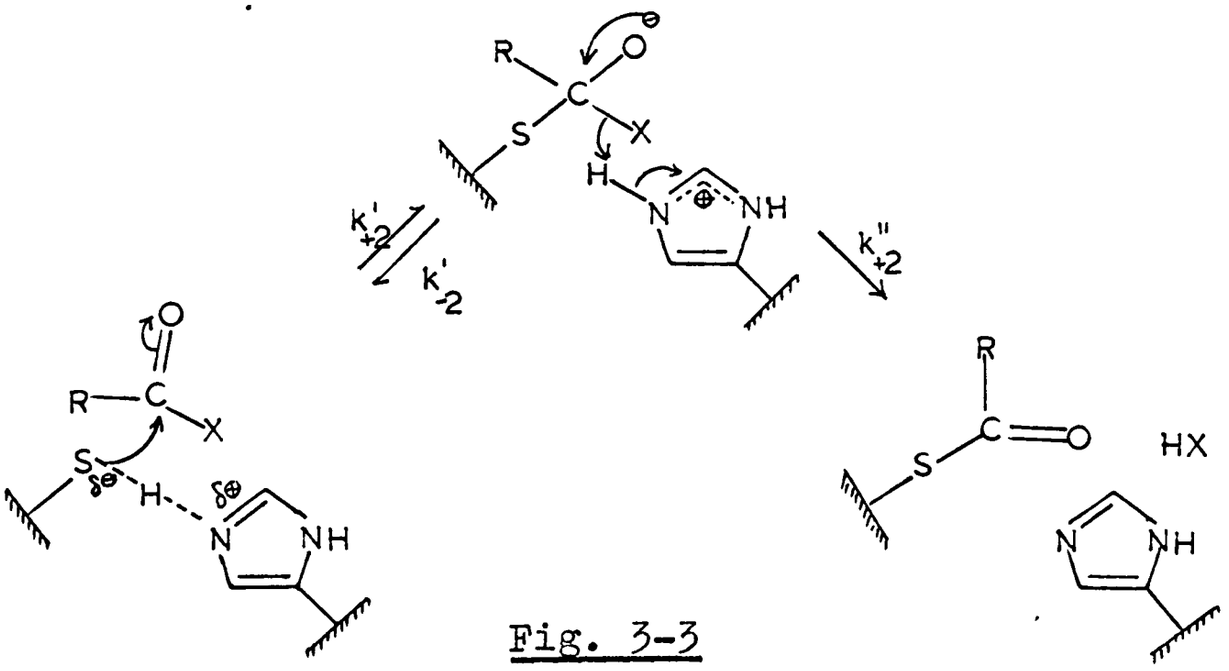



Fig. 3-3

possibility that the two processes occur simultaneously, or that pretransition-state protonation may occur (see below), is however not ruled out. If scheme (3-1) is valid, that is, protonation of the leaving group occurs in the $k''_{+2}$ step, then the fact that the two Hammett plots have the same slope although acylation rates for the two series of substrates differ by a factor of about 1000 confirms the suggestion that for both cases $k'_{-2} \gg k''_{+2}$ and $k_{+2} = k''_{+2} \cdot K/(K+1)$. Moreover, these results demand that the electronic effects on $k''_{+2}$ and K for both series are also similar. The electronic effects of a substituent on $k''_{+2}$ and K are in opposite directions. An electron-attracting substituent should facilitate formation of the tetrahedral intermediate and hence increase K, while $k''_{+2}$ which should depend on acid catalysis will be decreased. A negative ρ value indicates that the electronic effect on $k''_{+2}$ is stronger than on K.

## The Tetrahedral Intermediate

Bruice and Schmir[101] have pointed out that if an equilibrium K occurs prior to the rate-determining step in a steady-state enzyme reaction, this will shift the apparent $K_a$ of the latter to a higher value by a factor of (K+1).

Since $pK_1$ of the acylation step for APG p-nitroanilide is not significantly different from $pK_1$ of the free enzyme (see below), it is concluded that $K<1$. Also, if $K\geqslant 1$, in steady-state experiments where $(S)>(E)$ a substantial portion of the enzyme should exist as the tetrahedral intermediate. With hippuryl p-nitroanilide as substrate, an attempt to detect the tetrahedral intermediate has failed. (S) was approximately $3\times10^{-4}$M, (E) was $1.5\times10^{-4}$M. p-Nitroaniline has $\lambda_{max}$ at 380 mμ (log ε 4.0) in water.[102] The intermediate should have a similar ultraviolet absorption spectrum to p-nitroaniline, and from the known value of the Michaelis constant it can be calculated that the observed optical density at $\lambda_{max}$ should be approximately 0.1 if $K\gg1$. No new absorption region was observed immediately after the enzyme and the substrate had been mixed.

The conclusion that $K<1$ further simplifies the expression for acylation rates for anilides to eqn. (3-5)

$$k_{+2} = k''_{+2}.K. \qquad (3\text{-}5)$$

Arrhenius Parameters for Acylation by Anilides

The 1000-fold difference in the acylation rates of

Table 3-3

Effect of temperature on papain-catalysed hydrolysis of hippuryl p-chloroanilide. pH 6.0; $\mu$ 0.05M; phosphate-citrate buffer; 20% DMF; $10^{-3}$M EDTA; L-cysteine (0.01M) activated papain.

| Temperature (°C) | $10^3/T$ ($°K^{-1}$) | $k_0$ ($msec^{-1}$) | $K_m$ (mM) | $k_0/K_m$ ($M^{-1}sec^{-1}$) |
|---|---|---|---|---|
| 35 | 3.25 | 5.9 | 7.1 | 0.82 |
| 35 | 3.25 | 9.9 | 8.4 | 1.08±0.09 |
| 35 | 3.25 | 4.3 | 4.0 | 1.07±0.07 |
| 38 | 3.215 | 10.4 | 10.5 | 0.99±0.03 |
| 40 | 3.20 | 5.5 | 4.8 | 1.15±0.04 |
| 40 | 3.20 | 6.1 | 5.5 | 1.12±0.08 |
| 42 | 3.175 | 10.5 | 4.5 | 2.33±0.17 |
| 45 | 3.145 | 10.5 | 5.6 | 1.89±0.07 |
| 46 | 3.135 | 10.4 | 3.8 | 2.7 |
| 49 | 3.105 | 18.0 | 10.7 | 1.68±0.05 |
| 53 | 3.07 | 21.0 | 10.0 | 2.10±0.05 |
| 56 | 3.04 | 10.6 | 3.1 | 3.41 |

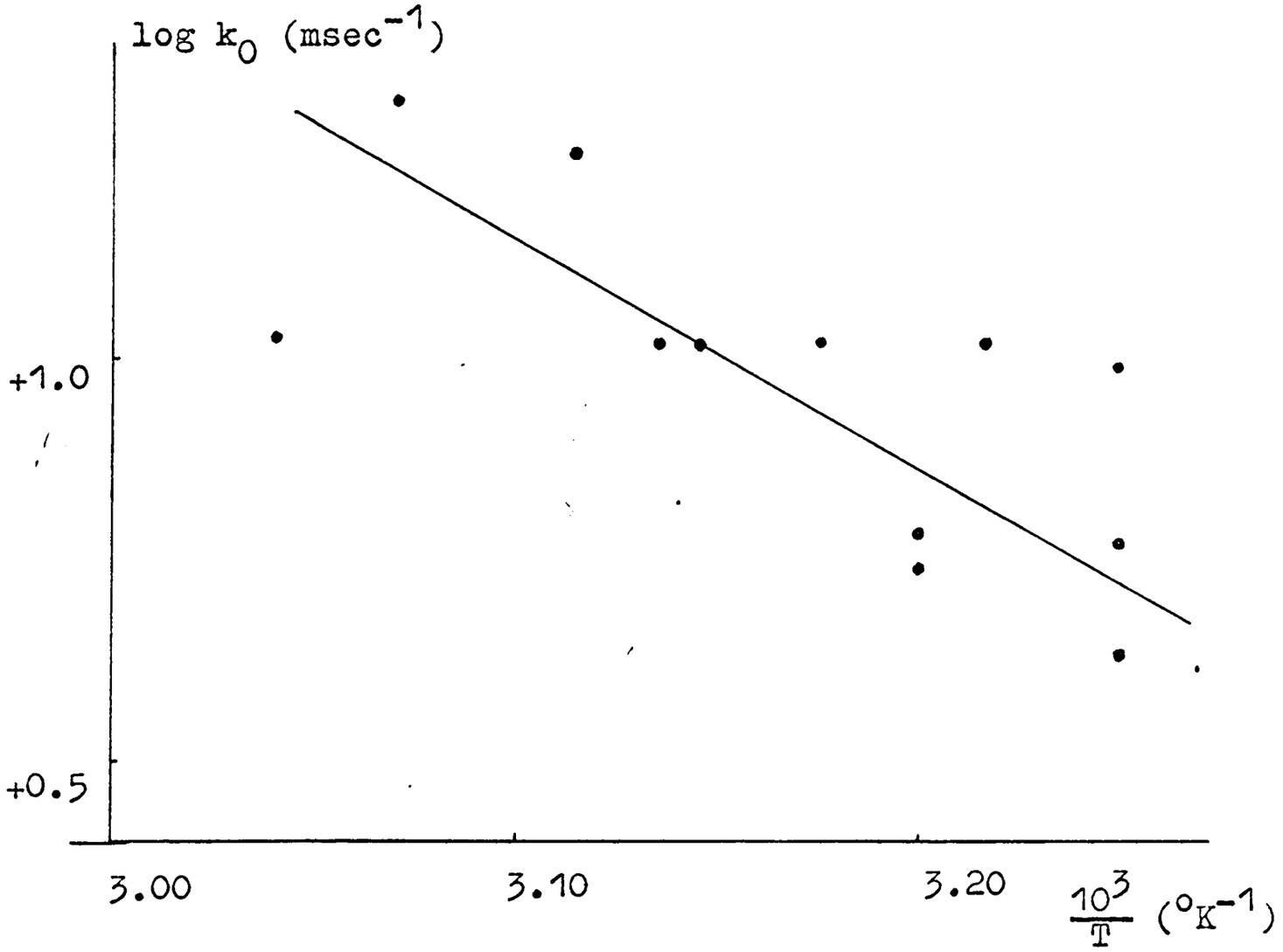



Fig. 3-4

Arrhenius plot of $k_0$ of hippuryl p-chloroanilide

Table 3-4

Effect of temperature on papain-catalysed hydrolysis of hippuryl p-bromoanilide. pH 6.0; $\mu$ 0.05M; phosphate-citrate buffer; 20% DMF; $10^{-3}$M EDTA; L-cysteine (0.01M) activated papain.

| Temperature(°C) | $10^3/T(°K^{-1})$ | $k_0(msec^{-1})$ | $K_m$(mM) | $k_0/K_m$ $(M^{-1}sec^{-1})$ |
|---|---|---|---|---|
| 35 | 3.25 | 3.09 | 1.82 | 1.70 |
| 39.5 | 3.20 | 3.8 | 2.2 | 1.76 |
| 44.5 | 3.15 | 5.9 | 2.7 | 2.18 |
| 50 | 3.10 | 8.3 | 2.8 | 2.95 |
| 55 | 3.05 | 9.2 | 2.4 | 3. 80 |

Fig. 3-5

Arrhenius plot of $k_0$ of hippuryl p-bromoanilide.

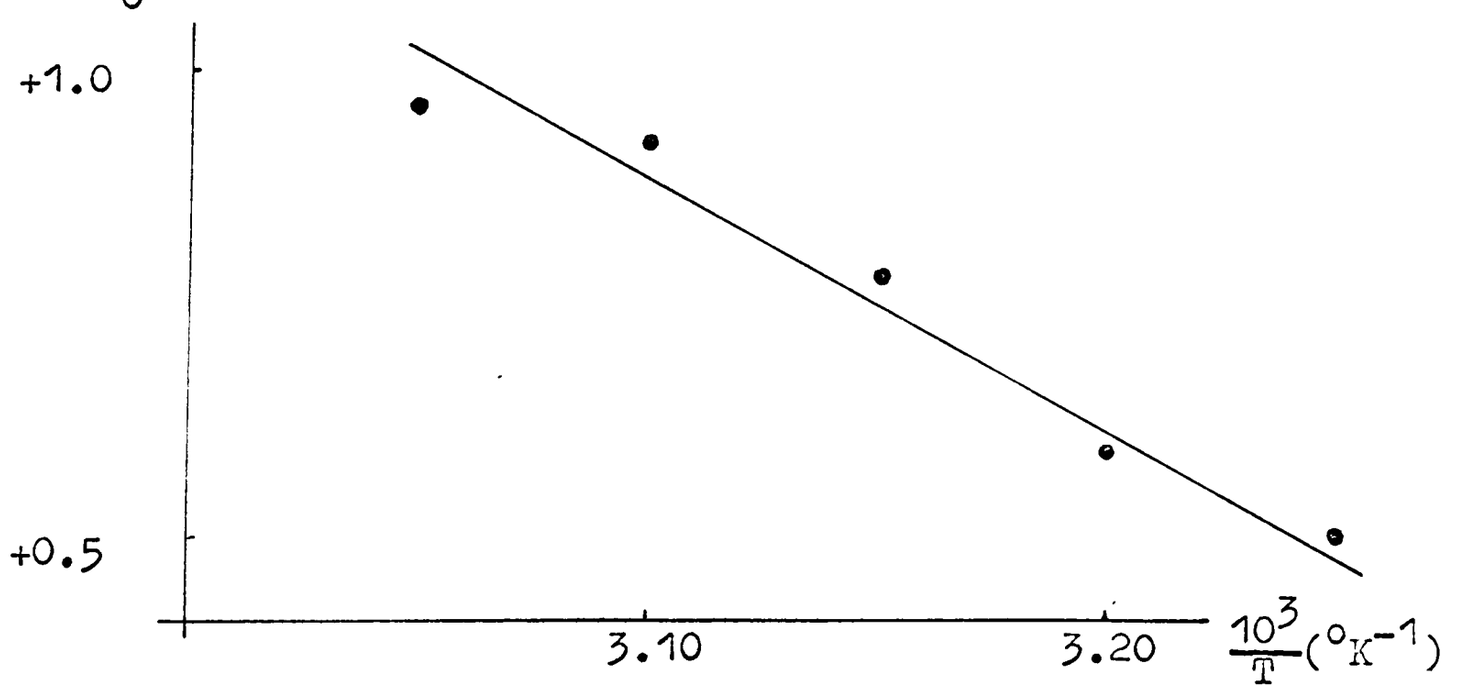


Table 3-5

Effect of temperature on papain-catalysed hydrolysis of acetyl-L-phenylalanylglycine p-chloroanilide. pH 6.0; u 0.3M; phosphate-citrate buffer; 10% DMF; $10^{-3}$M EDTA; $5 \times 10^{-4}$M L-cysteine.

| Temp. (°C) | $10^3/T$ ($°K^{-1}$) | $k_0$ ($sec^{-1}$) | $K_m$ (mM) | $k_0/K_m$ ($M^{-1}sec^{-1}$) | $k_{+2}$[a] ($sec^{-1}$) | $K_s$[b] (mM) |
|---|---|---|---|---|---|---|
| 29 | 3.31 | 2.99 | 0.83 | 3,600±100 | 7.8 | 2.17 |
| 35 | 3.25 | 3.69 | 0.79 | 4,700±200 | 8.5 | 1.81 |
| 40 | 3.20 | 4.90 | 0.89 | 5,500±200 | 11.7 | 2.13 |
| 41 | 3.185 | 6.13 | 1.12 | 5,500±300 | 18.8 | 3.42 |
| 45 | 3.145 | 6.55 | 1.02 | 6,400±200 | 15.8 | 2.47 |
| 50 | 3.10 | 8.12 | 1.04 | 7,800±200 | 19.3 | 2.47 |
| 54 | 3.06 | 10.64 | 1.31 | 8,100±200 | 27.9 | 3.45 |

(a) Calculated from the relationship $(1/k_0) = (1/k_{+2}) + (1/k_d)$, assuming $k_d$ values from the Arrhenius plot for p-nitrophenyl acetyl-L-phenylalanylglycinate (Table 4-3, p. 93)

(b) Calculated from the relationship $K_s = k_{+2}/(k_0/K_m)$.

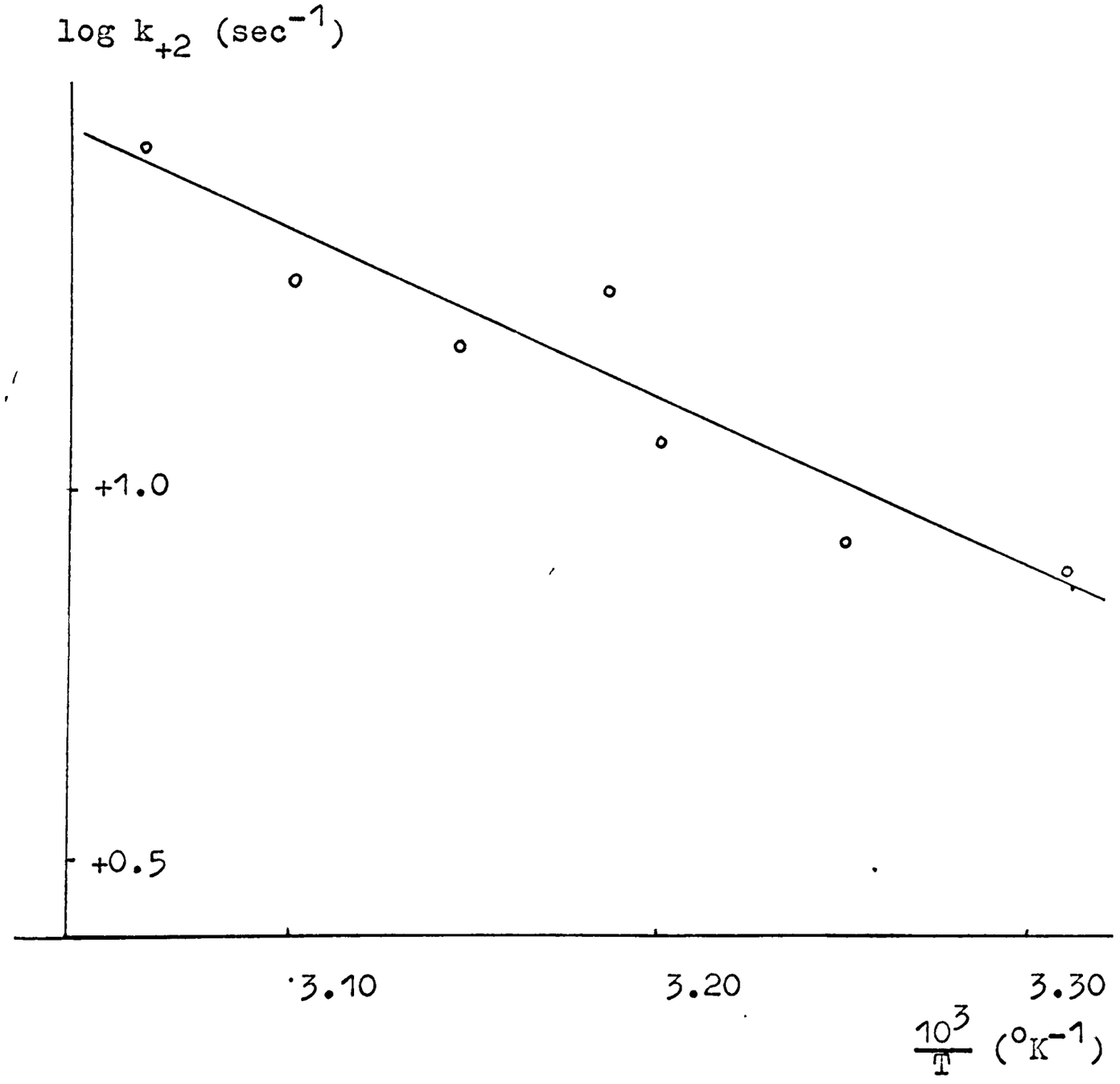



Fig. 3-6

Arrhenius plot of $k_{+2}$ of acetyl-L-phenylalanylglycine p-chloroanilide

the two series of anilides, corresponding to difference in free energies of activation of about 4.5 kcal$M^{-1}$, can be due to difference in either entropy or enthalpy of activation, or both. Activation energies for $k_{+2}$ of hippuryl p-chloroanilide, hippuryl p-bromoanilide and APG p-chloroanilide have been determined by studying the effect of variation in temperature between 30° and 55° (Tables 3-3, 3-4, 3-5, Fig. 3-4, 3-5, 3-6). The points on the Arrhenius plot for hippuryl p-chloroanilide are rather scattered since the accuracy of experimental $k_{+2}$ values ~~were~~ was limited by poor substrate solubility. Table 3-6 gives the values of Arrhenius parameters for the three substrates. It may be noted that the difference in acylation rates for the two series appear to stem from both entropy and enthalpy of activation.

Table 3-6

Rate constants and Arrhenius parameters for the acylation step of some anilides. $\Delta S^{\ddagger}$ was calculated from the equation $k_{+2}=(kT/h)\ \exp.(-(\Delta H^{\ddagger}-\Delta S^{\ddagger}T)/RT)$, where $kT/h=10^{12.81}$ at 35°.

| Substrate | $k_{+2}$ $sec^{-1}$ (35°) | $E_a$ kcal$M^{-1}$ | $\Delta S^{\ddagger}$ e.u.$M^{-1}$ |
|---|---|---|---|
| Hippuryl p-chloroanilide | $6.7\times10^{-3}$ | +13 | -30 |
| Hippuryl p-bromoanilide | $3.1\times10^{-3}$ | +12.4 | -33 |
| APG p-chloroanilide | 8.5 | +10.8 | -22 |

pH Dependence of $(k_0/K_m)$ and Acylation

The pH profile of $(k_0/K_m)$ should reveal the $pK_a$ values of prototropic groups of the free enzyme responsible for the subsequent rate step,[77,78] while the pH profile of $k_{+2}$ should yield similar information on these groups after the binding of the substrate has occurred. Various authors[1c,27,53,58,88] have reported the values of $pK_1$ of 4.0 - 4.4, and $pK_2$ of 8.0 - 8.5 for $(k_0/K_m)$ of neutral or positively charged substrates, the states of ionisation of which are not changed in the pH range studied. The results now reported for neutral substrates of various activities agree with the earlier results. The values of $pK_1$ of $(k_0/K_m)$ have been found for p-nitrophenyl esters of N-acetylglycine (Fig. 3-7), hippuric acid (Fig. 3-8), N-benzyloxycarbonylglycine (Fig. 3-9), α-N-acetyl-DL-tryptophan (Fig. 3-10; in a separate experiment it was confirmed that only the L-enantiomer was the substrate), and 3-benzoylpropionic acid (Table 3-7, Fig. 3-11).

Little information had so far been available on the pH dependence of $k_{+2}$ of neutral substrates, as distinct from that of $(k_0/K_m)$. $k_0$ for the positively charged

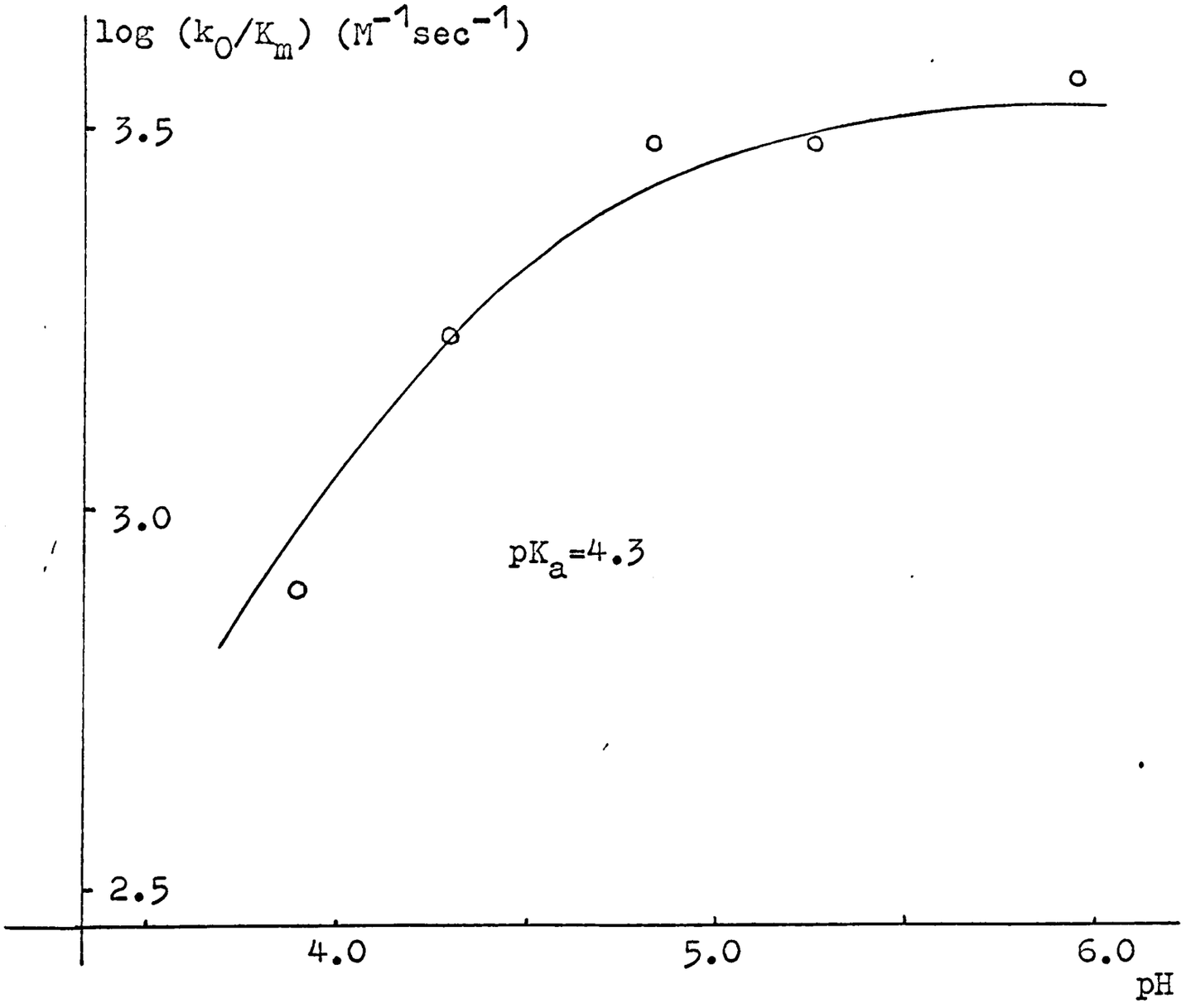



Fig. 3-7

pH dependence of ($k_o/K_m$) for p-nitrophenyl N-acetylglycinate; 40°;μ0.35M; phosphate-citrate buffer; 2%DMF; $10^{-3}$M EDTA; p-thiocresol-activated papain. For numerical values, see Table 4-17, p.110.

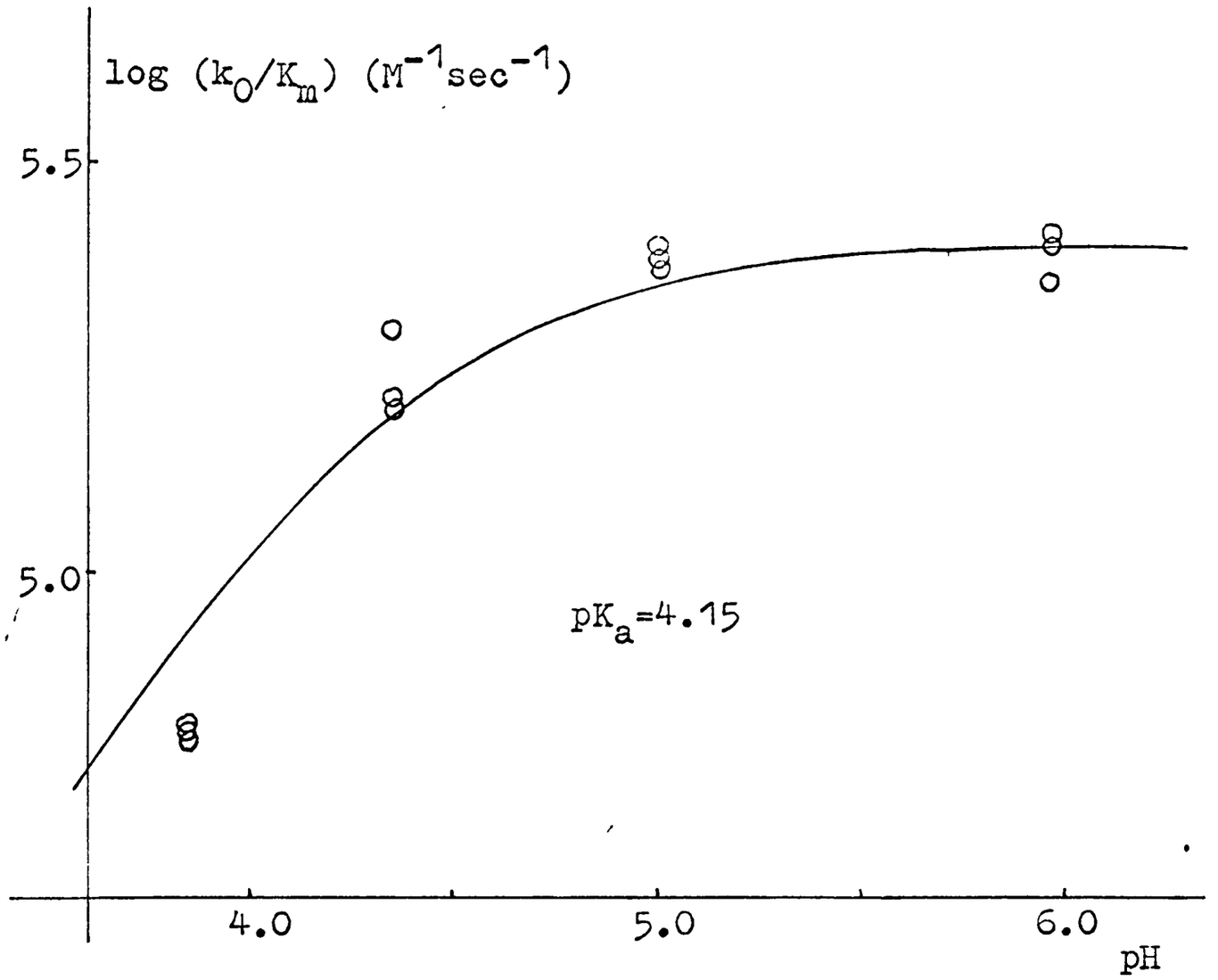



Fig. 3-8

pH dependence of ($k_0/K_m$) of p-nitrophenyl hippurate; 35°; $\mu$ 0.3M; phosphate-citrate buffer; 1.9% ethanol; $10^{-3}$M EDTA; p-thiocresol-activated papain. See Table 4-18, p.112.

