ON PROTEOLYTIC ENZYMES

XI. THE SPECIFICITY OF THE ENZYME PAPAIN PEPTIDASE I

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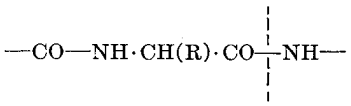
Papain Peptidase I was designated as that component enzyme of papain which splits hippurylamide, benzoylisoglutamine, and many other synthetic substrates and which is inhibited by phenylhydrazine (1–3). Papain Peptidase I occupies a singular position among the proteolytic enzymes for which synthetic substrates are known (4). It is the first enzyme of this group the specificity of which does not rigorously require the splitting of terminal peptide bonds, but operates instead in accordance with another more complicated principle.

Structural Specificity of Papain Peptidase I

Papain Peptidase I attacks only those substrates which contain two peptide bonds in the following arrangement.



I

The polar character of this atomic group results in an inequality in the two peptide bonds. The enzyme is, in fact, able to split only one of the two, namely, the one indicated by the broken line.

In many membered polypeptides and proteins, adjacent peptide bonds, as shown by formula (I), are present in a manifold repetition. If such substrates are subjected to the action of Papain

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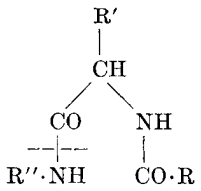
Peptidase I, then, as was shown by our first experiments, the enzyme selects particular peptide bonds from among those present for the splitting. The criteria which determine the nature of this selective process cannot be described by means of a simple chemical formulation. The basis of the nature of the selective process may be gleaned from the comparative enzymatic study of suitably chosen substrates. A number of such experiments are reported in this paper.

In the first place, there were studied a number of the simplest possible substrates of the general formula (II), in which R·CO was the benzoyl or carbobenzoxy group,[1] in order to determine the effect of variation of the substituents R′ and R″ in formula (II) upon the velocity of splitting by Papain Peptidase I.



II

The simplest compounds of formula (II) are of course the substituted hippurylamides (R = C_6H_5; R′ = H). Hippurylamide itself (R″ = H) is split quite rapidly by Papain Peptidase I. With our enzyme preparations, a splitting of nearly 100 per cent is obtained in 24 hours at 40°. Substituted hippurylamides, in which R″ is methyl, isoamyl, or phenyl, were split by the same enzyme solutions and under the same conditions only to a few per cent. Thus, neutral hydrocarbon residues, when acting as substituents in the amino group of hippurylamide, inhibit the splitting by Papain Peptidase I very strongly.

In order to extend the comparison to compounds having a

[1] We have repeatedly split benzoyl and carbobenzoxy derivatives of the same substance with Papain Peptidase I and have been unable to observe any marked difference in the velocity of splitting. It appears, therefore, permissible to compare the splitting by Papain Peptidase I of the benzoyl derivative with the carbobenzoxy derivative of another substance, as long as one compares only approximate reaction velocities in an extended series of experiments.

free carboxyl in the group R″ (II), carbobenzoxyglycylglycine, carbobenzoxydiglycylglycine, and carbobenzoxytriglycylglycine were subjected to enzymatic hydrolysis. All three of these acyl peptides are split by Papain Peptidase I with the formation of carbobenzoxyglycine and the splitting proceeds more rapidly as the length of the peptide chain in R″ is increased. It does not appear justifiable to interpret this finding as an indication of an inhibitory action of the free carboxyl group on the enzymatic hydrolysis. Upon passing successively from hippurylamide (III) to hippuryl methylamide (IV) and carbobenzoxyglycylglycine (V), *i.e.* upon the successive introduction of methyl and carboxyl groups, there results a marked inhibition of the enzymatic hydrolysis only after introduction of the methyl group but not following the carboxylation.

$C_6H_5 \cdot CO—NH \cdot CH_2 \cdot CO—NH_2$
III — Marked decrease in velocity of splitting

$C_6H_5 \cdot CO—NH \cdot CH_2 \cdot CO—NH \cdot CH_3$
IV — No decrease in velocity of splitting

$C_7H_7 \cdot O \cdot CO—NH \cdot CH_2CO—NH \cdot CH_2 \cdot COOH$
V — Marked increase in velocity of splitting

$C_6H_5 \cdot CO—NH \cdot CH_2 \cdot CO—NH \cdot CH_2 \cdot CO—NC_5H_{10}$
VI

In order further to supplement this series, benzoylglycylglycine piperidide (VI) was synthesized and its enzymatic hydrolysis compared with that of carbobenzoxyglycylglycine. The two compounds differ in the fact that (V) contains a free carboxyl group, while (VI) has an acid piperidide group in the corresponding position. The piperidide is split much more rapidly by Papain Peptidase I than is the acid (V). However, in this comparison also, it would be misleading to attribute the increase in the velocity of splitting to the substitution of the carboxyl group. It would be worth while to investigate the replacement of the carboxyl by an acid amide group.

