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THE MECHANISM OF ACTION AND THE ACTIVE CENTER OF PEPSIN

by

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PREFACE

The work described in the present thesis has been done with the object of gaining further knowledge of the mechanism of action of the proteolytic enzyme Pepsin. The studies described fall into four main steps: (1) Synthesis of radioactive substrates, (2) Isolation of intermediate complexes, (3) Chromatographic separation of peptides corresponding to the active center, (4) Identification of lower radioactive peptides.

Following an introductory section (Chapter 1), the thesis is divided into two main parts, the first (Chapter 2) is dealing with the experimental procedures, and the second (Chapter 3) with the results obtained and a discussion of the significance of the experimental results obtained.

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ABSTRACT

The C^{14}-labelled synthetic substrates, carbobenzoxy-L-glutamyl-L-tyrosine and its ethyl ester were synthesized by the condensation of carbobenzoxy-L-glutamic anhydride and L-tyrosine ethyl ester. C^{14} glutamic acid and C^{14} tyrosine were used in these syntheses giving substrate molecules labelled in the glutamic acid moiety or in the tyrosine moiety.

The various radioactive substrates were incubated at pH 4.0 with pepsin. After a given period of time, the pepsin was isolated from the incubation mixture and found to be radioactive only when substrates containing C^{14}-tyrosine were involved. This radioactive protein was found to liberate C^{14}-tyrosine ethyl ester upon incubation in acid solution. These facts were taken as evidence that the radioactive protein isolated is an intermediary complex pepsin-tyrosine ethyl ester formed during the hydrolysis of the peptide derivative by the proteolytic enzyme. A mechanism is proposed to explain the formation of such a complex.

After partial acid hydrolysis of this radioactive complex, paper chromatography and paper electrophoresis were used to isolate a radioactive peptide. The amino acid composition of this peptide was found to be the following: alanine, aspartic acid, serine, glutamic acid, threonine, glycine, arginine, valine, tyrosine and leucine. We claim that this peptide represents part of the active center of pepsin.

CHAPTER 1

1. Introduction

It is a key object of biological investigators to relate in a precise way the structure of a natural material to its function in a living system. The successful elucidation of the structure of insulins from several species, and of a number of smaller polypeptides, hormones, and antibiotics has stimulated efforts to understand the relationship between protein structure and biological activity. Enzymes are particularly well suited for such investigation inasmuch as their activity and specificity can be studied in vitro. The structure of several enzymes has been investigated by a number of investigators. However, a knowledge of amino acid sequence will not by itself solve the problem of correlating structure and function. Much more information will be necessary to enhance real understanding of the catalytic properties of enzymes. First of all, the region or regions of the protein which are involved in specific interaction with the substrate must be available. Secondly, the kinetic behavior and overall reaction mechanism must be explicable in terms of the enzyme structure.

2. The notion of active center

a. Definition

Since most enzymes are of large molecular weight compared to their substrates, it is clear that only a few of the amino acids of the enzyme can be in contact with the substrate during enzymatic action.

Even in the case of enzymes acting on high molecular weight substrates, such as the proteases and the nucleases, the fact that their specificity is the same for low molecular weight analogs and the sequential nature of their attack on the high molecular weight substrates, indicate that only a small portion of the large substrate is in contact with the enzyme at any one time. It is the amino acids in contact with the substrate and their immediate neighbors which we designate as the active center or active site of an enzyme.

It does not follow that such active parts would retain their activity if they could be split the remainder of the molecule, as the essential structure of an active center may be dependent on the primary and secondary valences which join it to the bulk of the molecule. The identification of active site for any particular enzyme is a matter of very recent history, none of the work done has yet permitted more than very general conclusions to be drawn.

b. Historical review

Information concerning the active center in enzymes can be traced back to 1934. Herriott (41) (42) showed that acetylation of the tyrosine phenolic group caused inactivation of pepsin. Later Herriott (43) inactivated pepsin by iodine and concluded that a substitution in the tyrosine of the enzyme was responsible for the loss of activity. Substitution undoubtedly occurs, for di-iodotyrosine was isolated.

Philpot and Small (71) found the partial inactivation of pepsin by nitrous acid to indicate that alteration of some of the tyrosine led to the loss of activity. However, Philpot and Small (72) measured the extent to

which iodine was introduced into the tyrosine of pepsin and found (at pH 5.4) that some other group present in the protein reacted more rapidly with iodine than the tyrosine did, when this group reacted the pepsin was inactivated.

Felix and Mager (24) found that dialyzed pepsin inactivated by standing in dilute sulfuric acid or in a trypsin solution gave a precipitate with clupein methyl ester. This precipitate together with casein showed a slight digestion, whereas the clupein ester alone and the inactivated pepsin alone gave nothing. The experiment may indicate the transfer of some sort of active group from the original pepsin to the modified clupein.

In 1946, Mayur and Bodansky (57) showed that both true and pseudo cholinesterases were readily inhibited by diisopropyl fluorophosphate (DFP). Later, Jansen and coworkers (45, 46, 47) showed that both the esterolytic and the proteolytic activities of trypsin and chymotrypsin are inhibited by DFP.

In 1951, Jansen, Curl and Balls (49) found that a reaction strictly analogous to that observed between α-chymotrypsin and diisopropyl-fluorophosphate also took place with a variety of similar reagents. The analysis of the crystalline-inhibited protein showed that in each case, the enzyme had undergone phosphorylation with the introduction of a single phosphate residue in the molecule. Hartley and Kilby (35) also found the inhibition of chymotrypsin by diethyl-p-nitrophenyl phosphate to follow the same course. Free nitrophenol was produced in this case, just as HF

was formed in the reaction of the enzyme with diisopropylfluorophosphate. Moreover, the liberation of nitrophenol paralleled the course of inhibition and stood in 1:1 equivalence to the enzyme inhibited.

The work of Michel and Krop (58) concerning choline esterase like that of Bourneil and Webb (14) on horse-liver esterase, can leave little doubt as to inhibitory action of these phosphates on the esterases in question. Jansen and Balls (50) found β and γ-chymotrypsin to react mole for mole with this inhibitor. Trypsin also reacted with diisopropyl-fluorophosphate to form an inhibited protein that on crystallization and analysis contained one mole of phosphate per 20,000 gm. of protein, the value of this equivalent weight comes close to the molecular weight of trypsin.

Hartley and Kilby (36) investigated the reaction of diethyl-p-nitrophenyl phosphate with α-chymotrypsin. One mole of nitrophenol was liberated per mole of enzyme, and hence the results support the hypothesis of a single active center in chymotrypsin.

In 1953, Schaffer, May and Summerson (74) reported that DFP-chymotrypsin prepared with phosphorous labelled DFP, upon partial hydrolysis by enzymes and acid, yielded serine phosphate in appreciable quantity (30%). The same compound (containing 46 per cent of the total radioactive phosphorus) was identified in a partial hydrolysate of DFP-inhibited eel cholinesterase (75). The fact that two esterases, so different in many respects, were both inhibited by organic phosphate and that both gave rise to O-phosphoryl serine on partial hydrolysis, is probably of the greatest impor-

tance. Hydrolysis of DFP and of diethyl-fluorophosphate is accelerated by histidine and imidazole. The catalytic effect is greatly enhanced by the copper chelated derivative of ethylene diamino-O-phenanthroline and α, α'-dipyridyl (78). It is tentatively explained by the formation of a quaternary ammonium compound $\geqslant N^{+}\text{-}PO(OR)_2$, with one of the cyclic nitrogens, in which the reactivity of the phosphoryl radical is high.

In 1954, the important studies of Hartley and Kilby (37) on the action of chymotrypsin on acetyl-p-nitrophenol led them to the conclusion that an acetyl chymotrypsin was formed. This was followed by the significant achievement of Balls and Aldrich (6) in the isolation of this postulated intermediate. These investigators took advantage of the fact that at pH 5 to 6, free p-nitrophenol is rapidly released and the chymotrypsin is inactive toward L-tyrosine ethyl ester. At pH 7.2, the inhibited chymotrypsin readily regains its original activity toward the synthetic substrate and in the clotting of milk, since the inhibited enzyme reacts with hydroxylamine to form one mole of hydroxamic acid per moles of protein, it is inferred that a mono acetyl-chymotrypsin was formed in the reaction at pH 5. That the acetylation involves a group concerned with the enzymic activity of chymotrypsin is indicated not only by the inhibition, but also by the fact that chymotrypsinogen, denatured chymotrypsin, and DP-chymotrypsin fail to cause the rapid liberation of p-nitrophenol from acetyl-p-nitrophenol at pH 5. The nature of the chemicalgroup in chymotrypsin that is acetylated is still unclear, but obviously presents a question of great

importance. Balls and Aldrich raise the possibility that it may be a sulfhydryl group (6), and Hartley (38) has suggested the imidazolyl of a histidine residue as the site of attack. Both thiolesters and acytimidazoles are known to be reactive acylating agents. Since DP-chymotrypsin is inactive in catalyzing the release of p-nitrophenol, it would appear that the diisopropylphosphoryl group may be attached to the same group in the protein as that which is acetylated.

