Acyl Intermediates in Penicillopepsin-Catalysed Reactions and a Discussion of the Mechanism of Action of Pepsins

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(Received 28 August 1974)

Penicillopepsin catalyses transpeptidation reactions involving the transfer of the *N*-terminal amino acids of suitable substrates via covalent acyl intermediates to acceptor peptides, usually the substrate. The major products obtained when Phe-Tyr-Thr-Pro-Lys-Ala and Met-Leu-Gly were used as substrates were Phe-Phe and Met-Met respectively. With Met-Leu-Gly the tetrapeptide Met-Met-Leu-Gly was observed as probable intermediate. Co-incubation of Leu-Tyr-Leu and Phe-Tyr-Thr-Pro-Lys-Ala led to the formation of Leu-Phe and Phe-Leu as well as Leu-Leu and Phe-Phe. No reaction was observed with tripeptides in which the first or second amino acid is glycine. It appears that two amino acids with large hydrophobic residues are needed for the transpeptidation reaction. Nucleophilic compounds other than peptides, such as hydroxylamine, aliphatic alcohols and dinitrophenylhydrazine, were not acceptors for the acyl group. Leucine, phenylalanine and leucine methyl ester also had no effect on the reaction. The transpeptidation reaction proceeded readily at pH 3.6 and 4.7. At pH 6.0 the reaction was slow and at pH 1.9 little or no transpeptidation was observed. Porcine pepsin catalyses similar transpeptidation reactions. Sequence studies show that porcine pepsin and penicillopepsin are homologous. The present study also suggests that they have a very similar mechanism. Evidence available at this time indicates that the mechanism of these enzymes is complex and may be modulated by secondary substrate-enzyme interactions. A hypothesis is presented which proposes that pepsin-catalysed reactions proceed via different covalent intermediates (amino-intermediates or acyl intermediates) depending on the nature of the substrate. The possibility that some reactions do not involve covalent intermediates is also discussed.

The central feature of a number of mechanisms of action of porcine pepsin that have been proposed in recent years is a covalent amino intermediate. These mechanisms have been reviewed by Clement (1973). The evidence for the amino intermediate comes from transpeptidation reactions catalysed by pepsin which involve the transfer of a *C*-terminal amino acid from a small substrate. Neumann *et al.* (1959) and Fruton *et al.* (1961) showed that these transpeptidation reactions required a covalently bound intermediate. The studies of Kitson & Knowles (1971*a*) indicate that in hydrolytic reactions there is a non-random release of products in an order that is consistent with the postulate of an amino enzyme. On the other hand, the possibility has been raised that acyl intermediates may also be involved in pepsin-catalysed reactions. Thus Sharon *et al.* (1962) showed that pepsin can catalyse ¹⁸O exchange between a virtual substrate and water and suggested that this provided evidence for the formation of an acyl enzyme. Shkarenkova *et al.* (1968) found that an

¹⁸O exchange catalysed by pepsin also occurs in the absence of a virtual substrate. Knowles (1970) proposed a mechanism which accounted for the observed ¹⁸O-exchange reactions without involving a covalent acyl intermediate. Recently we showed that both porcine pepsin and penicillopepsin, an evolutionary homologue of the porcine enzyme, catalyse transpeptidation reactions which involve the transfer of an *N*-terminal amino acid from the substrate Leu-Tyr-amide and Leu-Tyr-Leu (Takahashi *et al.*, 1974). These transpeptidation reactions occur in high yield and involve a covalent intermediate which in this case must be an acyl intermediate because of the nature of the transfer.

In the present report we describe in greater detail penicillopepsin-catalysed acyl-transfer reactions involving a number of substrates. Several properties of the transpeptidation reactions are also described and a proposal is made for the mechanism of pepsin-catalysed reactions which could account for the observed properties of the enzyme. Some preliminary results have been given elsewhere (Takahashi & Hofmann, 1972).

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Experimental

Materials

Penicillopepsin was prepared from *Penicillium janthinellum* essentially by the method of Sodek & Hofmann (1970a) and stored as a precipitate in 2.5M-LiSO₄ at pH4.5. This enzyme preparation was shown to be free (less than 1×10^{-6} contamination) of penicillocarboxypeptidase, an enzyme that has been isolated from the growth medium of the same organism (Jones & Hofmann, 1972). Porcine pepsin was prepared from pepsinogen as described by Rajagopalan *et al.* (1966). The suppliers of the di- and tri-peptides used were as follows. Miles-Yeda Ltd., Rehovot, Israel, provided Phe-Leu, Gly-Phe-Ala, DL-Leu-Gly-DL-Phe; Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. provided Leu-Tyr; Cyclo Chemical Corp., Los Angeles, Calif., U.S.A. provided leucine methyl ester hydrochloride; all others were from Schwartz-Mann, Orangeburg, N.Y., U.S.A.

Except where noted all amino acid residues were L-isomers. L-[¹⁴C]Leucine (10mCi/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K. Crystalline insulin was from British Drug Houses Ltd., Poole, Dorset, U.K. Leu-Tyr-Leu (Schwartz-Mann) was found to be heterogeneous by high-voltage electrophoresis. It was purified as follows: the peptide (20mg) dissolved in 4ml of water was spotted on Whatman 3MM filter paper (27cm × 57cm) as a 20cm long band, 12cm from the narrow edge. After electrophoresis at pH3.6 at 2000V for 90min, guide strips were cut and stained with a cadmium-ninhydrin reagent (Heilmann *et al.*, 1957). The major band, located between 10 and 14cm from the origin, was eluted with 0.01M-acetic acid and freeze-dried. It gave single spots on electrophoresis at pH3.6 and 3.1 (40V/cm, 120min).