Experiments similar to those with the substituted hippurylamides were performed with derivatives of carbobenzoxyisoglutamine (carbobenzoxyglutamyl-α-amide (VII)).

TABLE I

Hydrolysis of Substituted Acylamino Acid Amides by HCN-Papain Peptidase I

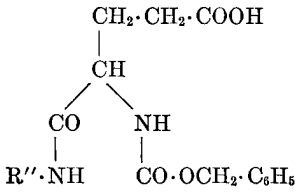
The splitting was measured in cc. of 0.01 N KOH per 0.2 cc. of test solution. 1 cc. increase represents 100 per cent splitting for one peptide bond in the synthetic substrates. The test solution was kept at pH 5 and 40° in all cases.

Substrate	Hydrolysis							Isolated products
	1 hr.	2 hrs.	3 hrs.	4 hrs.	24 hrs.	48 hrs.	144 hrs.	
Gelatin	0.79				1.32			
Hippurylamide				0.42	0.85			Hippuric acid
Hippuryl methylamide				0.06	0.15	0.18		
Hippurylisoamylamide*				0.02	0.04			
Hippurylanilide*				0.01	0.02			
Hippurylglycine piperidide				0.60	0.95			Hippuric acid (80% theory)
Carbobenzoxyglycylglycine					0.25			Glycine
Carbobenzoxydiglycylglycine					0.48			Glycylglycine
Carbobenzoxytriglycylglycine					0.85			Diglycylglycine
Gelatin	0.92				1.83			
Carbobenzoxyglutamyl-α-amide†	0.39				0.90			
" ‡		0.65		0.83	0.88			
Carbobenzoxyglutamyl-α-methylamide†			0.04		0.34			
" ‡		0.05		0.07	0.42			
Carbobenzoxyglutamyl-α-isoamylamide			0.18		0.77			
Gelatin		0.92			1.44			
Carbobenzoxyglutamyl-α-glycine					0.30	0.48	0.90	Carbobenzoxyglutamic acid

* Did not go into solution at start of reaction.

† 1 equivalent of 0.5 N ammonia was added.

‡ 1 equivalent of 0.5 N sodium acetate was added.



VII

Carbobenzoxyisoglutamine, like all the other test substances mentioned before, is based on the general formula (II). It differs from the hippurylamides in the fact that it contains as R′ an acidic group with a free carboxyl group. The enzymatic cleavage of carbobenzoxyisoglutamine was compared with the splitting of its derivatives which have a methyl or amyl group as R″. It will be noted from Table I that these substitutions cause a decrease in the velocity of the enzymatic hydrolysis, the methyl group being a stronger inhibitor than the isoamyl group. The inhibition becomes particularly clear if, for the purposes of the comparison, one considers the initial hours of the reaction in which the amounts of the inhibitory reaction products are still quite small. Furthermore, carbobenzoxyglutamylglycine, which has a carboxyl group in R″, was drawn into the comparative study and showed that no marked inhibition by the carboxyl group could be observed. A relationship similar to that for the hippurylamides is thus found here, the only difference being that the derivatives of carbobenzoxyisoglutamine mentioned above are split much more rapidly than are the corresponding derivatives of hippurylamide. This difference may be attributed to the presence of the side chain $CH_2 \cdot CH_2 \cdot COOH$ in the isoglutamine derivatives.

It was to be expected from experiences with other proteolytic enzymes that the action of Papain Peptidase I would be influenced by structural details of the substrate molecule. In the case of papain, however, what is surprising is the magnitude of this effect, since a very slight change, such as substitution of neutral hydrocarbon groups for a hydrogen group, produces a great difference in splitting. It is further noteworthy that such effects are not confined to those parts of the substrate molecule immediately adjacent to the peptide bond which is to be split. This great sensitivity of the enzyme toward structural influences and the

TABLE II

Hydrolysis of Acylated Peptides by HCN-Papain Peptidase I

The splitting was measured in cc. of 0.01 N KOH per 0.2 cc. of test solution. 1 cc. increase represents 100 per cent splitting for one peptide bond in the synthetic substrates. The test solution was kept at pH 5 and 40° in all cases.