Gutfreund (32) examined the effect of pH on the rate of hydrolysis of benzoyl-L-arginine ethyl ester by crystalline trypsin and interpreted the results to indicate that an uncharged imidazolyl group of histidine was essential for the catalysis.

Fraenkel-Conrat (28) compared the reactivity toward 1-fluoro-2, 4-dinitrobenzene (FDNB) of native chymotrypsin and of its diisopropyl phosphoryl derivative. The results indicated that one of the two histidine residues in the inhibited enzyme showed a decrease in reactivity, while the tyrosine and lysine groups reacted at similar rates in the two proteins, thus, this experimental evidence showed that an imidazole group was the site of attachment of the inhibitory phosphate, and most probably an important part of the enzymatic site. The same conclusion was reached by Gutfreund (32) for chymotrypsin and trypsin on the basis of similarities between the imidazole groups and the catalytic site in regard to their ionization constants as affected by temperature.

In a study of the effect of peroxidase and H_2O_2 on chymotrypsin

Wood and Balls (82) found that a derivative of about half the original activity resulted concomitantly with uptake of one mole of oxygen and destruction of one residue of tryptophan but not of histidine or tyrosine. Reactivity to, and inhibition by diisopropyl fluorophosphate was unimpaired. These results, in conjunction with those earlier obtained by Weil (80) with photooxidation, were in accord with the idea that a histidine residue was involved in the dialkyl phosphate binding by the enzymatic site, but they also suggested the presence of an indole group near that site. It now seems possible that the half active periodate oxidation product that had been crystallized by Jansen and coworkers (46) also was modified at the same indole group.

Massey and Hartley (56) indicated that a fluorescent dye, as the sulfonyl chloride, may also be introduced into the active site; these authors also used FDNB in conjunction with various inhibitors of chymotrypsin, and their results favored the idea that an imidazole group was involved. At the same time, Balls and Aldrich (6) isolated and crystallized the intermediate mono-acetyl-chymotrypsin formed from interaction with the nitrophenyl acetates. In the presence of ethanol, acetyl chymotrypsin formed ethyl acetate, so that the enzyme may be regarded as a transacetylase which transfers acetyl groups from nitrophenol to ethanol (7).

In 1956, Schaffer and his coworkers (76) established the sequence of one of the labelled peptides isolated from the acid hydrolysate of inactivated chymotrypsin. The sequence appeared to be Gly-Asp-Ser-Gly.

This report was confirmed by Oosterbaan and coworkers (65).

Dixon et al (21) obtained two labelled peptides from a chymotryptic hydrolysate of trypsin which had been inactivated with C^{14}-labelled DFP. After reaction with FDNB followed by acid hydrolysis, one of the peptides yielded Cyst-Asp-Glut-Gly-Ser-Ala-Prol-Val, but no dinitrophenyl derivative (DNP derivative). The other peptide contained the same amino acids and in addition E-DNP-lysine. Lysine was probably in C-terminal position, since α, E-diDNP was not formed. Schaffer and coworkers (76) also isolated the tripeptide Asp-Ser-Gly from sarin inactivated trypsin, while Oosterbaan and coworkers (65) isolated the peptide Asp, Ser, Prol, Val, Gly (2 or 3) from DIP-trypsin.

The behavior of phosphoglucomutase is not quite the same as that of the esterases. Larger amounts of organophosphate are required for complete inhibition of phosphoglucomutase (53), the peptides containing the active site were not obtained by reaction with DFP, but were labelled with P^{32} phosphate which is known to bind covalently with the enzyme (1). Koshland and Erwin (54) reported that all the labelled peptides contained aspartic acid, serine, glycine, and glutamic acid, although no sequences were determined, the authors concluded that phosphoglucomutase contained the same amino acid sequence in its active site as chymotrypsin and trypsin.

In 1958, analysis of the enzymically active region in trypsin was accomplished by labelling the enzyme with P^{32} organophosphate, and hydroly-

sis by enzyme or acid procedures, to yield P^{32} labelled peptides. Dixon, Kauffman and Neurath (23) obtained several peptides, one of which contained 15 residues in the sequence: Asp, Ser, $CySO_3H$, Glu, Gly, Gly, Asp, Ser (DIP), Gly, Pro, Val, $CySO_3H$, Ser, Gly, Lys., where DIP represents the diisopropyl phosphoryl moiety.

Oosterbaan and Van Adrichem (66) also examined peptides obtained by peptic digestion of acetyl chymotrypsin, prepared by treatment with p-nitro-phenyl acetate labelled with C^{14} in the carboxyl group of the acetate moiety. Five labelled peptides were isolated from the digests, and although no sequences were determined, the composition of the peptides were compatible with the sequence Gly, Asp, Ser, Gly, Gly, Pro, Leu., where the serine residue bears the acetyl label.

In 1958, Gladner and Laki (30) also identified the peptide Asp, Ser, Gly, Glu, Ala. as the site of DFP binding in DFP inhibited thrombin. Hartley and coworkers (39) determined the sequence Gly, Asp, Ser, Gly. as the site of DFP binding in DFP inhibited elastase. On the other hand, recent studies on inactivated pseudocholinesterase (52) and inactivated horse-liver ali esterase (51) have given the following peptide Gly, Glu, Ser, Ala, Gly. as part of the active site. Thus the sequence Asp, Ser, Gly., which is possibly an essential component of the active site, is identical in chymotrypsin, trypsin, phosphoglucomutase, thrombin, and elastase, while the sequence Gly, Glu, Ser, Ala, Gly is common to horse-liver ali esterase and pseudocholinesterase.

C. DISCUSSION

(1) General consideration

In no single case can a complete picture of an active center be given. The active center determines both the specificity and the catalytic activity, and it must be a structure of some complexity, adapted to a fairly close fitting of the substrate molecule, or at any rate of those parts of it which are concerned in the reaction.

The active center need not be considered to lie within one peptide chain only; it may be a pattern of groups extending transversely across two or more adjacent peptide chains or sections of one folded chain. If so, activity will depend not only on the existence of the combining groups, but also on the intactness and configuration of native protein molecule which determines the relative positions of those groups.

It is well known that when the adjoining peptide chains are separated in denaturation, the activity is lost in practically all cases. The fact that some power of combining with substrate has sometimes been found after denaturation although the activity has been completely lost, would suggest that the individual combining groups are still present although the pattern of the active center has been disrupted. This pattern may be reformed in certain cases where the denaturation can be reversed and the activity then returns. Such a picture is consistent with the fact that combination with the substrate often protects the enzymes from denaturation since it would tend to hold the chains together.

(2) Histidine as a part of active center

The catalysis of hydrolysis of p-nitrophenyl acetate by imidizole, Bender (8), Bruice and Schmir (17) have led to a theory of general base catalysis of ester hydrolysis by imidazole.

Bender and Turnquest have shown that the formation and disappearance of a species which absorbs at E_{245} (corresponding to N-acetyl imidazole) can be determined. Broumer et al (16) have demonstrated the presence of this intermediate in the imidazole-catalyzed hydrolysis of acetic anhydride, p-nitrophenyl acetate, and phenyl acetate, and have shown that its concentration reaches a level high enough to account for the entire hydrolysis in the initial steps. Brecher and Balls (15) have observed a similar intermediate in the hydrolysis of p-nitrophenyl acetate catalyzed by a α-N-benzoyl histidine methyl ester, and have isolated the α-N-benzoyl-1 or 3 N--acetyl histidine methyl ester as an intermediate. Bernhard and Gutfreund (10) have also demonstrated the appearance of an E_{245} intermediate with histidine methyl ester.

However, as Bender points out, even if the active site of chymotrypsin involves an imidazole ring, the mechanism is certainly more complex than a simple displacement by an imidazolyl side chain to give an acyl enzyme intermediate followed by hydrolytic cleavage, since in the catalytic hydrolysis, chymotrypsin is 10^5 times more efficient than imidazole. Also the following differences are evident:

(1) Aliphatic alcohol esters are not split by imidazole but by

chymotrypsin. In this connection, Broumer (16) has shown that imidazole can catalyze the hydrolysis of dimethyl oxalate, but this is rather a special case of a very unstable ester.

(ii) The second imidazole in chymotrypsin, i.e. that not in the active center, is catalytically equivalent to free imidazole, thus the primary imidazole must have a unique environment.

(iii) There is more selectivity in the deacylation step than would be expected from straight hydrolysis of acylimidazole.