The hexapeptide,* which represents the C-terminal of the B chain of insulin, was prepared from bovine insulin by digestion with penicillopepsin at pH1.9. At this pH peptide B₄ [identified by Mains *et al.* (1971) as the hexapeptide] is the major if not sole product representing the C-terminal of the B chain, whereas at pH3.6 peptide B₃, the pentapeptide Tyr-Thr-Pro-Lys-Ala is the major product (Mains *et al.*, 1971). Insulin (286mg = 50μmol) in 68ml of buffer, pH1.9, was incubated with 2.8mg of penicillopepsin (in 7ml of water) at 35°C for 6h. The reaction vessel was then immersed in a boiling-water bath for 10min. The solution was freeze-dried, the residue suspended in pyridine-acetic acid-water (100:4:900, by vol.), pH6.5, and the suspension centrifuged. The supernatant was applied to several sheets (46cm × 57cm) of Whatman 3MM paper. Methyl Green was spotted alongside as a marker. Electro-

phoresis was carried out at pH3.1 and 2000V for 90min. A ninhydrin-stained guide strip showed a major band between 11 and 14cm from the origin (Methyl Green was 20.5cm from the origin) and several weakly stained bands between the origin and 7cm. The major band was eluted with about 2.2ml of 0.01M-acetic acid. The composition and concentration of the peptide were determined by amino acid analysis. The composition showed the following molar ratios: lysine, 1.12; threonine, 1.09; proline, 0.77; alanine, 1.08; tyrosine, 0.91; phenylalanine, 1.03. The recovery was 16.5mg (44%). An analysis by the dansyl-Edman method showed that phenylalanine was N-terminal, followed by tyrosine. There was no evidence for the presence of the heptapeptide Phe-Phe-Tyr-Thr-Pro-Lys-Ala.

Methods

Peptide separations

Reaction products were separated on Whatman 3MM paper by high-voltage electrophoresis in volatile buffers at pH6.5, 3.6 (Bennett, 1968) or pH3.1 (acetic acid-90% formic acid-pyridine-water, 2.5:2.5:1:135, by vol.) or pH1.9 (acetic acid-formic acid-water, 15:1:250, by vol.), and by chromatography in butan-1-ol-*n*-butyl acetate-acetic acid-water (130:6:30:50, by vol.). For identification of the peptides enzyme digests were applied to the paper as a band. After separation, the peptides were located by spraying guide strips with a cadmium-ninhydrin reagent (Heilmann *et al.*, 1957). For two-dimensional separations the bands were sewn on to new sheets of paper and separated in the second system. After location, the peptides were eluted with 0.01M-acetic acid. For electrophoresis Methyl Green was added as a marker (Stevenson, 1971). Alternatively, separations were carried out in a Beckman-Spinco amino acid analyser with the buffer systems and conditions developed by Callahan *et al.* (1970) for the separation of dipeptides. Commercial dipeptides were used for determining elution times and colour values.

Amino acid analyses

Products of penicillopepsin-catalysed reactions were analysed for their amino acid composition, after hydrolysis under vacuum with 5.7M-HCl (0.2ml) at 107°C for 20h, by the method of Moore *et al.* (1958) in a Beckman-Spinco analyser, model 121C, fitted with a high-sensitivity colorimeter cell (18mm light-path; Evans Electro Selenium Ltd., Halstead, Essex, U.K.).

N-Terminal residues and sequences

These were determined by the dansyl and dansyl-Edman methods respectively as described by Gray

* Abbreviations: hexapeptide, Phe-Tyr-Thr-Pro-Lys-Ala; Cbz, benzyloxycarbonyl.

(1967*a,b*). Dansyl amino acids were identified on Ching-Chen polyamide sheets (5cm×5cm) as described by Hartley (1970).

Enzyme incubations

Incubations of penicillopepsin with various substrates were carried out at 37°C in volatile buffers containing pyridine and acetic acid (of the same composition as those used for electrophoresis). A buffer at pH4.7 (pyridine-acetic acid-water, 5:5:190, by vol.) was also used. For prolonged incubations toluene (0.1 ml) was added.

Results

Effect of proteinase inhibitors on transpeptidation by penicillopepsin

Since the demonstration of the formation of Leu-Leu from Leu-Tyr-Leu (Takahashi *et al.*, 1974) and Phe-Phe from the hexapeptide requires prolonged incubation with fairly high amounts of enzyme (0.2–0.5 mg/ml) it was possible that the transpeptidation was catalysed not by penicillopepsin itself but by a contaminating enzyme. To minimize the likelihood of this, penicillopepsin was preincubated with EDTA, *p*-hydroxymercuribenzoic acid or

Fig. 1. *Effect of various inhibitors on penicillopepsin*

(a) Penicillopepsin (0.07 mg in 0.3 ml of pyridine-acetate, pH3.6) was preincubated with EDTA (2mM) or *p*-hydroxymercuribenzoate (*p*-HOHgPhCO₂H) (2mM) for 10min at 35°C. Preincubation with di-isopropyl phosphorofluoridate (Dip-F) (1mM) was at pH6. The di-isopropyl phosphorofluoridate-treated enzyme was dialysed before incubation with Leu-Tyr-Leu. Treatment at pH8 was at 20°C for 3h in 0.1M-*N*-ethylmorpholine acetate (1.2mg of enzyme/ml). The pH was adjusted to 3.6 with acetic acid. Incubation of all solutions with Leu-Tyr-Leu (1.6μmol) was at pH3.6 for 24h at 35°C in a final volume of 0.4ml. The products were separated by electrophoresis at pH3.1, 2000V for 90min. (b) Penicillopepsin inhibited by diazoacetylnorleucine methyl ester (N₂AcNleOMe) was prepared as described (Sodek & Hofmann, 1970*b*) and incubated with Leu-Tyr-Leu under the conditions described above. Preincubations with *p*-hydroxymercuribenzoate and EDTA were also as above. Spots are identified as follows: L₁, tyrosine; L₂, leucine; L₃, Tyr-Tyr; L₄, Leu-Leu-Tyr-Leu; L₅, Leu-Tyr; L₆, Leu-Tyr-Leu; L₇, Tyr-Leu; L₈, Leu-Leu. Their intensity is indicated as follows: ●, strong; ○, intermediate; ◐, weak. M.G., Methyl Green.

di-isopropyl phosphorofluoridate (Fig. 1a). None of these inhibitors had any effect on the transpeptidation reaction. On the other hand, a short incubation of penicillopepsin at pH8 where the enzyme is rapidly and irreversibly denatured (Hofmann & Shaw, 1964) completely abolished the action of the enzyme on Leu-Tyr-Leu (Fig. 1a).