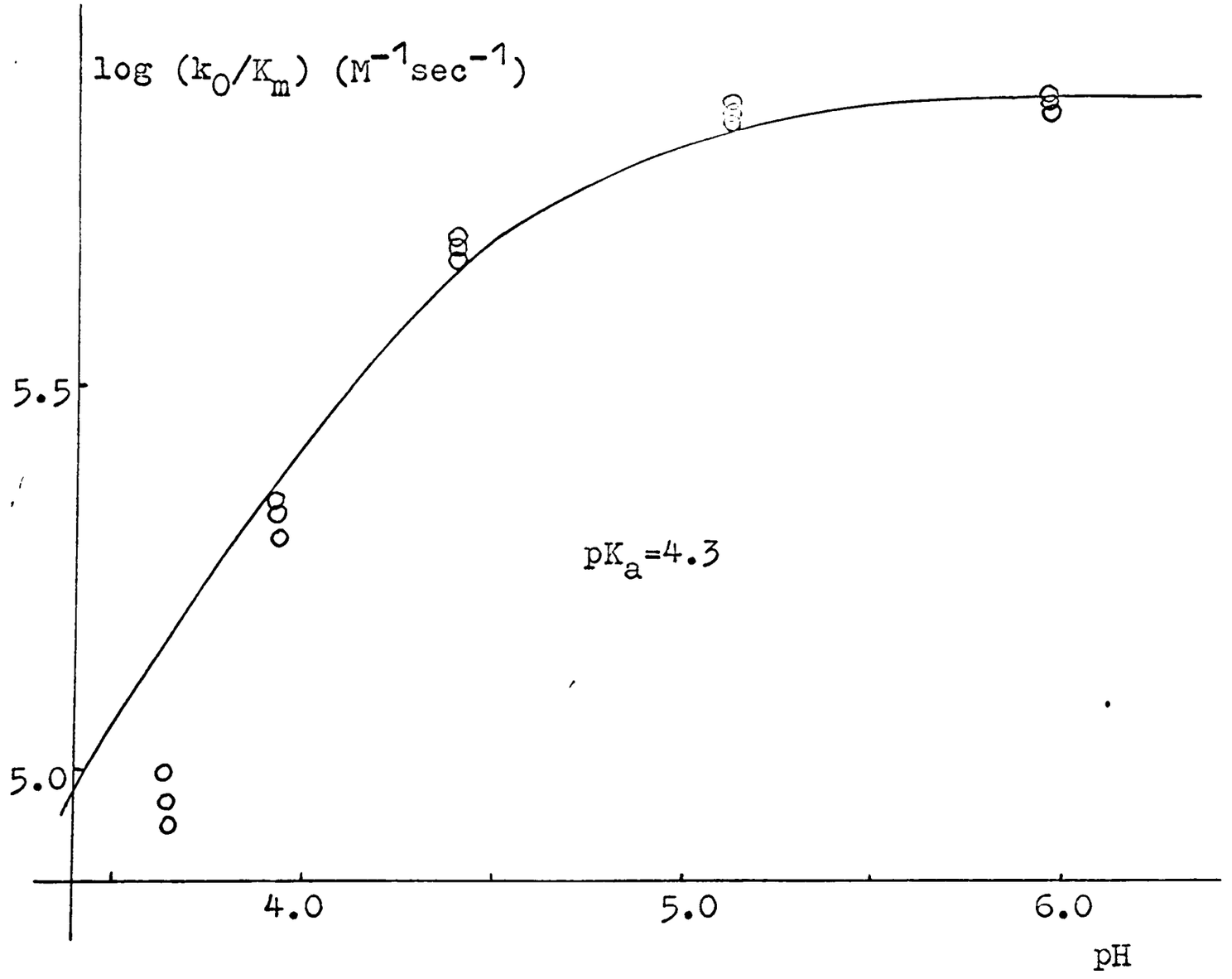



Fig. 3-9

pH dependence of ($k_0/K_m$) for p-nitrophenyl N-benzyloxycarbonyl-glycinate; 35°; μ 0.3M; phosphate-citrate buffer; 1.9% ethanol; p-thiocresol-activated enzyme. See Table 4-19, p. 114.

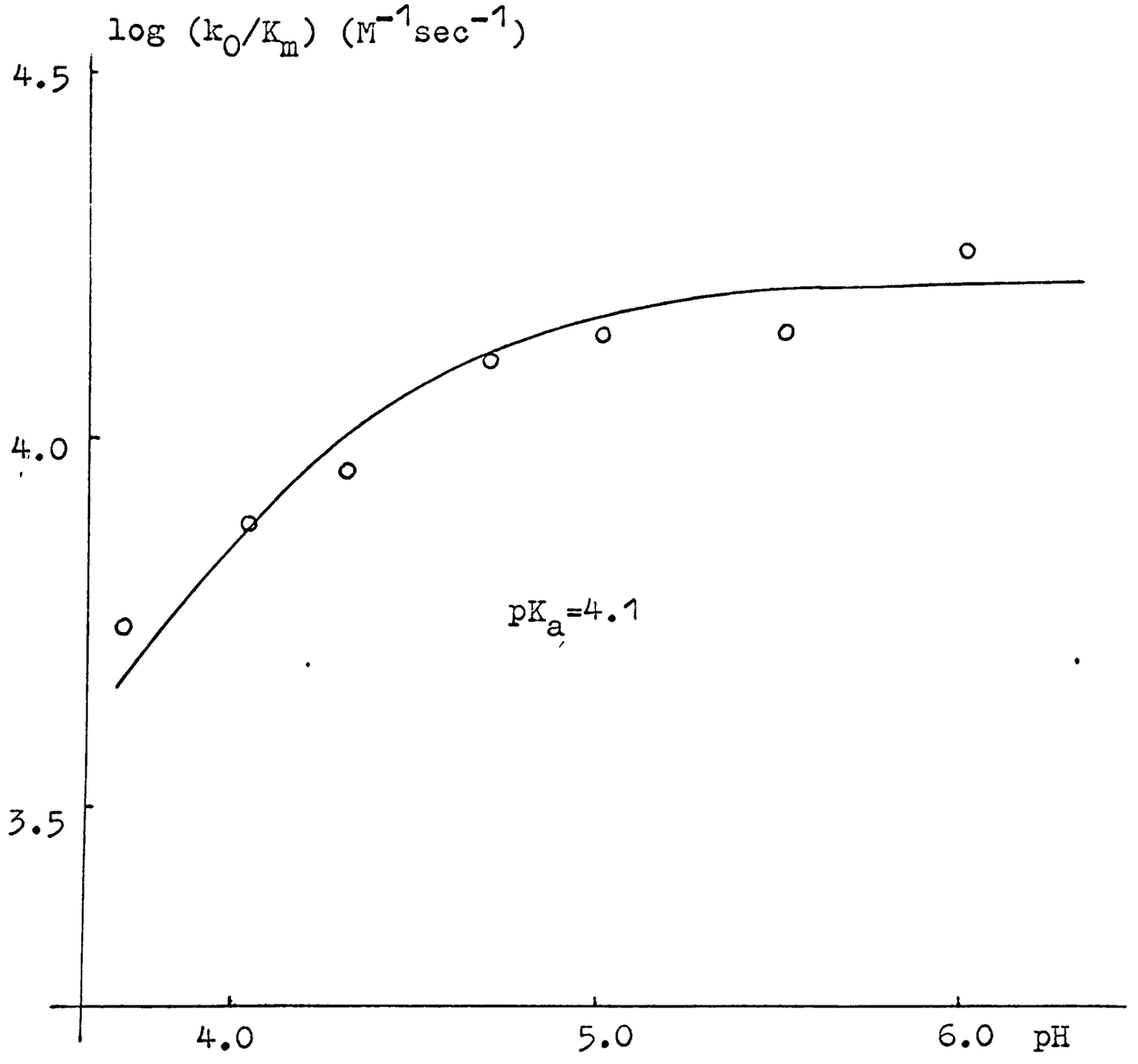



Fig. 3-10

pH dependence of ($k_0/K_m$) for p-nitrophenyl α-N-acetyl-DL-tryptophanate; 35°; μ 0.35M; phosphate-citrate buffer; 2% DMF; $10^{-3}$M EDTA; p-thiocresol-activated papain.

See Table 4-21, p. 118.

Table 3-7

The effect of pH on papain-catalysed hydrolysis of p-nitrophenyl 3-benzoylpropionate; 35°; μ 0.05M; citrate-phosphate buffer; 10% DMF; $10^{-3}$M EDTA; p-thiocresol-activated papain.

| pH | $(k_0/K_m)$ $M^{-1}sec^{-1}$ |
|---|---|
| 6.0 | 4670 ± 310 |
| 6.0 | 4320 ± 120 |
| 5.3 | 4000 ± 220 |
| 4.6 | 3370 ± 150 |
| 4.6 | 3460 ± 90 |
| 4.6 | 3430 ± 130 |
| 4.1 | 2750 ± 190 |
| 4.1 | 2470 ± 110 |
| 4.1 | 2380 ± 110 |
| 3.8 | 1850 ± 60 |
| 3.8 | 1880 ± 170 |
| 3.5 | 990 ± 180 |

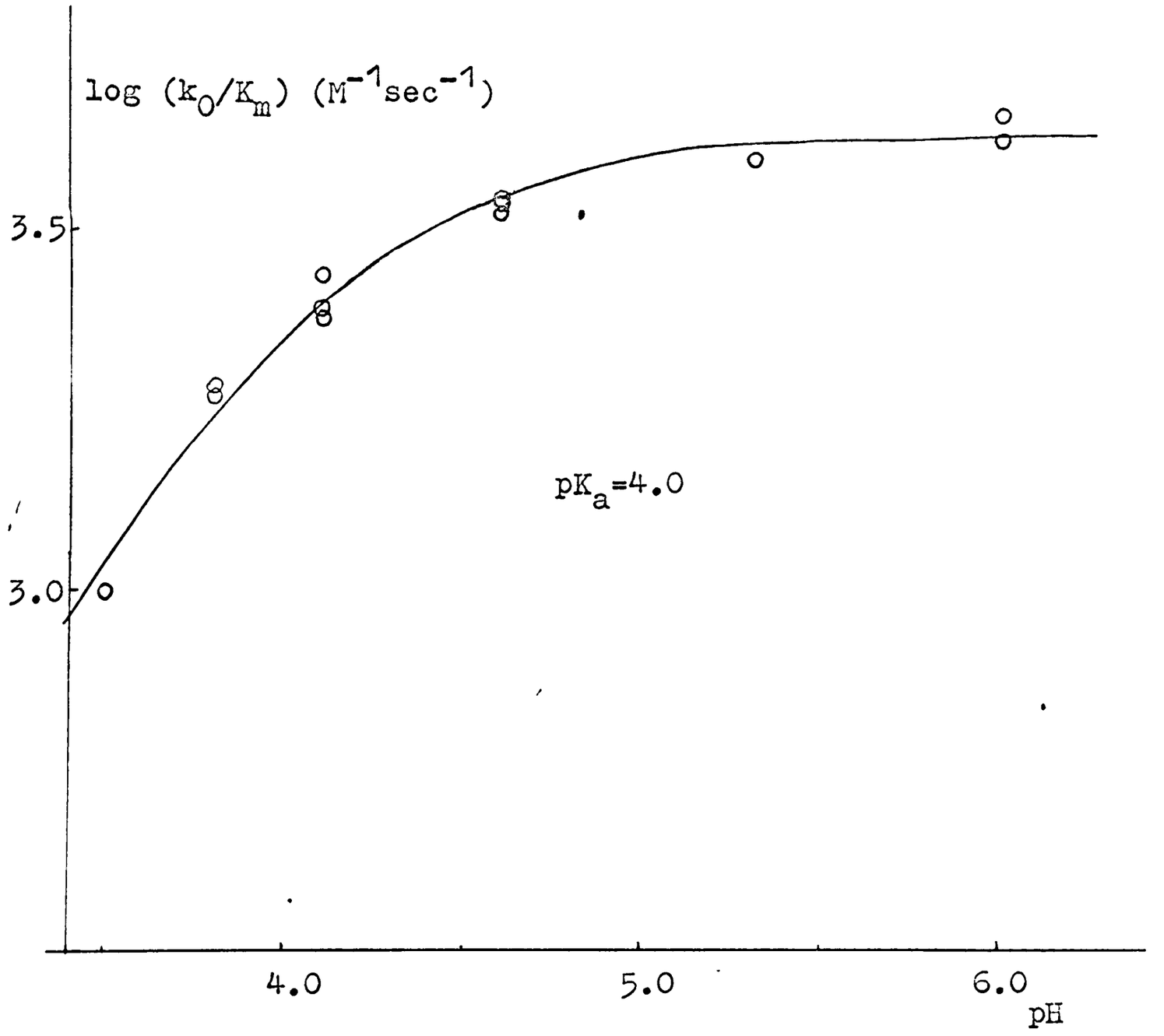



Fig. 3-11

pH dependence of ($k_0/K_m$) for p-nitrophenyl 3-benzoyl-propionate; 35°; μ 0.05M; phosphate-citrate buffer; 10% DMF; p-thiocresol activated enzyme; $10^{-3}$M EDTA.

Table 3-8

The effect of pH on papain-catalysed hydrolysis of acetyl-L-phenylalanylglycine p-nitroanilide; 35°; μ 0.1 - 0.3M; 10% DMF; $10^{-3}$M EDTA; $5\times10^{-4}$M L-cysteine.

| pH | $k_0$ $sec^{-1}$ | $K_m$ mM | $k_0/K_m$ $M^{-1}sec^{-1}$ | $k_{+2}$[a] $sec^{-1}$ | $K_s$[b] mM |
|---|---|---|---|---|---|
| 3.7[c] | 0.31±0.06 | 1.05±0.21 | 300±30 | 0.33 | 1.10 |
| 3.77[c] | 0.33±0.04 | 0.88±0.10 | 380±20 | 0.35 | 0.92 |
| 3.9[c] | 0.58±0.15 | 1.37±0.36 | 430±50 | 0.64 | 1.51 |
| 4.2[c] | 0.76±0.05 | 1.14±0.07 | 660±20 | 0.85 | 1.29 |
| 4.6[c] | 1.11±0.19 | 1.09±0.19 | 1,020±90 | 1.33 | 1.30 |
| 5.1[c] | 1.00±0.07 | 0.75±0.05 | 1,330±50 | 1.18 | 0.89 |
| 5.5[c] | 1.20±0.07 | 0.86±0.05 | 1,400±60 | 1.47 | 1.05 |
| 6.0[d] | 1.30±0.18 | 0.88±0.13 | 1,500±200 | 1.62 | 1.08 |
| 6.1[c] | 1.55±0.17 | 1.34±0.15 | 1,150±60 | 2.01 | 1.75 |
| 7.2[e] | 1.08±0.21 | 0.82±0.17 | 1,300±100 | 1.29 | 0.99 |
| 7.7[e] | 0.98±0.14 | 0.91±0.12 | 1,100±100 | 1.15 | 1.05 |
| 8.05[e] | 0.92±0.12 | 1.00±0.12 | 900±100 | 1.07 | 1.19 |
| 8.2[f] | 0.69±0.06 | 0.87±0.07 | 770±40 | 0.77 | 1.00 |
| 8.4[f] | 0.53±0.06 | 0.89±0.10 | 590±30 | 0.57 | 0.97 |
| 8.76[f] | 0.58±0.09 | 1.65±0.27 | 350±20 | 0.64 | 1.83 |
| 9.0[f] | 0.49±0.27 | 2.22±1.23 | 220±30 | 0.53 | 2.41 |
| 9.3[f] | 0.21±0.14 | 1.21±0.09 | 170±40 | 0.21 | 1.21 |

(a) Calculated from the relationship $(1/k_0)=(1/k_{+2})+(1/k_d)$, assuming $k_d=6.6$ $sec^{-1}$; (b) calculated from the relationship $K_s=k_{+2}/(k_0/K_m)$; (c), (d) and (e), phosphate-citrate buffer with μ=0.1, 0.3 and 0.2M respectively; (f) borate buffer with μ=0.1M. Lineweaver-Burk plots (6 points for each) were obtained by the initial rate method.

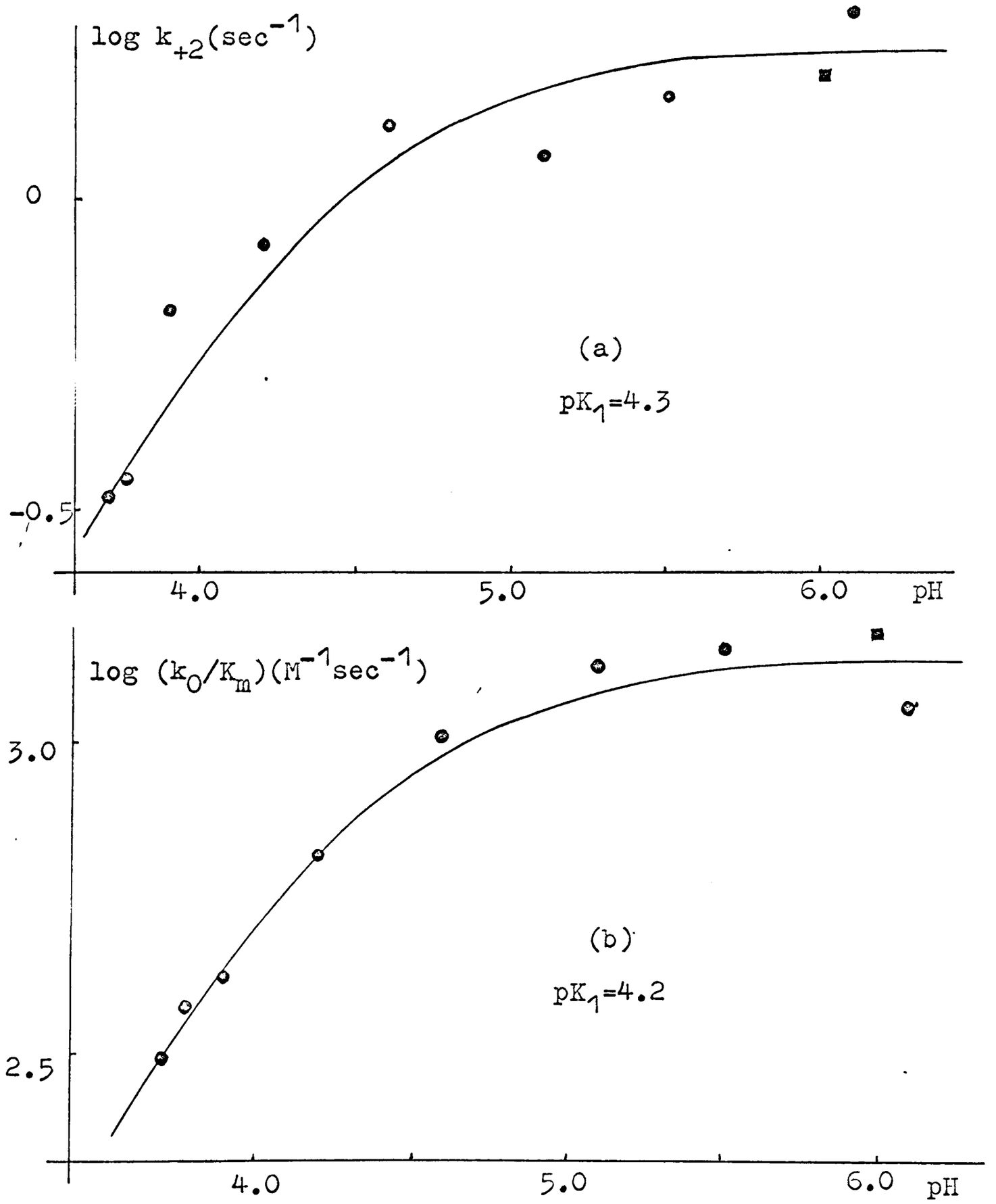



Fig 3-13 pH dependence of $k_{+2}$(a), and $k_0/K_m$ (b) of acetyl-L-phenylalanylglycine p-nitroanilide; 35°; phosphate-citrate buffer; ●,μ=0.1M; ■,μ=0.3M; 10% DMF; $10^{-3}$M EDTA; $5\times10^{-4}$M L-cysteine.

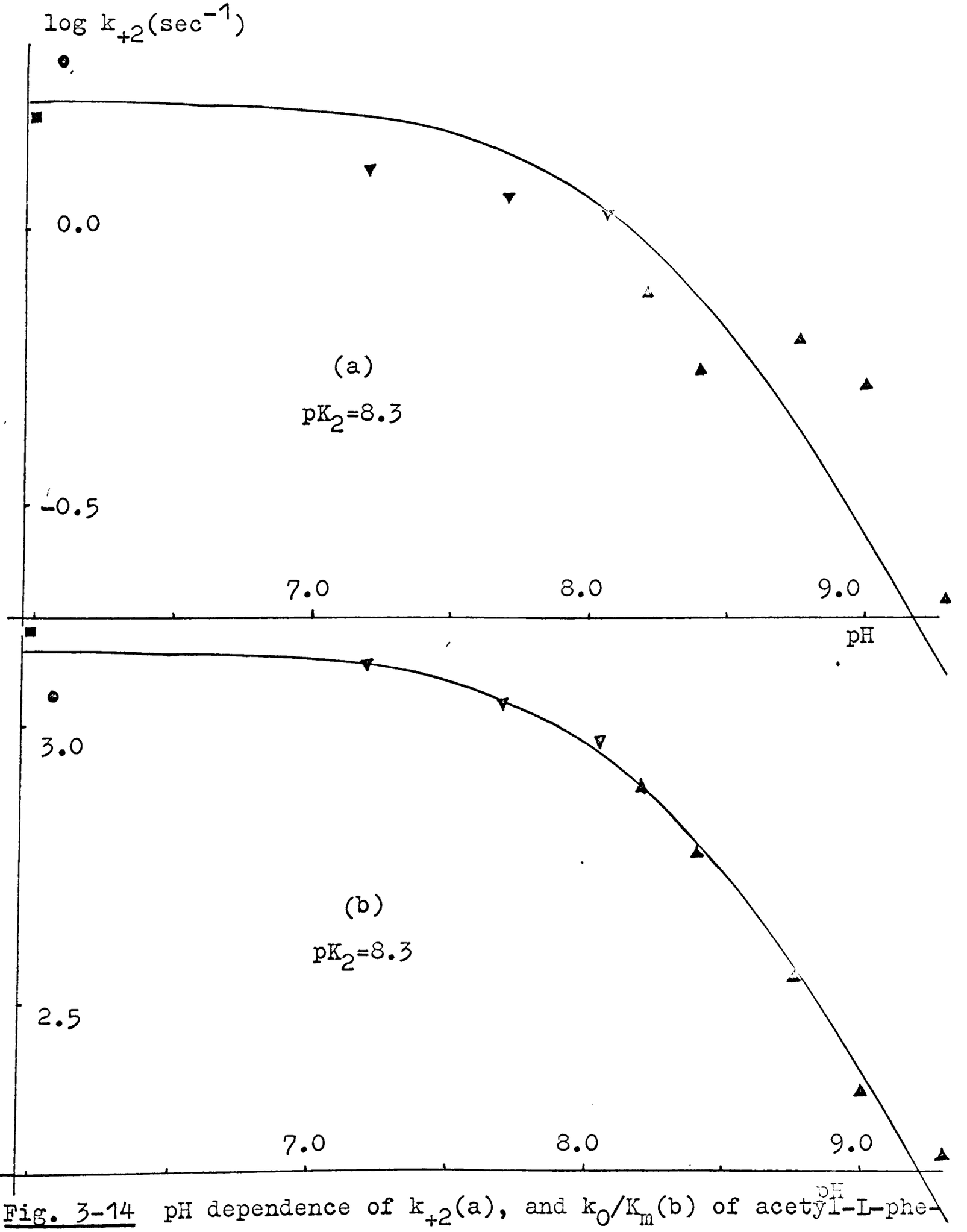



Fig. 3-14 pH dependence of $k_{+2}$(a), and $k_0/K_m$(b) of acetyl-L-phenylalanylglycine p-nitroanilide; ▼,μ 0.2M phosphate-citrate buffer; ▲, μ 0.1M borate buffer; ●,■ as in Fig. 3-13.

Table 3-9

Apparent $pK_a$ values of the prototropic groups of the enzyme essential to the acylation step.

| Substrate | Maximum value of $k_0/K_m$ ($M^{-1}sec^{-1}$)[a] | $pK_1$ of $k_0/K_m$ |
|---|---|---|
| p-Nitrophenyl N-acetylglycinate | 3,500 (40°) | 4.3 |
| p-Nitrophenyl hippurate | 250,000 (35°) | 4.15 |
| p-Nitrophenyl N-benzyloxycarbonylglycinate | 750,000 (35°) | 4.3[b] |
| p-Nitrophenyl α-N-acetyl-DL-tryptophanate | 17,000 (35°) | 4.1[c] |
| p-Nitrophenyl 3-benzoylpropionate | 4,400 (35°) | 4.0 |

(a) Reaction temperatures are indicated in parentheses.
(b) $pK_a$ 4.23 at 25° was reported in ref. 88.
(c) $pK_a$ 4.4 at 25° was reported in ref. 27.

For acetyl-L-phenylalanylglycine p-nitroanilide at 35°.

| Constant | Limiting value | $pK_1$(app) | $pK_2$(app) |
|---|---|---|---|
| $k_0$ | 1.35 $sec^{-1}$ | 4.2 | 8.5 |
| $K_m$ | 1.00mM | - | - |
| $k_0/K_m$ | 1,350 $M^{-1}sec^{-1}$ | 4.2 | 8.3 |
| $k_{+2}$ | 1.70 $sec^{-1}$ | 4.3 | 8.3 |
| $K_s$ | 1.26mM | - | - |

α-N-benzoyl-L-arginine ethyl ester and amide, which have been shown [71] to represent the acylation step, were found[53] to depend on prototropic groups of $pK_a$ 4.04 and 9.10, and 3.65 and 8.31 respectively. It appears therefore that the binding of these arginine derivatives results in substantial perturbation of the enzyme catalytic groups.

The effect of pH on papain-catalysed hydrolysis of acetyl-L-phenylalanylglycine p-nitroanilide has been studied in the pH range 3.7 - 9.3 (Table 3-8). $k_{+2}$ depends on $pK_1$ of 4.3 (Fig. 3-12a) and $pK_2$ of 8.3 (Fig. 3-13a). $(k_0/K_m)$ of this substrate has $pK_1$ of 4.2 and $pK_2$ of 8.3 (Figs. 3-12b and 3-13b). The observation that both $k_{+2}$ and $(k_0/K_m)$ have essentially the same pH dependence show that the essential groups of the enzyme involved in the acylation step are not perturbed by the binding of this substrate to any significant extent. If free papain has a hydrogen-bonded thiol-imidazole system it is unlikely that substrate binding in this case involves destruction of the hydrogen bond. It is likely that the same situation applies for neutral substrates generally, though possibly not for L-arginine derivatives.

The $pK_a$ values for $(k_0/K_m)$ and $k_{+2}$ are given in Table3-9.

A Comparison with α-Chymotrypsin

A ρ value of -1.63 was obtained from the plot of log $(k_0/K_m)$ versus σ for α-chymotrypsin-catalysed hydrolyses of anilides of α-N-benzoyl-L-tyrosine.[103] With the same enzyme anilides of α-N-acetyl-L-tyrosine[104] gave a ρ value of -2.0 when log $k_{+2}$ was plotted against σ. Both groups of workers concluded that the observed ρ values indicate electrophilic catalysis in the acylation step. A similar conclusion was drawn by Caplow[105] who argued further, from observation of difference in pH dependence of acylation by α-N-acetyl-L-tryptophan p-anisidide and p-chloroanilide, that for the latter $K \gg 1$. No direct spectroscopic evidence for the existence of the tetrahedral intermediate was given, however.

The experimental results on both papain and α-chymotrypsin show that for amide and anilide substrates protonation of the nitrogen atom of the leaving group is necessary during the course of acylation. In scheme (3-1) it has been assumed that protonation occurs in the $k''_{+2}$ step, but this is not the only possibility. Wang and Parker[106] have suggested that in α-chymotrypsin-catalysed hydrolysis

protonation of a substrate occurs prior to the formation of the transition-state of the acylation reaction. They assumed that the substrate is bound to the enzyme in such a stereo-specific way that the proton of the Ser-His hydrogen bond can jump to the N-atom of the leaving group of the substrate at a faster rate than the acylation step which follows (Fig. 3-17).