Phenylhydrazine per cc. of test solution	Substrate	Hydrolysis				Isolation of products
		2 hrs.	6 hrs.	24 hrs.	144 hrs.	
mM						
	Gelatin	0.92		1.44		
	Carbobenzoxy-*l*-glutamylglycine*		0.07	0.30	0.90	Carbobenzoxy-glutamic acid (70 % theory)
	Carbobenzoxy-*l*-glutamylglycylglycine*		0.38	0.96	1.58	Carbobenzoxy-glutamylglycine (55 % theory after 24 hrs.)
		1 hr.	5 hrs.	24 hrs.		
	Gelatin	0.81				
0.0025	"	0.47				
	Carbobenzoxy-glycyl-*l*-glutamylglycine		0.37	0.83		
0.0025	"		−0.01	0.02		
	Carbobenzoxy-*l*-glutamylglycylglycine			0.80		
0.0025	"			0.04		
		1 hr.	2½ hrs.	7 hrs.	4 hrs.	
	Gelatin	0.87			1.80	
	Benzoyl-*l*-leucyl-*l*-leucylglycine		0.50	1.03	1.20	Leucylglycine as carbobenzoxy derivative (67 % after 7 hrs.)

* 1 equivalent of 0.1 N NaOH was added; 1 equivalent of 0.5 N sodium acetate was added.

fact that the enzyme is not restricted to a certain fixed point of attack in the polypeptide molecule often lead to the result that the enzyme splits two quite similar substrates at different linkages in the molecule. It is therefore essential to investigate a greater number of substrates for Papain Peptidase I with respect to reaction velocity and point of attack. Only in this manner can one hope gradually to gain an insight into the rules of specificity which determine the details of the enzyme action.

TABLE III

Carbobenzoxyglycyl	glycine
"	glycylglycine
"	glycylglycylglycine
Benzoylglycyl	*l*-leucylglycine
"	glycyl-*l*-leucylglycine
Carbobenzoxyglycyl	glycylglycyl | *l*-leucylglycine
Carbobenzoxy-*l*-leucylglycyl	glycine
Benzoyl-*l*-leucylglycyl	glycine
Benzoyl-*l*-leucyl	*l*-leucylglycine
Carbobenzoxyglycyl	*l*-glutamylamide
"	*l*-glutamylglycine
Carbobenzoxy-*l*-glutamyl	glycine
"	$\vert^2$ glycyl $\vert^1$ glycine
Benzoyl-*l*-lysine	amide
Benzoylglycyl	*l*-lysylglycine
"	$\vert^2$ carbobenzoxy-*l*-lysyl $\vert^1$ glycine

The favored point of splitting is indicated by the numeral 1; the second point, by the numeral 2.

Table II shows the enzymatic hydrolyses of several newer substrates. These results are to be employed as a basis for further discussion.

In Table III there are collected a number of substrates for Papain Peptidase I. Each of these substrates offers the enzyme the choice of several possible points of attack. The actual position of splitting is indicated in Table III by vertical lines. In several substrates the splitting takes place at two linkages with a widely differing speed or in succession. The favored point of splitting is indicated by the numeral 1; the second point, by the numeral 2.

It is evident from Table III that Papain Peptidase I is not

limited in its action to the peptide linkages between particular amino acids. The enzyme is, on the contrary, able to hydrolyze the most varied of peptide bonds regardless of the presence of neutral, acidic, or basic amino acids on the carbonyl or imino side of the split linkage. However, when the enzyme acts on a substrate which contains several peptide linkages, it attacks them with widely different velocities. The splitting does not occur haphazardly at all the peptide linkages, but only certain ones are preferred. The characteristics of this relative specificity in the case of acylated peptides may be stated as follows:

The acylamino group of acylated peptides directs the splitting by Papain Peptidase I to the peptide linkage immediately adjacent to the acylamino group.