Further, when the enzyme was pre-treated with diazoacetyl norleucine methyl ester, an active-site-directed covalent inhibitor of penicillopepsin and other acid proteinases (Sodek & Hofmann, 1970b), no transpeptidation was observed (Fig. 1b), but surprisingly it hydrolysed Leu-Tyr-Leu as shown by the formation of leucine and Leu-Tyr (Fig. 1b). This, however, was explained when it was found that the preparation of inhibited enzyme which was used for this experiment differed from the enzyme used for the other experiments in that it contained a small contamination of penicillocarboxypeptidase S-1. This was demonstrated by the fact that the inhibited enzyme hydrolysed Cbz-Glu-Tyr, a peptide not hydrolysed by penicillopepsin (Mains *et al.*, 1971), but one which is an excellent substrate for the carboxypeptidase. Further, *p*-hydroxymercuribenzoic acid but not EDTA inhibited the action of the inhibited enzyme on Leu-Tyr-Leu. *p*-Hydroxymercuribenzoic acid was previously shown to be an inhibitor of the carboxypeptidase (Jones & Hofmann, 1972). When the experiment was repeated with inhibited enzyme which was prepared from penicillopepsin that was free of carboxypeptidase no hydrolysis of Leu-Tyr-Leu was observed.

Effect of penicillopepsin and porcine pepsin on Phe-Tyr-Thr-Pro-Lys-Ala

The effect of prolonged incubation of penicillopepsin with the hexapeptide is shown in Fig. 2 and in Table 1. Eight peptides were separated by high-voltage electrophoresis and paper chromatography (Fig. 2) and after elution were identified by their amino acid composition and *N*-terminal residues (Table 1). The transpeptidation product B_T was a major product and was identified as Phe-Phe by its electrophoretic mobility at pH3.1, where it migrated about twice as far as free phenylalanine, by its *N*-terminal residue and by the fact that after one cycle of Edman degradation free phenylalanine was obtained. Table 1 shows that the major products of the reaction are free phenylalanine and the pentapeptide Tyr-Thr-Pro-Lys-Ala, products obtained by hydrolysis of the *N*-terminal residue. Tyrosine and threonine are also cleaved off since tetrapeptide B_6 and the tripeptide B_7 were also obtained in reasonable yield. Free tyrosine was detected; however, no free threonine was found. It is not clear how the cleavage at -Thr-Pro- occurred since the peptides Phe-Tyr-Thr or Tyr-Thr were not observed either. Although the recoveries

Fig. 2. Action of penicillopepsin on Phe-Tyr-Thr-Pro-Lys-Ala; peptide map of products

Hexapeptide (2.7 μ mol in 0.5ml of water) and penicillopepsin (0.3mg in 0.3ml of water) were mixed with pyridine-acetate buffer, pH3.6 (1.2ml) and incubated for 24 h at 35°C. The reaction was stopped by heating in a boiling-water bath. A sample (50 μ l) was applied to Whatman 3MM paper and subjected to electrophoresis at pH3.1, 2000V for 90min followed by paper chromatography. Identification of peptides is given in Table 1. M.G. indicates position of Methyl Green. The intensity of spots is indicated as follows: ●, strong; ○, intermediate; ◐, weak.

are not quantitative since no corrections for losses were made, we feel nevertheless that they represent the relative proportion of the products formed since the overall recovery of the six amino acids were comparable and ranged from 32% for phenylalanine to 45% for the four *C*-terminal amino acids. With this in mind it can be seen that the ratio of transpeptidation to hydrolysis for the first amino acid is about 1 in 6. This contrasts with the high yield of transpeptidation that was obtained when penicillopepsin acted on Leu-Tyr-Leu (Takahashi *et al.*, 1974). In that case the ratio of transpeptidation/hydrolysis was approximately 2:1. The product peptide B_5 was obtained in low yield and had a composition that does not readily fit any likely product. Owing to lack of material it was not further investigated.

Fig. 3 shows that the action of porcine pepsin on the hexapeptide is similar to the action of penicillopepsin. The major products of incubation are again the free phenylalanine, the dipeptide Phe-Phe and the pentapeptide B_3 . However, there was no indication of the formation of peptides B_6 and B_7 , and another, unidentified, product B_8 was formed.

Effect of penicillopepsin on Met-Leu-Gly and other tripeptides

The effect of prolonged incubation of penicillopepsin with Met-Leu-Gly is shown in Fig. 4 and

Table 1. *Effect of penicillopepsin on Phe-Tyr-Thr-Pro-Lys-Ala*

The hexapeptide (2.7 μ mol) was incubated with penicillopepsin for 24h as described for Fig. 2. The products were separated by electrophoresis and chromatography (Fig. 2). They were analysed after separation and elution from the paper as described in the Methods section. Recoveries were calculated from the amino acid analyses. No corrections were made for mechanical losses (guide strips, etc.) and destructive losses. The overall recovery of phenylalanine was 32%, of tyrosine 40% and of the other four amino acids 48%. For details of peptide labelling see Fig. 2. *N*-Terminal residue was determined as dansyl amino acid, —.

Peptide	Composition (in molar ratios)	Recovery (nmol)	<i>N</i> -Terminal residue	Sequence deduced
B ₁	Phenylalanine	635	—	Free phenylalanine
B ₂	Tyrosine	51	—	Free tyrosine
B ₃	Tyr (0.76), Thr (1.06), Pro (1.07), Lys (1.04), Ala (1.08), no Phe	950	Tyr	Tyr-(Thr-Pro-Lys-Ala)
B _T	Phenylalanine	114*	Phe	Phe-Phe
B ₄	Phe (0.97), Tyr (0.80), Thr (1.3), Pro (1.03), Lys (0.83), Ala (1.12)	72	Phe	Phe-(Tyr-Thr-Pro-Lys-Ala)
B ₅	Phe (0.32), Tyr (0.52), Thr (1.0), Pro (0.6), Lys (lost), Ala (1.0)	30	—	Not identified
B ₆	Thr (0.87), Pro (1.08), Lys (1.0), Ala (1.03)	144	Thr	Thr-(Pro-Lys-Ala)
B ₇	Pro (0.92), Lys (1.04), Ala (1.05)	99	Pro	Pro-(Lys-Ala)

* As phenylalanine. This product yielded free phenylalanine after one cycle of Edman degradation.

Fig. 3. *Action of porcine pepsin on hexapeptide*

Pepsin (0.5mg in 0.05ml of water) and hexapeptide (4 μ mol in 0.1ml of water) were incubated with pyridine-acetate buffer, pH4.7, at 35°C for 24h. A sample was electrophoresed at pH3.6, 2000V for 100min. The products from a penicillopepsin digest (Fig. 2) were also run. The intensity of spots is indicated as follows: ●, strong; ○, intermediate; ◐, weak.