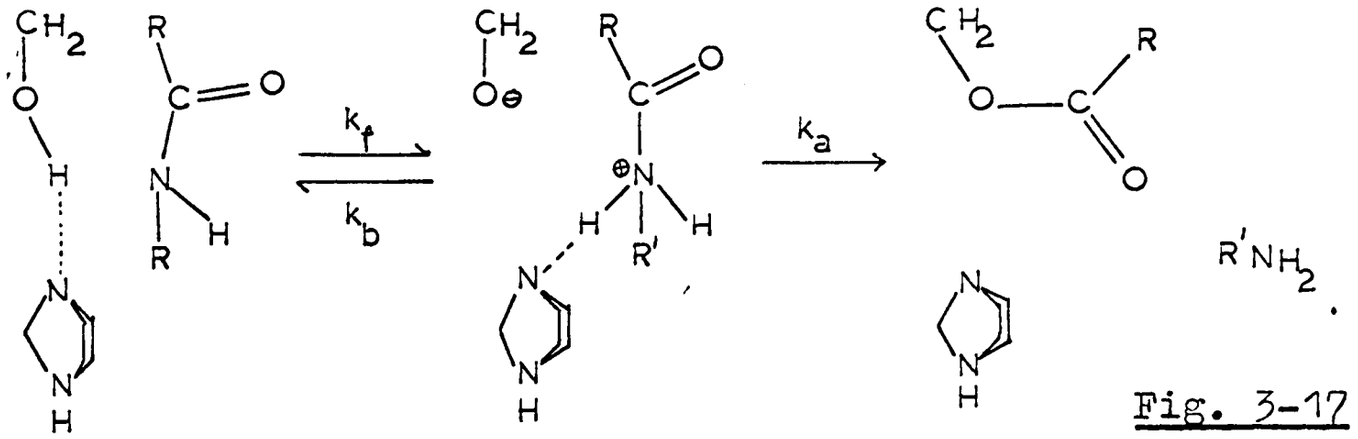


Fig. 3-17

$$k_{+2} = \frac{k_f}{k_b}.k_a \quad (\text{when } k_b \gg k_a)$$

$$= \frac{(K_a)_{Ser}}{(K_a)_{SH^+}}.k_a \qquad (3\text{-}6)$$

$(K_a)_{Ser}$ and $(K_a)_{SH^+}$ represent the acid dissociation constants of Ser-195 and the protonated substrate respectively. The authors proposed that $k_a$ for a series of α-N-acetyl-L-tyrosine substituted anilides are $\sim 10^{12}\,sec^{-1}$ and the observed $k_{+2}$ can be predicted from $K_a$ of substituted

acetanilides which are assumed to be approximately equal to $(K_a)_{SH^+}$.

It is not possible from available data, both for papain and α-chymotrypsin, to state definitely whether protonation of the leaving group occurs prior to formation of the transition state as in Fig. 3-17 (with Cys-25 in place of serine in the case of papain, which is probably engaged in an analogous hydrogen bond), or during the formation of the transition state, or during the breakdown of the tetrahedral intermediate as in Fig. 3-3.

# CHAPTER 4

# INVESTIGATION OF THE DEACYLATION STEP

The rate constants and Arrhenius parameters for deacylation of various acyl-papains are compared. Information on the mechanism of deacylation is obtained from studies on the effect of the presence of nucleophiles other than water. Variation of $pK_1$ of deacylation with the nature of the acyl moiety is discussed.

## The Influence of the Acyl Moiety on the Deacylation Rate

The rate-determining step for most specific ester substrates for papain is deacylation of the acyl-enzyme ($k_d$). Derivatives of acylglycines or related analogues have $k_d$ of 1 - 10 $sec^{-1}$ (Tables 2-1, 2-3 and 4-1), although $k_0/K_m$ may differ by a factor of $10^4$ (compare methyl O-benzoylglycollate with methyl acetyl-L-phenylalanylglycinate). Derivatives of other L-amino acids also have similar values of $k_d$ (Tables 4-2 and 4-21); for L-arginine[71] and L-lysine derivatives, however, $k_d > 10$ $sec^{-1}$. Apparently deacylation rates do not depend critically on the nature of interactions of the acyl moiety with the $\rho_1$ and $\rho_2$ sites. At least two non-specific substrates, however, are known to have very low values for

Table 4-1

Papain-catalysed hydrolyses of some esters of N-acyl- and sulphonyl-glycines at pH 6.0, 35° and $\mu$0.3M. The data are quoted from ref. 94 and should be compared with those in Tables 2-1 and 2-3.

| Substrate | $k_0 sec^{-1}$ | $k_0/K_m (M^{-1}sec^{-1})$ |
|---|---|---|
| p-Nitrophenyl N-trifluoroacetylglycinate | 4.92 ±0.49 | 10,500 |
| p-Nitrophenyl N-methanesulphonylglycinate | 2.34 ±0.42 | 1,300 |
| Methyl N-methanesulphonylglycinate | 1.87 ±0.18 | 21.1 |

Table 4-2

Papain-catalysed hydrolyses of p-nitrophenyl esters of some α-N-benzyloxycarbonylamino acids at pH 5 - 6.

| p-Nitrophenyl α-N-benzyloxycarbonyl- | $k_0 sec^{-1}$ | $k_0/K_m\ M^{-1}sec^{-1}$ |
|---|---|---|
| L-tryptophanate[a] | 10.3±0.6 | (1.70±0.21)x10$^6$ |
| L-phenylalaninate[a] | 3.36±0.05 | (6.38±0.33)x10$^5$ |
| L-tyrosinate[a] | 4.19±0.19 | (5.82±1.25)x10$^5$ |
| L-leucinate[a] | 4.76±0.06 | (2.21±0.06)x10$^6$ |
| L-alaninate[a] | 11.9±0.5 | 1.51x10$^6$ |
| DL-trifluoroalaninate[a] | 30.1±1.2 | 1.0x10$^7$ |
| L-lysinate[b] | 45.0±1.1 | (2.60±0.31)x10$^7$ |
| ε-formyl-L-lysinate[b] | 32.0 | 1.39x10$^7$ |

(a) Ref. 59; 35°; 14.6% DMF and 0.9% acetonitrile were present in all determinations except for the L-alanine and DL-trifluoroalanine derivatives (1% acetonitrile).

(b) Ref. 27; 25°; 1.6% acetonitrile; when different standardisation methods are taken into account (see Appendix 3), $k_0$ and $k_0/K_m$ for the two substrates must be multiplied by 9/16.

Table 4-3

The effect of temperature on papain-catalysed hydrolysis of p-nitrophenyl N-acetylglycinate. pH 6.0; $\mu$ 0.35M; phosphate-citrate buffer; 2% DMF; $10^{-3}$M EDTA; p-thiocresol-activated papain. About 4 points were used for each Lineweaver-Burk plot.

| Temperature(°C) | $10^3/T(°K^{-1})$ | $k_0(sec^{-1})$ | $K_m$(mM) | $k_0/K_m(M^{-1}sec^{-1})$ |
|---|---|---|---|---|
| 20 | 3.41 | 1.00±0.1 | 0.45 | 2,200 |
| 25 | 3.36 | 1.26±0.2 | 0.44 | 2,900 |
| 35 | 3.25 | 2.04±0.1 | 0.68 | 3,000 |
| 40 | 3.20 | 3.09±0.3 | 0.88 | 3,500 |

Table 4-4

The effect of temperature on papain-catalysed hydrolysis of APG p-nitrophenyl ester. pH 6.0; $\mu$ 0.25; phosphate-citrate buffer; 10% DMF; $10^{-3}$M EDTA; p-thiocresol activated papain. Hydrolysis was performed under $V_{max}$ conditions.

| Temperature(°C) | $10^3/T(°K^{-1})$ | $k_0(sec^{-1})$ |
|---|---|---|
| 29 | 3.31 | 4.6 |
| 35[a] | 3.25 | 6.6 |
| 40 | 3.20 | 9.2 |
| 45 | 3.145 | 11.3 |
| 53 | 3.07 | 15.8 |

(a) 2%DMF

Table 4-5

The effect of temperature on papain-catalysed hydrolysis of APG methyl ester at pH 6.0, $\mu$ 0.3M (sodium chloride), and without organic solvent, performed under $V_{max}$ conditions.

| Temperature(°C) | $10^3/T(°K^{-1})$ | $k_0(sec^{-1})$ |
|---|---|---|
| 17 | 3.45 | 1.80 |
| 35 | 3.25 | 5.43 |
| 40 | 3.20 | 6.20 |
| 45 | 3.145 | 8.49 |
| 50 | 3.09 | 9.63 |

$k_d$: trans-cinnamoylimidazole (0.004 $sec^{-1}$),[62] and methyl N-tosylsarcosinate.[85] Substrates derived from D amino acids such as p-nitrophenyl esters of α-N-benzyloxycarbonyl-D-phenylalanine, -D-tryptophan and -D-alanine also have relatively small values of $k_0$,[59] but there are no data to indicate whether $k_d$ is the rate-determining step.

Arrhenius parameters for deacylation of N-acetylglycyl- and acetyl-L-phenylalanylglycyl-papain have been determined using the corresponding p-nitrophenyl and methyl esters as substrates (Tables 4-3, 4-4, 4-5). The values found (Table 4-6) are not remarkably different from those for deacylation of hippuryl-papain[57] and for acylation of papain by anilides (Table 3-6).

Table 4-6

Rate constants and Arrhenius parameters for deacylation of some acyl-papains at pH 6.0, 35° and μ 0.3M.

| Acyl-enzyme[a] | $k_d$ $sec^{-1}$ | $E_a$ $kcalM^{-1}$ | $\Delta S^{\ddagger}$ $e.u.M^{-1}$ |
|---|---|---|---|
| N-Acetylglycyl-papain (p-nitrophenyl ester) | 2.04±0.07 | +10.0 | -28 |
| APG-papain (p-nitrophenyl ester) | 6.6±0.05 | +10.1 | -25 |
| (methyl ester) | 5.43±0.18 | +10 | -26 |
| Hippuryl-papain[b] (methyl ester) | 2.72±0.06 | +11.6 | -22[c] |

(a) Substrates are mentioned in parentheses. (b) ref. 57.
(c) Re-calculated; a value of -26 $e.u.M^{-1}$ was reported in ref. 57.

Is the deacylation rate controlled by a conformational change, by nucleophilic attack of the replacement agent or a reactive group of the enzyme on the carbonyl carbon, or by some other process? The observation of similar deacylation rates and their Arrhenius parameters for a wide range of substrates suggests that the possibility of a slow conformational change cannot be ruled out. While there have been no conclusive results to demonstrate a relationship between electron availability at the carbonyl carbon and the deacylation rate, it should be noted that $k_d$ for p-nitrophenyl N-trifluoroacetylglycinate[94] and N-benzyloxycarbonyl-DL-trifluoroalanine,[59] are more than twice as large as $k_d$ for the corresponding unfluorinated analogues.

## Elementary Steps in Deacylation

The nature of catalysis in the deacylation step can be partly studied by measuring the effects of added foreign nucleophiles. Lake and Lowe[83] argued from the results on papain- and ficin-catalysed hydrolyses of methyl hippurate (HME) and p-nitrophenyl hippurate (HNP) in the presence of methanol as a foreign nucleophile, that deacylation is a two-step process. A rate-determining acyl transfer from Cys-25 to

another residue in the enzyme, or a conformational change, is followed by rapid hydrolysis and methanolysis. The authors observed suppression of the rate of hippuric acid production in HME hydrolysis, and no rate increase in p-nitrophenol production in HNP hydrolysis, when methanol was present in both experiments. The observations are in accord with a limiting case of scheme B of Appendix 2, where $k'_{+4}+k_{+5}(N) \gg k_{+3}$.

$$k_{0,P_1} = \frac{k_{+2}k_{+3}}{k_{+2}+k_{+3}} \qquad (4-1)$$

$$(1/k_{0,P_2}) = \frac{k_{+2}+k_{+3}}{k_{+2}k_{+3}} + \frac{(k_{+2}+k_{+3}).k_{+5}(N)}{k_{+2}k_{+3}k'_{+4}} \qquad (4-2)$$

The results of Henry and Kirsch[84] do not support the suggested mechanism. Brubacher and Bender[62] had also previously found that deacylation of trans-cinnamoyl-papain was made faster by addition of nucleophilic agents, although the rate-determining step in this reaction could be different from that for acyl-papains derived from specific substrates.[83] Re-investigation and extension of experiments with added nucleophiles, the results of which are now reported, show that the rate of p-nitrophenol production in HNP hydrolysis is not unaffected by the presence of methanol, a conclusion in agreement with that of Henry and Kirsch.[84] The rate increase

was found to rise with increasing methanol concentration (Table 4-7)

Since methanol is a poor nucleophile, it was thought that more powerful nucleophiles such as amines would give less ambiguous results. Methylammonium chloride has been used, but found to show no nucleophilic behaviour: apparently a protonated ammonium derivative cannot act as a replacement agent. Aminoacetonitrile ($pK_a$ 5.3)[97] is almost completely unprotonated at neutral pH, and was found to be an excellent nucleophile, enhancing the rate of p-nitrophenol production in HNP hydrolysis much more markedly than methanol, and in a linear fashion (Table 4-8, Fig. 4-1). The nucleophile also increases the rates of p-nitrophenol production in hydrolyses of N-benzyloxycarbonylglycine p-nitrophenyl ester (Table 4-9, Fig. 4-2) and acetyl-L-phenylalanylglycine p-nitrophenyl ester (Table 4-10, Fig. 4-3). Glycinamide ($pK_a$ 8.0)[97], a more potent nucleophile due to favoured binding, was found, on the assumption that only the unprotonated species is the attacking agent, to increase the rate of p-nitrophenol production more strongly, again in a linear fashion (Table 4-11, Fig. 4-4). The results from experiments with p-nitrophenyl esters hence indicate that the rate-determining step in deacylation is the hydrolytic and aminolytic step (scheme A of Appendix 2). From eqn. (4-3),

$$k_{0,P_1} = k'_{+3} + k_{+4}(N) \qquad (4-3)$$

$$k_{0,P_2} = k'_{+3} \qquad (4-4)$$

the values of $k_{+4}$ for the reactions of nucleophiles with the acyl-enzymes can be found (Table 4-12). It may be noted that the ratio $k_{+4}:k_{+3}$ depends on the nature of the acyl moiety, indicating differences in specificity of the acyl-enzymes for the replacement agent. The results, when compared with those of Brubacher and Bender[62] for deacylation of trans-cinnamoyl-papain, appear to indicate that hippuryl-papain exhibits a stronger selectivity towards the replacement agent than trans-cinnamoyl-papain (Table 4-13).

The rate of hippuric acid production in hydrolysis of HME is decreased in the presence of aminoacetonitrile (Table 4-14a, Fig. 4-5). This result agrees with that of Lake and Lowe[83] for methanol nucleophile. The effect of aminoacetonitrile is less apparent on the hydrolysis of methyl N-benzyloxycarbonylglycinate (Table 4-14b), and less still on that of methyl acetyl-L-phenylalanylglycinate (Table 4-14c). Barring experimental errors, the results may be reconciled with scheme A by assuming that the nucleophiles can also behave as non-competitive inhibitors. A different mechanism from that in scheme A can be proposed which accommodates

all experimental results. One such mechanism is set in scheme C of Appendix 2; it permits alternative pathways, that is, schemes A and B are assumed to be operating simultaneously. The plot of $k_{0,P_1}$ versus (N) is linear provided $k'_{+6} \gg k_{+5}$. The plot of $k_{0,P_2}$ versus (N) has a negative slope and curves towards an asymptote, $k_{0,P_2}=k'_{+3}$.

$$k_{0,P_1} = k'_{+3} + k_{+4}(N) + k_{+5} \qquad (4\text{-}5)$$

$$k_{0,P_2} = k'_{+3} + \frac{k_{+5}k'_{+6}}{k'_{+6}+k_{+7}(N)} \qquad (4\text{-}6)$$

As is seen in Fig. 4-5, the upper limit for $k'_{+3}$ for HME hydrolysis is 1 $sec^{-1}$. Assuming $k'_{+3}=1$ $sec^{-1}$ other kinetic constants can be calculated from the intercepts and slopes of the two plots (Table 4-15). The values reported are only tentative, mainly because of uncertainty in the experimental values of $k_{0,P_1}$ and $k_{0,P_2}$.

The deacylation scheme proposed for hippuryl-papain may not be universally applicable, or may indeed be a special case. Results with acetyl-L-phenylalanylglycyl-papain indicate that the major pathway for its deacylation is through the rate-determining hydrolytic step (scheme A). From the principle of simplicity of mechanism, scheme A is preferable to scheme C.

Table 4-7

The effect of methanol on papain-catalysed hydrolysis of p-nitrophenyl hippurate, p-nitrophenol ($P_1$) production being followed. pH 7.0; 35°; $\mu$ 0.1M; phosphate-citrate buffer; $10^{-3}$M EDTA; p-thiocresol-activated papain.

| Methanol concentration(M) | $k_0(sec^{-1})$ | $K_m(mM)$ | $k_0/K_m(M^{-1}sec^{-1})$ |
|---|---|---|---|
| 0[a] | 2.14 | 0.026 | 82,000 |
| 0.79 | 3.29 | 0.038 | 86,000 |
| 1.27 | 5.19 | 0.080 | 65,000 |

(a) 5.9% acetonitrile.

Table 4-8

The effect of aminoacetonitrile on papain-catalysed hydrolysis of p-nitrophenyl hippurate, p-nitrophenol ($P_1$) production being followed. pH 7.0; 35°; $\mu$ 0.1M; phosphate-citrate buffer; $10^{-3}$M EDTA; p-thiocresol-activated papain; 5.9% acetonitrile. Each value is an average of two determinations.

| Aminoacetonitrile[a] concentration(M) | $k_0(sec^{-1})$ | $K_m(mM)$ | $k_0/K_m(M^{-1}sec^{-1})$ |
|---|---|---|---|
| 0 | 2.14 | 0.026 | 82,000 |
| 0.022 | 5.35 | 0.070 | 77,000 |
| 0.044 | 11.4 | 0.148 | 77,000 |
| 0.080 | 15.0 | 0.181 | 83,000 |
| 0.106 | 18.4 | 0.222 | 83,000 |

(a) Concentration of the unprotonated form; $pK_a$ 5.3 was assumed.

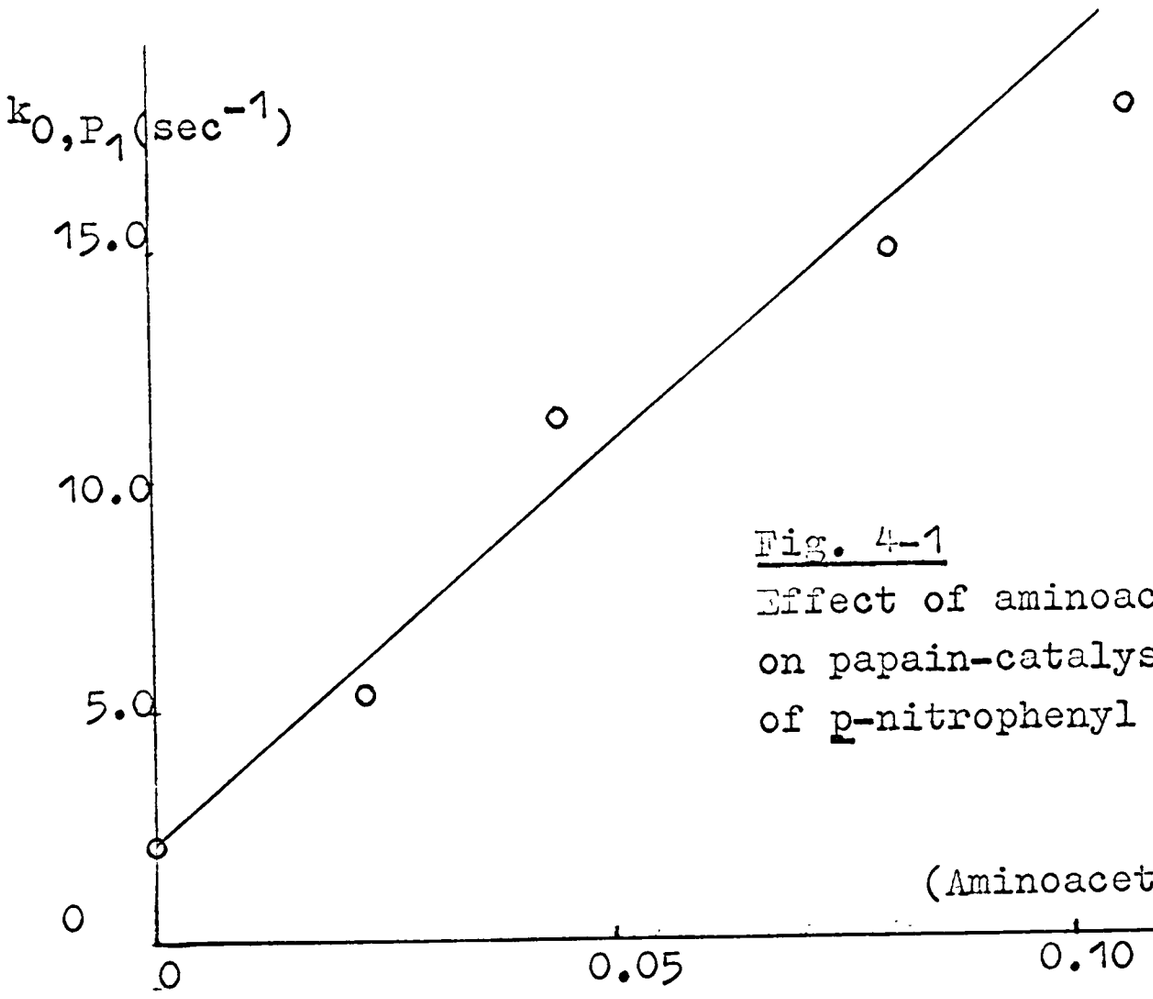



Fig. 4-1
Effect of aminoacetonitrile on papain-catalysed hydrolysis of p-nitrophenyl hippurate.

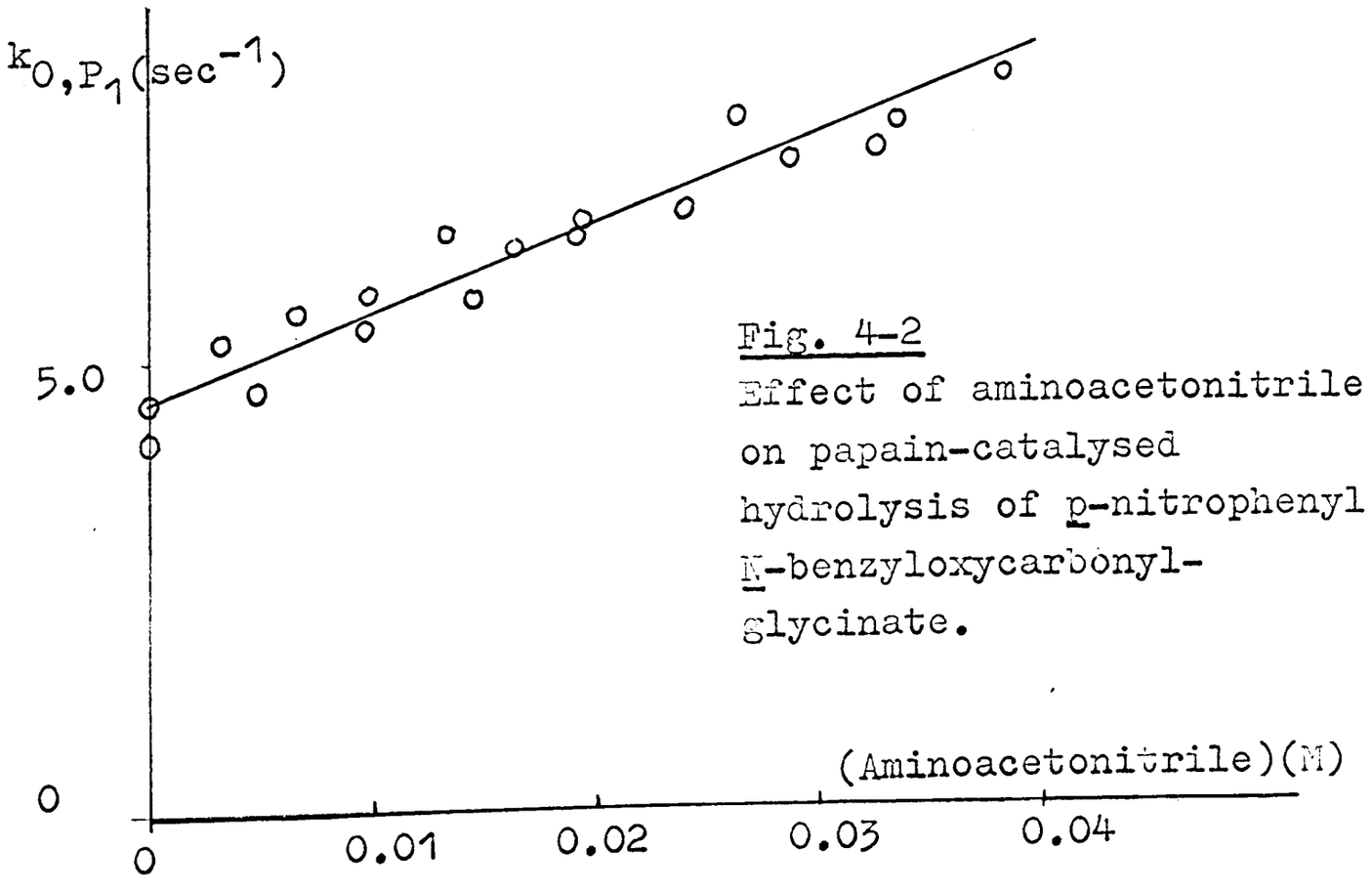



Fig. 4-2
Effect of aminoacetonitrile on papain-catalysed hydrolysis of p-nitrophenyl N-benzyloxycarbonyl-glycinate.

## Table 4-9

The effect of aminoacetonitrile on papain-catalysed hydrolysis of p-nitrophenyl N-benzyloxycarbonylglycinate, p-nitrophenol ($P_1$) production being followed. pH 7.0; 35°; μ0.3M; phosphate-citrate buffer; 2.3% ethanol; p-thiocresol-activated papain.

| [Aminoacetonitrile](M)[a] | $k_0$($sec^{-1}$) | $K_m$(mM) |
|---|---|---|
| 0 | 4.53 | 0.0085 |
| 0 | 4.07 | 0.0097 |
| 0.0032 | 5.21 | 0.0096 |
| 0.0047 | 4.64 | 0.0109 |
| 0.0065 | 5.46 | 0.0107 |
| 0.0096 | 5.37 | 0.0125 |
| 0.0098 | 5.66 | 0.0135 |
| 0.0131 | 6.37 | 0.0124 |
| 0.0144 | 5.72 | 0.0136 |
| 0.0163 | 6.23 | 0.0121 |
| 0.0191 | 6.28 | 0.0159 |
| 0.0195 | 6.65 | 0.0124 |
| 0.0240 | 6.58 | 0.0152 |
| 0.0262 | 7.69 | 0.0175 |
| 0.0288 | 7.14 | 0.0187 |
| 0.0326 | 7.28 | 0.220 |
| 0.0335 | 7.70 | 0.201 |
| 0.0384 | 8.13 | 0.300 |

(a) Concentration of the unprotonated form, calculated by assuming $pK_a$=5.3.