(3) Serine as a part of active center

All the work on the degradation of proteolytic enzymes, labelled with diisopropyl phosphoryl-P^{32}, tends to confirm the observation that the aliphatic hydroxyl of a seryl residue bears the phosphoryl group. Results reported in part b indicate that Asp, Ser (PO_4), Gly is a sequence common to the active centers of several esterases, including chymotrypsin, trypsin and cholinesterase. The singularity of this observation is reemphasized by the demonstration of Koshland and Erwin (54) that phosphoglucomutase, which binds phosphate in covalent linkage during its catalytic transfer from glucose-1-phosphate to glucose-6-phosphate, also possesses an active center with a sequence Asp, Ser, Gly, with phosphate esterified on to the seryl. As a result, Koshland has suggested that phosphoglucomutase and other esterases possess the same basic bond-breaking mechanism. The common amino acid sequence being important in the activation of the seryl hydroxyl, clearly however this basic bond-breaking mechanism is

overlaid with other structural factors which determine the substrate specificity, and since in the case of phosphoglucomutase there is no hydrolysis, they also prevent a nucleophilic attack by water on the enzyme bond phosphoryl. Dixon and Neurath (61) have reported the isolation of a number of large labelled peptides from tryptic hydrolysate of diisopropylphosphoryl (P^{32}) labelled trypsin, oxidized with performic acid. The largest peptide isolated is a peptide containing 55 residues which possesses five cysteic acid residues and no histidine.

(4) Proposed mechanisms

Many workers have assumed that serine hydroxyl is the secondary point of attachment of phosphate, as the result of an N $\longrightarrow$ O migration, the favorite mechanism being the initial formation of an unstable N-phosphoryl imidazole which subsequently donates the phosphoryl to serine. A major piece of evidence for this view has been the two phase nature of the reactivation of organo phosphorus inhibited esterases by nucleophilic reagents, there being an initial reactivable phase immediately after inhibition (44) passing into a non-reactivable phase after several hours. The first stage is equated with phosphoryl imidazole, and the second with phosphoryl serine. However, most of the evidence has been gathered from cholinesterase, diisopropyl phosphoryl chymotrypsin and trypsin have never been reactivated to a significant extent. Dixon and Neurath (22) showed previously that the diisopropyl phosphoryl group was immediately attached to trypsin to form a stable intermediate without any unstable intermediate

being detectable.

Gutfreund and Sturtevant (33) have proposed a general mechanism to account for the hydrolysis of p-nitrophenyl acetate and other esters by α-chymotrypsin (Fig. I). ES stands for the rapidly formed adsorption complex. ES' is formed from it by the splitting off of one reaction product P_1 (in this case p-nitrophenol), therefore ES' would represent the mono-acetyl chymotrypsin. The last step involves liberation of the acyl group from the enzyme to give acetic acid (P_2). Rate constants were determined. It was concluded that the second step involved acetylation of the OH of a serine residue in the enzyme, and that the last step involved the imidazole group of a histidine residue.

Cunningham (19) has suggested a more elaborate mechanism whereby many of these observations are correlated. The existence of a hydrogen bond between serine and a conjugate base form of histidine, whereby the seryl oxygen is activated, accounts well for the pH dependence of activity, phosphorylation of DFP, and acetylation by p-nitrophenyl acetate as well as for the immediate, stable binding of diisopropylphosphoryl or acetyl (at low pH).

The observed dependence of the activity, DFP-inhibition and acetylation by p-nitrophenyl acetate upon the structural integrity of the molecule, is explained by the assumption that the histidine and serine are in different chains (or portions of chains) and would be separated by the disorientation in urea solution. The observation that a large peptide containing the serine but no histidine may be derived from trypsin lends

weight to the assumption that the histidine and serine are well separated in the sequence, but not in space.

The reactivity of the acetyl group toward hydroxylamine is also dependent on the structural integrity and is lost reversibly upon urea denaturation, and this would be explained by assuming that the acetyl imidazolyl complex, seen in hydrolytic deacetylation, is also the reactive intermediate in reaction with hydroxyl amine, and would not be formed when the imidazole is spatially separated from the acetylated seryl side chain.

3. Pepsin

a. Introduction

From the previous discussion, it seems clear that in the case of proteolytic enzymes, such as chymotrypsin and trypsin which have also an esterolytic activity, the active center of the esterolytic function of the enzyme involves at least a histidine and a serine residue; whether both activities are mediated by a single site or a number of separated sites on the enzyme surface, is a problem of current biochemical interest and importance. Some workers concluded that the esterolytic and the proteolytic activities of trypsin and chymotrypsin must be located at a single site, with the following evidence: (I) inhibitors of fairly large molecular weight produce equivalent decreases in the two activities (45); (II) the enzymes hydrolyze either type of substrate at a decreased rate in the presense of the other type (27); (III) photooxidation of chymotrypsin with visible light results in rapid inactivation of both activities (80).

These evidences do not preclude the existence of closely adjacent or overlapping sites.

On the other hand, the same two activities are inactivated unequally when chymotrypsin is x-irradiated in dilute solution (2), or oxidized with sodium periodate (48). This could be interpreted as meaning that two active sites are involved. Recent studies (3), using x-ray inactivation and ultraviolet inactivation, have been interpreted as showing the existence of overlapping sites, where the hydrolytic apparatus would be common but the elements which could form specific attachment would vary with the substrate.

Pepsin is a proteolytic enzyme which is completely devoid, as far as we know, of esterolytic activity. We thought it would be very interesting to gain some knowledge about the mechanism of action and also about the amino acid composition at the active site of this enzyme in order to compare it with the active center of enzymes having two activities.

(B) The Chemistry of Pepsin

(1) Historical

Pepsin is the proteolytic enzyme of gastric juice which starts the digestion of proteins. It was discovered by Schwann in 1836. In 1882, Langley (55) was able to obtain pepsinogen, the inactive precursor, by extracting the gastric mucosa of a pig with alkaline solutions, and to convert the pepsinogen into pepsin by acidification. In 1930 pepsin was obtained in crystalline form by Northrop (63).

(2) Method of preparation

Swine pepsin was first crystallized from commercial preparations which still serve as the most convenient starting material; the enzyme may also be obtained from gastric juice or from pepsinogen isolated from the gastric mucosa. Purification is accomplished by precipitation with magnesium sulfate (Ca. 0.6 saturated) at acid pH, dissolution of the precipitate with alkali (pH less than 4) and reprecipitation by acidification to pH 2.5. Crystallisation occurs when this precipitate is dissolved at pH 4, and 45°C, and the solution allowed to cool. Pepsin may also be crystallized from twenty percent ethanol (64).

Pepsin is produced from pepsinogen autocatalytically. Acidification of pepsinogen solutions is sufficient to bring about activation, since traces of pepsin are always present. During this process, a polypeptide (molecular weight, Ca. 5000) is split from the pepsinogen molecule, and in solution more alkaline than pH 5.4 it combines with pepsin and causes inhibition.

(3) Physical properties

Molecular weight determinations by a variety of methods seem to point toward 35,000 as the most probable value. The results obtained by sendimentation and diffusion measurements are most reliable and yield the value 35,500 (70). The pure protein is believed to have an isoelectric point below pH 1, a value which is in accord with the low content in basic amino acid residues.

(4) Chemical composition

The pepsin molecule appears to be composed of single polypeptide chain containing three disulfide bridges. The most recent values for the amino acid composition are included in Table I (13), which shows that pepsin has only 4 basic amino acid residues (one each of lysine, histidine and 2 arginine residues). The pepsin molecule has a single phosphate ester of -O-p-O- diester type (67), this phosphorus can be removed enzymatically by potato phosphatase at pH 5.6. The dephosphorylated pepsin has a slightly higher isoelectric point than pepsin itself and is still fully active. It was shown by Flavin (26) that the phosphate residue was bound to a serine residue in the sequence -threonyl-seryl-glutamyl-.

The N-terminal groups of pepsin have been found to be isoleucyl-glycyl- (77). The action of carboxypeptidase on pepsin gives alanine as the C-terminal residue (51).

(5) Stability

Pepsin is optimally active at pH 2 and is unstable in this pH region due to autolysis. It reveals maximum stability at pH 5-5.5 and at higher pH becomes highly sensitive to alkali. This inactivation is partly reversible. Pepsin is irreversibly inactivated by urea at temperature above 20°C; the rate of inactivation is function of urea concentration, pH, and composition of solvent (69).

(6) Enzymatic activity

Using various protein substrates, Northrop (62) observed a broad pH region of peptic activity extending from below pH 2 to above pH 4 with a maximum at pH 1.8. In contrast, when synthetic substrates were used, maximum hydrolytic activity occurred at pH 4 (29).

Pepsin hydrolyzes most of the high molecular proteins and a number of synthetic substrates. The synthetic substrates are listed in Table II.

(7) Mechanism of action and active center

Little is known about the mechanism of action and the active center of pepsin. In 1954, Baker (5) suggested a mechanism identical with the one presented in Figure I to explain results obtained with acetyl-L-phenylalanine-L-tyrosine. However, it was later shown that her results could not support such a mechanism (31). Perlmann (68) reported the formation of enzymatically active dialysable fragments during the autodigestion of pepsin. The pH studies of Bull and Currie (18) suggested that a carboxyl group was involved in the active center. Recently, Neumann and coworkers (60) presented a general mechanism to explain the pepsin catalyzed transpeptidation they have observed during the hydrolysis of carbobenzoxy-L-glutamyl-L-tyrosine. This mechanism would involve the formation of an intermediate complex pepsin-tyrosine.