Fig. 4. *Effect of penicillopepsin on Met-Leu-Gly*

Met-Leu-Gly (1.88 μ mol) in 0.6ml of pyridine-acetate buffer, pH3.6, was incubated with 0.6mg of penicillopepsin in 0.6ml of water at 35°C for 24h. For pH4.7, 0.16 μ mol of Met-Leu-Gly was incubated with 0.1mg of penicillopepsin in pyridine-acetate buffer, pH4.7. The products were separated by electrophoresis at pH1.9 for 90min. Further separation and identification of the peptides are given in Table 3. M.G., Methyl Green. The intensity of spots is indicated as follows: ○, strong; ◐, weak.

Table 2. The two main products of interest are Met-Met (III_a) and Met-Met-Leu-Gly (VI). By analogy with the experiments with Leu-Tyr-amide (Takahashi *et al.*, 1974) it is reasonable to conclude that these are the products of an acyl transfer and that the tetrapeptide is an intermediate product. Unfortunately the recovery of methionine is low (8%) compared with that of leucine and glycine (34%). The low recovery is probably due to oxidation of methionine and Met-Met; both of these required several electrophoresis and chromatography steps for isolation. As determined from recovered methionine the ratio of transpeptidation/hydrolysis is about 2.5:1, similar to that observed for Leu-Tyr-Leu, but higher than that of the hexapeptide.

The origin of a trace of Leu-Leu is not clear. Since Leu-Gly peptides do not appear to be sub-

strates it is difficult to see how this could have arisen from the action of penicillopepsin on Leu-Gly. Peptide VII could not be identified. It had the same composition as the substrate, but a very different mobility at pH1.9. The difference indicates that it is either larger or more acidic. If it was larger it could be the product of a condensation of two substrate molecules. Observations have recently been made with porcine pepsin which would support this possibility (T. Wang & T. Hofmann, unpublished work). Alternatively it could be more acidic

Table 2. *Effect of penicillopepsin on Met-Leu-Gly*

Met-Leu-Gly was incubated with penicillopepsin as described for Fig. 4. The reaction products (pH 3.6) were separated as indicated below and analysed after elution from the paper. Recoveries were calculated from the amino acid analyses. No corrections were made for mechanical losses (guide strips, trial maps, etc.) and destructive losses. The overall recovery of methionine was 8%, that of leucine and glycine 34%. Peptides are numbered as shown in Fig. 3. They were purified by electrophoresis (twice) at pH 1.9. Band III was further separated by paper chromatography into two bands. Band V was separated by electrophoresis at pH 3.1 into six bands. Two of these were identical with bands IV and VI respectively; two were isolated in trace amounts only and could not be identified. Band VII was further purified by electrophoresis at pH 3.1.

Peptide	Composition (in molar ratios)	Recovery (nmol)	N-terminal residue	Sequence deduced
I	Glycine	78	—	Free Glycine
II	Leu (0.97), Gly (1.03)	5450	—	Leu-Gly
III _a	Methionine	484	Met	Met-Met*
III _b	Leucine	25	—	(Leu-Leu)†
IV	Met (0.93), Leu (1.01), Gly (1.06)	628	—	Met-Leu-Gly
V _a	Methionine	208	—	Free methionine
V _b	Met (2.34), Leu (0.87), Gly (1.13)	22	—	(Met, Met, Met, Leu, Gly)‡
VI	Met (1.7), Leu (1.0), Gly (1.05)	68	Met	Met-Met-Leu-Gly§
VII	Met (1.04), Leu (1.07), Gly (0.90)	52	—	?

* This product yielded free methionine after one cycle of Edman degradation. The mobility at pH 3.1 (relative to Methyl Green) was 0.68 compared with 0.17 for free methionine.

† Deduced from mobility at pH 3.1 relative to Leu-Leu and leucine.

‡ This peptide differed clearly from peptide VI and from band V_a in mobility and composition. Insufficient material was available for further analysis.

§ The sequence of this was established by the dansyl-Edman procedure.

due to oxidation of methionine to the sulfoxide. Insufficient material was available for a more detailed analysis.

Fig. 4 also shows that qualitatively the reaction products appear to be the same for the two pH conditions used for the incubation.

The following peptides were not acted on by penicillopepsin: Gly-Phe-Ala, Gly-Leu-Tyr, Leu-Gly-Phe, Leu-Gly-Leu, Gly-Leu-Tyr, Gly-Phe-Phe and Gly-Leu-Gly.

Acceptors for transpeptidation

The results presented previously (Takahashi *et al.*, 1974) and in Tables 1 and 2 and Figs. 2 and 4 show that the peptide that acts as donor of the N-terminal amino acid also acts as acceptor. This is further demonstrated in an experiment in which two substrates, Leu-Tyr-Leu (2.5 mm) and the hexapeptide (5 mm) are incubated together with the enzyme (Table 3). In addition to the expected transpeptidation products Leu-Leu and Phe-Phe, the peptides Leu-Phe and Phe-Leu were also formed, showing that either substrate can act as the acceptor of the N-terminal amino acid of the other substrate. Interestingly the formation of the heteropeptides is favoured over the formation of the homopeptides. The presence of the second peptide also appears to favour the transpeptidation reaction. Thus in the experiment with hexapeptide alone (Table 1) 114 nmol of phenylalanine were recovered as Phe-Phe from 2.7 μ mol of

substrate (equivalent to 82 nmol from 2 μ mol) whereas with the mixed substrates 2 μ mol of the hexapeptide yielded 433 nmol of transpeptidation products of phenylalanine. Even taking into account that the recovery of the products was far from quantitative it is highly unlikely that it was over five times as high with the mixed substrates as with the hexapeptide alone. In fact, the recoveries were probably similar

Table 3. *Action of penicillopepsin on mixed substrates*

Penicillopepsin (0.1 mg) was incubated at 35°C for 24 h in pyridine-acetate buffer, pH 3.6, with a mixture of Leu-Tyr-Leu (1 μ mol) and hexapeptide (2 μ mol); total volume 0.4 ml. The products were applied as a 8 cm long band on Whatman 3MM paper and separated by electrophoresis at pH 3.1, 2000 V, for 90 min. The region of products with mobilities between 0.32 and 0.54 (relative to Methyl Green) known to contain the dipeptides was eluted and applied to the amino acid analyser. Separation was by the method of Callahan *et al.* (1970).

Elution time (min)	Peptide deduced	Elution time of standard	Yield (nmol)
267	Leu-Leu	267	166*
341	Leu-Tyr	340	134
394	Leu-Phe	402	143
531	Tyr-Leu	538	301
622	Phe-Leu	631	141
941	Phe-Phe	950	150*

* As amino acid residue.