Table 4-10

The effect of aminoacetonitrile on papain-catalysed hydrolysis of p-nitrophenyl acetyl-L-phenylalanylglycinate, p-nitrophenol($P_1$) production being followed. pH 7.0; 35°; μ 0.3M; phosphate-citrate buffer; 2% DMF; $10^{-3}$M EDTA; p-thiocresol-activated papain.

| [Aminoacetonitrile](M)[a] | $k_0$($sec^{-1}$) | $10^4 K_m$(mM) |
|---|---|---|
| 0 | 6.39 | 6.4 |
| 0.057 | 26.0 | 29.2 |
| 0.080 | 33.8 | 29.9 |
| 0.181 | 58.6 | 96 |
| 0.240 | 95.6 | 147 |

(a) Concentration of the unprotonated form, calculated by assuming $pK_a$=5.3

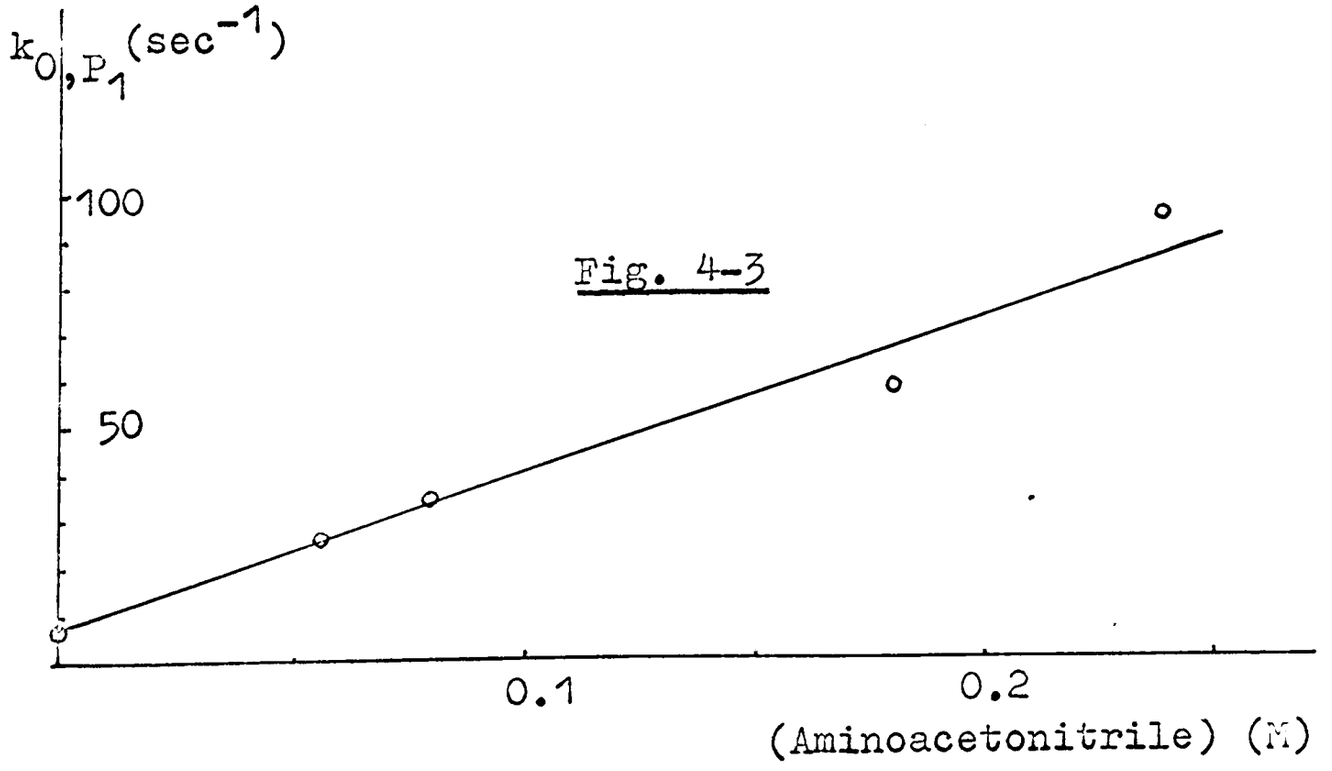



Fig. 4-3

Table 4-11

The effect of glycinamide on papain-catalysed hydrolysis of p-nitrophenyl hippurate, p-nitrophenol ($P_1$) production being followed. pH 7.0; 35°; μ0.3M; phosphate-citrate buffer; 4.5% acetonitrile; $10^{-3}$M EDTA; p-thiocresol-activated papain.

| [Glycinamide] (M)[a] | $k_0$($sec^{-1}$) | $K_m$(mM) |
|---|---|---|
| 0 | 3.00 | 0.039 |
| 0.0027 | 4.98 | 0.043 |
| 0.0055 | 7.15 | 0.059 |
| 0.0057 | 7.35 | 0.035 |
| 0.0082 | 10.3 | 0.070 |
| 0.0109 | 11.2 | 0.080 |
| 0.0136 | 10.5 | 0.069 |
| 0.0164 | 14.0 | 0.113 |
| 0.0171 | 12.9 | 0.083 |
| 0.0191 | 16.7 | 0.0113 |
| 0.0200 | 19.3 | 0.0115 |

(a) Concentration of the unprotonated form, calculated by assuming that $pK_a$=8.0

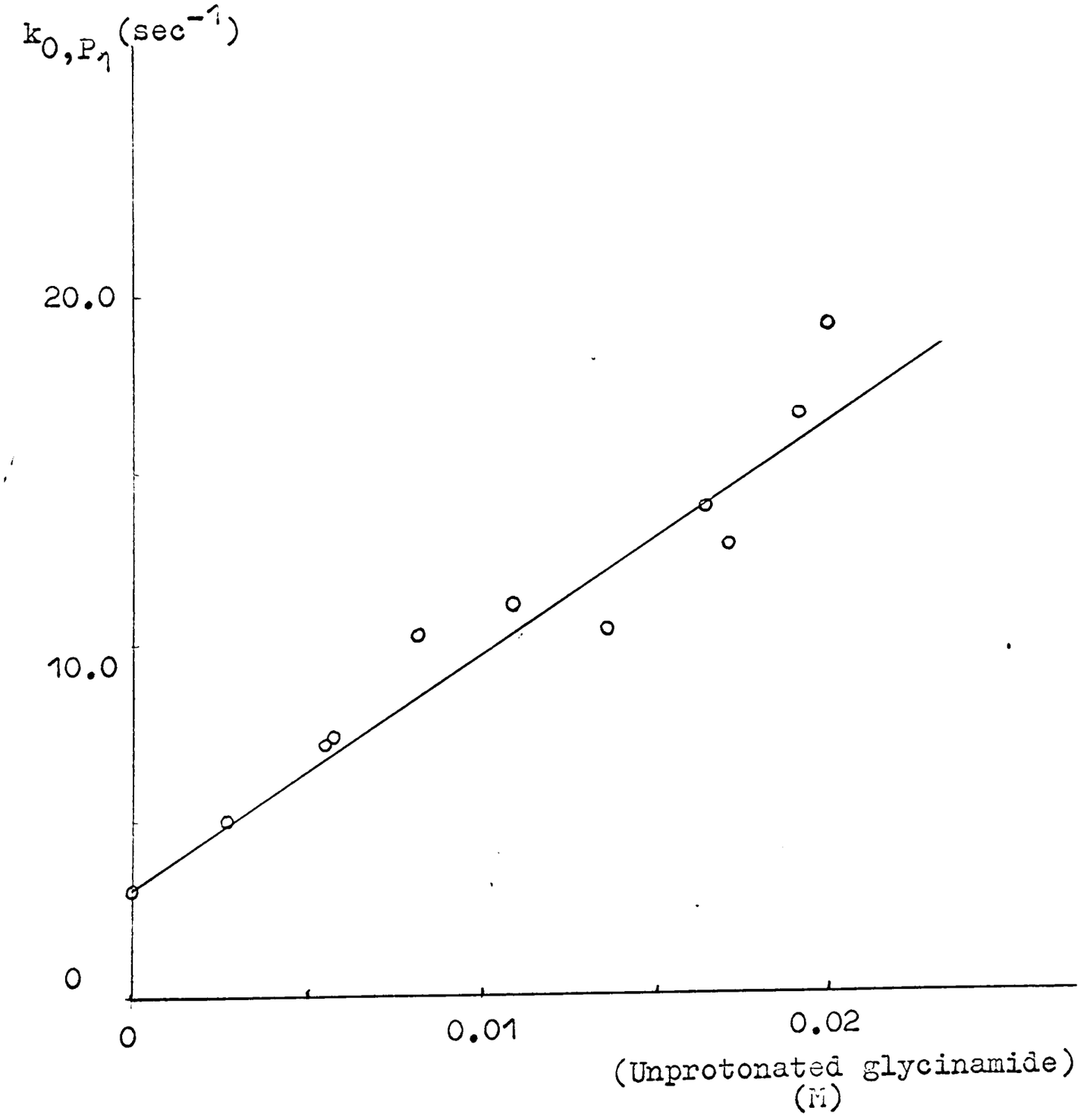



Fig. 4-4

The effect of glycinamide on papain-catalysed hydrolysis of p-nitrophenyl hippurate.

Table 4-12

Kinetic constants for the reaction of nucleophiles with acyl-papains at pH 7.0 and 35°; $k'_{+3}$ and $k_{+3}$ are pseudo-first-order and second-order rate constant respectively for the nucleophile water; $(H_2O)=55M$; $k_{+4}$= second-order rate constant for the amine nucleophile.

| Acyl-enzyme | $k'_{+3}(sec^{-1})$ | $k_{+3}(M^{-1}sec^{-1})$ | $k_{+4}(M^{-1}sec^{-1})$ | $\frac{k_{+4}}{k_{+3}}$ |
|---|---|---|---|---|
| Nucleophile=aminoacetonitrile | | | | |
| Hippuryl-papain | 2.2 | 0.040 | 175 | 4,300 |
| N-benzyloxycarbonylglycyl-papain | 4.3 | 0.078 | 100 | 1,300 |
| Acetyl-L-phenylalanyl-glycyl-papain | 6.4 | 0.117 | 330 | 2,800 |
| Nucleophile=glycinamide | | | | |
| Hippuryl-papain | 2.2 | 0.040 | 780 | 19,500 |

Table 4-13

Kinetic constants for the reaction of nucleophiles with trans-cinnamoyl-papain at pH 9.0 and 25°(Brubacher and Bender[62]).

| Nucleophile | $k_{+3}(M^{-1}sec^{-1})$ | $k_{+4}(M^{-1}sec^{-1})$ | $\frac{k_{+4}}{k_{+3}}$ |
|---|---|---|---|
| Water | 0.0000665 | | - |
| Aminoacetonitrile | | 0.476 | 7,200 |
| Glycinamide | | 0.533 | 8,200 |

Table 4-14a

The effect of aminoacetonitrile on papain-catalysed hydrolysis of methyl hippurate, hippuric acid ($P_2$) production being followed. pH 7.0; 35°; $\mu$ 0.3M (sodium chloride); $10^{-3}$M EDTA; p-thiocresol-activated papain.

| Aminoacetonitrile (M) | $k_0$($sec^{-1}$) | $K_m$(mM) |
|---|---|---|
| 0 | 2.72 | 23.6±1.0 |
| 0.0043 | 2.61±0.09 | 27.5±0.9 |
| 0.0057 | 2.45±0.12 | 27.2±1.4 |
| 0.0072 | 2.16±0.13 | 23.0±1.3 |
| 0.0085 | 2.24±0.06 | 25.9±0.6 |
| 0.0101 | 2.31±0.07 | 29.0±0.8 |
| 0.0115 | 2.16±0.12 | 27.8±1.5 |
| 0.0144 | 2.00±0.11 | 26.8±1.5 |
| 0.018 | 1.94 | 33.1 |
| 0.026 | 1.81 | 36.4 |
| 0.044 | 1.19 | 26.2 |
| 0.054 | 1.09 | 25.8 |
| 0.088 | 1.21 | 46.1 |

(a) Concentration of the unprotonated form, calculated by assuming $pK_a$=5.3.

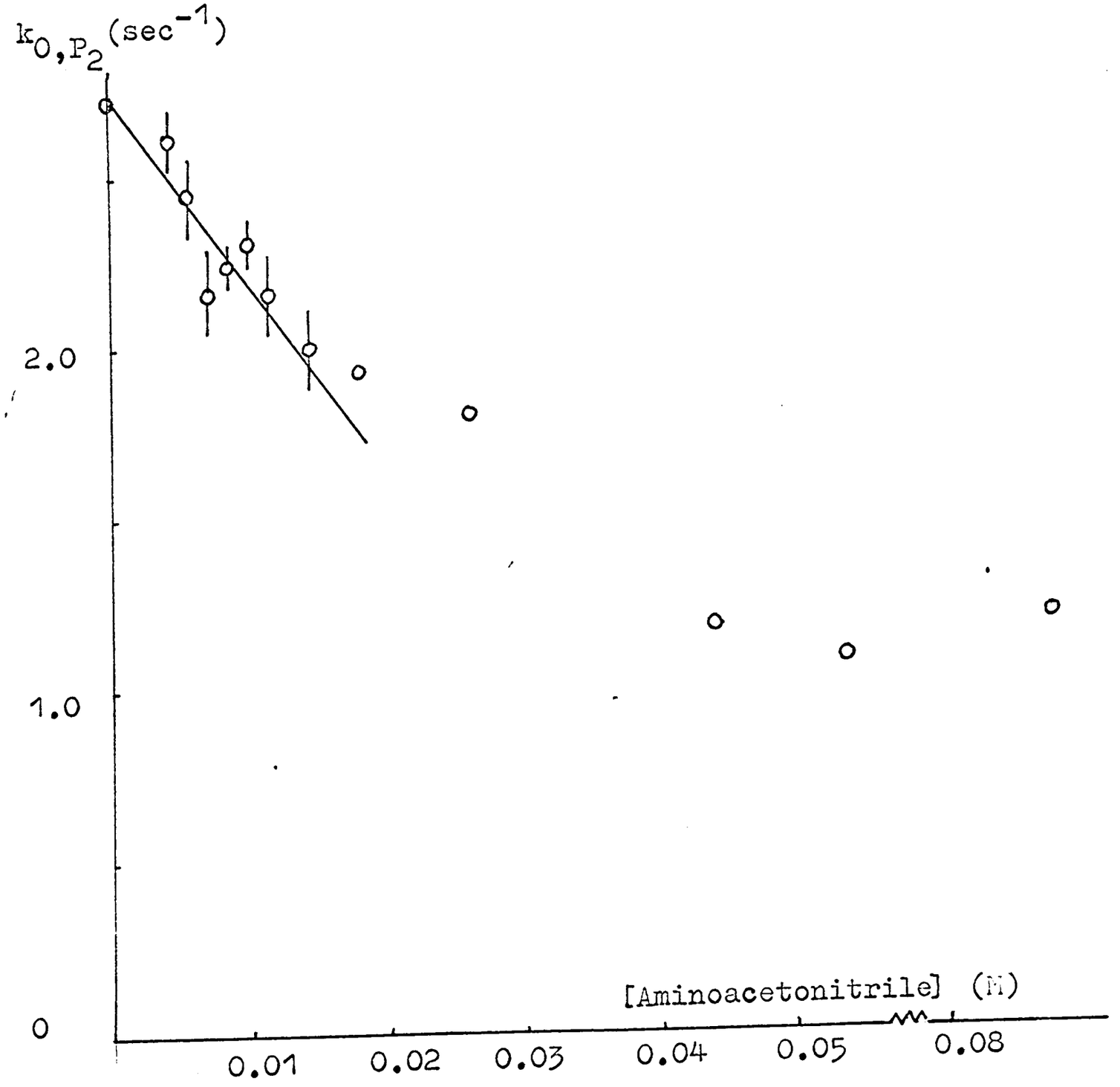



Fig. 4-5 The effect of aminoacetonitrile on papain-catalysed hydrolysis of methyl hippurate.

Table 4-14b

The effect of aminoacetonitrile on papain-catalysed hydrolysis of methyl N-benzyloxycarbonylglycinate, the acid ($P_2$) production being followed. pH 7.0; 35°; μ 0.3M (sodium chloride); 2.5% ethanol; $10^{-3}$M EDTA; p-thiocresol-activated papain.

| [Aminoacetonitrile] (M)[a] | $k_0$($sec^{-1}$) | $K_m$(mM) |
|---|---|---|
| 0 | 3.76±0.04 | 10.08±0.14 |
| 0.0043 | 3.30±0.06 | 9.37±0.18 |
| 0.0072 | 3.76±0.04 | 11.61±0.16 |
| 0.0101 | 3.27±0.09 | 10.35±0.25 |
| 0.0144 | 3.43±0.19 | 11.98±0.66 |
| 0.0187 | 2.99±0.16 | 10.68±0.56 |

Table 4-14c

The effect of aminoacetonitrile on papain-catalysed hydrolysis of methyl acetyl-L-phenylalanylglycinate, the acid ($P_2$) production being followed. pH 7.0; 35°; μ 0.3M (sodium chloride); p-thiocresol-activated papain. Hydrolyses were performed under $V_{max}$ conditions.

| [Aminoacetonitrile] (M)[a] | $k_0$($sec^{-1}$) |
|---|---|
| 0 | 6.2 |
| 0.011 | 6.2 |
| 0.016 | 5.8 |
| 0.022 | 5.8 |
| 0.044 | 4.4 |
| 0.130 | 4.9 |

(a) Concentration of the unprotonated form; $pK_a$ 5.3 was assumed.

---

Table 4-15

Kinetic constants calculated from scheme C of Appendix 3 for deacylation of hippuryl-papain. Nucleophile=aminoacetonitrile.

| $k'_{+3}sec^{-1}$ | $k_{+3}M^{-1}sec^{-1}$ | $k_{+4}M^{-1}sec^{-1}$ | $k_{+5}sec^{-1}$ | $k_{+4}/k_{+3}$ | $k_{+7}/k_{+6}$ |
|---|---|---|---|---|---|
| ~1 | ~0.018 | ~170 | ~1.2 | ~9300 | ~1800 |

Table 4-16

The effect of pH on papain-catalysed hydrolysis of methyl acetyl-L-phenylalanylglycinate. 35°; μ0.3M (sodium chloride); $10^{-3}$M EDTA; p-thiocresol-activated papain. Lineweaver-Burk plots were obtained by the total progress method. Each value is an average of three determinations.

| pH | $k_0(sec^{-1})$ | $K_m$(mM) | $k_0/K_m(M^{-1}sec^{-1})$ |
|---|---|---|---|
| 6.0 | 5.43 | 0.032 | 170,000 |
| 7.0 | 5.68 | 0.073 | 78,000 |
| 7.9 | 5.09 | 0.121 | 42,000 |
| 8.5 | 5.13 | 0.143 | 36,000 |
| 8.9 | 5.68 | 0.284 | 20,000 |
| 9.5 | 3.49 | 0.47 | 7,500 |

Table 4-17

The effect of pH on papain-catalysed hydrolysis of p-nitrophenyl N-acetylglycinate. 40°; μ 0.35M; phosphate-citrate buffer; 2% DMF; $10^{-3}$M EDTA; p-thiocresol-activated papain. Lineweaver-Burk plots were obtained by the initial rate method.

| pH | $k_0(sec^{-1})$ | $K_m$(mM) | $k_0/K_m(M^{-1}sec^{-1})$ |
|---|---|---|---|
| 5.95 | 3.22±0.17 | 0.865±0.045 | 3,720±170 |
| 5.25 | 3.14±0.30 | 1.308±0.101 | 3,000±200 |
| 4.83 | 2.13±0.09 | 0.716±0.031 | 3,000±100 |
| 4.30 | 1.28±0.06 | 0.781±0.039 | 1,700±70 |
| 3.90 | 0.845±0.09 | 1.083±0.116 | 780±40 |

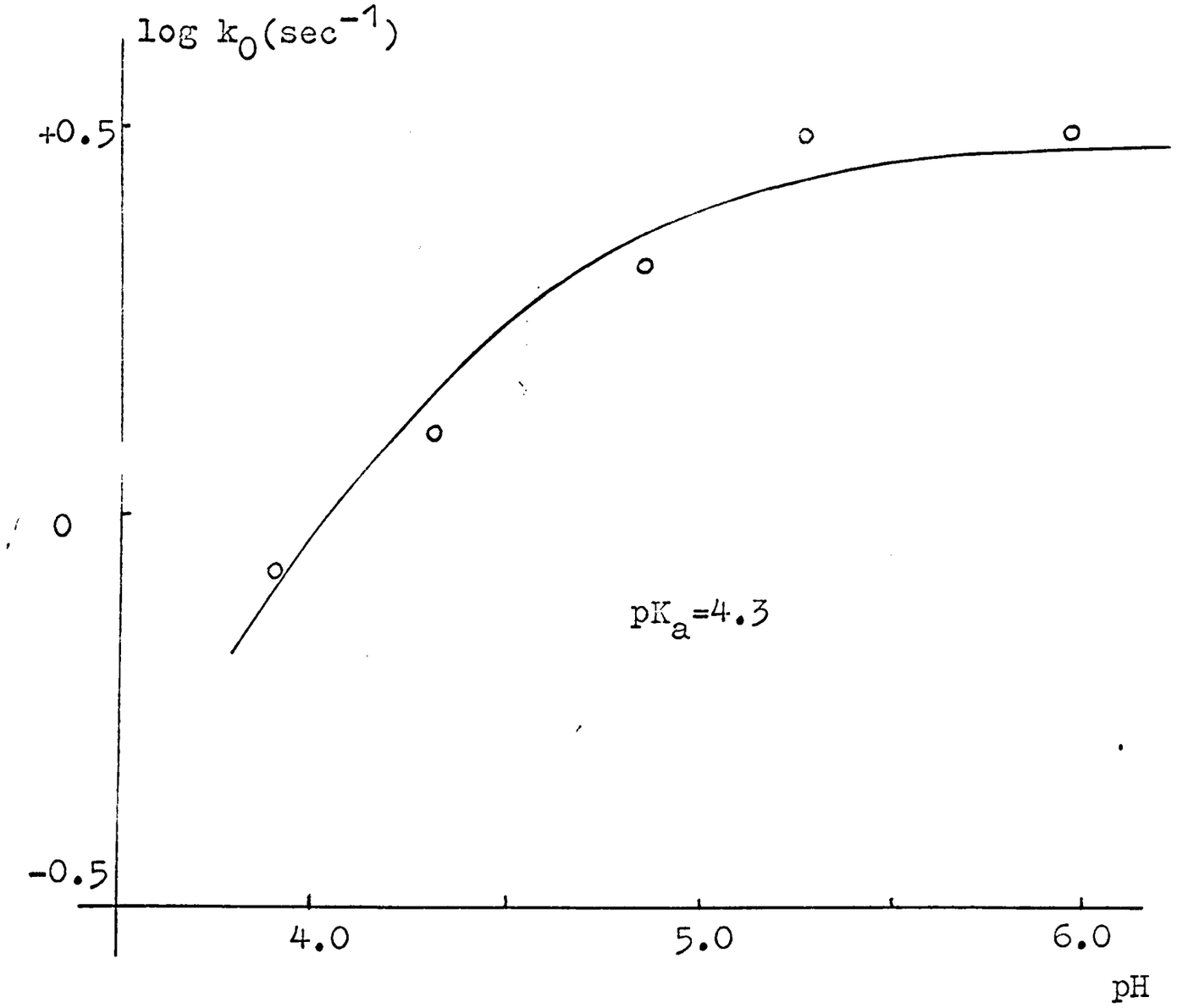



Fig 4-6

pH dependence of $k_0$ for p-nitrophenyl N-acetylglycinate; 40° μ 0.35M; phosphate-citrate buffer; 2% DMF; $10^{-3}$M EDTA; p-thiocresol-activated papain.

Table 4-13

The effect of pH on papain-catalysed hydrolysis of p-nitrophenyl hippurate. 35°; μ 0.3M; phosphate-citrate buffer; 1.9% ethanol; $10^{-3}$M EDTA; p-thiocresol-activated papain. Lineweaver-Burk plots were obtained by the total progress method.

| pH | $k_o$ ($sec^{-1}$) | $K_m$ (mM) | $k_o/K_m$ ($M^{-1}sec^{-1}$) |
|---|---|---|---|
| 5.95 | 3.00±0.10 | 0.0115±0.0005 | 260,000±4,000 |
| 5.95 | 3.40±0.10 | 0.0137±0.0005 | 248,000±5,000 |
| 5.95 | 3.80±0.17 | 0.0170±0.0008 | 224,000±4,000 |
| 5.0 | 3.38±0.06 | 0.0140±0.0003 | 241,000±4,000 |
| 5.0 | 3.35±0.16 | 0.0141±0.0007 | 237,000±6,000 |
| 5.0 | 3.00±0.24 | 0.0120±0.0010 | 250,000±10,000 |
| 4.35 | 3.12±0.13 | 0.0195±0.0008 | 160,000±2,000 |
| 4.35 | 2.95±0.10 | 0.0182±0.0006 | 165,000±2,000 |
| 4.35 | 2.75±0.15 | 0.0138±0.0008 | 198,000±7,000 |
| 3.85 | 3.04±0.14 | 0.0452±0.0023 | 65,000±1,000 |
| 3.85 | 3.55±0.43 | 0.0590±0.0071 | 61,000±2,000 |
| 3.85 | 3.02±0.10 | 0.0420±0.0014 | 63,000±1,000 |

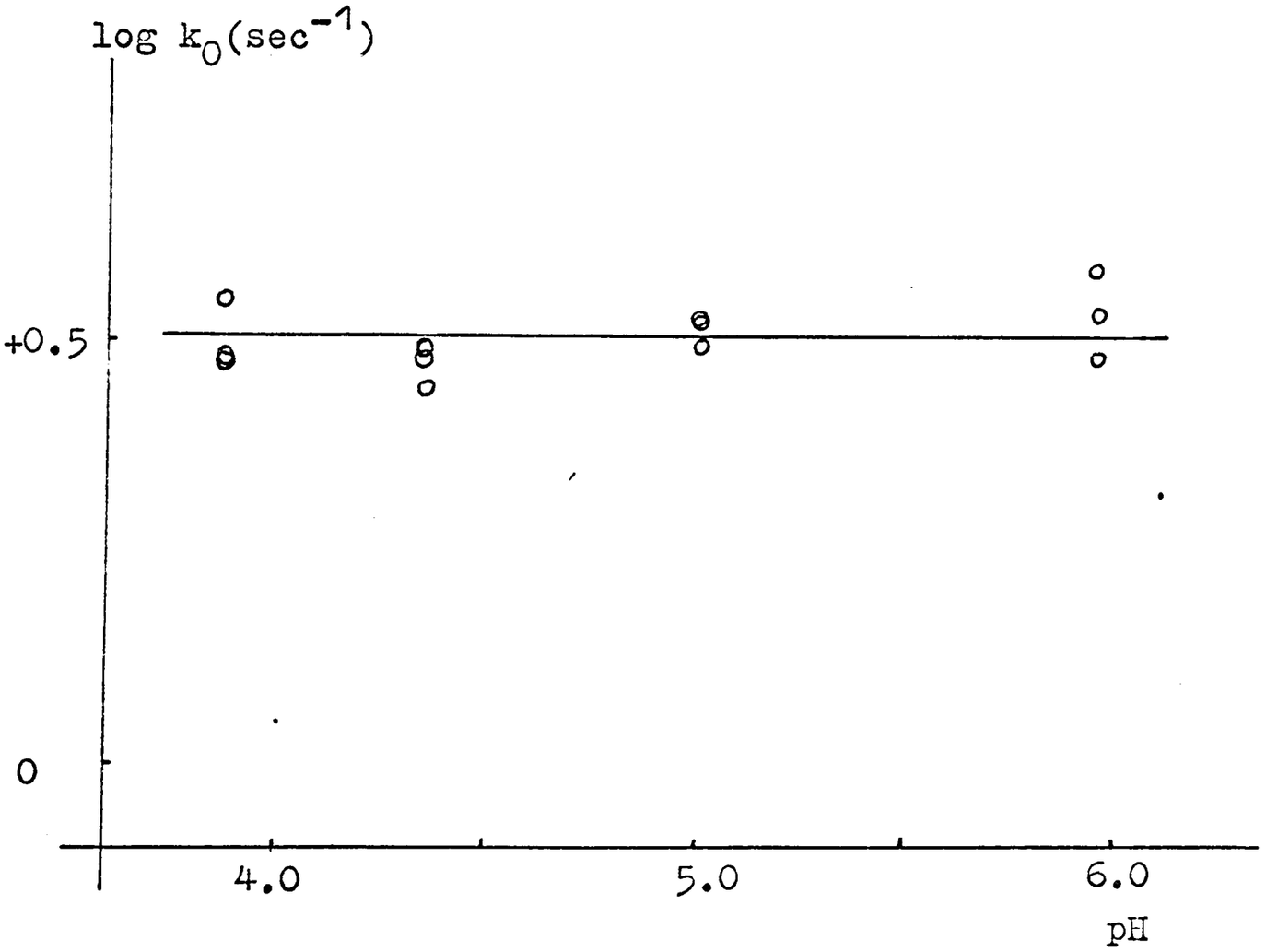



Fig. 4-7

pH dependence of $k_0$ for p-nitrophenyl hippurate; 35°; $\mu$ 0.3M; phosphate-citrate buffer; 1.9% ethanol; $10^{-3}$M EDTA; p-thiocresol-activated papain.