CHAPTER 2

EXPERIMENTAL PROCEDURES

I. Preparations

a. C^{14}-L-tyrosine (I)

Uniformly labelled C^{14}-L-tyrosine (0.01 millicuries in 0.18 mg.) was diluted with unlabelled L-tyrosine (62 mg.). The mixture was crystallized from ethanol to give C^{14}-L-tyrosine having a radioactivity of 51,800 counts per minute per mg.

b. C^{14}-L-Glutamic acid (II)

Uniformly labelled C^{14}-L-glutamic acid (0.01 millicuries in 0.17 mg.) was diluted with unlabelled L-glutamic acid (60 mg.). The mixture was crystallized from ethanol to give C^{14}-L-glutamic acid having a radioactivity of 47,700 counts per minute per mg.

c. C^{14}-L-Tyrosine ethyl ester hydrochloride (III)

C^{14}-L-tyrosine (36 mg., 0.2 mM) was dissolved in dry ethanol, saturated with dry HCl (20 ml.). The mixture was left overnight at room temperature, then evaporated to dryness under reduced pressure, this procedure was repeated twice. Finally, the residue was dissolved in the minimum amount of ethanol, and ether was added till the solution became turbid. Crystals separated out upon cooling.

Yield: 43 mg. (87.5%)

M.P. : 166°C; Lit. M.P. 166°C (73).

d. C^{14}-L-Tyrosine ethyl ester (IV)

C^{14}-L-Tyrosine ethyl ester hydrochloride (43 mg., 0.17 mM) was suspended in ether saturated with dry ammonia (30 ml.). The mixture was stirred vigorously for 30 minutes, then filtered. The filtrate was evaporated to dryness. The residue was recrystallized from ether-petroleum ether.

Yield: 37 mg. (100%)

M. P.: 103°C; Lit. M.P. 108°C (25).

e. Carbobenzoxy-L-glutamyl-C^{14}-L-tyrosine ethyl ester (VII)

To an ethyl acetate solution (5 ml.) of C^{14}-L-tyrosine ethyl ester (37 mg. 0.17 mM), carbobenzoxy-L-glutamic acid anhydride (48 mg., 0.18 mM) was added. The reaction mixture was stirred for 24 hours at room temperature and then washed twice with dilute hydrochloric acid and twice with water. The ethyl acetate layer was dried over anhydrous magnesium sulfate, and then evaporated to dryness, the resulting material was recrystallized from ether-petroleum ether.

Yield: 36 mg. (43%)

M.P. : 176°C; Lit M.P. 176°C. (9)

Radioactivity: 30,700 C/mdn./mg.

Overall yield based on C^{14}-L-tyrosine: 38%.

f. Carbobenzoxy-L-glutamyl-C^{14}-L-tyrosine. (VIII)

Carbobenzoxy-L-glutamyl-C^{14}-L-tyrosine ethyl ester (36 mg., 0.08 mM) was dissolved in acetone (2 ml.), and 0.9 N NaOH (0.25 ml.) was added.

The mixture was stirred at room temperature for 20 minutes, acidified with 0.9 N HCl (0.28 ml.), and the acetone removed under vacuum. An oil was formed which crystallized after cooling for a few days. The product was recrystallized from acetone-water.

Yield: 20 mg. (59%)

M.P. : 185°C; Lit. M.P.: 185°C (9)

Radioactivity: 30,900 C/min./mg.

Overall yield based on C^{14}-L-tyrosine: 22%.

g. Carbobenzoxy-C^{14}-L-glutamic acid (V)

C^{14}-L-glutamic acid (30 mg., 0.2 mM) and magnesium oxide (25 mg.) were dissolved in water (2 ml.), and ether (2 ml.) was added. The mixture was cooled in an ice bath and stirred, 13 drops of carbobenzoxy-chloride were added within half an hour. The mixture was then stirred for six hours, the solid formed was centrifuged down and the liquid phase was washed three times with 2 ml. aliquots of ether. The water layer was acidified with dilute HCl, and then extracted with ethyl acetate. The ethyl acetate solution was dried over anhydrous $MgSO_4$ and evaporated to dryness, the residue was recrystallized from chloroform-petroleum ether.

Yield: 25.5 mg. (44.5%)

M.P. : 115°C. Lit. M.P.: 117-118°C (11)

h. Carbobenzoxy-C^{14}-L-glutamic anhydride (VI)

Carbobenzoxy-C^{14}-L-glutamic acid (25.5 mg., 0.09 mM) was added to acetic anhydride (0.2 ml.), the mixture was heated on a water bath for a

few minutes, and then evaporated to dryness under reduced pressure, the residue was recrystallized from chloroform-petroleum ether.

Yield: 17.6 mg. (74%)

M.P. : 94°C; Lit. M.P.: 94°C. (11)

i. Carbobenzoxy-C^{14}-L-glutamyl-L-tyrosine ethyl ester (VII)

This compound was prepared according to the procedure described in (e).

Radioactivity: 26,600 C/min./mg.

Overall yield based on C^{14}-L-glutamic acid: 14%.

j. Carbobenzoxy-C^{14}-L-glutamyl-L-tyrosine (VIII)

This compound was prepared according to the procedure described in (f).

Radioactivity: 26,700 C/min./mg.

Overall yield based on C^{14}-L-glutamic acid: 8%.

II. Enzymatic Reaction

a. Pepsin

The crystalline pepsin was obtained from Worthington Biochemical Corporation. This pepsin (0.1 mg. protein N per mg. of enzyme) is twice crystallized from dilute alcohol according to the method of Northrop (64).

Fresh solutions of pepsin were prepared in 0.1 N acetate buffer, pH 4.0. High enzyme concentration was used in order to be able to isolate substantial quantities of enzyme from the incubation mixture using a minimum amount of C^{14}-labelled substrate.

b. Substrate

Because of very low solubility of the compounds, the concentration of substrate employed in the experiment was made low enough to avoid any precipitation during the experiment.

The dipeptide is very insoluble in water. In order to get it into solution at pH 4.0, a weighted amount was dissolved in a small amount of dilute NaOH for two minutes, then dilute HCl was added to neutralize the NaOH.

c. Incubation

(1) Carbobenzoxy-L-glutamyl-L-tyrosine

Carbobenzoxy-L-glutamyl-L-tyrosine (1.713 mg., 4 mM) was dissolved in 0.1 N NaOH (0.25 ml.) and pH 4.0 acetate buffer solution (0.5 ml.) was added, then 0.1 N HCl (0.25 ml.) was added. After the addition of a pepsin solution (1 ml., 13 mg. of crystalline pepsin per 1 ml. of solution) the mixture was incubated at 37°C in a constant temperature bath.

Samples were taken out at different time intervals. The percentage of hydrolysis was determined colorimetrically using the Moore and Stein ninhydrin method (59).

(2) Carbobenzoxy-L-glutamyl-L-tyrosine ethyl ester

Same procedure as in (1).

(3) Controls

The control experiments were run using same conditions as in (1) but no substrate was added to the incubation mixture.

d. Isolation of intermediate complex from the reaction of carbobenzoxy-L-glutamyl-C^{14}-L-tyrosine or carbobenzoxy-C^{14}-L-glutamyl-L-tyrosine with pepsin.

The substrate (0.2 mg.) was dissolved in 0.1 N NaOH (0.25 ml.), and pH 4.0 acetate buffer (0.5 ml.) was added, then 0.1 N HCl (0.25 ml.) and pepsin (1 ml., 13 mg. of crystalline pepsin in pH 4.0 acetate buffer) were added, the mixture was incubated at 37°C for 5 hours, then, the reaction mixture was transferred into a 15 ml. centrifuge tube, 1 ml. of ethanol and two drops of conc. HCl were added. After cooling at 0°C overnight, the precipitate formed was centrifuged down, washed twice with ethanol and twice with ethyl ether, then suspended in acetone and filtered on weighted paper disk (40), dried and counted to a ±2% standard error. The results were corrected for back ground and self-absorption. The apparatus used was Gas-flow-end-window counter (Nuclear Chicago, Model D-47).

e. Isolation of intermediate complex from the reaction of Carbobenzoxy-L-glutamyl-C^{14}-L-tyrosine ethyl ester or carbobenzoxy-C^{14}-L-glutamyl-L-tyrosine ethyl ester with pepsin.

The substrate (0.2 mg.) was dissolved in water (2 ml.), and the pH of the solution was adjusted to pH 4.0. Crystalline pepsin (15 mg.), dissolved in 2 ml. of H_2O and adjusted to pH 4.0, was added, this mixture was incubated at 37°C for 8 hours.

After incubation, the reaction mixture was cooled at 2°C, and extracted six times with 1 ml. portions of cold ethyl acetate, the water

layer was then lyophilised. The solid residue was suspended in acetone and filtered off on a filter paper disk and counted as described in (d).

f. Liberation of L-tyrosine ethyl ester from the intermediate complex

The intermediate complex, isolated as described in (e), was dissolved in acetic acid or distilled water (adjusted to pH 4.0). It was incubated at 37°C in a constant temperature bath.