Table 4. *Leu-Tyr as acceptor in transpeptidation*

Penicillopepsin (0.3 mg) was incubated at 35°C, pH 3.6, for 24 h with Leu-Tyr-Leu (1.23 μ mol) and Leu-Tyr (6.1 μ mol) as indicated (total vol. 0.5 ml). Products were separated on an amino acid analyser by the method of Callaghan *et al.* (1970). Controls were incubations without enzyme.

Substrate	Yield of products (nmol)				
	Leu-Leu	Leu-Tyr	Tyr-Leu	Leucine	Tyrosine
Expt. 1. Leu-Tyr-Leu + Leu-Tyr	432	3520	680	336	314
Expt. 2. Leu-Tyr-Leu	262	190	546	180	49
Expt. 3. Leu-Tyr	0	6400	0	56	53
Controls					
Leu-Tyr-Leu	0	0	0	0	0
Leu-Tyr	0	5830	0	34	23

Table 5. *Effect of various compounds on reaction of penicillopepsin with Leu-Tyr-Leu*

Penicillopepsin (0.1 mg) was incubated at pH 3.6 or 4.7, 35°C for 24 h with Leu-Tyr-Leu (2–2.5 mM) and the compounds listed; final vol. 0.3 ml. The digests were separated by electrophoresis at pH 3.1. The effect on hydrolysis was estimated by eye from the amount of free leucine, that on transpeptidation from the amount of Leu-Leu.

Compound	Concn.	Inhibition of	
		Hydrolysis	Transpeptidation
Phenylalanine	29 mM	None	None
Leucine methyl ester	20 mM	None	None
Methanol	2.2 M	None*	None*
Ethanol	1.5 M	None*	None*
Propanol	1.2 M	Partial	Partial
Butanol	0.9 M	Partial	Partial
Isobutanol	0.9 M	Partial	Partial
NH ₂ OH-HCl, pH 3.6	2 M	None*	None*
NH ₂ OH-HCl, pH 4.7	1.6 M	None*	None*
Aniline, pH 3.6	Approx. 0.5 M	Complete	Complete
Aniline, pH 4.7	Approx. 0.5 M	Complete	Complete
Dinitrophenylhydrazine, pH 3.6	7 mM	Slight	Slight
Dinitrophenylhydrazine, pH 4.7	7 mM	Slight	Slight

* Recently we observed a slight inhibition of the penicillopepsin acting on Leu-Tyr-amide by these compounds (T. Wang & T. Hofmann, unpublished work).

since the recovery of Tyr-Leu was comparable in the experiment with mixed substrates (201 nmol from 1 μ mol of Leu-Tyr-Leu) and with Leu-Tyr-Leu alone (722 nmol from 3.2 μ mol) (Takahashi *et al.*, 1974). The overall recovery in the latter experiment and in Table 1 was comparable. A 1.7-fold difference in enzyme concentration between the two experiments is probably also not responsible for the difference in yield of transpeptidation products since the results represent final yields rather than rates of reaction.

Although the results presented so far show that the substrate molecules act as ready acceptors for the acyl group one peptide has so far been found which is only an acceptor and not a substrate (Table 4). Incubation of Leu-Tyr-Leu with and without Leu-Tyr (Expts. 1 and 2, Table 4) shows a 65% increase in the formation of Leu-Leu in the presence of Leu-Tyr and a 90%

increase in free leucine. These increases are not primarily due to a stimulation of the action on Leu-Tyr-Leu because of the sixfold increase in free tyrosine. In the absence of Leu-Tyr only a small amount of tyrosine is formed (Expt. 2; Takahashi *et al.*, 1974). Expt. 3 shows that Leu-Tyr is at best a very poor substrate; after subtraction of the control values only some 0.5% of the Leu-Tyr added appears to have been hydrolysed.

A variety of other compounds were also tested for their ability to act as acceptors for the leucyl group. The results are summarized in Table 5. It was shown previously that [¹⁴C]leucine was not incorporated to any significant extent into Leu-Leu when incubated with Leu-Tyr-Leu and penicillopepsin. The results presented in Table 5 are only semi-quantitative but confirm the lack of effect of free amino acids and

leucine methyl ester. Other nucleophilic compounds at high concentration either had no effect on the reaction or inhibited both hydrolysis and transpeptidation to the same extent. An acceptor of the leucyl group would be expected to decrease the formation of Leu-Leu without affecting hydrolysis. It is noteworthy that the reagents that inhibit the enzyme are hydrophobic; for the alcohols, they are those with the longer side chains, whereas hydroxylamine and the lower alcohols even at high concentrations have no effect.

Effect of pH

The effect of pH on the transpeptidation reaction using Leu-Tyr-Leu and the hexapeptide is shown in Figs. 5 and 6 respectively. Incubation with Leu-Tyr-Leu for 6h showed that both leucine and Leu-Leu were formed most rapidly at pH3.6 and nearly as rapidly at pH4.7. At pH6.0 there was evidence for some hydrolysis, but no transpeptidation, and at pH1.9 there appeared to be no reaction. With the hexapeptide, too, the reaction was most rapid at pH3.6, and slightly slower at pH4.7 (Fig. 6). On prolonged incubation at pH6.0 (24h) there was evidence for a new unidentified product (B_x). A very slow reaction was also observed at pH1.9; another unidentified product (B_y) was also formed.

Discussion

The experiments reported here add further evidence to the previous report on the involvement of acyl

intermediates in pepsin- and penicillopepsin-catalysed reactions. Of the inhibitors directed against the major classes of proteolytic enzymes (di-isopropyl phosphorofluoridate, *p*-hydroxymercuribenzoic acid, EDTA and diazoacetyl norleucine methyl ester) only diazoacetyl norleucine methyl ester inhibited. This shows that the transpeptidation is not caused by contaminating enzymes but by the pepsins themselves. The observation that a hydrolytic action of the first preparation of inhibited enzyme could be inhibited by *p*-hydroxymercuribenzoic acid, indicating the presence of carboxypeptidase, strengthens this conclusion and demonstrates the effectiveness of the specific inhibitors. Additional support, if such is needed, comes from the fact that both pepsins catalyse similar reactions and it is highly unlikely that both pepsins would be contaminated by similar enzymes.