## Table 4-19

The effect of pH on papain-catalysed hydrolysis of p-nitrophenyl N-benzyloxycarbonylglycinate. 55°; μ 0.3M; phosphate-citrate buffer; 1.9% ethanol; $10^{-3}$M EDTA; p-thiocresol-activated papain. Lineweaver-Burk plots were obtained by the total progress method.

| pH | $k_0(sec^{-1})$ | $K_m(mM)$ | $k_0/K_m(M^{-1}sec^{-1})$ |
|---|---|---|---|
| 5.95 | 4.98±0.20 | 0.0068±0.0003 | 719,000±35,000 |
| 5.95 | 6.00±0.14 | 0.0078±0.0002 | 768,000±20,000 |
| 5.95 | 6.15±0.15 | 0.0083±0.0007 | 743,000±13,000 |
| 5.11 | 5.68±0.19 | 0.0073±0.0002 | 776,000±32,000 |
| 5.11 | 5.75±0.26 | 0.0081±0.0004 | 708,000±43,000 |
| 5.11 | 5.62±0.25 | 0.0074±0.0004 | 758,000±37,000 |
| 4.40 | 5.10±0.20 | 0.0105±0.0004 | 488,000±12,000 |
| 4.40 | 4.90±0.20 | 0.0101±0.0004 | 486,000±15,000 |
| 4.40 | 5.44±0.18 | 0.0116±0.0004 | 468,000±10,000 |
| 3.93 | 4.04±0.23 | 0.0181±0.0011 | 224,000±10,000 |
| 3.93 | 4.36±0.34 | 0.0205±0.0017 | 212,000±8,000 |
| 3.93 | 4.86±0.47 | 0.0247±0.0024 | 197,000±7,000 |
| 3.65 | 3.69±0.43 | 0.0439±0.0053 | 84,000±2,000 |
| 3.65 | 3.22±0.18 | 0.0355±0.0020 | 90,000±2,000 |
| 3.65 | 3.03±0.18 | 0.0310±0.0016 | 97,000±3,000 |

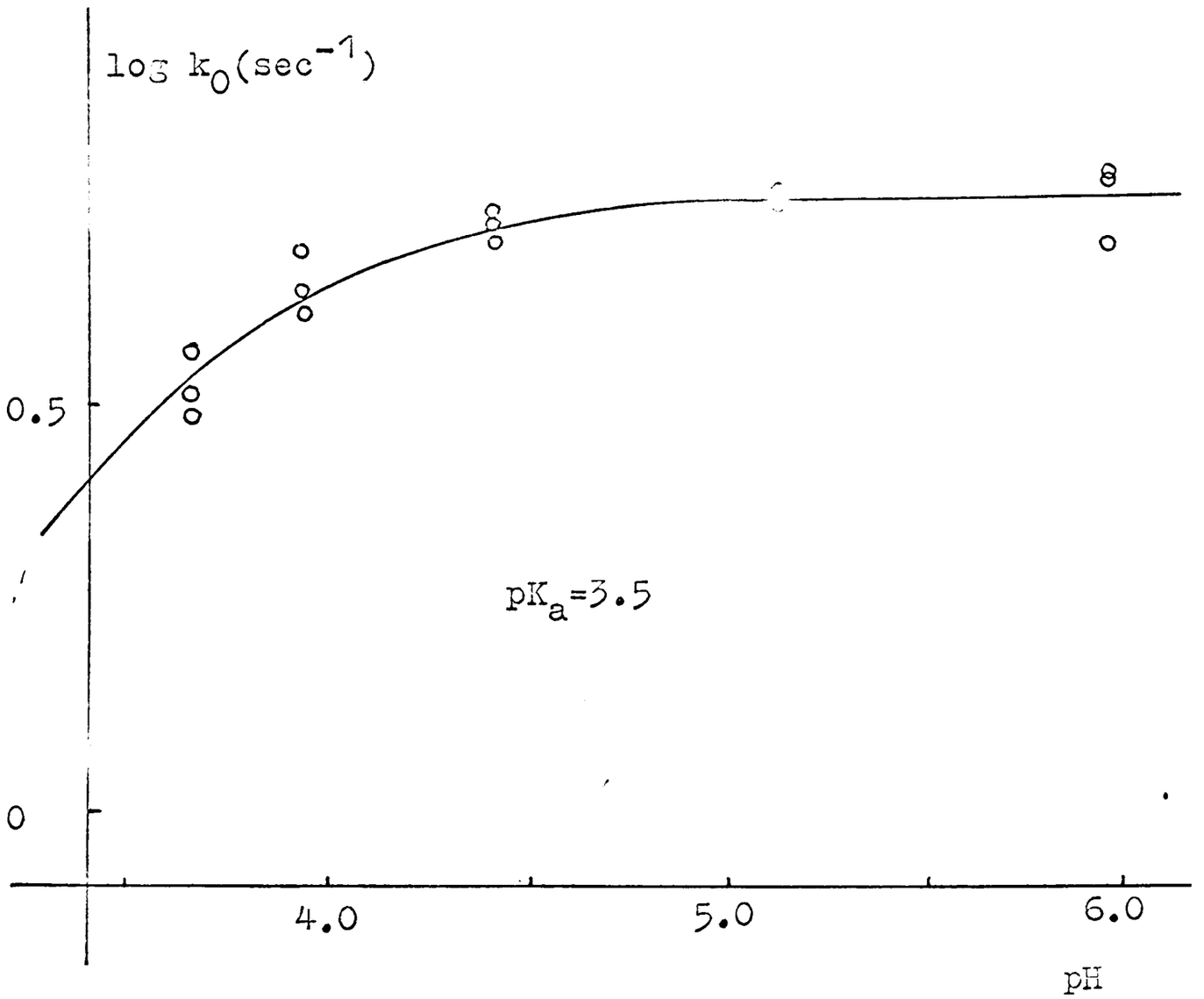


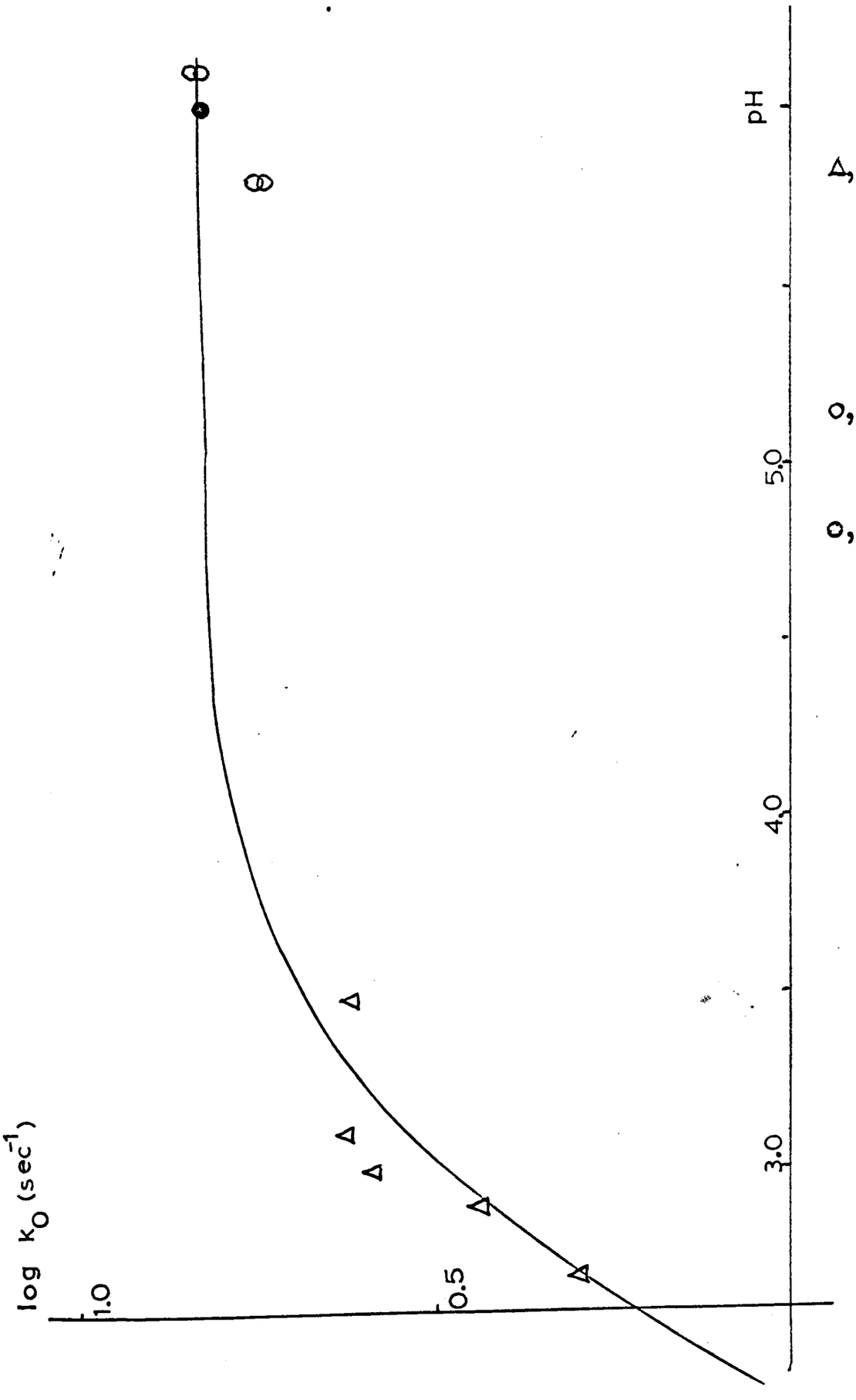

Fig. 4-8

pH dependence of $k_0$ for p-nitrophenyl N-benzyloxycarbonyl-glycinate; 35°; μ 0.3M; phosphate-citrate buffer; 1.9% ethanol; $10^{-3}$M EDTA; p-thiocresol-activated papain.

## Table 4-20

The effect of pH on papain-catalysed hydrolysis of p-nitrophenyl acetyl-L-phenylalanylglycinate. 35°; μ 0.3M; phosphate-citrate buffer; p-thiocresol-activated papain. (a) Lineweaver-Burk plots were obtained by the total progress method; (b) by the initial rate method. (c) Hydrolysis was performed under $V_{max}$ conditions. In all determinations 2% DMF was present, except for (d), where 10% DMF was present.

| pH | $k_0(sec^{-1})$ | $10^4 K_m(mM)$ | $10^7 k_0/K_m(M^{-1}sec^{-1})$ |
|---|---|---|---|
| 6.1[a] | 6.88±0.03 | 3.07 | 2.21 |
| 6.1[a] | 6.66±0.07 | 5.12 | 1.30 |
| 6.0[c,d] | 6.6 | - | - |
| 5.8[a] | 5.50±0.25 | 3.13 | 1.76 |
| 5.8[a] | 5.57±0.02 | 3.51 | 1.59 |
| 3.48[b] | 4.15±0.12 | 10.2 | 0.41 |
| 3.1[b] | 4.24±0.34 | 42.9 | 0.103 |
| 3.0[b] | 3.81±0.16 | 37.2 | 0.102 |
| 2.9[b] | 2.73±0.07 | 28.5 | 0.10 |
| 2.7[b] | 1.99±0.34 | 65 | 0.03 |




Δ, the value was obtained by the initial rate method;

o, the total progress method;

o, hydrolysis was performed under $V_{max}$ conditions. For Δ and o, 2% DMF was present; for ●, 10% DMF.

Fig.4-9 pH dependence of $k_O$ for p-nitrophenyl acetyl-L-phenylalanylglycinate; 35°;μ0.3M; phosphate-citrate buffer.

Table 4-21

The effect of pH on papain-catalysed hydrolysis of p-nitrophenyl α-N-acetyl-DL-tryptophanate. 35°; μ 0.35M; phosphate-citrate buffer; 2% DMF; $10^{-3}$ M EDTA; p-thiocresol-activated papain. The Lineweaver-Burk plots were obtained by the total progress method. Each value is an average of 3 - 4 determinations.

| pH | $k_0$ ($sec^{-1}$) | $K_m$ (mM) | $k_0/K_m$ ($M^{-1}sec^{-1}$) |
|---|---|---|---|
| 6.0 | 2.27±0.3 | 0.128 | 17,700 |
| 5.5 | 2.21±0.3 | 0.153 | 14,400 |
| 5.0 | 2.21±0.2 | 0.159 | 13,900 |
| 4.7 | 1.59±0.3 | 0.126 | 12,600 |
| 4.3 | 1.34±0.1 | 0.149 | 9,000 |
| 4.03 | 0.69±0.1 | 0.086 | 8,000 |
| 3.7 | 0.235±0.01 | 0.042 | 5,600 |

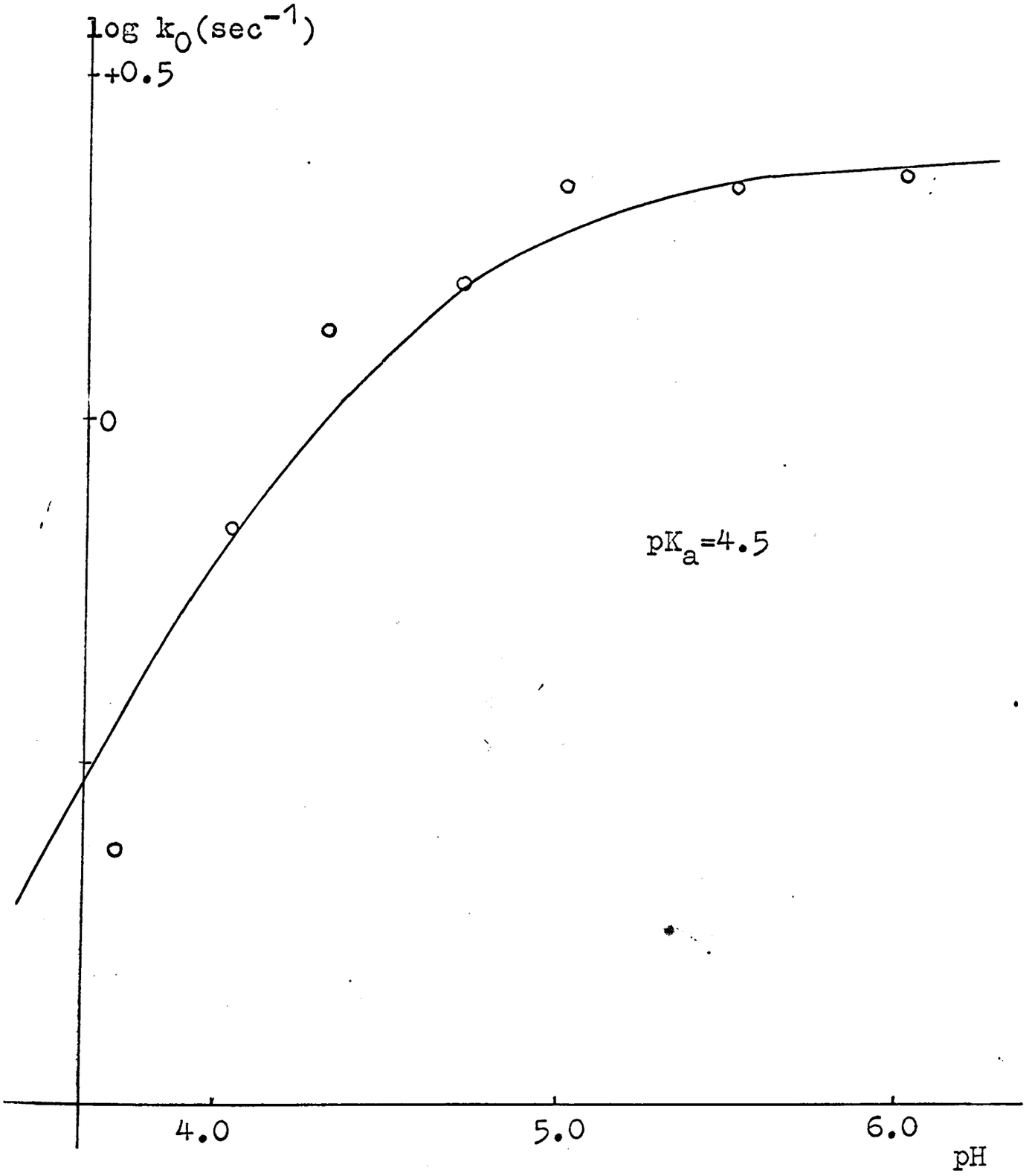



Fig.4-10

pH dependence of $k_0$ for p-nitrophenyl α-N-acetyl-DL-tryptophanate; 35°; μ 0.35M; phosphate-citrate buffer; 2% DMF; $10^{-3}$M EDTA; p-thiocresol-activated papain.

Table 4-22

Apparent $pK_1$ values of the prototropic group of the enzyme essential to the deacylation step.

| Substrate | Temperature | Maximum values of $k_0$ ($sec^{-1}$) | Maximum values of $k_0/K_m$ ($M^{-1}sec^{-1}$) | $pK_1$ of $k_0$ |
|---|---|---|---|---|
| p-Nitrophenyl α-N-acetyl-DL-tryptophanate | 35° | 2.50 | 17,000 | 4.5[a] |
| p-Nitrophenyl N-acetyl-glycinate | 40° | 3.1 | 3,500 | 4.3[b] |
| p-Nitrophenyl hippurate | 35° | 3.2[c] | 250,000 | 3.8[d] |
| p-Nitrophenyl N-benzyloxy-carbonylglycinate | 35° | 5.7 | 750,000 | 3.5[e] |
| p-Nitrophenyl acetyl-L-phenyl-alanylglycinate | 35° | 6.6 | $1.7 \times 10^7$ | 3.1 |

(a) A value of 4.70 at 25° for $pK_1$ was reported in ref. 27

(b) At 35°, maximum values of $k_0$ and $k_0/K_m$ were found to be 2.0 $sec^{-1}$ and 3,000 $M^{-1}sec^{-1}$ respectively; an approximate value of 4.2 for $pK_1$ was obtained. A $pK_1$ of 3.8 at 35° was reported, however, in ref. 90.

(c) $k_0$ is slightly higher in 1.9%-ethanol solutions than in aqueous acetonitrile solutions ($k_0$=2.5 $sec^{-1}$).

(d) $k_0$ was found to be independent of pH in the acid range down to pH 3.5 in ref. 59.

(e) A value of 3.85 at 25° was reported in ref. 86. However, a value of 3.4 at 35° was reported in ref. 90.

pH Dependence of Deacylation

In agreement with results of other workers,[27,58,62,86] it has been found that the deacylation rate is independent of pH in the alkaline pH region. $k_0$ for methyl acetyl-L-phenylalanylglycinate is constant with change in pH up to pH 8.9 (Table 4-16). The observed decrease in $k_0$ at pH 9.5 may be due to experimental errors, denaturation of the enzyme or the acylation rate becoming rate-determining.

pH dependence of deacylation in the acid pH region is, in sharp contrast with that of acylation, sensitive to the nature of the acyl moiety. Kinetic parameters for deacylation of acyl-papains as functions of pH, with p-nitrophenyl N-acetylglycinate, hippurate, N-benzyloxycarbonylglycinate, acetyl-L-phenylalanylglycinate and α-N-acetyl-DL-tryptophanate as substrates are given in Tables 4-17 - 4-21 and Figs. 4-6 - 4-10 respectively. The apparent $pK_1$ values for deacylation are given in Table 4-22. It is notable that as the acyl portion of the substrate in the $\rho_1$ site becomes more hydrophobic or specific, $pK_1$ tends to become lower. Hence the deacylation step in papain-catalysed hydrolysis of p-nitrophenyl α-N-acetyl-DL-tryptophanate, in which it was found that only the L-enantiomer was hydrolysed, depends on

a group with $pK_a$ 4.5, while deacylation of acetyl-L-phenylalanylglycyl-papain depends on a group with $pK_a$ 3.1.

The influence of the acyl moiety on $pK_1$ may be explained in terms of shielding of the essential basic group, so that it becomes less accessible to protons. The efficiency of shielding depends on hydrophobicity of the portion of the acyl group in the $\rho_1$ site as well as the nature of its interactions with the enzyme. Apart from possible steric effects, shielding by hydrophobic groups also results in decrease in local dielectric constant, which tends to depress the $pK_a$ of a cationic acid such as the imidazolium ion, but would raise the $pK_a$ of a neutral acid such as the carboxyl group, as Bateman[89] has shown from experiments with model systems. The shielding effect may be regarded as further evidence against participation of a carboxyl group in the deacylation step. An alternative explanation to shielding is that an equilibrium is formed between the acylthiol-papain and another intermediate of a comparable concentration, the breakdown of which is rate-determining; the apparent $pK_a$ will then be lower than the real $pK_a$ to an extent which depends on the position of the equilibrium. This intermediate cannot be the acylimidazole-papain, since it should be

detectable if it existed, but might another conformer of the acylthiol-enzyme with a lower $pK_a$. The argument involving shielding appears more attractive for its simplicity.

# CHAPTER 5

# GENERAL DISCUSSION

## Specificity

Experimental observation leads to a tentative generalisation that specificity of papain is better measured in terms of acylation rates than substrate constants or deacylation rates. With a few exceptions deacylation rates are typically 1 - 10 $sec^{-1}$ regardless of the nature of the substrates, and their entropies and enthalpies of activation are similar. Substrate constants for specific substrates have been found to be in the range of 1 - 200 mM, and although the values tend to be smaller for more specific substrates they cannot be regarded as reliable indicators of specificity. That the specificity of an enzyme is often manifested in the reaction rate after a substrate has been bound rather than in strength of binding has already been noted by many authors including Jencks[107]. Examples can be found in the behaviour of liver esterase and α-chymotrypsin towards substituted phenyl esters $CH_3(CH_2)_nCOOC_6H_4COO^-$, increase in chain length being accompanied by larger maximal rates but relatively little change in Michaelis constants.[108]

The nature of binding is of course important since it determines the mode in which the enzyme and the substrate molecules are relatively oriented for subsequent reactions, but this is not necessarily reflected in the magnitude of the substrate constant. On the one hand, there may be several binding modes, not all of which leading to catalytic activity, and the apparent substrate constant will be artificially small. On the other hand, the strength of binding for the productive mode may be poor because a part of the free energy of enzyme-substrate interactions is utilised to bring the complex to an active state. Reactivity of the enzyme-substrate complex originates from either 'entropic strain' or 'enthalpic strain' or both. It is unfortunately hazardous to estimate the amounts of the two types of strain by comparing the entropies and enthalpies of activation of the next reaction step of two substrates different in activity, since other unrelated factors such as solvation energies are also involved.

For substrates of the same type such as anilides, if it is assumed that other factors remain constant, some conclusion on the nature of binding may, it was expected, be drawn from the study of Arrhenius parameters of the

acylation step. It was found that both the entropy and enthalpy of activation for the acylation step of acetyl-L-phenylalanylglycine p-chloroanilide were more favourable than those of hippuryl p-chloroanilide and p-bromoanilide.

The experimental results on papain can be explained by several theories of specificity. The theory of 'induced fit', proposed by Koshland,[109] assumes that the enzyme conformation is flexible, and binding of a substrate induces a conformation, probably energetically unfavourable, in which the catalytic groups have been brought into proper positions for rate enhancement. A specific substrate induces a good 'fit' between the catalytic groups and the susceptible bond, that is, an orientation similar to that in the transition state. The large acylation rates for specific substrates of papain is expected to arise mainly from favourable entropy of activation, although a contribution from enthalpy of activation is no doubt possible. The 'rack' theory, proposed by Eyring and Lumry[110] and closely similar to an earlier suggestion by Haldane[111], states that the enzyme-substrate complex is made reactive by a strain in bond angles and bond lengths. Reactivity is expected to result from a decrease in enthalpy of activation, compared to the complex formed from a less

specific substrate. It is possible, however, that by being bound on the protein 'rack' the substrate is strained into a conformation resembling that in the transition state, and a contribution to reactivity comes from favourable entropy of activation. Another theory somewhat similar to this aspect of the 'rack' theory was proposed by Bender and Kézdy, and explains the reactivity of a specific substrate of α-chymotrypsin as the result of restricted rotation around single bonds of the substrate in the enzyme-substrate complex.[112]

It is possible that more than one theory of specificity are applicable to the case of papain, but unlikely that all of them are of comparable importance. The following observations can be explained in terms of a strain theory:

1) Methoxycarbonyl-L-phenylalanylaminoacetonitrile is an excellent competitive inhibitor ($K_i$=0.0018mM, 35°), the binding with the enzyme of which being 500 - 2,400 times more favourable than acetyl-L-phenylalanylglycine anilides.

2) The binding of hippuronitrile ($K_i$=0.46mM, 35°), also a good competitive inhibitor, is only 6 - 120 times more favourable than hippuryl anilides.

3) Methoxycarbonyl-L-phenylalanylaminoacetonitrile is a far better inhibitor than hippuronitrile, and yet the binding of acetyl-L-phenylalanylglycine anilides is comparable in

strength with that of hippuryl anilides.

4) The acylation rates for acetyl-L-phenylalanylglycine anilides are approximately 1,000 times larger than the corresponding rates for hippuryl anilides. It appears that reduction in activation energies for the former must arise at least partly from utilisation of their greater inherent binding capacity.

5) The binding of a specific substrate hippurylamide[56] ($K_s$= 202mM) is very much poorer than hippuryl thioamide ($K_i$=2.3mM) and hippuronitrile, although the sizes of the groups in the $\rho_3$ site are similar for all three compounds. (The strain theory cannot explain the poor activity of methoxycarbonyl-L-phenylalanylglycine thioamide, however, since its $K_s$(0.61mM) is much larger than that of the corresponding nitrile.)

6) $K_i$ for undissociated acetyl-L-phenylalanylglycine has been found to be $\geqslant$5mM from an observation that at pH 3.1 $k_0$ and $K_m$ for its p-nitrophenyl ester are not affected by its presence (5.2mM). According to the strain theory, this relatively large value of $K_i$ implies that the acid is activated in the enzyme active site in a manner similar to other acyl derivatives. This prediction agrees well with results from $^{18}O$ exchange studies[67], which indicate that acylamino acids behave as 'virtual' substrates for papain.

It is proposed that the anilide, amide or carboxyl group

undergoing hydrolysis is detrimental to binding because a part of the free energy of binding must be utilised to induce a strain in it or in the portion of the enzyme in its vicinity. No satisfactory explanation of these results can be derived from the 'induced fit' theory, which claims that conformational distortion occurs only to bring the catalytic groups into proper positions. The anilide groups should be capable of more interactions with the enzyme, and hence promote better binding, than the nitrile group, should there be no strain in the induced conformation. Additional evidence is needed, however, before any theory can be accepted or discarded.

## Deduction of a Plausible Mechanism of Action of Papain

The existence of covalent acyl-enzyme intermediates suggests a symmetry in the reaction path of papain-catalysed hydrolyses. If the substrate and the product can be treated as equivalent chemical entities - and the fact that papain catalyses transpeptidation, aminolysis, alcoholysis and other analogous reactions suggests that such is the case - then by the principle of microscopic reversibility the mechanism of deacylation is necessarily the exact reverse

of that of acylation. If there are multiple mechanisms for deacylation, this will be true also for acylation, and the major pathways for the two steps may not be the same. Consequently the observed mechanism for acylation is not necessarily the reverse of that of deacylation. It has been shown that there is a possibility of alternative pathways in the deacylation of hippuryl-papain.

Important features in the observed mechanism of acylation are nucleophilic attack of the active thiol group on the carbonyl carbon of the substrate, and general acid catalysis for departure of the leaving group by a functional group of the enzyme, probably the imidazolium ion previously engaged in hydrogen bonding of the thiol-imidazole system. The exact sequence of the reaction steps is not known. Although pretransition-state protonation of the leaving group followed by nucleophilic attack of the thiolate ion, or simultaneous occurrence of the two processes, cannot both be ruled out, a plausible suggestion is that general acid catalysis occurs after formation of the tetrahedral intermediate. The microscopic reverse of general acid catalysis is general base catalysis. Therefore eqn. (5-1) can be formulated:

$$\text{Tetrahedral intermediate (T)} \underset{\text{general base catalysis}}{\overset{\text{general acid catalysis}}{\rightleftharpoons}} \text{Acyl-enzyme} \qquad (5\text{-}1)$$

It is reasonable to assume that the Michaelis complex and the acyl-enzyme are mechanistically equivalent, both being acyl derivatives. Eqn. (5-2) is then derived:

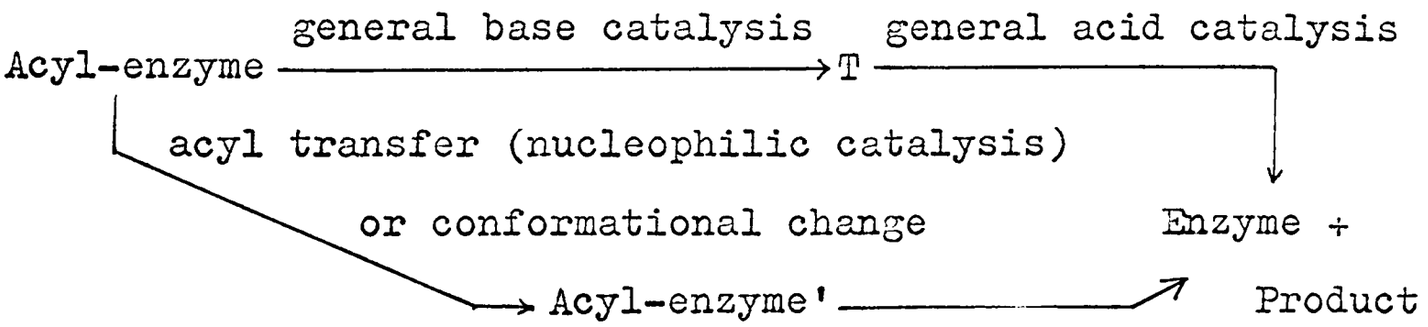
$$\text{Michaelis complex} \underset{\text{general acid catalysis}}{\overset{\text{general base catalysis}}{\rightleftharpoons}} \text{(T)} \underset{\text{general base catalysis}}{\overset{\text{general acid catalysis}}{\rightleftharpoons}} \text{Acyl-enzyme} \qquad (5\text{-}2)$$

In the acylation reaction, when the leaving group is an amine or an aniline, general-acid-catalysed breakdown of the tetrahedral intermediate is the slowest step, but when the leaving group is an alcohol or a phenol, formation of the intermediate through general base catalysis becomes rate-determining.

Eqn. (5-2) is valid for both acylation and deacylation, the Michaelis complex for the former being the enzyme-substrate complex, and for the latter, the enzyme-acid product complex. The subsequent ionisation of the acid at neutral pH is virtually irreversible and drives the hydrolysis to completion. Experiments with added nucleophiles show that the slowest step in deacylation is the nucleophilic attack of water or the nucleophiles on the acyl-enzyme,

presumably to form another tetrahedral intermediate. From eqn. (5-2) it can be deduced that this step must be general-base-catalysed. Independent evidence that the deacylation step involves general base catalysis has been given by other workers.[62]

In order to explain all experimental results on deacylation in the presence of added nucleophiles, another pathway has been incorporated for hippuryl-papain and possibly for other acyl-papains as well: a rate-determining acyl transfer or conformational change followed by rapid hydrolysis which may of course be assisted by general base, general acid or other types of catalysis. The deacylation scheme as set in (5-3) violates the principle of simplicity of mechanism, and requires support from further results.




(5-3)

Effective catalysis must depend to a considerable

extent on orientation of the substrate molecule with respect to the essential groups of the enzyme. $pK_1$(4.2) and $pK_2$ (8.3) of the free enzyme are not much affected by binding of acetyl-L-phenylalanylglycine p-nitroanilide. The enzyme catalytic groups are therefore not much perturbed by the presence of this substrate and probably other neutral substrates in the active site. If these groups are imidazole of a histidine and thiol of a cysteine, then any mode of substrate binding which would result in destruction of the hydrogen bond, such as that represented in Fig. 5-1, would be expected to result in substantial perturbation of pK values.