Samples were taken out at different intervals of time, cooled in an ice bath, and washed six times with ethyl acetate. The resulting aqueous solution was lyophilized, and then the solid residue was suspended in acetone, filtered and counted as described in (d).

These ethyl acetate extracts were evaporated to dryness, the residues were spotted on Whatman paper No. 1 and the chromatogram developed in a mixture of Butanol-acetic acid-water (4:1:5). When the run was finished, the paper was air dried and sprayed with a 0.1% ninhydrine solution. Spots corresponding to L-tyrosine or L-tyrosine ethyl ester were cut out and their radioactivity was determined in a gas-flow counter.

III. Studies on the Active Center of Pepsin

a. Partial hydrolysis of enzyme complex

The intermediate complex (30 mg.) was dissolved in conc. HCl (4 ml.) and let standing for 48 hours at room temperature, then it was put into a vacuum dessicator over P_2O_5 and solid NaOH until dry.

b. Separation of peptides by paper chromatography

The partial hydrolysate (a) was spotted as a streak on Whatman

No. 1 filter paper, the chromatogram was run in butanol-acetic acid-water (4:1:5). An half inch longitudinal strip was cut out from the dry sheet, cut down in small half inch long pieces and each piece was counted to locate the radioactive peptides.

c. Elution of radioactive peptides

Sections of the paperchromatogram containing radioactive substances (as determined in (b)) were cut out and the substances eluted from the paper with distilled water.

d. Paper electrophoresis

The radioactive substances eluted from the paper were spotted again on Whatmann paper No. 1., the paper was then subjected to high voltage electrophoresis (800 volts, 1.5 milliamperes) in 0.05 M acetic acid pH 3.0 for 5 hours. Radioactive regions were located on the paper using the method described in (b).

e. Complete hydrolysis

The purified C^{14}-peptide was completely hydrolyzed with 6 N HCl in a sealed tube at 100°C for 24 hours, and the amino acids in the hydrolysate were determined by means of two-dimensional paper chromatography. (a) butanol-acetic acid-water (4:1:5) (b) phenol-water (4:1 w/w).

f. Semi-quantitative estimation of the amino acid composition of the C^{14}-peptide

The paper chromatogram obtained in (d) was sprayed with a 2% alcoholic ninhydrin solution. After 30 hours in the dark, all the spots

on the paper were cut out. A piece of paper of the same size was also cut out to be used as a blank. The color was eluted from the paper squares with ethyl alcohol and the clear solutions read against the blank at 570 μμ. The amino acid concentration is directly proportional to the optical density.

g. Determination of N-terminal residue of the C^{14}-peptide

The purified C^{14}-peptide was dissolved in water and a small quantity of $NaHCO_3$ was added. To this solution was added 1 ml. of ethanol and one drop of 1-fluoro-2, 4-dinitrobenzene (FDNB). The mixture was left for 2 hours at room temperature. It was then extracted with several 2 ml. portions of ether, the aqueous solution was acidified with a few drops of 6N HCl, then extracted with ethyl acetate; the ethyl acetate layer was evaporated to dryness. The residue was dissolved in 2 ml. of 6 N HCl, and hydrolyzed in sealed tube for 24 hours at 100°C. The hydrolysate was transferred to a centrifuge tube and diluted with 2 ml. of water. The N-terminal amino acid was extracted with three 2 ml. portions of ether.

The ether extract was evaporated to dryness, and the residue was quantitatively transferred to a paper chromatogram with a small portion of acetone, the paper used was Whatmann No. 3 MM paper which was buffered with pH 6 phthalate buffer (12). The chromatogram was developed with tert-amyl alcohol, saturated with pH 6 phthalate buffer. Pure DNP-amino acids were spotted on the same paper for reference.

CHAPTER 3

RESULTS AND DISCUSSION

Pepsin is a proteolytic enzyme which has no esterolytic activity. Pepsin is not inhibited by diisopropyl-fluorophosphate or other inhibitors of that type which were used in the work on esterases. We therefore had to use another approach to study the mechanism of action and the active center of pepsin. The isolation of mono-acetyl-chymotrypsin during the hydrolysis of p-nitrophenyl acetate by chymotrypsin was favored by the fact that the reaction was carried out at pH 5. At this pH value the activity of the enzyme is very low, the release of the acetyl group from the enzyme being the rate limiting step. The hydrolysis of substrates, such as carbobenzoxy-L-glutamyl-L-tyrosine, (equation a, Figure III) by pepsin at pH 4 is a very slow reaction (Figure IV) as compared with other enzymatic reactions. Pepsin hydrolyses proteins or substrates such as acetyl-L-tyrosyl-L-tyrosine at pH 2 much more rapidly than the former substrate. Therefore we thought that the study of the slow hydrolysis at pH 4 could provide us with an answer to our problem. The first step was the synthesis of the labelled substrates. As C^{14}-amino acids are very expensive, the main problem was to prepare the peptide derivatives on a micro scale.

We used a 0.2 millimolar scale and the overall yields obtained in the case of the C^{14}-L-tyrosine labelled substrates (38% and 22%) can be considered as very good on such a scale. In the case of the C^{14}-glutamic acid labelled substrates, lower yields were obtained (14% and 8%), mainly

because the preparation of carbobenzoxy-L-glutamic acid gives a low yield. The reactions used in these synthesis were all well known reactions. The conditions were sometimes slightly modified to suit our needs. The overall yields could be raised by 10 to 15 per cent if the complete synthesis was done in the same flask without isolating and crystallising any of the intermediary compounds, but then the melting points of the final products were always a few degrees lower. As it was impossible to say in advance which part of the peptide would remain bound to the enzyme, we had to synthesize two types of substrates: 1. labelled with C^{14}-L-tyrosine, 2. labelled with C^{14}-L-glutamic acid.

The first substrate used was the original substrate used by Fruton and Bergmann (29) carbobenzoxy-L-glutamyl-L-tyrosine, this substrate is more soluble than its ester, and can therefore be used in higher concentration. In order to isolate the protein from the reaction mixture after a few hours of incubation, we have tried many different techniques, such as alcohol precipitation, TCA precipitation, purification on ion exchange resins. Results obtained by all these methods were comparable, but the first one proves to be the most convenient to use. Typical results are given in Table III. It can be seen that only one substrate gives appreciable incorporation, carbobenzoxy-L-glutamyl-C^{14}-L-tyrosine; with the substrate labelled with C^{14}-L-glutamic acid, no incorporation whatsoever was obtained. The time zero controls did not show any incorporation, this rules out contamination of the precipitate by the substrate. The C^{14}-L-tyrosin control showed little incorporation; this shows that contamination

by the product of the reaction C^{14}-L-tyrosine is not important. Therefore, this can be taken as an indication that most of the incorporation obtained with the C^{14}-tyrosine labelled substrate is due to the binding to the enzyme molecule of C^{14}-L-tyrosine as a result of the enzymatic hydrolysis. As a conclusion, we assume that there is formation of a pepsin-tyrosine complex during the hydrolysis due to the transfer of tyrosine from the substrate to some unknown group in the protein molecule, followed by the liberation of tyrosine and regeneration of the free active enzyme. The amount of such a complex we were isolating at that moment was very small, probably due to the method used for the isolation, we then decided to use a method identical to the one used to isolate mono-acetyl-chymotrypsin (7). We used carbobenzoxy-L-glutamyl-L-tyrosine ethyl ester as substrate because the substrate and the products of the reaction, carbobenzoxy-L-glutamic acid and L-tyrosine ethyl ester (equation by Figure III) are all soluble in organic solvents and can be extracted from the reaction mixture, leaving the pure protein which can be isolated by lyophilization, the results obtained are given in Table III. Here again there is a strong indication that a complex pepsin-tyrosine ethyl ester is formed as a result of the enzymatic hydrolysis, the incorporation obtained was much higher than that obtained in the previous experiment, the values obtained were between 26 and 54 counts per minutes after 8 hours (26, 41, 54, 35 and 32 counts/min.), showing that the complex is not very stable. Much higher incorporation was obtained later for longer incubation periods (Figure II). This figure shows that the radioactivity of the protein precipitate increases up to about 24

hours after the beginning of the incubation and then remains at the same level for quite a long time. If we compare this curve to curve B (Figure III) which gives the percent of hydrolysis in function of time, we see that it is between the 20th and 24th hours that there is a change in the slope of the hydrolysis curve. Afterward the rate of hydrolysis is much slower. At about the same time, the incorporation of tyrosine ethyl ester into pepsin or the formation of pepsin-tyrosine ethyl ester complex reaches a plateau. The procedure used to isolate this enzyme precipitate: extraction with ethylacetate, freeze drying, washing with acetone, leave no doubt that the tyrosine ethyl ester is firmly bound to the enzyme, otherwise it would have been washed away with the organic solvents.