Although a limited number of substrates have been studied so far the acyl-transfer reaction has been observed only with substrates which have: (a) a free *N*-terminal ammonium group and (b) two amino acids with large hydrophobic side chains (leucine, phenylalanine, tyrosine and methionine) in the *N*-terminal positions. Transpeptidation substrates so far found are Leu-Tyr-Leu, which gives Leu-Leu and a small amount of Tyr-Tyr; Met-Leu-Gly which gives Met-Met; and Phe-Tyr-Thr-Pro-Lys-Ala which gives Phe-Phe, but no Tyr-Tyr, although some hydrolysis at the Tyr-Thr and Thr-Pro bonds was observed. In addition Leu-Tyr acts as acceptor, but not as substrate, whereas Leu-OMe is neither.

Scheme 1 summarizes the major features of the active site of porcine pepsin and penicillopepsin as they relate to the acyl intermediate. Initial binding to form the enzyme-substrate complex occurs through

Fig. 5. Effect of pH on the effect of penicillopepsin on Leu-Tyr-Leu

Penicillopepsin (0.067mg) was incubated for 6h at 35°C with Leu-Tyr-Leu (0.85μmol) with volatile buffers as used for electrophoresis (diluted 1:1 with water) at pH1.9, 3.6 and 4.7 in a final volume of 0.2ml. The buffer at pH6.0 was obtained by additions of acetic acid to the pH6.5 buffer. The products were separated by electrophoresis at pH3.6, 2000V for 90min. Spots are identified in Fig. 1. M.G., Methyl Green. The intensity of spots is indicated as follows: ●, strong; ○, intermediate; ◐, weak.

Fig. 6. Effect of pH on penicillopepsin effect on Phe-Tyr-Pro-Lys-Ala

Penicillopepsin (0.05mg) was incubated for 6h at 35°C with the hexapeptide (0.5μmol) as described in Fig. 5. The products were separated at pH3.1, 2000V for 90min. Spots are identified in Table 2. M.G., Methyl Green. The intensity of spots is indicated as follows: ●, strong; ○, intermediate; ◐, weak.

Scheme 1. Pepsin-catalysed reactions involving covalent acyl intermediate

X, -NH₂ or other amino acids; S₁, S₁', primary binding sites (hydrophobic); S₂', secondary binding site.

the hydrophobic side chains in subsites S₁ and S₁' (as described by Berger & Schechter, 1970). The acyl intermediate, which is envisioned as an anhydride with one of the active site aspartic acids, forms through a nucleophilic attack on the carbonyl group of the substrate leaving the amino moiety of the peptide bond free to form an ion pair with the other active site aspartic acid.*

* Evidence for two active-site aspartic acids in porcine pepsin has been obtained by chemical modification with trimethyloxonium fluoroborate by Paterson & Knowles (1972) by reaction with diazoacetyl norleucine methyl ester [porcine pepsins, (Rajagopalan *et al.*, 1966) and penicillopepsin (Sodek & Hofmann, 1970b)] and 1-(*p*-nitrophenoxy)-2,3-propoxide [porcine pepsin (Hartsuck & Tang, 1972) and penicillopepsin (Mains & Hofmann, 1974)].

The acyl intermediate once formed appears to be stabilized as indicated by the high yield of the transfer reaction which can exceed hydrolysis [as with leucine peptides (both pepsins) and methionine peptides (Takahashi *et al.*, 1974; the present study)]. This stability is surprising considering that the optimum of the reaction is at pH 3–4. Two major factors could be responsible for this stability. One is a hydrophobic environment as indicated by the specificity for amino acids with large hydrophobic side chains and by the inhibition by aniline, propanol and butanol. The other is the possibility of a neighbouring aspartic acid forming an ion pair with the *N*-terminal ammonium group. In this connexion it is noteworthy that sequence identities and similarities between porcine and penicillopepsin are especially pronounced near at least four aspartic

acid residues. In porcine pepsin evidence is available for the presence of three aspartic acids near or at the active site (Hartsuck & Tang, 1972).

For the transfer reaction a new molecule of substrate binds with its hydrophobic side chains into subsites S'_1 and S'_2 and its *N*-terminal interacts with the carboxylate on the enzyme. The partial withdrawal of the proton from the nitrogen enables the amino group to exert a nucleophilic attack on the carbonyl carbon of the acyl group thus forming the transeptidation product. The requirement for two hydrophobic side chains is indicated from the fact that Leu-Tyr is an acceptor but not a substrate and Leu-Gly-Leu is neither (Wang *et al.*, 1974). In contrast, Leu-Tyr-Leu is a good substrate and acceptor. Leu-Gly-Leu in fact acts as activator of Leu-Tyr-amide cleavage (Wang *et al.*, 1974) presumably by binding elsewhere into the extended binding site.

The failure of hydroxylamine and alcohols to act as acceptors for the acyl group agrees with the observation of Cornish-Bowden *et al.* (1969) who used [^{14}C]methanol as a nucleophilic agent and failed to obtain evidence in porcine pepsin for an acyl intermediate. [^{14}C]Methanol acts as an acceptor for the acyl group of the substrate in chymotrypsin (Inward & Jencks, 1965). The high yield of transfer when substrates at millimolar concentrations act as acceptors suggests that a high degree of binding specificity is required for a compound to act as acceptor.

Silver & Stoddard (1972) questioned the relevance of the amino intermediate in pepsin-catalysed transeptidation reactions to hydrolytic reactions. One of the arguments used was that the pH-dependence of transeptidation does not follow that of hydrolysis and that at low pH no transeptidation occurs. The pH-dependence of the acyl transfer shown in this paper for penicillopepsin follows that of the hydrolytic activity more closely. Optimum reaction was observed at pH 3.6 which is close to the observed optimum for trypsinogen activation by penicillopepsin (pH 3.4) and within the optimum range for bovine serum albumin hydrolysis (pH 2–3.5; Mains *et al.*, 1971). However, pH does influence the enzyme qualitatively since in the action of penicillopepsin on the B chain of insulin at pH 1.9 the major product from the C-terminal region is the hexapeptide, whereas at pH 3.6 the pentapeptide Thy-Thr-Pro-Lys-Ala is mainly formed (Mains *et al.*, 1971).