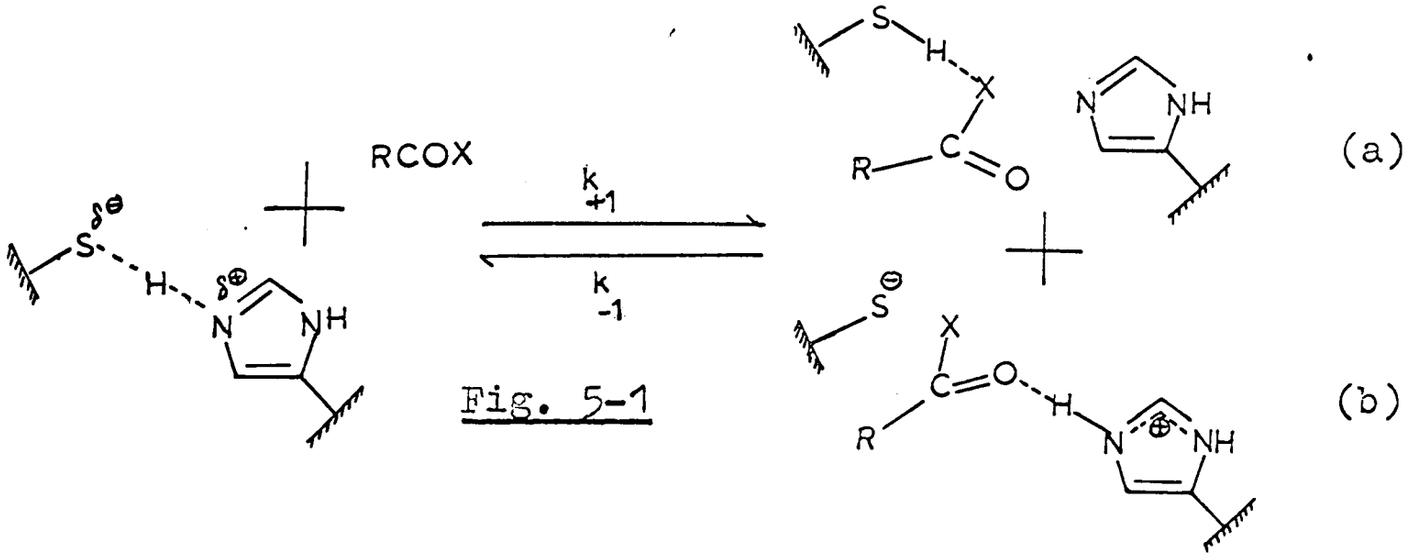



Fig. 5-1

There are other criticisms for this mode of binding. In (a) the role of imidazole in catalysis is not apparent. A mechanism involving (b) is kinetically indistinguishable from one involving neutral imidazole and neutral thiol, or a hydrogen-bonded thiol-imidazole system. Starting with (b)

a possible mechanism can be envisaged (Fig. 5-2). In this

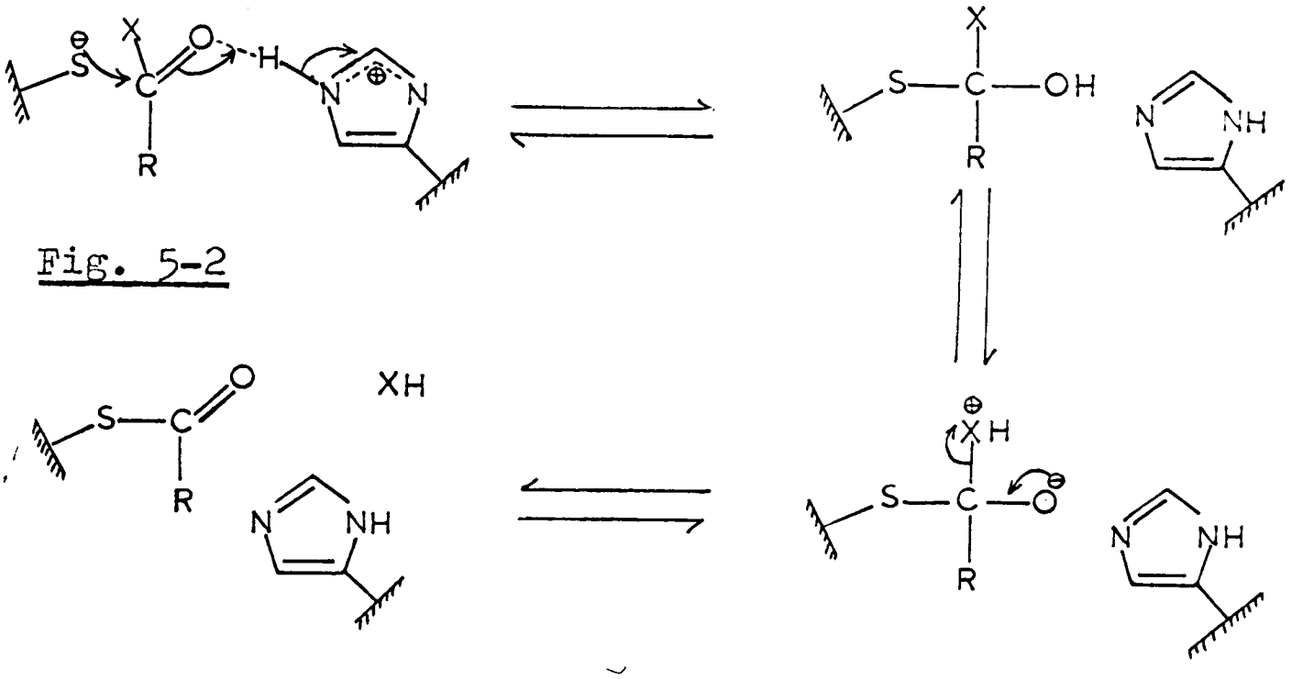


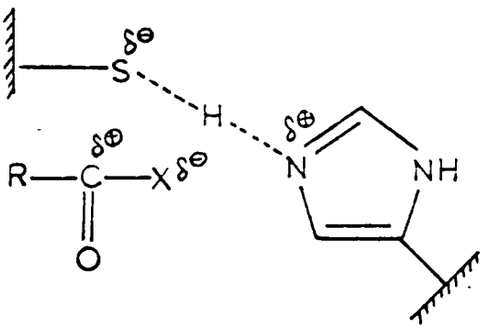
Fig. 5-2

mechanism it is necessary to postulate existence of more than one tetrahedral intermediate, and rapid establishment of equilibrium between them. Another mechanism can be speculated in which nucleophilic attack of the thiolate ion is followed by departure of the leaving group, probably general-acid-catalysed by the imidazolium ion. Unlike the mechanism in Fig. 5-2, in this mechanism there is no 'push-pull' effect which might explain the efficiency of the catalytic groups which are present in exceedingly minute quantities at neutral pH.

Another mode of binding, analogous to that suggested

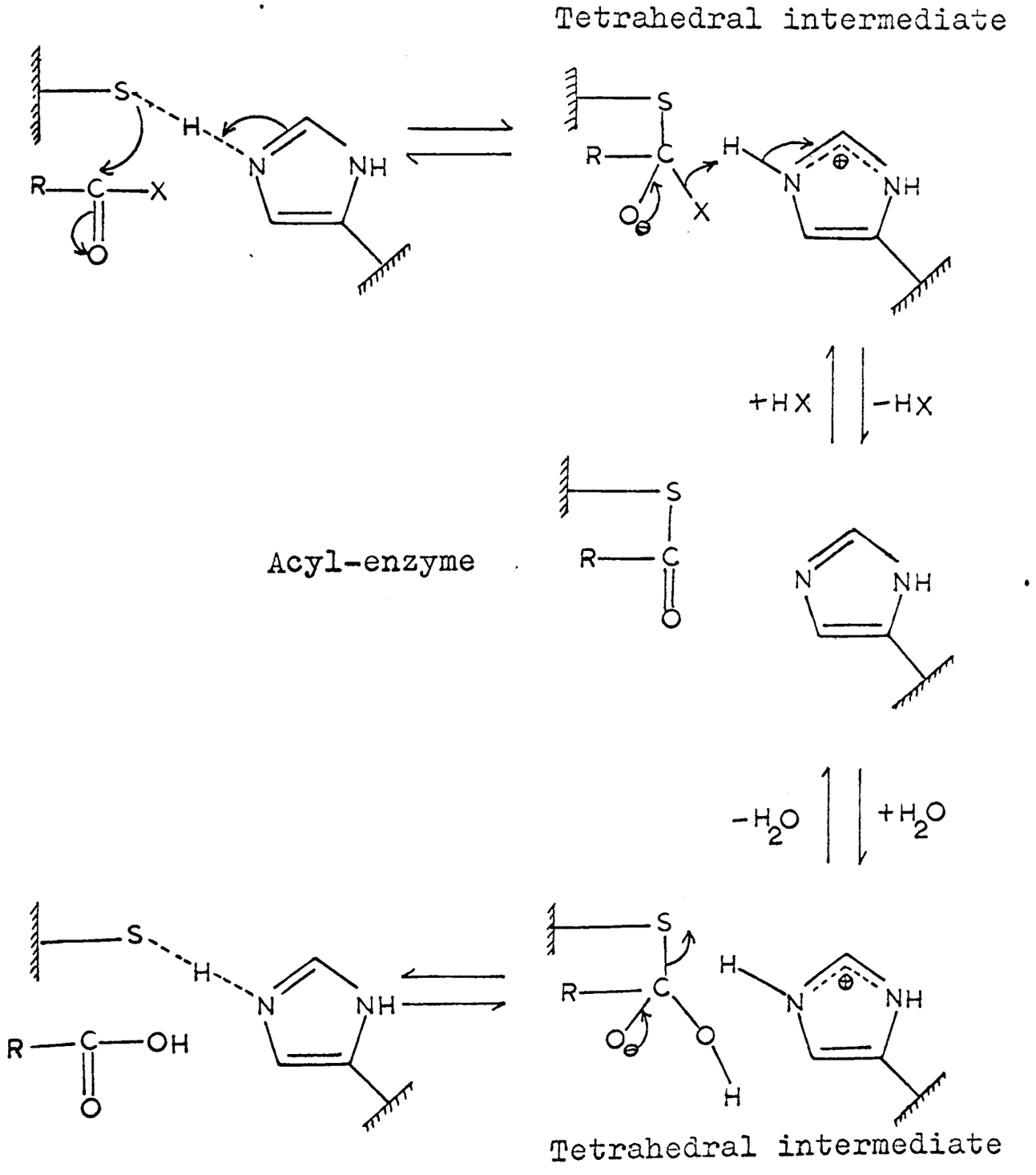
by Wang and Parker[106] for α-chymotrypsin, is shown in Fig. 5-3. The hydrogen bond of the thiol-imidazole system is preserved and can play a crucial role in the acylation step.

Fig. 5-3




A mechanism compatible with experimental evidence, and based on plausible catalytic properties of the thiol-imidazole system is proposed in Fig. 5-4.

Fig. 5-4


Tetrahedral intermediate
+HX
-HX
Acyl-enzyme
-H2O
+H2O
Tetrahedral intermediate

# CHAPTER 6

# EXPERIMENTAL

Melting points were determined on a Kofler block or with a Gallenkamp melting point apparatus and are uncorrected.

Microanalyses were performed by Dr. F. B. Strauss and Dr. G. Weiler.

Infrared spectra were recorded on a Unicam SP. 200 infrared spectrophotometer or a Perkin-Elmer 257 spectrophotometer.

Ultraviolet spectra were recorded on a Unicam SP. 800 spectrophotometer or a Cary 14 M spectrophotometer.

Thin layer chromatography was performed with Kieselgel plates which fluoresce at 254 mμ and/or 366 mμ. The spots were detected either by fluorescence or by developing the plates in an iodine tank. Chromedia SG 31 silica gel was used for column chromatography.

## Materials

Sodium chloride, disodium hydrogen phosphate, citric

acid, boric acid and ammonium sulphate were AnalaR grade reagents obtained from British Drug Houses Ltd., Poole,Dorset.

Acetonitrile was dried over phosphorus pentoxide and redistilled over another fresh quantity of the same material, b.p. 81°.

Dimethylformamide was redistilled over dried magnesium sulphate under reduced pressure, b.p. 55°/15mm.

Ethyl hippurate. This was prepared as described by Franzen.[113] Ethyl glycinate hydrochloride (20 g, 0.14M) and benzoyl chloride (22 g, 0.17M) were refluxed together in benzene (100 ml) for 20 hr. The solution was filtered, and benzene was evaporated off *in vacuo*. On cooling and scratching the residue gave a solid mass which was filtered off and collected; crude yield, 29 g (100%). The solid was recrystallised from ether-petrol ether, m.p. 57 - 59° (lit.[113] m.p. 60.5°). The crude product was used for a subsequent synthesis.

Methyl hippurate. Hippuric acid (36 g, 0.2M) was dissolved in dry methanol (400 ml). Conc. sulphuric acid

(20 ml) was slowly added and the mixture, protected from atmospheric moisture, was refluxed gently for 24 hr. Methanol was distilled off, leaving a syrup (approximately 100 ml). This was neutralised by carefully adding sat. potassium bicarbonate solution. A white precipitate appeared which was filtered off and kept. The filtrate was extracted with ether (800 ml). After being dried over anhydrous sodium sulphate, the ether solution was evaporated to dryness in vacuo. The solid residue and the first crop were combined; crude yield, 28 g (72%). The compound was recrystallised from methanol-water; m.p. 81 - 83° (lit.[114] m.p. 80 - 81.5°). Found: C, 62.2%; H, 5.8%; N, 7.8%. Calc. for $C_{10}H_{11}NO_3$: C, 62.1%; H, 5.75%; N, 7.75%.

p-Nitrophenyl hippurate. This was prepared as described by Lowe and Williams.[57] Hippuric acid (9.0 g, 0.05M) and p-nitrophenol (7.0 g, 0.05M) were mixed and treated with phosphoryl chloride (10 g, 6 ml). The mixture was warmed in a water bath until effervescence had subsided and a brown solution was obtained. This was cooled in an ice bath, and water (75 ml) was carefully added. The oily layer solidified after a vigorous stirring. The solid was re-crystallised from methanol; yield, 4.5 g (30%); m.p. 171 -

172.5° (lit.[57] m.p. 170 - 171°). $\lambda_{max}^{EtOH}$ 225 mμ (ε 12,500), 268 mμ (ε 8,600); $\nu_{max}$(nujol) 3280 (NH), 1790 (COO) and 1655 $cm^{-1}$ (CONH). Found: C, 60.0%; H, 3.8%; N, 9.55%. Calc. for $C_{15}H_{12}N_2O_5$: C, 60.0%; H, 4.0%; N, 9.3%.

Hippuryl hydrazide. This was prepared as described by Curtius.[115] A solution of ethyl hippurate (21.5 g, 0.10M) in ethanol (50 ml) was added drop by drop, over a period of 1 hr, into hydrazine hydrate (5.3 g, 0.11M) which was stirred and warmed in a water bath. The mixture was refluxed for 1 hr. A crystalline solid was obtained on cooling to room temperature and after 2 hr was filtered off; yield, 14 g, (72%). The crude material was used for a subsequent synthesis.

Hippuryl anilide. Hippuryl hydrazide (2 g, 0.01M) was dissolved in 2N hydrochloric acid (5 ml) at 0°. A solution of sodium nitrite (0.8 g, 0.01M) in water (5 ml) was added drop by drop to the stirred hydrazide solution, temperature being kept below 10°. A white precipitate of hippuryl azide was formed which after about 5 min was filtered off quickly and suspended in ether (50 ml) at 0°. Aniline (1 g, 1 ml, 0.01M) was dissolved in ether (10 ml) and the solution added drop by drop to the cold, stirred suspension of hippuryl azide. Stirring was continued overnight, the solution being

allowed to warm gradually to room temperature. A white precipitate was collected; crude yield, 1.6 g (65%). Recrystallisations from acetone gave platelets of m.p. 211 - 213° (lit.[116] m.p. 212.5°); $\lambda_{max}^{EtOH}$ 285 mμ (ε 1,700), 232 mμ (ε 8,500); $\nu_{max}$(nujol) 1690 (CONHC$_6$H$_5$), 1650 cm$^{-1}$(C$_6$H$_5$CONH). Found: C, 71.1%; H 5.6%; N, 11.1%. Calc. for $C_{15}H_{14}N_2O_2$: C, 70.8%; H, 5.55%; N, 11.0%.

Hippuryl p-anisidide. Powdered hippuric acid (3.6 g, 0.02M) was suspended in methylene chloride (10 ml) in a three-necked flask. Triethylamine (2.8 ml, 2 g, 0.02M) was added, and the clear solution formed was stirred and cooled to about -10°, the mixture being protected from atmospheric moisture. A solution of ethyl chloroformate (2 ml, 2.2 g, 0.02M) in methylene chloride (10 ml) was added dropwise over 15 min. After another 15 min a solution of p-anisidine (2.5 g, 0.02M) in methylene chloride (15 ml) was added dropwise over 30 min to the stirred solution maintained at -5°. Stirring was continued for 1 hr, after which the white precipitate of hippuryl p-anisidide was filtered off, washed with sat. sodium bicarbonate solution and water, and recrystallised from acetone-water; yield, 3 g (50%); m.p. 210 - 212°; $\lambda_{max}^{EtOH}$ 249 mμ (ε 20,000); $\nu_{max}$(nujol) 3370 (NH),

1730 ($\underline{CO}NHC_6H_4\underline{p}$-$OCH_3$), 1690 ($C_6H_5\underline{CO}NH$), 880 $cm^{-1}$($\gamma$CH of $NH\underline{C_6H_4}\underline{p}$-$OCH_3$). Found: C, 68.4%; H, 5.7%; N, 9.55%. $C_{16}H_{16}N_2O_3$ requires C, 68.7%; H, 5.6%; N, 9.85%.

Hippuryl p-bromoanilide. Powdered hippuric acid (2 g, 0.011M) and phosphorus pentachloride (2.4 g, 0.011M) were stirred together in anhydrous ether (40 ml) cooled in an ice bath. A solution of p-bromoaniline (2 g, 0.013M) in anhydrous ether (25 ml) was added dropwise over 10 min. The mixture was stirred for 4 hr in the cold, and then overnight at room temperature. A white precipitate was filtered off, washed with ether, sat. sodium bicarbonate solution and water; crude yield, 1.7 g. (45%). White platelets were obtained from two recrystallisations from ethanol; m.p. 229 - 231° (lit.[117] m.p. 228 - 229°); $\lambda_{max}^{EtOH}$ 251 mμ (ε 24,400); $\nu_{max}$(nujol) 3300 (NH), 1680 ($\underline{CO}NHC_6H_4\underline{p}$-Br), 1640 ($C_6H_5\underline{CO}NH$), 840 ($\gamma$CH of $NH\underline{C_6H_4}\underline{p}$-Br). Found: C, 54.4%; H, 3.9%; Br, 24.1%; N, 8.4%. Calc. for $C_{15}H_{13}BrN_2O_2$: C, 54.1%; H, 3.9%; Br, 24.0%; N, 8.4%.

Hippuryl p-chloroanilide. This was prepared by the same method as that described for hippuryl anilide. The quantities of reagents were the same, except that p-chloroaniline (1.3 g, 0.01M) was used in place of aniline; crude

yield, 1 g (30%). The product was recrystallised from ethanol-acetone; m.p. 223 - 224° (lit.[117] m.p. 222 - 223°); $\lambda_{max}^{EtOH}$ 249 mμ (ε 19,700); $\nu_{max}$(nujol) 3300 (NH), 1680 (CONH-$C_6H_4$p-Cl), 1640 ($C_6H_5$CONH), 840 $cm^{-1}$(γCH of $NHC_6H_4$p-Cl). Found: C, 62.4%; H, 4.8%; Cl, 12.1%; N, 9.6%. Calc. for $C_{15}H_{13}ClN_2O_2$: C, 62.2%; H, 4.5%; Cl, 12.3%; N, 9.7%.

Hippuryl p-hydroxyanilide. This was prepared by the same method as that described on p. 141 for hippuryl p-anisidide. The quantities of reagents were doubled, and p-aminophenol (4.4 g, 0.04M) was used in place of p-anisidine; crude yield of hippuryl p-hydroxyanilide, 5 g (45%). The product was recrystallised several times from acetone-water; m.p. 235 - 237°; $\lambda_{max}^{EtOH}$ 249 mμ (ε 22,900); $\nu_{max}$(nujol) 3480 (OH), 3380 (NH), 1700 (broad, CONH$C_6H_4$p-OH), 890 (γCH of $NHC_6H_4$p-OH). Found: C, 66.7%; H, 5.3%; N, 10.4%. $C_{15}H_{14}N_2O_3$ requires C 66.7%; H, 5.22%; N, 10.4%.

Hippuryl p-nitroanilide. This was prepared by the same method as that described on p. 142 for hippuryl p-bromoanilide. A solution of p-nitroaniline (1.5g, 0.011M) in anhydrous ether (75 ml) was used in place of p-bromoaniline solution. Recrystallisations of the crude product from

ethanol gave pale yellow needles of hippuryl p-nitroanilide; m.p. 248 - 250°; $\lambda_{max}$(aqueous 8% acetonitrile) 231 mμ (ε 13.300), 317 mμ (ε 10,000); $\nu_{max}$(nujol) 1705 ($\underline{CO}NHC_6H_4$-p-$NO_2$), 1635 ($C_6H_5\underline{CO}NH$), 870 $cm^{-1}$(γCH of $NH\underline{C_6H_4}$p-$NO_2$). Found: C, 60.4%; H, 4.3%; N, 14.0%. $C_{15}H_{13}N_3O_4$ requires C, 60.2%; H, 4.4%; N, 14.05%.

Hippuronitrile. This was a kind gift from E. D. Sinclair.

p-Nitrophenyl N-acetylglycinate. This was a kind gift from E. C. Lucas.

N-Acetylglycine p-nitroanilide. This was prepared by the same method as that described on p. 142 for hippuryl p-bromoanilide; crude yield of N-acetylglycine p-nitroanilide, 30%. The product was recrystallised three times from ethanol; m.p. 262 - 264°; $\lambda_{max}^{EtOH}$ 222 (ε 5,100), 313 mμ (ε 7,200); $\nu_{max}$(nujol) 1690 ($\underline{CO}NHC_6H_4$p-$NO_2$), 1650 ($CH_3\underline{CO}NH$), 860 $cm^{-1}$(γCH of $NH\underline{C_6H_4}$p-$NO_2$). Found: C, 50.6%; H, 4.8%; N, 17.9%. $C_{10}H_{11}N_3O_4$ requires C, 50.7%; H, 4.7%; N, 17.7%.

α-N-benzoyl-DL-arginine p-nitroanilide. This was purchased from Sigma (London) Chemical Co.

p-Nitrophenyl N-benzyloxycarbonylglycinate. This was purchased from Sigma (London) Chemical Co.

Methyl N-benzyloxycarbonylglycinate. Methyl glycinate hydrochloride (25.1 g, 0.20M) was dissolved in water (25 ml) and the solution was brought to pH 8 by addition of solid sodium carbonate. This was cooled in an ice bath and stirred while benzyl chloroformate (30 ml, 36.0 g, 0.21M) and sat. sodium carbonate solution (about 60 ml) were added separately over 20 min, pH being maintained at 8. After 1 hr of stirring the mixture was extracted three times with ether (3x100 ml). The combined ether extract was dried over dried magnesium sulphate. A colourless oil obtained after evaporation of the ether was subjected to column chromatography (silica gel, petrol ether-ether (4:1), t.l.c. $R_f$ 0.043); yield,39 g (90%). Attempts failed to obtain the compound in the crystalline form (lit.[118] m.p. 24.5 - 25.5°). $\nu_{max}$ (liquid film) 3340 (NH), 1745 (COOCH$_3$), 1715, 1705 cm$^{-1}$ (OCONH). Found: C, 59.0%; H, 6.1%; N, 6.2%. Calc. for $C_{11}H_{13}NO_4$: C, 59.2%; H, 6.0%; N, 6.3%.

O-Benzoylglycollic acid. This was prepared as described by Strecker and Sokoloff.[119] Hippuric acid (15 g, 0.08M)

was dissolved in conc. nitric acid (40 ml). Nitric oxide produced from the action dil. nitric acid on copper (15 g) was passed through the solution for 4 hr. The green solution obtained was left overnight. A white precipitate appeared when the solution was diluted with water (80 ml). This was filtered off, washed with water, dried and recrystallised from petrol ether; m.p. 109 - 112° (lit.[119] m.p. 112°); yield, 5 g (30%); $\nu_{max}$(chloroform) 1720 $cm^{-1}$(COOH). N.m.r. spectrum shows a singlet at τ-1.13 (1H, COOH), a multiplet at τ1.9 (2 H, o-protons), a multiplet at τ2.5 (3 H, m- and p-protons) and a singlet at τ5.1 (2 H, $OCH_2COOH$). Mass spectrum shows peaks at m/e 180 (parent peak), 135 ($C_6H_5$-$COOCH_2$), 105 ($C_6H_5CO$) and 77 ($C_6H_5$). Found: C, 59.8%; H, 4.5%. Calc. for $C_9H_8O_4$: C, 60.0%; H, 4.4%.

Methyl O-benzoylglycollate. Diazomethane was prepared by adding, in portions, N-nitrosomethylurea (2.0 g) to a cooled mixture of 50% aqueous potassium hydroxide (6 ml) and ether (20 ml). The ether layer was dried over solid potassium hydroxide and maintained at 0°. O-Benzoylglycollic acid (0.45 g, 2.5mM) was dissolved in dry methanol (5 ml). The ethereal diazomethane solution was added in small portions until the yellow colour persisted. A few drops of

acetic acid were added to destroy excess of diazomethane. The solution was concentrated *in vacuo*, and the *methyl O-benzoylglycollate* obtained as a syrup was purified by column chromatography (silica gel, 10% acetic acid in chloroform, t.l.c. $R_f$ 0.7); yield, 0.4 g (80%); attempts failed to obtain the compound in a crystalline form. Found: C, 61.6%; H, 5.4%. $C_{10}H_{10}O_4$ requires C, 61.9%; H, 5.15%.

*p-Nitrophenyl O-benzoylglycollate*. O-Benzoylglycollic acid (0.9 g, 5mM) and p-nitrophenol (0.7 g, 5mM) were dissolved in ethyl acetate (5 ml) and the solution was cooled in an ice bath. N,N'-Dicyclohexylcarbodiimide (1.1 g, 5mM) was added and the mixture was stirred for 2 hr, the solution being allowed to warm gradually to room temperature. After a filtration, the filtrate was evaporated *in vacuo*. The yellow residue *p-nitrophenyl O-benzoylglycollate* was recrystallised from benzene, and then ethanol; m.p. 101 - 103°; yield, 0.9 g (60%); $\lambda_{max}^{EtOH}$ 230 mμ (ε 16,200), 269 mμ (8,900); $\nu_{max}$(nujol) 1780 ($\underline{C}OOC_6H_4p\text{-}NO_2$), 1730 $cm^{-1}$($C_6H_5\underline{C}OO$). Found: C, 60.0%; H, 3.45%; N, 4.9%. $C_{15}H_{11}NO_6$ requires C, 59.8%; H, 3.7%; N, 4.7%.

*O-Benzoylglycollyl p-anisidide*. This was prepared by

the same method as that described for hippuryl p-anisidide on p. 141. O-Benzoylglycollic acid (0.89 g, 5mM) and equivalent amounts of p-anisidine and other reagents were used; crude yield of O-benzoylglycollyl p-anisidide, 1.25 g. (88%). The product was recrystallised from acetone-ether-petrol ether; m.p. 140 - 140.5°; $\lambda_{max}^{EtOH}$ 239 mμ (ε 17,000, a shoulder at 248 mμ); $\nu_{max}$(nujol) 3270 (NH), 1740 ($C_6H_5\underline{COO}$), 1670 ($\underline{CONHC_6H_4}$p-$OCH_3$), 830 $cm^{-1}$(γCH of $NH\underline{C_6H_4}$p-$OCH_3$). Found: C, 67.5%; H, 5.43%; N, 5.1%. $C_{16}H_{15}NO_4$ requires C, 67.4%; H, 5.3%; N, 4.9%.

3-Benzoylpropionic acid. This was prepared as described by Somerville and Allen.[120] Succinic anhydride (34 g, 0.34M) and benzene (175 g, 2.25M) were placed in a three-necked flask fitted with a mechanical stirrer and two reflux condensers. Crushed, dry aluminium chloride (100 g, 0.75M) was added to the stirred mixture in one portion. The stirred mixture was warmed in a water bath for 30 min, a gentle reflux being maintained. After cooling to room temperature, water (150 ml) was added slowly to the stirred mixture. Excess of benzene was removed by steam distillation. The remaining hot solution was poured into a one-litre beaker, and supernatant liquid was decanted when the mixture had cooled to room temperature. Conc hydrochloric acid (50 ml)

was added and the white precipitate formed was filtered off, washed with water, and recrystallised from benzene; m.p. 113 - 116° (lit.[120] m.p. 116°).

p-Nitrophenyl 3-benzoylpropionate. 3-Benzoylpropionic acid (3.56 g, 0.02M) and triethylamine (2.8 ml, 2.0 g, 0.02M) were dissolved in chloroform (20 ml) and cooled to -5°. Ethyl chloroformate (2.0 ml, 2.2 g, 0.02M) was added dropwise over 10 min to the stirred solution. A solution of p-nitrophenol (2.8 g, 0.02M) and triethylamine (2.8 ml, 2.0 g, 0.02M) in chloroform (10 ml) was added dropwise over another 10 min. The mixture was stirred in the cold for another 30 min, at room temperature for 2 hr, and at 40° for 15 min. The solution was washed with dil. hydrochloric acid (50 ml), sat. sodium carbonate solution (50 ml) and water, and dried over anhydrous sodium sulphate. Plateletn of p-nitrophenyl 3-benzoylpropionate was obtained on addition of petrol ether; yield, 2.4 g (40%). The compound was recrystallised from acetone-water, and from benzene; m.p. 124 - 126°(decomp.); $\lambda_{max}^{EtOH}$ 244 mμ (ε 15,800), 270 mμ (ε 10,400); $\nu_{max}$(nujol) 1770 ($\underline{C}OOC_6H_4$p-$NO_2$), 1670 ($C_6H_5\underline{C}OCH_2$), 865 $cm^{-1}$(shoulder, γCH of $OC_6H_4$p-$NO_2$). Found: C, 64.2%; H, 4.3%; N, 4.7%. $C_{16}H_{13}NO_5$ requires C, 64.25%; H, 4.35%; N, 4.7%.

3-Benzoylpropionyl p-anisidide. This was prepared by the same method as that described for hippuryl p-anisidide on p. 141. 3-Benzoylpropionic acid (0.88g, 5mM) and equivalent amounts of p-anisidine (0.61 g) and other reagents were used; crude yield of 3-benzoylpropionyl p-anisidide, 1.39 g (98%). The product was recrystallised from acetone-petrol ether; m.p. 125 - 126.5°; $\lambda_{max}^{EtOH}$ 248 mμ (ε 22,000); $\nu_{max}$(nujol) 3300 (NH), 1685 ($\underline{CO}NHC_6H_4\underline{p}$-$OCH_3$), 1660 ($C_6H_5\underline{CO}NH$), 840 $cm^{-1}$ ($\gamma$CH of $NH\underline{C_6H_4}\underline{p}$-$OCH_3$). Found: C, 72.0%; H, 6.1%; N, 5.0%. $C_{17}H_{17}NO_3$ requires C, 72.0%; H, 6.0%; N, 4.9%.