We then made a few experiments to see if we could liberate the C^{14}-tyrosine ethyl ester from the enzyme. This series of experiments was not entirely successful, because we were not able to liberate completely all the radioactivity from the protein in water solution. There is a rapid initial liberation as soon as the precipitate is dissolved in water, and then the process becomes very slow (Table V). It is possible that the enzyme has been partially denatured during the isolation, organic solvents are known to denature proteins, and that part of the tyrosine ethyl ester is irreversibly bound to the enzyme. But the examination by paper chromatography of the ethyl acetate extracts from these experiments has clearly shown the presence of free C^{14}-tyrosine ethyl ester (Table V). The only explanation possible for the presence of this compound in these extracts

is that it was liberated from the enzyme intermediate complex. In acetic acid solution the liberation is much more rapid and most of the radioactivity can be liberated from the enzyme. It was also possible to show the presence of C^{14}-tyrosine in addition to C^{14}-tyrosine ethyl ester on the paper chromatogram. The amount of tyrosine seems to increase with time and with the temperature. It is therefore possible that in acetic acid solution the tyrosine ethyl ester liberated is slowly hydrolysed to free tyrosine. The values reported in Table V for the radioactivity of the tyrosine or tyrosine ethyl ester spots are not very high. This is to be expected because the filter paper is known to absorb the C^{14} radiations.

The next step in our investigation was an attempt at elucidating the structure of the active center. If the C^{14}-tyrosine ethyl ester is bound to the active center of the enzyme it is reasonable to expect that upon partial decomposition of this complex, some peptides containing the C^{14} label will be formed. We therefore hydrolyzed partially the complex with concentrated hydrochloric acid, and the resulting peptide mixture was fractionated first by paper chromatography. This process gave rise to a separation into 9 to 10 components (blue bands on paper after spraying with ninhydrin), and only one of these components was strongly radioactive. From time to time, some radioactivity was also found associated with the component remaining at the origin on the paper, and with the component having the same R_f value as tyrosine ethyl ester but always the major fraction of the radioactivity was associated with the component having a

R_f value of 0.35. This component cannot be free tyrosine (R_f value: 0.27), free tyrosine ethyl ester (R_f value: 0.68) or carbobenzoxy-L-glutamyl-L-tyrosine ethyl ester (R_f value: 0.90), we consider these results as a very strong proof that tyrosine ethyl ester is really bound to the enzyme to form a complex, and that this component is a peptide coming from the active center of the enzyme.

The radioactive component was further purified by paper electrophoresis to give a single radioactive component moving by about six centimeters toward the negative electrode in acetic acid buffer 0.05 M, pH 3.0 after being subjected for 5 hours to 800 volts and 1.5 milliamperes. This single radioactive component was analyzed for amino acid by semiquantitative paper chromatography, the results are given in Table VI. Alanine, being an amino acid rather stable to acid hydrolysis, was given the value of 1. It can be seen that alanine, aspartic acid, serine, glutamic acid, threonine, arginine, valine and leucine are present in equimolecular amount while there is twice as much tyrosine; this tyrosine spot is radioactive. The value for serine is rather low, but serine is not very stable during acid hydrolysis. We believe that this peptide, containing aspartic acid, serine, glycine, alanine, glutamic acid, valine, threonine, arginine, leucine, and tyrosine to which is bound tyrosine ethyl ester, is part of the active center of pepsin.

We have not yet been able to determine the amino acid sequence of this peptide. Some preliminary work has been done, using 1-fluoro-2,

4-dinitrobenzene (FDNB) to determine the N-terminal residue. Unfortunately, this peptide does not give easily a N-terminal residue; this may be an indication that the N-terminal residue is either aspartic acid, glycine or serine. Dinitrophenyl-glycine (DNP-glycine) and DNP-serine are easily destroyed during acid hydrolysis. On the other hand, peptides and proteins having aspartic acid as N-terminal amino acid, are known to give very low yield of DNP-aspartic acid. We have also found that the radioactive DNP-peptide is extractable in ethyl acetate from the alkaline dinitrophenylation solution; this is rather unusual. On the other hand, no free DNP-tyrosine or DNP-tyrosine ethyl ester have been isolated by paper chromatography from these reaction mixtures, this is one more proof in favor of the existence of a pepsin-tyrosine ethyl ester intermediate complex.

The existence of such a complex is also in accord with the work of Neumann and coworkers (60) on pepsin-catalysed transpeptidation of the amino-transfer type. They found that during the hydrolysis of carbobenzoxy-L-glutamyl-L-tyrosine, the peptides carbobenzoxy-L-glutamyl-L-tyrosyl-L-tyrosine and L-tyrosyl-L-tyrosine were formed as well as free tyrosine and carbobenzoxy-L-glutamic acid. In order to explain these results they suggested a mechanism involving the formation of an intermediate enzyme-tyrosine complex, then the tyrosine could be transferred to water to give free tyrosine, to tyrosine to give tyrosyl-tyrosine or to the substrate to give carbobenzoxy-L-glutamyl-L-tyrosyl-L-tyrosine; they did not isolate the intermediate complex. Our work, in which the intermediate complex was isolated, suggests an identical mechanism (Figure VI). It should be noted that

it is the first time that a complex between an enzyme and an amino acid or peptide substrate is isolated. According to this mechanism pepsin would act as a transferring enzyme which would transfer an amino group from the substrate first to an unknown group in the enzyme itself, and then to water or to an other receptor having a free carboxyl group. The overall reaction could be considered then as a transpeptidation. This mechanism would also fit the scheme presented in Figure I. We have a stepwise liberation of the products of the reaction with formation of an intermediate ES' complex.

During the last few years several papers have established that in esterases a histidine and a serine residues are involved during the hydrolysis and that in the stable complex isolated the foreign group is bound to the serine residue. It has also been established that the sequence -glycyl-aspartyl-seryl- is common to many esterases. In our work we have not yet established the amino acid sequence of the radioactive peptide isolated, but the amino acid composition (Table VI) is entirely compatible with the amino acid sequence found in esterases. Naturally in pepsin it is difficult to conceive how the hydroxyl group of serine could serve as an acceptor for the amino group of tyrosine, the free carboxyl group arising for the side chain of a dibasic amino acid is much more indicated for such a role. In the radioactive peptide we have two such groups: the β-carboxyl group of aspartic acid and the γ-carboxyl group of glutamic acid. It should be remembered that the pH studies of Hull and Currie (18)

suggested that a carboxyl group is involved at the active center of pepsin. It is also interesting to note that in all the sequences determined in esterases an aspartic acid or glutamic acid residue is next to the active serine residue. The optimum activity of pepsin in acid solution could also be explained by the fact that a carboxyl group is involved at the active center. It is also very interesting to note that the amino acid composition of the radioactive peptide is compatible with the sequence -threonyl-O-phosphoseryl-glutamyl- found in pepsin (26). The solution to this problem must await the determination of the sequence of the radioactive peptide isolated.

CLAIMS TO ORIGINAL RESEARCH

1. The synthesis of C^{14}-labelled pepsin substrates have been accomplished for the first time.

2. C^{14}-labelled peptide substrates were used for the first time to study the mode of action of a proteolytic enzyme.

3. A mechanism for the enzymatic hydrolysis of a peptide by pepsin has been proposed.

4. Intermediate complexes pepsin-tyrosine and pepsin-tyrosine ethyl ester have been isolated for the first time.

5. The rate of formation of the complex has been studied.

6. The decomposition of the complex has been studied.

7. A peptide from the active center of pepsin was isolated for the first time.

8. The amino acid composition of this peptide was determined.

TABLE I

Amino Acid Composition of Pepsin (13)

Amino acid	Residues per mole	Amino acid	Residues per mole
Lysine	1	Half-Cystine	~6
Histidine	1	Valine	1
Arginine	2	Methionine	~5
Aspartic acid	44	Isoleucine	27
Threonine	28	Leucine	28
Serine	44	Tyrosine	18
Glutamic acid	27	Phenylalanine	14
Proline	15	Tryptophan	6
Glycine	38	Hydroxyproline	0.1
Alanine	18	Amide NH_3	36

TABLE II

Pepsin Substrates

Substrates	Refs.
I	
Acetyl-L-phenylalanyl-L-phenyl alanine Acetyl-L-tyrosyl-L-tyrosine Acetyl-L-phenylalanyl-L-tyrosine Carbobenzoxy-L-tyrosyl-L-phenylalanine	(4)
II	
Carbobenzoxy-L-glutamyl-L-tyrosine Carbobenzoxyglycyl-L-glutamyl-L-tyrosine Carbobenzoxy-L-glutamyl-L-tyrosyl glycine Glycyl-L-glutamyl-L-tyrosine Carbobenzoxy-L-glutamyl-L-phenylalanine	(29)
Carbobenzoxy-L-methionyl-L-tyrosine Methionyl-L-tyrosine Carbobenzoxy-L-cysteinyl-L-tyrosine	(34)
III	
Carbobenzoxy-L-glutaminyl-L-phenylalanine Carbobenzoxyglycyl-L-tyrosine Carbobenzoxy-L-glutamyl-L-tyrosinamide L-glutamyl-L-tyrosine	(29)
Carbobenzoxy-L-tyrosyl-L-cysteine L-Tyrosyl-L-cysteine L-Cysteinyl-L-tyrosine	(34)

The substrate have been divided into three classes according to their approximate ease of hydrolysis I > II > III.