If, in addition to peptide bonds with glycine-carbonyl, an acylated polypeptide contains peptide linkages with leucine-, glutamic acid-, or lysine-carbonyl, then papain peptidase splits the peptide bond with the glycine-carbonyl. It appears, furthermore, that in all of the cases studied, the directing influence of the glycine-carbonyl is stronger than that of the acylamino group. Thus, if the peptide bond adjacent to the acylamino group has no glycine-carbonyl, but another peptide bond of the molecule does contain one, then the point of splitting is shifted away from its proximity to the acylamino group.

It might be thought that perhaps this preference for glycine was connected with its low molecular weight; *i.e.*, that papain peptidase chose those peptide bonds the carbonyl of which belonged to the amino acid with the lowest molecular weight. However, from the last two examples in Table III it appears that this is not the case. In benzoylglycyl-*l*-lysylglycine the enzyme splits the peptide bond which is adjacent to the acylamino group and which also contains a glycine-carbonyl. In benzoylglycyl-ε-carbobenzoxy-*l*-lysylglycine, however, that peptide bond is split which contains the carbonyl of the lysine (the amino acid with the highest molecular weight). The introduction of an acid amide group at the end of the long side chain of lysine has so powerful an effect that the combined influence of the acylamino group and the glycine residue is overcome.

It has hitherto never been found that the lysine residues in proteins are acylated. On the other hand, it is known that the

side groups of glutamic and aspartic acids are in the amide form. One might expect, in connection with the experiences mentioned above, that such acid amide groups in the side groups of proteins play a rôle in directing the enzymatic hydrolysis.

Hydrolysis of Free Polypeptides by Papain Peptidase I

The hitherto mentioned experiments on the specificity of Papain Peptidase I are concerned with the splitting of acylated peptides.

TABLE IV

Hydrolysis of Polypeptides by HCN-Papain Peptidase I

The splitting was measured in cc. of 0.01 N KOH per 0.2 cc. of test solution. 1 cc. increase represents 100 per cent splitting for one peptide bond in the synthetic substrates. The test solution was kept at pH 5 and 40° in all cases.

Phenylhydrazine per ml. of test solution	Substrates	Hydrolysis						
		1 hr.	1 day	2 days	3 days	4 days	5 days	6 days
mM								
	Gelatin	1.00	1.86					
0.0025	"	0.54	1.16					
0.005	"	0.45	0.94					
	Diglycyl-*l*-leucylglycine			0.47			0.73	
	"		0.21	0.42		0.68		0.75
	Triglycyl-*l*-leucylglycine		0.52	0.86	1.02			1.32
	"		0.58	0.91	1.07	1.12		1.46
0.0025	"				0.08*			
0.005	"				0.11*			

* The presence of phenylhydrazine caused the test solution to turn brownish yellow; these titration values are therefore probably too high because of overtitration.

The action of the enzyme is, however, not confined to acylated peptides. It was found that papain splits free peptides as well (5). In Table IV are given the rates of splitting of diglycyl-*l*-leucylglycine and of triglycyl-*l*-leucylglycine. It will be noted that the enzyme action on the pentapeptide involves the splitting of more than one peptide linkage. It was found further that the splitting of the two polypeptides which were studied is inhibited completely by phenylhydrazine. There can therefore be no doubt

that the hydrolysis of these free polypeptides is performed by the same enzyme as the one which splits the acylated peptides, and which is similarly inhibited by phenylhydrazine; namely, Papain Peptidase I (6). Bergmann and Ross have shown recently that the splitting of gelatin by activated papain is strongly inhibited by phenylhydrazine, and concluded from this finding that Papain Peptidase I participates significantly in the papain digestion of gelatin. This conclusion is supported by the finding that the enzyme attacks free polypeptides. Furthermore, the earlier observation of Willstätter and Grassmann (7) that papain is able to split the tripeptide, leucylglycylglycine, finds a wider significance.

One of the immediate problems is to investigate a large number of polypeptides of various amino acids in their behavior toward Papain Peptidase I, in order to collect further material regarding

TABLE V

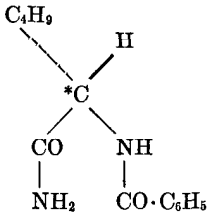
Hydrolysis of d- and l-Benzoylleucineamide at 40° and pH 5

	24 hrs.	48 hrs.
	per cent	*per cent*
l-Amide	79	84
d-Amide	0	0

the specificity of the enzyme. It is not improbable that the enzyme acts differently toward free and acylated peptides.