S-Benzoyl-2-thiolacetic acid. Thiobenzoic acid (14 g, 0.1M) and bromoacetic acid (14 g, 0.1M) were mixed, and sat. potassium carbonate solution was added until effervescence ceased. The mixture was left at room temperature for 1 hr, and acidified with dil. hydrochloric acid. A white precipitate was filtered off, dissolved in acetone, and dried over anhydrous sodium sulphate. Acetone was evaporated off *in vacuo*, and the product was recrystallised from benzene-petrol ether; m.p.105 - 107°(lit.[121] m.p. 105.5 - 106°). Found: C, 55.2%; H, 4.2%; S, 16.0%. Calc. for $C_9H_8O_3S$: C, 55.1%; H, 4.1%; S, 16.3%.

p-Nitrophenyl S-benzoyl-2-thiolacetate. S-Benzoyl-2-

thiolacetic acid (0.4 g, 2 mM) and p-nitrophenol (0.28 g, 2mM) were dissolved in ethyl acetate (2 ml). A solution of N,N'-dicyclohexylcarbodiimide (0.42 g, 2mM) in ethyl acetate (2 ml) was added, and the mixture was stirred for 5 min. The precipitate was filtered off and the filtrate was concentrated *in vacuo*. The residue, p-nitrophenyl S-benzoyl-2-thiolacetate, crystallised from benzene-petrol ether; yield, 0.5 g (75%); m.p. 89 - 91°; $\lambda_{max}^{EtOH}$ 267 mμ (ε 7,800, a shoulder at 247 mμ); $\nu_{max}$(nujol) 1760 ($\underline{CO}OC_6H_4p\text{-}NO_2$), 1660 $cm^{-1}$($C_6H_5\underline{CO}S$). Found: C, 56.7%; H, 3.5%; N, 4.4%; S, 10.25%. $C_{15}H_{11}NO_5S$ requires C, 56.7%; H, 3.5%; N, 4.4%; S, 10.1%.

Methyl N-benzylglycinate hydrochloride. Methyl N-benzylglycinate was prepared by a similar method to that of King and McMillan,[122] except that methyl bromoacetate was used in place of methyl chloroacetate, resulting in less formation of by-products. Benzylamine (11.0 ml, 10.7 g, 0.05M) was added to a solution of methyl bromoacetate (4.6 ml, 7.6 g, 0.05M) in methanol (10 ml). The solution heated up spontaneously and was left for 1 hr to cool to room temperature. Ether (100 ml) was added, and the mixture was filtered. The filtrate was evaporated *in vacuo*, and the

remaining syrup was purified by column chromatography (10% deactivated silica gel, ether, t.l.c. $R_f$ 0.42). A slightly yellow liquid product was obtained; yield, 4.0 g (40%). A portion (2.1 g) of the liquid was dissolved in 2N hydrochloric acid (6 ml) and evaporated to dryness *in vacuo*. The remaining hydrochloride was recrystallised several times from methanol-acetone; m.p. 144 - 145°; $\nu_{max}$(chloroform) 1730 (COO), 2700 $cm^{-1}$($NH_2^+$). Found: C, 55.6%; H, 6.2%; Cl, 16.4%; N, 6.55%. $C_{10}H_{14}ClNO_2$ requires C, 55.7%; H, 6.5%; Cl, 16.5%; N, 6.5%. The ester is unstable at room temperature, gradually forming a diketopiperazine ($\nu_{max}$ 1660 $cm^{-1}$) over a few days, and must therefore be stored as a hydrochloride.

p-Nitrophenyl 4-phenylbutyrate. 4-Phenylbutyric acid (1.64 g, 0.01M, Koch-LightLtd.) and p-nitrophenol (1.4 g, 0.01M) were dissolved in ethyl acetate (30 ml) at 0°. N,N'-Dicyclohexylcarbodiimide (2.1 g, 0.01M) was added and the mixture was stirred overnight, the solution being allowed to warm up gradually to room temperature. The precipitate was filtered off and the filtrate was concentrated *in vacuo*. The residue, after recrystallisations from ether-petrol ether, gave platelets of p-nitrophenyl 4-phenylbutyrate; yield,

2.1 g (73%); m.p. 43 - 44°; $\lambda_{max}^{EtOH}$ 269 mμ (ε 7,200); $\nu_{max}$ (nujol) 1760 $cm^{-1}$(COO$C_6H_4$p-$NO_2$). Found: C, 67.35%; H, 5.5%; N, 4.6%. $C_{16}H_{15}NO_4$ requires C, 67.4%; H, 5.3%; N, 4.9%.

4-Phenylbutyryl p-anisidide. This was prepared by the same method as that described on p. 141 for hippuryl p-anisidide. 4-Phenylbutyric acid (1.64 g, 0.01M, Koch-Light Co.) and equilvalent amounts of p-anisidine (1.2 g) and other reagents were used; crude yield of 4-phenylbutyryl p-anisidide, 2.45 g (92%). The compound was recrystallised from methanol-water; m.p. 97 - 98°; $\lambda_{max}^{EtOH}$ 251 mμ (ε 13,400); $\nu_{max}$(nujol) 3280 (NH), 1655 $cm^{-1}$(CONH$C_6H_4$p-$OCH_3$). Found: C, 75.6%; H, 7.3%; N, 5.25%. $C_{17}H_{19}NO_2$ requires C, 75.8%; H, 7.1%; N, 5.2%.

Methyl N-thiobenzoylglycinate. This was prepared as described by Kurzer.[123] A solution of methyl glycinate hydrochloride (1.26 g, 0.01M) in 2N sodium hydroxide (5 ml, 0.01M) was mixed with a solution of S-thiobenzoyl-2-thiolacetic acid (2.12 g, 0.01M) in 2N sodium hydroxide(5 ml, 0.01M). After 1 hr the mixture yielded a brown oil which solidified when the flask was scratched. The solid was filtered off, taken up in a minimum of benzene, and purified by column

chromatography (silica gel, ether, t.l.c. $R_f$ 0.6). The purified product was recrystallised as yellow needles from benzene-hexane; yield, 0.6 g (28%); m.p. 68 - 69°( lit.[123] m.p. 68°); $\lambda_{max}^{EtOH}$ 242 mμ (ε 8,700), 288 mμ (ε 5,700); $\nu_{max}$ (chloroform) 3400 (NH), 1740 $cm^{-1}$(COO). Found: C, 57.4%; H, 5.2%; N, 6.6%; S, 15.45%. Calc. for $C_{10}H_{11}NO_2S$: C, 57.4%; H, 5.3%; N, 6.7%; S, 15.3%.

p-Nitrophenyl α-N-acetyl-DL-tryptophanate. α-N-acetyl-DL-tryptophan (2.46 g, 0.01M, Sigma (London) Chemical Co.) and p-nitrophenol (1.39 g, 0.01M) were mixed in ethyl acetate (40 ml) at room temperature. N,N'-dicyclohexylcarbodiimide (2.06 g, 0.01M) was added and the mixture was stirred for 5 hr. The precipitate was filtered off and the filtrate was evaporated *in vacuo*. The syrupy residue was dissolved in acetone (5 ml) and decolorised by active charcoal. The product was recrystallised several times from acetone-ether-petrol ether; yield, 1.3 g (35%); m.p. 132 - 133° (lit.[124] m.p. 136 - 137°); t.l.c. shows the product to be homogeneous, $R_f$(silica gel, ether) 0.12; $\nu_{max}$(nujol) 3290 (NH), 1780 (COO), 1620 (shoulder, CONH). Found: C, 62.0%; H, 4.9%; N, 11.4%. Calc. for $C_{19}H_{17}N_3O_5$: C, 62.1%; H, 4.7%; N, 11.4%.

N-Benzyloxycarbonyl-L-phenylalanine. A sample of this

compound was a kind gift from P. Greenwell. L-phenylalanine (100 g, 0.605M) was dissolved in 4N sodium hydroxide solution (150 ml, 0.605M) and the mixture was stirred and cooled in an ice bath. Benzyl chloroformate (86 ml, 103 g, 0.605M) and 4N sodium hydroxide solution (130 ml, 0.52M) were added in portions over 30 min, the mixture being kept always slightly alkaline to phenolphthalein. After a further 30 min, excess of benzyl chloroformate was removed by extraction with ether (200 ml). The product solution was acidified by slowly adding to a vigorously stirred 10N sulphuric acid (200 ml). After about 1 hr the solid formed was filtered off and washed with water. This was then taken up in ethyl acetate (400 ml) and dried over dried magnesium sulphate. After evaporation of the solution to dryness *in vacuo*, the product was recrystallised from benzene-hexane; yield, 123 g (69%); m.p. 84 - 87°(lit.[125] m.p. 88 - 89°).

Methyl benzyloxycarbonyl-L-phenylalanylglycinate. N-Benzyloxycarbonyl-L-phenylalanine (30 g, 0.1M) was dissolved in chloroform (200 ml) and cooled to -5°. Methyl glycinate hydrochloride (12.5 g, 0.1M) and triethylamine (10 g, 0.1M) were dissolved in chloroform (200 ml) and cooled to -5°. N,N'-Dicyclohexylcarbodiimide (20.6 g, 0.1M) was added in

four portions at intervals of about 5 min to the stirred solution of N-benzyloxycarbonyl-L-phenylalanine, the solution of methyl glycinate and triethylamine being added after the addition of the first portion of the carbodiimide. The mixture was stirred overnight, the solution being allowed to warm gradually to room temperature. The mixture was filtered and the filtrate was washed with N/2 hydrochloric acid (40 ml) and water (40 ml) and dried over dried magnesium sulphate. Evaporation to dryness yielded a white solid residue; yield, 36.5 g (99%). The solid was recrystallised from ethyl acetate-ether; m.p. 119° (lit.[126] m.p. 122 - 123°)

Methyl L-phenylalanylglycinate hydrobromide. Methyl benzyloxycarbonyl-L-phenylalanylglycinate (25 g, 67.5mM) was dissolved in a sat. solution of hydrogen bromide in glacial acetic acid (70 ml). After 30 min, when evolution of carbon dioxide had subsided, ether (700 ml) was added. A syrup obtained under the ether layer was stirred vigorously until it hardened. Supernatant ether was discarded, another portion of ether (700 ml) was added, and the process was repeated until a solid product was obtained; yield, 21.6 g (100%). The crude material melted gradually above 100° and

decomposed at 180° (Cornish-Bowden reported complete melting at 135° for the compound obtained by the same method.[127]) $\nu_{max}$(nujol) 3310 ($NH_3^+$), 1745 (COO) and 1675 $cm^{-1}$(CONH).

Methyl acetyl-L-phenylalanylglycinate. Methyl L-phenylalanylglycinate hydrobromide (15.15 g, 50 mM) was suspended in chloroform (250 ml) and the mixture was cooled in an ice bath with stirring. Triethylamine ( 7.0 ml, 5.1 g, 50 mM) was added, and a solution of acetic anhydride (4.7 ml, 5.1 g, 50 mM) in chloroform (50 ml) was then added dropwise over 30 min. After stirring overnight, the mixture was washed with N/2 hydrochloric acid (40 ml) and water (40 ml) and dried over dried magnesium sulphate. After the solvent was removed *in vacuo*, the product was recrystallised from acetone-ether; crude yield, 9.2 g (66%); m.p. 141 - 142° (after four recrystallisations); $[\alpha]_D^{20}$ -12.6°(*c* 1 in dimethylformamide). Determann *et al*. reported m.p. 139°; $[\alpha]_D^{22}$ -14.5° (*c* 10 in dimethylformamide).[128] $\nu_{max}$(nujol) 3305, 3290 (NH), 1755 (COO), 1640 $cm^{-1}$(CONH). Found: C, 60.4%; H, 6.4%; N, 10.3%. Calc. for $C_{14}H_{18}N_2O_4$: C, 60.4%; H, 6.5%; N, 10.1%.

Acetyl L-phenylalanylglycine. Methyl acetyl-L-phenyl-

alanylglycinate (8.3 g, 30mM) was dissolved in warm acetone (250 ml). N sodium hydroxide (30 ml) was added in parts over 15 min, a small amount of water being added in order to prevent formation of two phases. After standing for 2 hr at room temperature, the mixture was slightly alkaline. Acetone was evaporated off *in vacuo*, and the aqueous residue was acidified with conc. hydrochloric acid to pH 5. The solution was concentrated *in vacuo* until about 20 ml remained. A white precipitate appeared which was filtered off, washed with a small amount of water, and dried in air; crude yield, 5 g (64%). The product was recrystallised from ethyl acetate; m.p. 163 - 165° (lit.[127] m.p. 164.5 - 165.5°). Found: C, 59.1%; H, 6.0%; N, 10.3%. Calc. for $C_{13}H_{16}N_2O_4$: C, 59.0%; H, 6.1%; N, 10.6%.

*p*-Nitrophenyl acetyl-L-phenylalanylglycinate. Acetyl-L-phenylalanylglycine (0.2 g, 0.76mM) was dissolved in warm ethyl acetate (30 ml). The solution was cooled in an ice bath, and *p*-nitrophenol (0.105 g, 0.76mM) and *N*,*N*'-dicyclohexylcarbodiimide (0.15 g, 0.76mM) were added successively. The mixture was stirred in the cold for 30 min, and at room temperature for 5 hr. The precipitate was filtered off and the filtrate was concentrated *in vacuo* until a yellow syrup

was left. Recrystallisations from acetone-ether yielded colourless crystals of p-nitrophenyl acetyl-L-phenylalanyl-glycinate; yield, 0.11 g (38%); m.p. 192 - 193°; $[\alpha]_D^{20}$ -9.1° (c 0.3 in dimethylformamide); $\lambda_{max}^{EtOH}$ 269 mμ (ε 8,700); $\nu_{max}$ (nujol) 3290 (NH), 1760 (COO), 1650 $cm^{-1}$(CONH). Found: C, 59.1%; H, 5.0%; N, 10.8%. $C_{19}H_{19}N_3O_6$ requires C, 59.2%; H, 5.0%; N, 10.9%.

Acetyl-L-phenylalanylglycine anilide. This was prepared by the same method as that described on p. 141 for hippuryl p-anisidide. Acetyl-L-phenylalanylglycine (0.4 g, 1.5mM) and equivalent amounts of aniline (0.14 g) and other reagents were used; crude yield of acetyl-L-phenylalanylglycine anilide, 0.29 g (57%). The product was recrystallised from acetone-ether-petrol ether and from acetone-water; m.p. 218 - 210°(at 198° the crystals softened but became solid again at a few degrees higher); $[\alpha]_{365\ mu}^{20}$ +35.6° (c 0.3 in dimethyl-formamide); $\lambda_{max}^{EtOH}$ 242 mμ (ε 13,000); $\nu_{max}$(nujol) 1670 $cm^{-1}$ (broad, CONH). Found C, 64.15%; H, 6.4%; N, 12.1%. $C_{19}H_{21}N_3O_3$ requires C, 67.2%; H, 6.25%; N, 12.4%. $C_{19}H_{21}N_3O_3.H_2O$ requires C, 63.9%; H, 6.5%; N, 11.8%.

Acetyl-L-phenylalanylglycine p-anisidide. This was

prepared by the same method as that described on p. 141 for hippuryl p-anisidide. Acetyl-L-phenylalanylglycine (0.45 g, 1.7mM) and equivalent amounts of p-anisidine (0.21 g) and other reagents were used; crude yield of acetyl-L-phenylalanylglycine p-anisidide, 0.44 g (70%). The product was recrystallised from acetone-water and acetone-ether-petrol ether; m.p. 211 - 212.5°; $[\alpha]_D^{20}$ +49.7 (c 0.3 in ethanol); $\lambda_{max}^{EtOH}$ 251 mμ (ε 11,600); $\nu_{max}$ 3310, 3280 (NH), 1640 $cm^{-1}$ (shoulder, CONH). Found: C, 65.0%; H, 6.2%; N, 11.4%. $C_{20}H_{23}N_3O_4$ requires C, 65.0%; H, 6.3%; N, 11.4%.

Acetyl-L-phenylalanylglycine p-chloroanilide. This was prepared by the same method as that described on p. 141 for hippuryl p-anisidide. Acetyl-L-phenylalanylglycine (0.4 g, 1.5mM) and equivalent amounts of p-chloroaniline (0.17 g) and other reagents were used; crude yield of acetyl-L-phenylalanylglycine p-chloroanilide, 0.28 g (50%). The product was recrystallised from acetone-water and acetone-ether-petrol ether; m.p. 234 - 235°; $[\alpha]_D^{20}$ +11.0 (c 0.3 in dimethylformamide); $\lambda_{max}^{EtOH}$ 249 mμ(ε17,100); $\nu_{max}$(nujol) 1700 (CONH$C_6H_4$p-Cl), 1630 $cm^{-1}$(shoulder, CONHCH). Found: C, 60.85%; H, 5.4%; Cl, 9.5%; N,11.1%. $C_{19}H_{20}ClN_3O_3$ requires C, 61.0%; H, 5.4%; Cl, 9.5%; N, 11.25%.

Acetyl-L-phenylalanylglycine m-nitroanilide. This was prepared by the same method as that described on p. 141 for hippuryl p-anisidide. Acetyl-L-phenylalanylglycine (0.4 g, 1.5mM) and equivalent amounts of m-nitroaniline (0.19 g) and other reagents were used; crude yield of acetyl-L-phenylalanylglycine m-nitroanilide, 0.4 g (67%). The product was recrystallised from acetone-water and acetone-ether-petrol ether; m.p. 168 - 170°; $[\alpha]_D^{20}$ +16.7°(c 0.3 in dimethylformamide); $\lambda_{max}^{EtOH}$ 241 mμ (ε 21,800, a shoulder at 272 mμ); $\nu_{max}$ (nujol) 3360 (NH), 1710 (shoulder, CONHC$_6$H$_4$m-NO$_2$), 1630 cm$^{-1}$ (shoulder, CONHCH). Found: C, 59.3%; H, 5.3%; N, 14.4%. $C_{19}H_{20}N_4O_5$ requires C, 59.3%; H, 5.25%; N, 14.6%.

Acetyl-L-phenylalanylglycine p-nitroanilide. This was prepared by the same method as that described on p, 141 for hippuryl p-anisidide. Acetyl-L-phenylalanylglycine (0.66 g, 2.5mM) and equivalent amounts of p-nitroaniline (0.35 g) and other reagents were used; crude yield of acetyl-L-phenylalanylglycine p-nitroanilide, 0.52 g (54%). The product was recrystallised several times from acetone-ether-petrol ether; m.p. 221 - 223°; $[\alpha]_D^{20}$ +117°(c 0.3 in dimethylformamide); $\lambda_{max}^{EtOH}$ 313 mμ (ε 13,300); $\nu_{max}$(nujol) 1720 (CONHC$_6$H$_4$pNO$_2$) 1670, 1650 (broad, CONHCH) cm$^{-1}$. Found: C, 59.4%; H, 5.4%; N, 14.4%. $C_{19}H_{20}N_4O_5$ requires C, 59.3%; H, 5.25%; N, 14.6%.

N-(Acetyl-L-phenylalanylglycyl)sulphanilamide. This was prepared by the same method as that described on p. 141 for hippuryl p-anisidide. Acetyl-L-phenylalanylglycine (0.4 g, 1.5mM) was used in place of hippuric acid, and a solution of sulphanilamide (0.26 g, 1.5mM) in dry ether (50 ml) was used in place of p-anisidine solution; the other reagents were used in equivalent quantities; crude yield of N-(Acetyl-L-phenylalanylglycyl)sulphanilamide, 0.49 g (78%). The product was recrystallised from methanol-water and acetone-methanol-water; m.p. 270 - 272°; $[\alpha]_D^{20}$ +10.2°(c 0.3 in dimethylformamide); $\lambda_{max}^{EtOH}$ 259 mμ (ε 18,200); $\nu_{max}$(nujol) 3320, 3300 (NH), 1700 ($\underline{CO}NHC_6H_4\underline{p}\text{-}SO_2NH_2$), 1645 $cm^{-1}$(CONHCH). Found: C, 54.4%; H, 5.2%; N, 13.6%; S, 8.0%. $C_{19}H_{22}N_4O_5S$ requires C,54.4%; H, 5.3%; N,13.4%;S,7.7%.

N-Methoxycarbonyl-L-phenylalanine. This was prepared by the same method as that described on p. 155 for N-benzyloxycarbonyl-L-phenylalanine. L-Phenylalanine (30 g, 0.2M) and methyl chloroformate (20 g, 0.21M) were used; crude yield, 34 g (76%). The product was a colourless syrup which solidified slowly; m.p. 72 - 75°. Leuchs and Geiger[129] obtained a syrup of the racemic mixture of this compound.

N-Methoxycarbonyl-L-phenylalanylaminoacetonitrile.

N-methoxycarbonyl-L-phenylalanine (10.6 g. 0.05M) and triethylamine (5.0 g, 0.05M) were dissolved in methylene chloride (100 ml). The solution was stirred at -5° while a solution of ethyl chloroformate (5.4 g, 0.05M) in methylene chloride (20 ml) was added over 15 min. After a further 15 min, a solution of aminoacetonitrile bisulphate (7.7 g, 0.05M, Koch-Light Ltd.) in 2N sodium hydroxide (50 ml), pH 7, was added over 30 min, the temperature being maintained at -5°. The mixture was stirred overnight, and allowed to warm up gradually to room temperature. The solid nitrile was filtered off; yield, 7.5 g (58%). This was recrystallised from methylene chloride; m.p. 174 - 175°; $[\alpha]^{20}_{365m\mu}$ +19.3 (c 0.5 in ethanol); $\nu_{max}$ 1670 $cm^{-1}$(broad,CONH). Found: C, 59.5%; H, 5.8%; N, 15.7%. $C_{13}H_{15}N_3O_3$ requires C, 59.8%; H, 5.8%; N, 16.1%.

N-Methoxycarbonyl-L-phenylalanylglycine thioamide.

The nitrile obtained from above (2.0 g, 7.6mM) was dissolved in a mixture of dry pyridine (5 ml) and triethylamine (3 ml). Dry hydrogen sulphide was passed through the solution at room temperature for 90 min, and the solution was kept in a closed vessel for a further 60 min. The solution was

poured into water (100 ml), and the product was extracted with chloroform (3x100 ml). The thioamide crystallised from chloroform-petrol ether; yield, 0.9 g (40%). It was purified by column chromatography (silica gel, ether, tl.c. $R_f$ 0.12) and recrystallised three times from chloroform-petrol ether; m.p. 139 - 141°; $[\alpha]_D^{20}$ +30° (c 1 in ethanol); $\lambda_{max}^{EtOH}$ 267 mμ (ε 14,000); $\nu_{max}$(chloroform) 1710 $cm^{-1}$(broad,OCONH). Found: C, 52.6%; H, 5.8%; N, 14.1%; S, 11.0%. $C_{13}H_{17}N_3O_3S$ requires C, 52.8%; H, 5.8%; N, 14.3%; S, 10.9%.

Aminoacetonitrile. This was prepared from its bisulphate by the method of Cook et al.[130] The bisulphate (40 g, 0.26M, Koch-Light Ltd.) was covered with methanol (50 ml) containing a trace of phenolphthalein. Sodium methoxide solution made by dissolving sodium wire (10.5 g, 0.46M) in methanol (300 ml) was added to the stirred bisulphate suspension. The final mixture, remaining colourless, was filtered and the filtrate was evaporated in vacuo at room temperature. The remaining liquid was distilled under reduced pressure; b.p. 56°/1-2 mm; yield, 5 g (40% based on sodium). The colourless liquid gradually turns brown on storage and must be kept under nitrogen in the cold. Found: C, 42.55%; H, 7.9%; N, 49.55%. Calc. for $C_2H_4N_2$: C, 42.8%; H, 7.2%; N, 49.55%.

Glycinamide hydrochloride. This was purchased from Koch-Light Ltd.

Methylammonium hydrochloride. This was purchased from British Drug Houses Ltd. and recrystallised once from ethanol.

## Crystalline Papain

Papain was obtained from two sources. British Drug Houses Ltd. supplied twice-recrystallised papain as a suspension in 0.05M sodium acetate solution. Papain was also obtained in this laboratory from dried papaya latex ( a gift from the Wallerstein Co., New York, U.S.A.) by the method of Kimmel and Smith[7]. Some papain was a kind gift from Dr. S. S. Husain.

Extraction of crystalline papain from dried papaya latex. The dried latex (180 g), celite (100 g) and washed sand (150 g) were ground thoroughly for 30 min with 0.04M L-cysteine solution (250 ml, made from L-cysteine hydrochloride (6.3 g, British Drug Houses Ltd.), N sodium hydroxide (54 ml) and water (946 ml)). Another portion of the L-cysteine solution (250 ml) was added and the mixture was filtered under suction, the filter paper being covered with a layer of

celite. The residue was ground with another portion of the L-cysteine solution (500 ml) and filtered. The pH of the combined filtrates was adjusted to pH 9.0 by slowly adding N sodium hydroxide (about 120 ml). The slightly green and cloudy solution was centrifuged at 4° and 2,000 r.p.m. for about 1 hr. The precipitate was discarded. Ammonium sulphate (320 g, AnalaR) was slowly added to the stirred solution, so that the final solution became 40% saturated. This was left overnight at 4°. The mixture was centrifuged at 4° for 90 min and the supernatant was discarded. The solid was washed with 40% saturated ammonium sulphate solution (400 ml) and the mixture was centrifuged at 4° for 30 min. The precipitate was dissolved in 0.02M L-cysteine solution of pH 7.3 (600 ml, made from dissolving L-cysteine hydrochloride (3.15 g) in water (1000 ml) and adjusting the pH with N sodium hydroxide). Sodium chloride (60 g, AnalaR) was added slowly to the stirred solution, which was then left overnight at 4°. The suspension was centrifuged at 4° for 1 hr. The solid was suspended in 0.02M L-cysteine solution of pH 6.5 (400 ml). The pH, slightly raised by this operation, was adjusted back to pH 6.5 with dil. hydrochloric acid. The mixture was left overnight at 4°, after which it was centrifuged for 6 hr at the same tempe-

rature and the supernatant discarded. The solid was further recrystallised as follows: It was dissolved in a minimum of water (about 300 ml), and the slightly cloudy solution was centrifuged at 4° for 30 min. Sat. sodium chloride solution (about 36 g in 100 ml of water) was added slowly (10 ml per 300 ml of the enzyme solution). The solution was left overnight, and centrifuged for 6 hr at 4°. The solid was dissolved in a minimum of water (about 200 ml) and sat. sodium chloride solution (10 ml) was added slowly. The enzyme suspension, containing about 8 mg of papain per ml of 0.3M sodium chloride solution can be stored for many months, under refrigeration, without appreciable loss of activity.

Preparation of Active Papain Solution

A. Activation by p-thiocresol.[131] Active papain solutions of about $10^{-5}$M concentration or less were obtained by this method. In a typical preparation, twice-recrystallised papain (0.1 ml of the sodium acetate suspension from British Drug Houses Ltd.) was dissolved in deionised water (8.8 ml) and the ionic strength was adjusted to 0.3M by the addition of 3M sodium chloride solution (1 ml). 0.1M EDTA (ethylenediamine tetraacetic acid disodium salt) solution (0.1 ml)

was added, so that $10^{-3}$M of this compound were present in the enzyme solution. A few crystals of p-thiocresol were added and the mixture was left for about 30 min, with occasional swirling. The p-thiocresol was extracted with toluene (10 ml), the toluene layer being separated by filtering the mixture through a filter paper (Whatman no. 1 or 42) moistened with deionised water, which retained the toluene. The extraction procedure was repeated twice. The concentration of p-thiocresol in the final preparation was estimated to be less than 10 uM using a similar procedure.[131] No loss of enzyme activity could be detected for at least 10 hr at room temperature. The solution tends however to become slightly cloudy on prolonged standing, especially at enzyme concentrations higher than $10^{-5}$M

B. Activation by L-cysteine. Active papain solutions of about $10^{-4}$M concentration were prepared by this method. Twice-recrystallised papain suspension (1.0 ml from British Drug Houses Ltd. or 3.0 ml of the enzyme prepared as above) was diluted with deionised water and the ionic strength was adjusted if necessary with 3M sodium chloride solution. The clear solution obtained should have an ionic strength less than ~0.11M. 0.1M EDTA solution (0.1 ml) was added. 0.1M

neutralised L-cysteine solution (1 ml) was then added to the enzyme solution (9 ml). The slightly cloudy solution was filtered through a filter paper (Whatman no. 42). The final preparation (10 ml), containing 0.05 - 0.1M sodium chloride, $10^{-3}$M EDTA and 0.01M L-cysteine, remained fully active for at least 24 hr. Note: For enzyme concentrations of about $10^{-4}$M the ionic strength must not exceed 0.1M, as otherwise the enzyme will be salted out.

The enzyme concentration was estimated by rate assays with methyl hippurate as the substrate and assuming kinetic data from Lowe and Williams.[57] (See Appendix 3.)

Kinetic Studies

Two general methods were employed to measure reaction rates: spectrophotometry and titrimetry.

A. Spectrophotometry. Hydrolysis of a substrate such as an anilide or an aryl ester can be followed by this method if the substrate and the products have sufficiently different ultraviolet absorption spectra. A fixed wavelength was chosen where the extinction coefficient of the substrate

is negligible, or nearly so. In a typical run a buffer solution (1.8 ml) was incubated in a 1 cm silica cell for 15 min at the required temperature in the cell compartment of a Unicam SP. 500 or SP. 800 spectrophotometer. Both instruments were capable of recording automatically the transmission at a fixed wavelength against time. A solution of the substrate in an organic solvent (0.10 ml) was added with a micropipette. After about 5 min, the stock enzyme solution (0.10 ml) was added and the change in optical transmission against time was followed. Final optical transmission was noted when the reaction was completed in a few minutes. For slow reactions, the extinction coefficients were measured independently with authentic compounds. The wavelengths chosen for following production of substituted anilines and p-nitrophenol, and their extinction coefficients, are given in Table 6-1.