TABLE III

Reaction of Pepsin with Carbobenzoxy-L-glutamyl-L-tyrosine at pH 4.0 and at 37°C

Substrates	time (hrs.)	counts per minute per mg. protein +
Carbobenzoxy-L-glutamyl-C^{14}-L-tyrosine	0	0
Carbobenzoxy-L-glutamyl-C^{14}-L-tyrosine	8	12
C^{14}-L-tyrosine	8	2
Carbobenzoxy-C^{14}-L-glutamyl-L-tyrosine	0	0
Carbobenzoxy-C^{14}-L-glutamyl-L-tyrosine	8	0

+ Results are corrected for selfabsorption and back ground.

TABLE IV

Reaction of Pepsin with Carbobenzoxy-L-glutamyl-L-tyrosine ethyl ester at pH 4.0 and 37°C

Substrates	time (hrs.)	counts per minute per mg. protein +
Carbobenzoxyl-C^{14}-L-glutamyl-L-tyrosine ethyl ester	0	0
Carbobenzoxyl-C^{14}-L-glutamyl-L-tyrosine ethyl ester	8	0
Carbobenzoxyl-L-glutamyl-C^{14}-L-tyrosine ethyl ester	8	26-54

+ Results are corrected for selfabsorption and background.

TABLE V

Liberation of C^{14}-Tyrosine ethyl ester

Time (hrs.)	Medium	pH	Temperature °C.	Radioactivity			
				Initial precipitate + C/min./mg. protein	Final precipitate + C/min./mg. protein	Tyrosine spot C/min.	Tyrosine ethyl ester spot C/min.
96	acetic acid	4	22°	125	2	23	33
20	acetic acid-HCl	2	22°	107	4	21	29
2	acetic acid	4	22°	107	7	3	29
2	acetic acid	4	37°	97	1	10	17
0.5	water	4	22°	102	52		
2	water	4	22°	102	40		
5	water	4	37°	112	99		12
10	water	4	37°	112	82		14
5	water	2	37°	100	90		
10	water	2	37°	100	88		
24	water	2	37°	100	82		

+ Initial precipitate is the pepsin-tyrosine ethyl ester complex which is incubated in the medium. Final precipitate is the pepsin-tyrosine ethyl ester complex which is reisolated after incubation. For more experimental details please see Experimental Part II, F.

TABLE VI

Amino Acid Composition of the Radioactive Peptide from the Active Center

Amino acids	Relative concentration in mole
Alanine	1.00
Aspartic acid	0.70
Serine	0.57
Glutamic acid	0.85
Glycine	0.85
Threonine	0.85
Arginine	0.85
Valine	0.85
Tyrosine	2.42
Leucine	1.14

Mechanism of Enzymatic Ester Hydrolysis

$$E + S \rightleftharpoons E S$$

$$E S \rightleftharpoons E S' + P_1$$

$$E S' \rightleftharpoons E + P_2$$

FIGURE I

Synthesis of Substrates

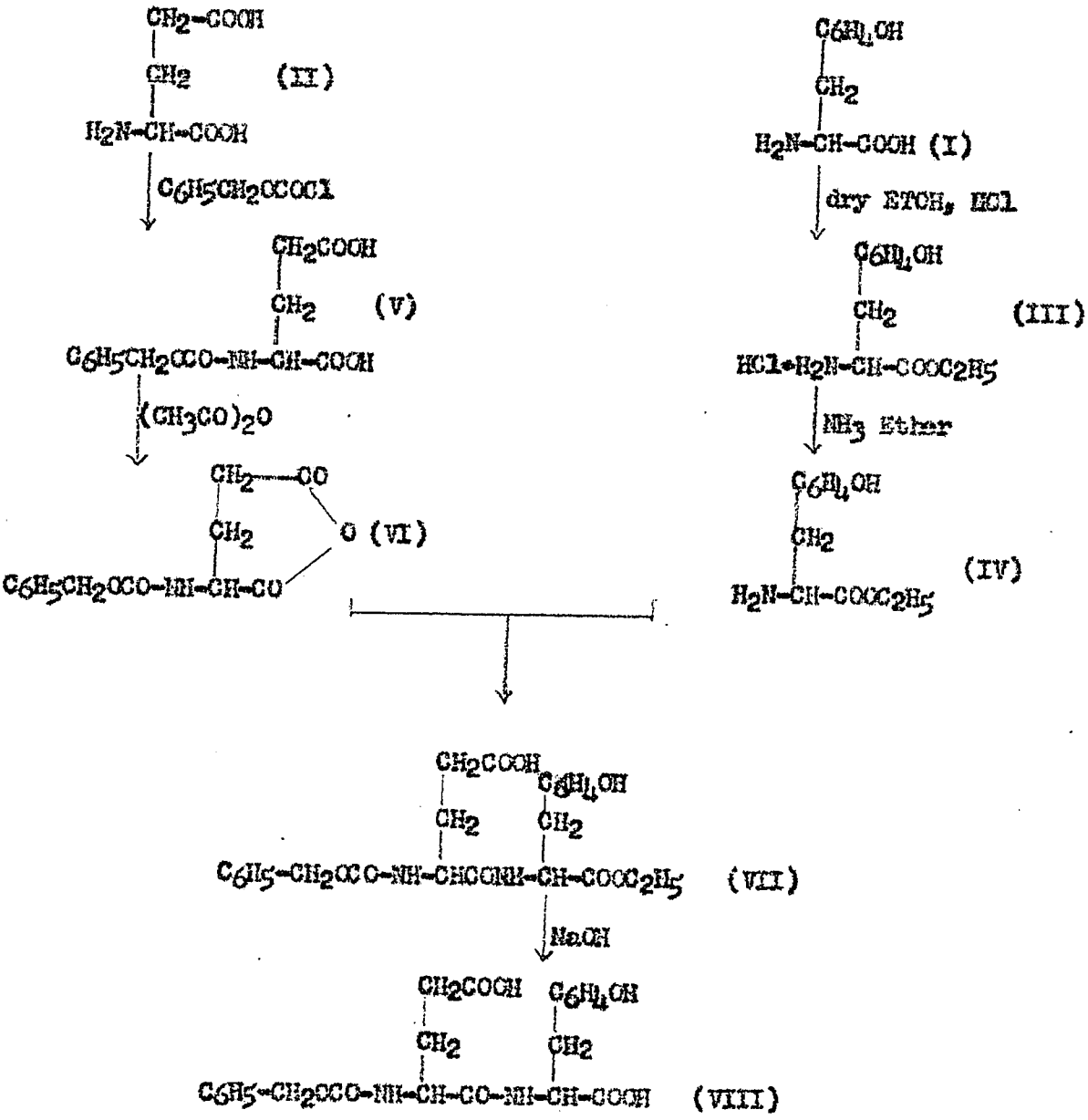
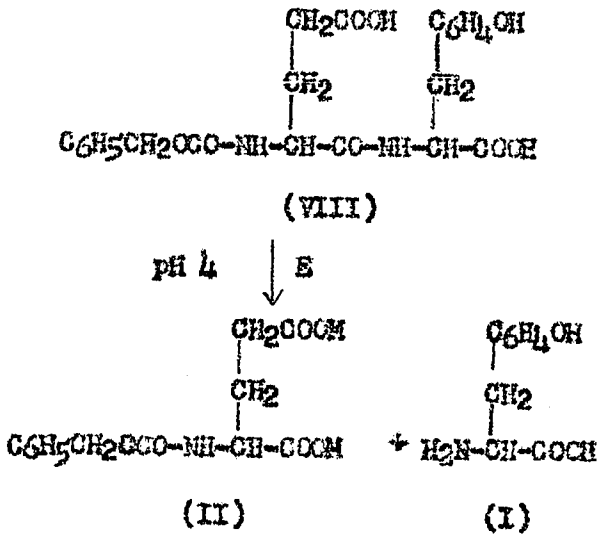


FIGURE II.

Hydrolysis of Substrates by Pepsin

a.



b.