*Implication for the mechanism of pepsin-catalysed hydrolytic reactions**

The evidence presented by Takahashi *et al.* (1974) and in this paper shows conclusively that certain

* This has also been discussed in part elsewhere (Hofmann, 1974).

pepsin- and penicillopepsin-catalysed transeptidation reactions involve a covalent acyl intermediate. Analogous evidence from a number of laboratories (Neumann *et al.*, 1959; Fruton *et al.*, 1961; Terada *et al.*, 1971) has been obtained for an amino intermediate in transeptidation reactions where the C-terminal of *N*-blocked peptides is transferred. This evidence has been used by Knowles (1970) and Delpierre & Fruton (1965) to postulate that the pepsin-catalysed reactions involve an amino intermediate. Evidence has earlier been obtained for an acyl intermediate from experiments that show that pepsin catalysed the exchange of ^{18}O between the free carboxyl group of an acyl amino acid product and water (Sharon *et al.*, 1962). However, Shkarenkova *et al.* (1968) have subsequently shown that the ^{18}O is incorporated from H_2^{18}O into the active-site carboxyl group of pepsin in the absence of an acyl amino acid and can be transferred via the enzyme to the product. The ^{18}O -exchange experiments therefore do not require the formation of an acyl intermediate. With the demonstration of acyl intermediates in this work, however, it becomes clear that mechanistic proposals that do not involve an acyl intermediate are insufficient for a complete description of pepsin-catalysed reactions.

A key question in this connexion is whether the transeptidation reactions which are confined to free *N*-terminal or C-terminal residues respectively are representative of all pepsin-catalysed reactions. Especially important is the question 'Which is the pathway of hydrolytic reactions catalysed by pepsin involving good substrates?' Almost all the mechanistic experiments, including the kinetic experiments of Hollands & Fruton (1969), Greenwell *et al.* (1969), Kitson & Knowles (1971b), Silver & Stoddard (1972) and others, have so far been carried out with poor substrates (low k_{cat}). Fruton (1970) has clearly demonstrated that secondary binding with larger substrates has a dramatic effect on the catalytic constant and experiments with penicillopepsin (Wang *et al.*, 1974) and porcine pepsin (T. Wang & T. Hofmann, unpublished work) have shown conformational changes directly associated with binding in a secondary site. It is therefore possible that not only the rate of hydrolysis but also its pathway is affected by the secondary binding.

There are five possibilities for the pathway of the hydrolytic reaction:

- (1) Only an amino intermediate forms in hydrolytic reactions whereas acyl intermediates are involved only in transeptidation reactions of the type demonstrated in this paper.
- (2) Only the acyl intermediate is involved in hydrolytic reactions whereas amino intermediates are involved only in transeptidation reactions of the type observed by Neumann *et al.* (1959) and Fruton *et al.* (1961).

Scheme 2. Possible pathways of transpeptidation by amino transfer; double intermediate common to all reactions

Reactions I-IV are described in the Discussion section.

- 3) Appropriate covalent intermediates are necessary only for transpeptidation reactions; hydrolytic reactions of good substrates proceed without forming covalent intermediates.
- (4) Pepsin-catalysed reactions involve both an amino and an acyl intermediate as proposed by Bender & Kezdy (1965). The order of the release of the intermediate would be determined by the nature of the substrate.
- (5) Any reaction, hydrolytic or transpeptidation, can proceed via one intermediate or the other (but not both). The nature of the substrate would determine which intermediate is on the pathway of any particular reaction.

Possibilities (1) and (2) are unlikely on the following grounds. The evidence for the amino intermediate comes from the transpeptidation reactions and from kinetic studies. In both types of studies poor substrates were used and as Silver & Stoddard (1972) have pointed out transpeptidation of the amino-transfer type proceeds only at high pH values and with substrates with a free C-terminal carboxyl group, and not with peptides in which the C-terminal is blocked by an amide or methyl group. No evidence for an amino intermediate has as yet been obtained from good substrates (high k_{cat}). Similarly the evidence for the acyl intermediate comes from the transpeptidation studies discussed in this paper. So far these reactions, too, have been observed only with poor substrates which in addition have a free N-terminal ammonium group. There is therefore no

logical reason for preferring one of the intermediates over the other for a rapid hydrolytic reaction.

The possibility that hydrolysis proceeds without the formation of covalent intermediates has been mentioned by Silver & Stoddard (1972) and must be given serious attention in future studies. Because of the very large effect of the secondary binding sites on the catalytic efficiency as demonstrated by Fruton (1970) it is essential that mechanistic studies be carried out with good substrates. This possibility can therefore at present not be ruled out.

Bender & Kezdy (1965) suggested that two carboxylic acid groups on the enzyme could reversibly form an anhydride which could then undergo an exchange reaction with the peptide substrate to form an amino intermediate from the amino moiety, and an acyl intermediate from the acyl moiety of the substrate. Clement (1973) (equation 30) proposed the formation of the double intermediate without the necessity of an anhydride in the enzyme. The proposal for a double intermediate is attractive because it allows all three reactions (hydrolysis, amino transpeptidation and acyl transpeptidation) to proceed through a common initial step (reaction I in Schemes 2 and 3). In the double intermediate formed the acyl moiety of the peptide bond of the substrate would be linked to one carboxyl group of the enzyme through a carboxylic anhydride and the amino moiety to another carboxyl group through an amide bond. The subsequent step

Scheme 3. Possible pathways of transpeptidations by acyl transfer: double intermediate common to all reactions

Reactions I-IV are described in the Discussion section.

would be the loss of the acyl group (reaction II, Scheme 2) to give an amino intermediate or the loss of the amino moiety to give an acyl intermediate (reaction II, Scheme 3). The first of these was originally proposed by Bender & Kezdy (1965). According to these authors the anhydride bond could readily cleave by general acid catalysis. At this point we would be left with an amino intermediate which could be hydrolysed by internal catalysis by the neighbouring carboxyl group on the enzyme. The net result would be substrate hydrolysis. A model compound for such internally catalysed hydrolysis is phthalamic acid in which a carboxyl group in the *ortho* position to a carboxamide group catalyses the hydrolysis of the amide (Bender *et al.*, 1958). Alternatively the intermediate could react with suitable acceptors. In this case the result would be transpeptidation of the amino-transfer type (Scheme 2). Two pathways are possible for this reaction. In the first (reactions II and III, Scheme 2) it would be assumed that reaction II was essentially irreversible and that the acceptor acyl group R_4CO_2^- could act as acceptor without forming a new covalent double intermediate. Transpeptidation would be essentially irreversible and asymmetrical. This is clearly unlikely. The second pathway would be symmetrical. Reaction IV (Scheme 2) would be analogous to reaction II (Scheme 2) and would involve the formation of the double intermediate by reaction of a carboxylate ion of the

acceptor (R_1CO_2^- or R_4CO_2^-) with a carboxy group on the enzyme. [Amino transpeptidation has not been observed at low pH and is optimal at pH 4-4.6 (Kitson & Knowles, 1971*b*; T. Wang & T. Hofmann, unpublished work). The acceptor peptide would therefore have to be in the carboxylate form.] Such a reaction would be energetically highly unfavourable, but cannot be ruled out completely.