B. Titrimetry. Hydrolysis of an ester substrate produces an acid, which in an unbufferred solution tends to depress the pH. If the pH is to remain constant alkali has to be added in order to neutralise the acid produced. The pH-stat is an instrument which can keep the pH constant by automatic addition of alkali or acid. In a typical run, an

## Table 6-1

Extinction coefficients of the substrates and the products at wavelengths chosen for kinetic studies. pH 6.0 (except (a), for which pH=pH 7.0); for other experimental conditions, see under appropriate sections below.

| Compound | Wavelength mμ | ε |
|---|---|---|
| Aniline | 285 | 1260 |
| | 295 | 630 |
| p-Anisidine | 305 | 1570 |
| | 315 | 560 |
| | 320 | 300 |
| p-Aminophenol | 310 | 1150 |
| pBromoaniline | 300 | 1100 |
| p-Chloroaniline | 300 | 1050 |
| m-Nitroaniline | 400 | 840 |
| Sulphanilamide | 300 | 1120 |
| p-Nitrophenol | 340 | 6400 |
| | 450 | 2000[a] |
| Hippuryl anilide | 285 | 170 |
| | 295 | 0 |
| Hippuryl p-anisidide | 305 | 100 |
| Hippuryl p-hydroxyanilide | 310 | 30 |
| Hippuryl p-bromoanilide | 300 | 0 |
| Hippuryl p-chloroanilide | 300 | 0 |
| Hippuryl p-nitroanilide | 410 | 0 |
| APG anilide | 295 | 0 |
| APG p-anisidide | 315 | 0 |
| | 320 | 0 |
| APG p-chloroanilide | 300 | 70 |
| APG m-nitroanilide | 400 | 30 |
| APG p-nitroanilide | 450 | 0 |
| APG sulphanilamide | 300 | 0 |
| p-Nitrophenyl esters | 340 | 0 |
| | 450 | 0[a] |

unbuffered solution containing the substrate (5 ml) was equilibrated to the required temperature in a thermostat-controlled cell of a pH-stat (main components: a Radiometer titrator type TTT 1C, a titrigraph recorder type SBR 2C, and a syringe burette type SBU 1a, delivering 0.5 ml of alkali or acid at full scale deflection of the recorder, all purchased from Radiometer Ltd., Copenhagen). An enzyme solution (0.5 ml) was added, and the reaction rate was determined from a recorded plot of 0.02N sodium hydroxide added against time to maintain the pH of the stirred solution at a previously fixed value.

Experimental Conditions and Evaluation of Results

In all kinetic studies deionised or distilled water was used and all solutions had $10^{-3}$M EDTA, in order to remove traces of heavy metal ions, and 0.05 - 0.3M ionic strength (sodium chloride or buffer ions), the enzyme activity being independent of the ionic strength in this range[13]. Except for the experiments performed to determine the Arrhenius parameters, hydrolyses were carried out at 35°. Except for the experiments performed to determine the pH profiles, the solutions had pH 6.0 or 7.0, both in the optimum pH range.

McIlvaine's (citric acid-disodium hydrogen phosphate) buffer[132] was used for the pH range 2.5 - 8.0 and Clark and Lub's (boric acid-sodium hydroxide) buffer[132] was used for the pH range 8.0 - 9.2 in spectrophotometric studies.

A KDF 9 digital computer (Leo-Marconi) calculated by means of a least squares programme (FADAB 01) the values of the intercepts and slopes of the Lineweaver-Burk plots, from which the Michaelis-Menten parameters could be determined (see Appendix 1).

Papain-catalysed hydrolyses of hippuryl anilides and analogues. These were studied by spectrophotometry. Buffer solutions of ionic strength 0.05 - 0.1M (McIlvaine's buffer diluted 3 - 5 times) and containing 20% dimethylformamide were used. Papain was activated by 0.01M L-cysteine. Under the usual conditions, pH 6.0, 35°, there was no non-enzymatic hydrolysis. (S)= 0.1 - $1 \times 10^{-2}$M. (E)= 0.5 - $1.5 \times 10^{-4}$M. 6 - 10 points were used for each Lineweaver-Burk plot. The points were determined from initial rates at various substrate concentrations.

Papain-catalysed hydrolyses of acetyl-L-phenylalanyl-glycine anilides. These were studied by spectrophotometry.

The solutions were McIlvaine's (pH 3.7 - 8) or Clark and Lubs' (pH 8 - 9.3) buffers containing sodium chloride, total ionic strength 0.3M, and 10% dimethylformamide. Papain was activated by 0.01M cysteine. Under the experimental conditions there was no detectable non-enzymatic hydrolysis. (S)= 0.1 - $1 \times 10^{-2}$M. (E)= $10^{-5}$M. About 5 points were used for each Lineweaver-Burk plot. These were obtained either from initial rates at different substrate concentrations or from the total progress curve of a single run (see Appendix 1). The results from the two methods were found not to be substantially different if the rates were measured within 3 min of addition of the enzyme. The rates after 3 min were sometimes found to be smaller than expected from Lineweaver-Burk plots obtained from initial rates, indicating a possibility of either back reaction or product inhibition. Ultraviolet spectra of the products were found to be identical with authentic substituted anilines.

Papain-catalysed hydrolyses of p-nitrophenyl esters.

These were studied by spectrophotometry. McIlvaine's buffer of ionic strength 0.05 - 0.35M were used. Experimental conditions for variuos substrates are summarised in Table 6-2. Unless otherwise stated, hydrolyses were carried out at pH 6

Table 6-2

Some experimental conditions for papain-catalysed hydrolyses of p-nitrophenyl esters.

| p-Nitrophenyl ester | Organic solvent | Ionic strength M | (S) mM | $10^6$(E) M | Method[a] |
|---|---|---|---|---|---|
| Hippurate[b,c] | 1.9% or 5.9% aceto-nitrile | 0.1-0.3 | 0.01-0.1 | 1 | T.p.(6) |
| O-Benzoylglycollate[c] | 25% acetone | 0.05 | 0.1-1 | 1 | I.r.(20) |
| S-Benzoyl-2-thiol-acetate | 25% acetone | 0.05 | 0.1-0.4 | 1 | I.r.(5) |
| 3-Benzoylpropionate | 10% DMF | 0.05 | 0.07-0.2 | 20 | T.p.(8) |
| 4-Phenylbutyrate | 10% DMF | 0.1 | 0.02-0.07 | 10 | I.r.(7) |
| N-Acetylglycinate[c,d] | 2% DMF | 0.35 | 0.7-3 | 10 | I.r.(4) |
| N-Benzyloxycarbonyl-glycinate[b,c] | 2.3% ethanol | 0.3 | 0.01-0.05 | 0.2 | T.p.(10) |
| Acetyl-L-phenyl-alanylglycinate[b,c,d] | 2% DMF | 0.3 | 0.004-0.02 | 0.2 | T.p.(5) |
| α-N-Acetyl-DL-tryptophanate[c] | 2% DMF | 0.35 | 0.01-0.05 | 0.2 | T.p.(5) |

(a) T.p.=total progress method; i.r.=initial rate method; the number of points for a Lineweaver-Burk plot are given in parentheses.

(b) Effect of the presence of aminoacetonitrile was determined.

(c) A pH profile was determined.

(d) Temperature effect was determined.

and 35°. Papain was activated by p-thiocresol. Non-enzymatic hydrolysis is significant at pH above pH 5 (especially in the presence of L-cysteine which was therefore not used as the activator). The blank rates determined in the absence of papain, were subtracted from the total observed rates to give the enzymic rates.

Papain-catalysed hydrolyses of methyl esters. These were studied by the titrimetric method. Unless otherwise stated, hydrolyses were carried out at pH 6.0 and 35°. Papain was activated by p-thiocresol. Non-enzymatic hydrolysis is negligible at pH below pH 7. Table 6-3 gives some details of the experimental conditions.

Table 6-3

| Methyl ester | Organic solvent | Ionic strength M | (S) mM | $10^6$(E) | Method[a] |
|---|---|---|---|---|---|
| Hippurate[b] | None | 0.3 | 10-30 | 1 | I.r.(6) |
| O-Benzoylglycollate[c] | 3.6% acetonitrile | 0.3 | 1-4 | 10 | I.r.(5) |
| N-Benzylglycinate | None | 0.1 | 60 | 20 | - |
| S-Thiobenzoylglycinate | 2.5% acetone | 0.05 | 3-10 | 10 | I.r.(13) |
| N-Benzyloxycarbonylglycinate[b,e] | 2.5% ethanol | 0.3 | 7-17 | 3 | I.r.(5) |
| Acetyl-L-phenylalanylglycinate[b,c,d] | None | 0.3 | 0.05-0.5 | 0.5 | T.p.(4) |

(a), (b), (c) and (d): As in table 6-2. (e) pH 7.0

Effects of nucleophiles on papain-catalysed hydrolysis. Fresh stocks of aqueous aminoacetonitrile solution were prepared and used on the same day. Glycinamide (Koch-Light Ltd.) was used without further purification. All hydrolyses of p-nitrophenyl esters were studied spectrophotometrically (340 mμ or 450 mμ) and by the total progress method. The rates of non-enzymatic hydrolyses of p-nitrophenyl esters are slightly increased in the presence of aminoacetonitrile and glycinamide and had therefore to be determined for each concentration. Hydrolyses of methyl esters were followed by pH-stat.

An attempt to detect a tetrahedral intermediate in papain-catalysed hydrolysis of hippuryl p-nitroanilide. The experimental arrangements were essentially the same as those of Lowe and Williams[65] for the detection of an acyl-papain. $(S)=3\times10^{-4}M$. $(E)=1.5\times10^{-4}M$. The reaction mixture was at pH 5.4 and 0.1M ionic strength (McIlvaine's buffer diluted 3 times), and contained 10% dimethylformamide. Ultraviolet spectra were scanned immediately and occasionally after the enzyme and the substrate solutions had been mixed. There was a gradual increase in absorption at 385 mμ due to p-nitroaniline, but no absorption region between 290 and 450 mμ appeared immediately after the mixing.

Determination of inhibition constants of competitive inhibitors. In a typical run, a stock solution of the inhibitor in acetonitrile was prepared and added (0.1 ml) with a micropipette to an incubated solution of methyl hippurate (5 ml, 35°) of a known concentration. The enzyme solution (0.2 ml) was added and the rate of hydrolysis was followed at pH 6.0. A Lineweaver-Burk plot for a given inhibitor concentration was obtained from rate determination at various methyl hippurate concentrations. The inhibition constant, $K_i$, can be calculated from a plot of the apparent Michaelis constants, $K_m(app)$, versus inhibitor concentrations, (i),

$$K_m(app) = K_m + \frac{K_m}{K_i}.(i), \qquad (6-1)$$

where $K_m$ = Michaelis constant determined in the absence of the inhibitor under otherwise identical conditions.

Identification of the L-enantiomer as the only substrate in papain-catalysed hydrolysis of p-Nitrophenyl α-N-acetyl-DL-tryptophanate. The racemic ester (0.06 g) was dissolved in acetonitrile (50 ml) and the solution added to p-thiocresol-activated papain solution ($10^{-5}$M, 950 ml) of pH 6.5, ionic strength 0.07M (McIlvaine's buffer diluted 5 times), and containing $10^{-3}$ M EDTA, at 35°. After 3 min, the remaining ester was extracted with chloroform (3x300 ml). The

chloroform solution was dried over anhydrous sodium sulphate and evaporated *in vacuo* at room temperature. The residue was recrystallised three times from acetone-ether-petrol ether; m.p. 138°; $[\alpha]_D^{20}$ +45.3° (*c* 0.3 in chloroform). Ingles and Knowles reported m.p. 130 - 130.5° and $[\alpha]_D^{20}$ +46.6° (*c* 1 in chloroform) for the D-enantiomer.[93] This must therefore be the remaining ester.

# APPENDIX 1

## STEADY-STATE KINETICS FOR PAPAIN-CATALYSED HYDROLYSIS

Papain-catalysed hydrolysis follows the Michaelis-Menten scheme[134]:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_0} E + P \qquad (A1\text{-}1)$$

$$V = \frac{k_0(E)(S)}{K_m + (S)} \qquad (A1\text{-}2)$$

V is the rate of formation of the product P. $k_0$ and $K_m$ are Michaelis-Menten constants. Eqn. (A1-2) can be obtained from the steady-state assumption, which requires that $V \gg -\underline{d}(ES)/\underline{d}(\text{time})$. This assumption is valid when either $(S) \gg (E)$ or $(E) \gg (S)$. In the present work the former condition was employed.

Eqn. (A1-2) can be rearranged to give (A1-3),

$$\frac{1}{V} = \frac{1}{k_0(E)} + \frac{K_m}{k_0(E)} \cdot \frac{1}{(S)} \qquad (A1\text{-}3)$$

The Lineweaver-Burk plots,[135] those of 1/V against 1/(S), are straight lines whose intercepts and slopes are functions of the Michaelis-Menten parameters. A necessary condition for an accurate determination of the intercepts and slopes is that $(S) \sim K_m$.

Lineweaver-Burk plots in the present work were obtained from two different methods. In the 'initial rate' method, the rates of a reaction were measured at the beginning of various kinetic runs, each with a different initial substrate concentration. The method is suitable for slow reactions. In the absence of product inhibition, back reaction or enzyme deactivation during the course of the reaction, the 'total progress' method can be used. The Lineweaver-Burk plot is obtained from only one kinetic run, in which the change in substrate or product concentration has been continuously followed. The 'total progress' method was found to be applicable to many papain-catalysed reactions, provided that these were virtually complete in 5 min or less, and the solutions were at near neutral pH values. At pH less than pH 3.8 papain is gradually deactivated especially at high temperature, and many acid products are competitive inhibitors, and this method is therefore not suitable.

# APPENDIX 2

## EVALUATION OF MICHAELIS-MENTEN PARAMETERS FOR VARIOUS MECHANISTIC SCHEMES OF PAPAIN-CATALYSED HYDROLYSIS

In papain-catalysed hydrolysis at least two steps follow the formation of the Michaelis complex: acylation of the enzyme and deacylation respectively.

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} \underset{+P_1}{ES'} \xrightarrow{k_d} E + P_2 \qquad \text{(A2-1)}$$

It can easily be shown from the steady-state assumption that

$$k_0 = \frac{k_{+2}k_d}{k_{+2}+k_d} \qquad \text{(A2-2)}$$

and

$$K_m = \frac{k_{-1}+k_{+2}}{k_{+1}} \cdot \frac{k_d}{k_{+2}+k_d} . \qquad \text{(A2-3)}$$

For specific ester substrates deacylation is the rate-determining step. Predictions of the effects of added nucleophiles can be made from various mechanistic schemes and compared with experimental results. Three possible schemes are discussed below.

### Scheme A

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} \underset{+P_1}{ES'} \begin{cases} \xrightarrow{k'_{+3}} E + P_2 \\ \xrightarrow[k_{+4}(N)]{} E + P_3 \end{cases} \qquad \text{(A2-4)}$$

$k'_{+3}=k_{+3}(H_2O)$; (N)=added nucleophile; $k_{+4}$=rate constant for the reaction of (N) with the acyl-enzyme, and $P_3$=the product from this reaction. From the steady-state assumption,

$$(E)_0=\frac{1}{k_{+2}}\left[\frac{K_m}{(S)}\cdot(k'_{+3}+k_{+4}(N)) + k_{+2} + k_{+3} + k_{+4}(N)\right](ES'), \quad (A2\text{-}5)$$

where $(E)_0$=total enzyme concentration. If $P_1$ production is followed, $V_{P_1}$ being the rate of the production,

$$V_{P_1} = (k'_{+3} + k_{+4}(N))(ES')$$

$$= \frac{(k'_{+3} + k_{+4}(N)).\ k_{+2}(E)_0}{\frac{K_m}{(S)}.(k'_{+3} + k_{+4}(N)) + k_{+2} + k'_{+3} + k_{+4}(N)}, \quad (A2\text{-}6)$$

and $k_0$ for the production of $P_1$ is given by eqn. (A2-7):

$$k_{0,P_1} = \frac{k_{+2}(k'_{+3} + k_{+4}(N))}{k_{+2} + k'_{+3} + k_{+4}(N)} \quad (A2\text{-}7)$$

If $P_2$ production is followed,

$$V_{P_2} = k'_{+3}(ES'), \text{ and}$$

$$k_{0,P_2} = \frac{k_{+2}k'_{+3}}{k_{+2} + k'_{+3} + k_{+4}(N)}\ . \quad (A2\text{-}8)$$

For both cases, $K_m(app) = \frac{k'_{+3} + k_{+4}(N)}{k_{+2} + k'_{+3} + k_{+4}(N)} \cdot K_m$ (A2-9)

Scheme B

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow[+P_1]{k_{+2}} ES' \xrightarrow{k_{+3}} ES'' \begin{cases} \xrightarrow{k'_{+4}} E + P_2 \\ \xrightarrow[k_{+5}(N)]{} E + P_3 \end{cases} \quad (A2\text{-}10)$$

$k_{+3}$=rate constant for an acyl transfer or a conformational change; $k'_{+4}=k_{+4}(H_2O)$, rate constant for hydrolysis; $k_{+5}$=rate constant for the reaction of the nucleophile (N) with the acyl-enzyme. From the steady-state assumption,

$$(E)_0 = \frac{1}{k_{+2}k_{+3}}\left[\frac{K_m}{(S)}\cdot k_{+3}(k'_{+4}+k_{+5}(N)) + k_{+3}(k'_{+4}+k_{+5}(N)) + k_{+2}(k'_{+4}+k_{+5}(N)) + k_{+2}k_{+3}\right](ES'). \quad \text{(A2-11)}$$

If $P_1$ production is followed,

$$V_{P_1} = (k'_{+4} + k_{+5}(N))(ES')$$

$$= \frac{k_{+2}k_{+3}(k'_{+4} + k_{+5}(N))\,(E)_0}{\left[\frac{K_m}{(S)}\cdot k_{+3}(k'_{+4}+k_{+5}(N)) + k_{+3}(k'_{+4}+k_{+5}(N)) + k_{+2}(k'_{+4}+k_{+5}(N)) + k_{+2}k_{+3}\right]} \quad \text{(A2-12)}$$

$$k_{0,P_1} = \frac{k_{+2}k_{+3}(k'_{+4}+k_{+5}(N))}{k_{+2}k_{+3}+(k_{+2}+k_{+3})(k'_{+4}+k_{+5}(N))}. \quad \text{(A2-13)}$$

If $P_2$ production is followed,

$$V_{P_2} = k'_{+4}(ES')$$

$$k_{0,P_2} = \frac{k_{+2}k_{+3}k'_{+4}}{k_{+2}k_{+3} + (k_{+2}+k_{+3})(k'_{+4}+k_{+5}(N))}. \quad \text{(A2-14)}$$

For both cases, $K_m(app) = K_m \dfrac{k_{+3}(k'_{+4}+k_{+5}(N))}{k_{+2}k_{+3} + (k_{+2}+k_{+3})(k'_{+4}+k_{+5}(N))}$ (A2-15)

Scheme C

Schemes A and B are assumed to operate simultaneously.

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} ES' \xrightarrow{+P_1} \quad ES' \xrightarrow{k_{+5}} ES'' \xrightarrow{k'_{+6}} E + P_2$$

$$ES' \xrightarrow{k'_{+3}} E + P_2 \qquad ES' \xrightarrow{k_{+4}(N)} E + P_3 \qquad ES'' \xrightarrow{k_{+7}(N)} E + P_3 \qquad \text{(A2-16)}$$

$k_{+5}$=rate constant for an acyl transfer or a conformational change; $k'_{+3}=k_{+3}(H_2O)$ and $k'_{+6}=k_{+6}(H_2O)$, rate constants for hydrolyses of ES' and ES" respectively; $k_{+4}$ and $k_{+7}$ are rate constants for reactions of the nucleophile (N) with ES' and ES" respectively. From the steady-state assumption,

$$(E)_0 = \frac{1}{k_{+2}(k'_{+6}+k_{+7}(N))}\left[\left(\frac{K_m}{(S)}+1\right)(k'_{+6}+k_{+7}(N))(k'_{+3}+k_{+4}(N)+k_{+5}) + k_{+2}(k'_{+6}+k_{+7}(N)) + k_{+2}k_{+5}\right](ES'). \qquad \text{(A2-17)}$$

If $P_1$ production is followed,

$$V_{P_1}=(k'_{+3}+k_{+4}(N)+k_{+5})(ES')$$

$$k_{0,P_1}=\frac{k_{+2}(k'_{+3}+k_{+4}(N)+k_{+5})(k'_{+6}+k_{+7}(N))}{(k'_{+3}+k_{+4}(N)+k_{+5})(k'_{+6}+k_{+7}(N)) + k_{+2}(k_{+5}+k'_{+6}+k_{+7}(N))}. \qquad \text{(A2-18)}$$

If $P_2$ production is followed,

$$V_{P_2}=\left[k_{+3} + \left(k'_{+6}\cdot\frac{k_{+5}}{k'_{+6}+k_{+7}(N)}\right)\right](ES')$$

$$k_{0,P_2}=\frac{k_{+2}\left[k'_{+3}(k'_{+6}+k_{+7}(N)) + k_{+5}k'_{+6}\right]}{(k'_{+3}+k_{+4}(N)+k_{+5})(k'_{+6}+k_{+7}(N)) + k_{+2}(k_{+5}+k'_{+6}+k_{+7}(N))}. \qquad \text{(A2-19)}$$

For both cases,

$$K_m(app)= K_m\cdot\frac{(k'_{+6}+k_{+7}(N))(k'_{+3}+k_{+4}(N)+k_{+5})}{(k'_{+6}+k_{+7}(N))(k'_{+3}+k_{+4}(N)+k_{+5}) + k_{+2}(k_{+5}+k'_{+6}+k_{+7}(N))}. \qquad \text{(A2-20)}$$

## APPENDIX 3

## ENZYME STANDARDISATION AND THE VALUES OF KINETIC CONSTANTS

The values of kinetic constants reported in this work were obtained from steady-state kinetic studies, from which the maximal velocity, $V_{max}$, and $K_m$ were derived. $K_m$ is independent of the enzyme concentration, whereas $V_{max}$ is linearly related to it,

$$V_{max} = k_0(E). \qquad (A3\text{-}1)$$

Enzyme concentrations were determined from rate assays at pH 6.0, 35° and 0.3M ionic strength, using methyl hippurate as the substrate. A $k_0$ value of 2.72 $sec^{-1}$ was assumed for this substrate. The value was ontained by Lowe and Williams[57] who in turn determined enzyme concentrations by assuming $k_0$ for α-N-benzoyl-L-arginine ethyl ester to be 9.0 $sec^{-1}$ at 25°, a value found by Smith and Parker[54]. By means of the 'burst' titration, that is, a stoichiometric titration of the active-site concentration at pH 3.2 using p-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate as the titrant, Bender et al.[66] found that papain was normally only 58 - 75% pure, and that $k_0$ for α-N-benzoyl-L-arginine ethyl ester would be 16.0 $sec^{-1}$ if the presence of impurities and inactive enzyme were taken into account.

For the purpose of comparison with the results of other workers in this laboratory, the values of the kinetic constants reported in this thesis are based on the 'methyl hippurate standard' ($k_0$=2.72 $sec^{-1}$). The 'absolute' values can be obtained by multiplication by a factor of 16/9.

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# ABSTRACT

The mechanism of papain-catalysed hydrolyses of N-acyl-amino acid derivatives is known to involve initial formation of an enzyme-substrate complex, followed by acylation of the active-site thiol of Cys-25 by the substrate to give a covalent acyl-enzyme intermediate, and subsequent deacylation of this intermediate:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} ES' \xrightarrow[+P_1]{k_{+3}} E + P_2.$$

This thesis is concerned with an investigation of the various steps in catalysis, mainly by a study of steady-state kinetics of hydrolyses of specific substrates. The acylation step for a number of anilide substrates was found to be completely or partially rate-determining. The acylation rate constants ($k_{+2}$) and the substrate constants ($K_s$) can be evaluated from the determination of Michaelis-Menten parameters ($k_0$ and $K_m$). For hippuryl p-substituted anilides and closely related analogues, $k_0=k_{+2}$ and $K_m=K_s$. For m- and p-substituted anilides of acetyl-L-phenylalanylglycine (APG), $k_{+2}$ were found to be comparable with the deacylation rate ($k_d$); $k_{+2}$ and $K_s$ were calculable once $k_d$ was known. The deacylation step for the ester substrates studied was

found to be the rate-determining step ($k_0=k_d$).

The specificity of the enzyme for the N-acyl moiety of the substrate is probably determined to a large extent by hydrophobic interactions between the enzyme and the substrate, since ($k_0/K_m$) for N-acylglycine esters and anilides increase in magnitude in the order acetyl-<benzoyl-<benzyloxycarbonyl<acetyl-L-phenylalanyl-glycine derivatives. The greater reactivity of APG esters and anilides, despite the fact that hydrophobicity of the acyl moiety is comparable with benzoyl- and benzyloxycarbonyl-glycyl moieties, however, indicates that polar interactions are also significant. This conclusion is supported by the observation of differences of ($k_0/K_m$) values of analogues of hippuric acid esters and anilides, in which the amide link has been modified. These differences are not solely determined by the inherent electronic properties of the acyl group, as measured by hydroxide-ion-catalysed rates of p-nitrophenyl esters. The amide link of N-acylamino acid derivatives may interact through its carbonyl oxygen with a polar group of the enzyme, possibly the γ-amide group of Gln-19, and the interaction may be important for the catalytic activity.

As has been found by other workers, the enzyme interacts

specifically with the side chain of L-arginine and it has been found that ($k_o/K_m$) for α-N-benzoyl-DL-arginine p-nitroanilide is 150 times that for hippuryl p-nitroanilide.

The values of $K_s$ for hippuryl anilides are in the range of 2 - 60 mM; those for APG anilides, 1 - 4 mM. Binding specificity is not a reliable indicator of papain activity for a given substrate, although frequently $K_s$ is smaller for a more specific substrate. Free energy of binding interactions must be utilised in order to bring the enzyme-substrate complex to an active state for subsequent reactions. Comparison ~~and~~ of activity among inhibitors and substrates derived from APG, methoxycarbonyl-L-phenylalanylglycine and hippuric acid indicates that the enzyme-substrate complex is subject to a strain around the susceptible bond, the amount of which accounts for its lability. An 'induced-fit' mechanism, involving conformational adjustment of the enzyme in order to bring the catalytic groups into proper positions, does not provide a satisfactory explanation for the experimental results.

Among individual rate steps represented by $K_s$, $k_{+2}$ and $k_d$, the best measure of specificity is $k_{+2}$, as demons-

trated by comparison between anilides of APG, hippuric acid and analogues. $k_{+2}$ values for APG anilides are 2 - 20 $sec^{-1}$, while for hippuryl anilides, 3 - 50 $msec^{-1}$. The 1,000-fold difference is due to both enthalpy and entropy of activation, as concluded from studies of the effect of temperature on the acylation rates of APG p-chloroanilide, hippuryl p-chloroanilide and hippuryl p-bromoanilide. In contrast with $k_{+2}$, $k_d$ is not very sensitive to the nature of the N-acyl moiety. For acylglycine esters $k_d$ values lie normally between 1 and 10 $sec^{-1}$, although $(k_O/K_m)$ may differ by a factor of more than $10^4$. Therefore, like $K_s$, $k_d$ is not a good indicator of specificity.

A Hammett plot of log $k_{+2}$ versus $\sigma$ for hippuryl anilides gives a $\rho$ value of -1.2, indicating electrophilic catalysis in the acylation step. The same $\rho$ value is obtained from a similar Hammett plot for APG anilides. Electronic effects on $k_{+2}$ for both series of substrates are therefore similar. It is proposed that in the acylation of papain by anilide substrates the leaving groups are protonated through general acid catalysis. The donated proton is probably that originally forming a hydrogen bond between the essential imidazole group of His-159 and the essential thiol of Cys-25, which

therefore may attack the carbonyl carbon of the substrate in the form of a thiolate anion. Available results do not allow a determination of the exact sequence of these processes. From the principle of microscopic reversibility the same processes in reverse must take place in the deacylation step.

Experiments on kinetics of papain-catalysed hydrolyses of methyl and p-nitrophenyl esters in the presence of nucleophiles (methanol, aminoacetonitrile, glycinamide) support the suggestion of various workers that the rate-determining step in deacylation is the general-base-catalysed nucleophilic attack of water or other nucleophiles on the thiolester intermediate. A tetrahedral intermediate formed is rapidly decomposed to give the acid or other acyl derivatives. For hippuryl-papain and possibly for other acyl-papains, experimental results on the effects of aminoacetonitrile can be interpreted as indicating the existence of an alternative pathway. This may involve an acyl transfer to another group of the enzyme, or a conformational change, followed by relatively rapid hydrolysis and aminolysis.

The acylation step depends on two prototropic groups

with $pK_a$(app) of approximately 4.2 and 8.3. The $pK_a$ values of these groups in the free enzyme, as determined by the pH profile of $(k_o/K_m)$ for p-nitrophenyl esters and APG p-nitroanilide, are very similar to those in the enzyme-substrate complex, as determined by the pH profile of $k_{+2}$ for APG p-nitroanilide. If the two $pK_a$ values represent protonation and deprotonation of the hydrogen-bonded thiol-imidazole system respectively, the binding of the substrate cannot result in the destruction of this hydrogen bond.

As expected, $pK_2$ was not observed in deacylation of APG-papain. In the deacylation step, $pK_1$ is sensitive to the nature of the acyl moiety and varies in the range 3.1 - 4.7. The $pK_1$ value tends to be low for a specific substrate or a substrate with hydrophobic groups in the acyl moiety. This observation can be interpreted in terms of shielding of the thiol-imidazole system by the acyl moiety from hydronium ions. Apart from steric effects, shielding by hydrophobic groups also gives rise to a decrease in local dielectric constant, which could further depress the $pK_a$ of a cationic acid such as the thiol-imidazolium system.