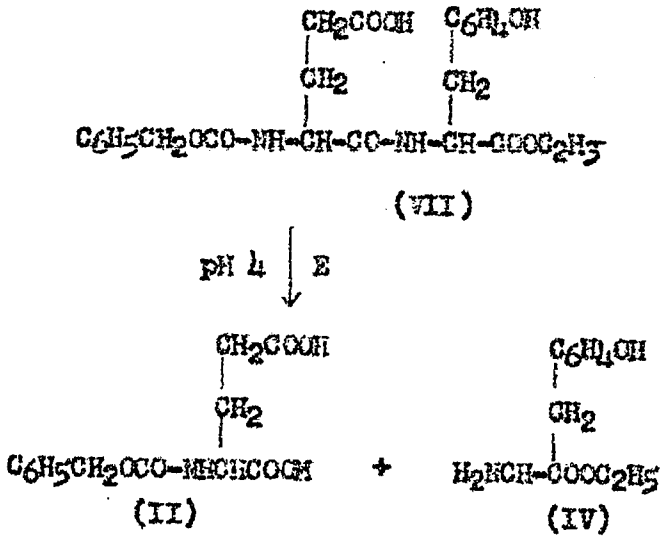


FIGURE III

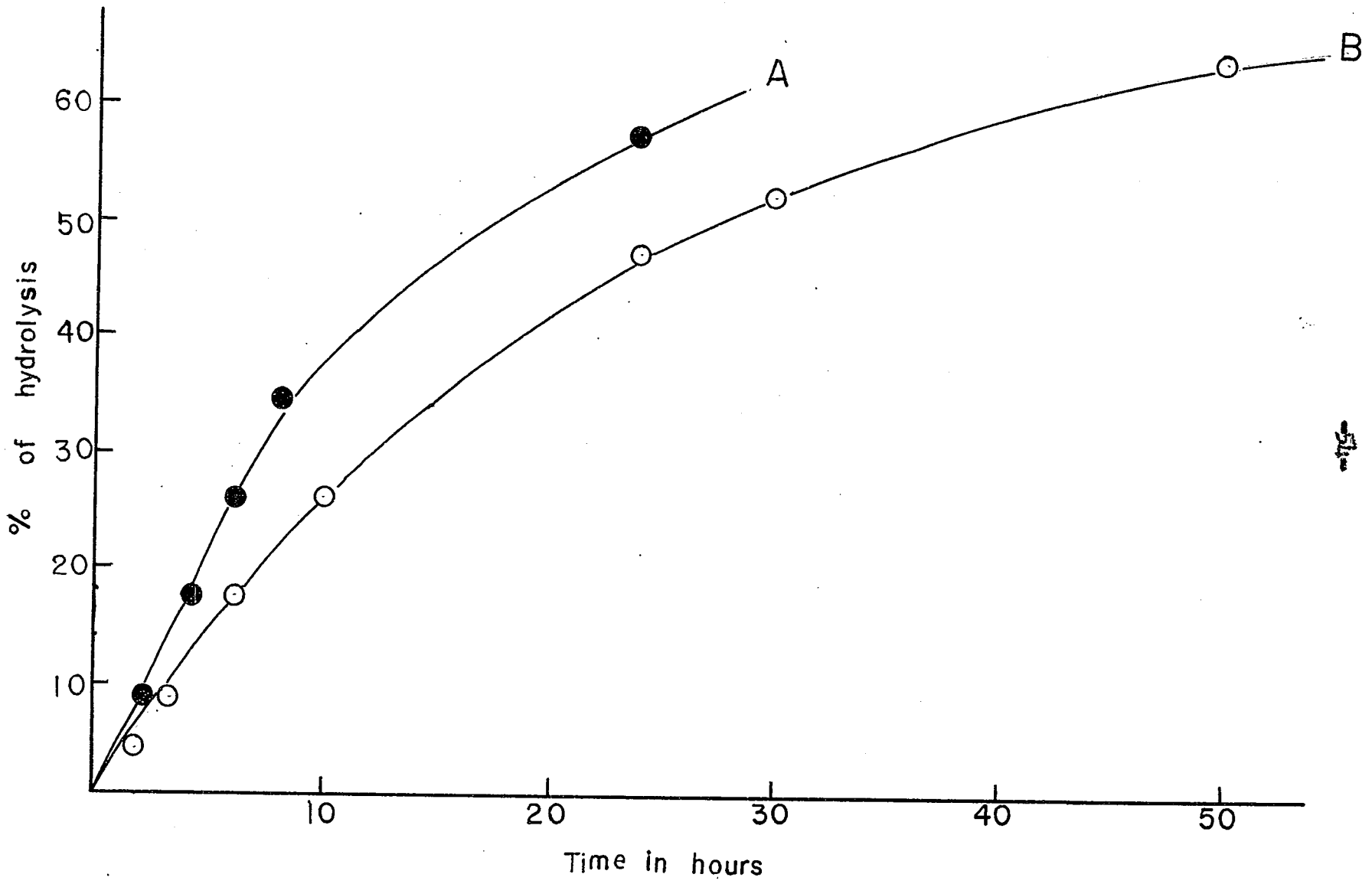


FIGURE IV. Hydrolysis of carbobenzoxy-L-glutamyl-L-tyrosine (A) and carbobenzoxy-L-glutamyl-L-tyrosine ethyl ester (B).

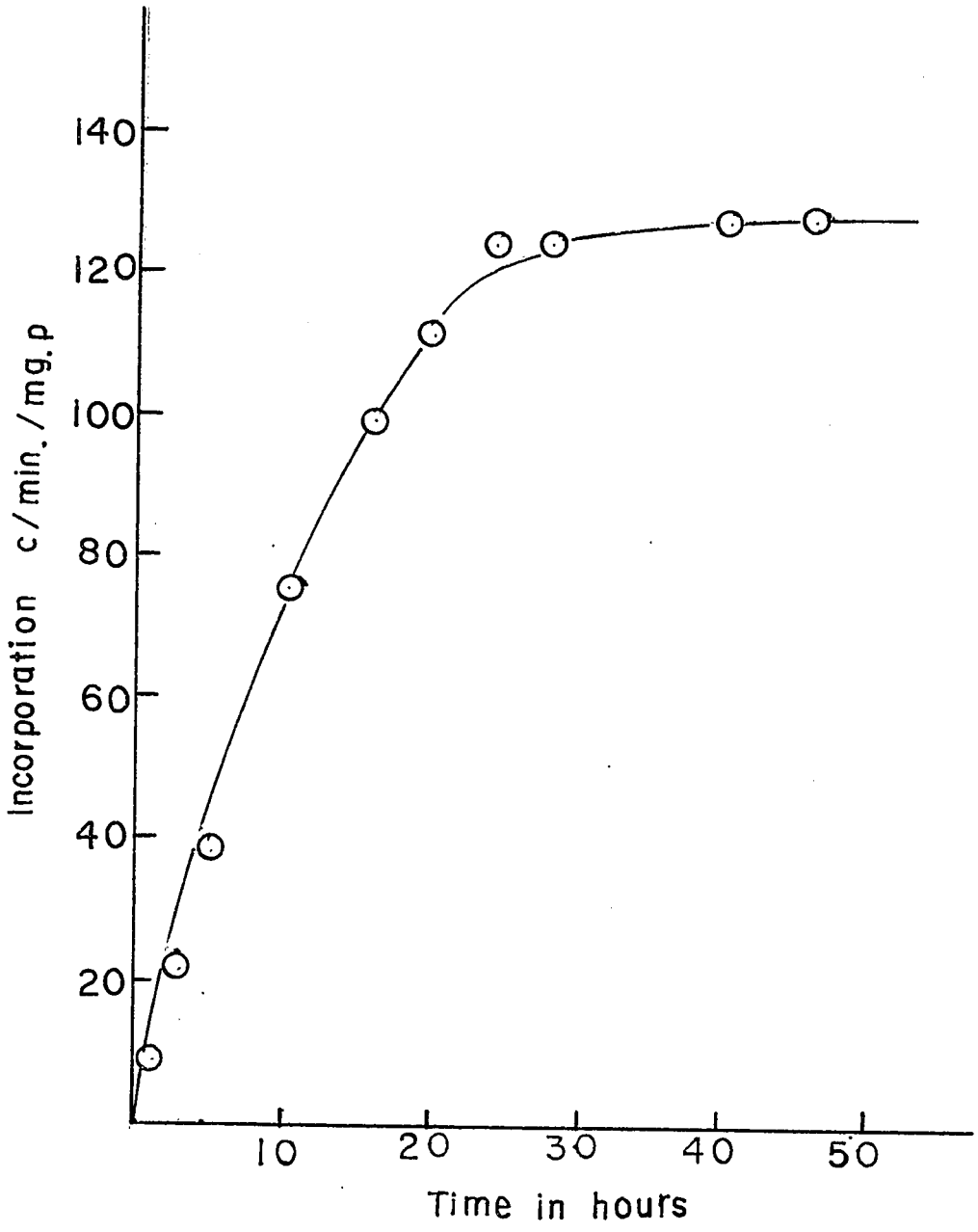


FIGURE V. Incorporation of C^{14}-L-tyrosine ethyl ester into pepsin as a result of enzymatic hydrolysis.

Mechanism of Action of Pepsin

$$R\text{-}CO\text{-}NH\text{-}R' + E\text{-}COOH \longrightarrow R\text{-}COOH + E\text{-}CO\text{-}NHR'$$

$$E\text{-}CO\text{-}NH\text{-}R' + H_2O \longrightarrow E\text{-}COOH + NH_2\ R'$$

FIGURE VI

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