Scheme 3 shows the analogous situation involving the acyl intermediate. The same double intermediate as in Scheme 2 would be formed (reaction I). However, the subsequent reaction (II) presents a difficulty. It would necessitate the hydrolysis of the amide bond of the amino intermediate while retaining the acyl bond. In this case internal catalysis by the neighbouring carboxyl group would not be possible and it would be necessary to postulate a new catalytic group or groups to bring about the hydrolysis of this amide bond. Whereas the hydrolysis of the acyl intermediate would present no difficulties the transpeptidation reaction would present problems similar to those outlined for the amino intermediate (Scheme 2). Reaction III shows the formation of the product without re-forming the double intermediate. This would again present the improbable irreversibility and asymmetry of the transpeptidation. The path through reactions II and IV on the other hand would be symmetrical and fully reversible, but it would require the formation of

Scheme 4. *Alternative pathways for pepsin-catalysed reactions*

Roman numerals are reaction numbers; see the text.

the amino intermediate from the carboxyl group on the enzyme (which could be either charged or uncharged) and the charged ammonium group of the acceptor $\text{R}_3\text{-NH}_3^+$. This reaction, too, would be energetically unfavourable. For a number of reasons therefore the proposal for a common covalent double intermediate is not attractive.

If we, however, assume that not all pepsin-catalysed reactions involve the same covalent double intermediate then the energetic and mechanistic difficulties outlined above largely disappear. We should like therefore to postulate that not all pepsin- and penicillopepsin-catalysed reactions proceed via the same mechanism. We propose that the nature of the substrate will determine the type of covalent intermediate that will be in the pathway of the reaction. The proposed pathways are summarized in Scheme 4.

The first step would, of course, be the formation of an enzyme-substrate complex as in all other mechanisms (reaction I). Reaction II shows the formation of the acyl intermediate which then reacts with an acceptor, $^+\text{NH}_3\text{-R}_3$ (reaction V) or is hydrolysed (as shown also in Scheme 1). Reaction III shows the possibility of hydrolysis without formation of a covalent intermediate. Reaction IV shows the formation of an amino intermediate. Detailed mechanisms for such a reaction have been proposed by numerous authors (e.g. Clement, 1973). The amino intermediate then hydrolyses (reaction VII) or reacts with an acceptor $\text{R}_4\text{-CO}_2^-$ (reaction VIII) to give transpeptidation of the amino-transfer type. With this proposal the transpeptidation reactions of both acyl- and amino-transfer types are symmetrical and completely reversible without requiring energetically unfavour-

Scheme 5. *Substrate binding in extended binding site of pepsin and penicillopepsin*

able reactions of the type shown in Schemes 2 and 3.

What the proposal does require is that the nature of the catalytic groups can be modified as a result of substrate binding. This hypothesis receives support from the fact that the catalytic centre of the enzyme in its native state is in a state of very low catalytic efficiency as shown by the low values of k_{cat} towards small substrates. As has been stressed by Fruton (1970) a secondary interaction is required (which presumably induces a conformational change at the catalytic apparatus) to generate the high catalytic efficiency observed when pepsin acts on longer-chain substrates or on proteins. The evidence from Fruton's (1970) extensive studies with peptides of increasing chain lengths shows that the secondary interactions can take place on both sides of the sensitive bond and lead to increases in k_{cat} without appreciable increases in K_M [see Tables III and IV in Fruton (1970)]. This suggests strongly that there are in fact two secondary binding sites, A and B, when we let A be the site that would be occupied by residues P_2-P_n and B that occupied by residues $P_2'-P_n'$ [according to Berger & Schechter (1970)]. Work from this laboratory (Wang *et al.*, 1974; T. Wang & T. Hofmann, unpublished work) has shown directly that binding of non-substrate peptides causes conformational changes, increases the catalytic activity towards small substrates and probably occurs in the secondary binding site. This is indicated in Scheme 5. It is likely that the conformational changes induced by binding in site A differ from those induced by binding in site B although binding in either increases catalytic efficiency. Those substrates with which amino transpeptidation has been observed will bind in the primary site S_1 and S_1' (*N*-substituted dipeptides; Neumann *et al.*, 1959) and in site A with larger substrates (Terada *et al.*, 1971). The latter authors showed that transpeptidation occurred more readily with the substrate Gly-Tyr-Tyr than with the substrate Gly-Tyr-Tyr although hydrolysis was observed with both substrates. Conversely, substrates

that give acyl-transpeptidation reactions bind in the primary sites S_1 and S_1' and, if long enough, in site B, (Leu-Tyr-Leu and Phe-Tyr-Thr-Pro-Lys-Ala). We postulate therefore that the changes induced by binding in site A lead to a configuration of the catalytic apparatus that favours formation of the amino intermediate whereas binding in site B favours the formation of the acyl intermediate. In addition it seems that for the formation of the amino intermediate the presence of the free carboxyl group of the substrate is also required and conversely the presence of a free amino group for the formation of the acyl intermediate. So far no transpeptidation reaction has been observed in which more than one amino acid is transferred. Hydrolysis of substrates with longer peptide chains which bind efficiently on both sides of the catalytic site could proceed via one intermediate or the other depending on the exact local conformation of the catalytic apparatus induced by the binding. Alternatively a conformation could be induced by such substrates that would not require a covalent intermediate. It is clear that a careful study of a number of good substrates is required before evidence for these alternatives can be obtained, but this proposal of alternative intermediates is certainly compatible with the available evidence pertaining to the mechanism of action of these enzymes.

In a review, Clement (1973) suggested that there is as yet no consistent mechanism for pepsin and that more research incorporating new approaches is an absolute requirement. The experiments on the acyl intermediates (Takahashi *et al.*, 1974; and the present paper) and on the activating peptides (Wang *et al.*, 1974) show that Clement's (1973) remarks have validity.

This work was supported by the Medical Research Council of Canada (Grant MT-1982). M. T. was the holder of an M.R.C. (Canada) Fellowship. We thank Mr. C. K. Yu for the amino acid analyses, and Dr. K. G. Scrimgeour for his critical review of the manuscript.

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