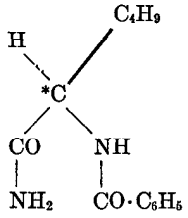
Antipodal Specificity of Papain Peptidase I

The discovery of synthetic substrates for Papain Peptidase I made it possible to study the antipodal specificity of the enzyme. The simplest substrates for such experiments are the two antipodes of benzoylleucineamide. If the formulas of the two antipodes are reproduced in spatial projection, the C·N·C·C·N— chain, the essential grouping of peptide bonds which both antipodes contain, is to be visualized as lying in the plane of the paper (VIII and IX). In this chain the asymmetric carbon lies between the two peptide bonds. It is indicated by an asterisk. The two substituents of the asymmetric carbon atom, H and C_4H_9, do not lie in the plane of the paper but in front of, or behind it.



VIII

l-Benzoylleucineamide. (The H at the asymmetric carbon atom is to be visualized as being in front of the plane of the paper, the C_4H_9 behind the plane)



IX

d-Benzoylleucineamide. (The H at the asymmetric carbon atom is to be visualized as being behind the plane of the paper, the C_4H_9 in front of the plane)

It will be seen from Table V that Papain Peptidase I splits only one of the antipodes; namely, the one derived from the natural *l*-leucine. Papain Peptidase I therefore shows antipodal specificity and is restricted to the natural *l* configuration of the substrates just as are dipeptidase and the other known digestive enzymes.

In order to explain the antipodal specificity of certain enzymes, Emil Fischer made the assumption that these enzymes were themselves built asymmetrically. This generally accepted concept may be more closely defined as follows: If an enzyme catalyzes only one of two antipodes, then it must contain at least three different atoms or atomic groups which are fixed in space with respect to one another, these groups entering during the catalysis into relation with a similar number of different atoms or atomic groups of the substrate.[2] Through this polyaffinity relationship, the active groups of the enzyme force the active groups of the substrate into a definite spatial arrangement with respect to each other and to the enzyme. One might imagine in the enzyme-substrate combination a plane formed by the active groups of the enzyme (binding plane of the enzyme) and a plane formed by the active groups of the substrate (binding plane of the substrate). Then the requirements for the formation of an enzyme-substrate combination and for the appearance of antipodal specificity may be formulated as follows: During enzymatic catalysis the binding

[2] The peptide bond is considered double because of its dipole character.

plane of an enzyme with polyaffinity approaches within a few Ångström units of the binding plane of the substrate. If the substrate is so constructed that one or more large atomic groups[3] jut out from the binding plane into the space between the binding planes of substrate and enzyme, then the approach of the binding plane of the enzyme toward the binding plane of the substrate is prevented. The catalysis does not occur as a result of stearic hindrance.

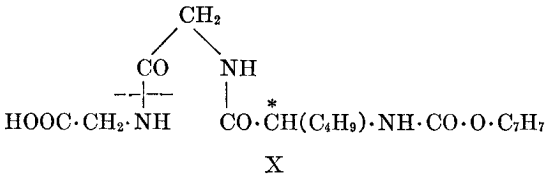
Antipodal specificity has been found for all the proteolytic enzymes the specificity requirements of which could be investigated with substrates of known structure, and in all these cases it was possible to confirm the polyaffinity relationship. If one allows such an enzyme to act on the two antipodes of a substrate and determines which antipode is split, it is possible to decide which spatial arrangement of the active groups of the substrate is required by the enzyme and from which side the enzyme approaches the binding plane of the substrate during catalysis. The spatial picture obtained in this way allows one to predict the enzymatic behavior of the antipodes of all possible substrates of the same enzyme, always assuming that the center of antipodal asymmetry lies within the binding plane of the substrate. For Papain Peptidase I it may be concluded from the splitting of benzoyl-*l*-leucineamide that the enzyme splits only that antipode of an optically active compound with a large R′ in which the arrangement NH·CO·CH(R′)·NH·CO is present in a clockwise order as viewed from the binding plane of the enzyme, and in which the H of the CHR′ group points toward the enzyme. In the case of benzoyl-*d*-leucineamide and other derivatives of *d*-amino acids, these two requirements are not fulfilled simultaneously; these compounds cannot therefore be split by Papain Peptidase I.

Von Euler and Josephson have set up a two affinity theory of enzyme action (9). This theory requires two different groups in the enzyme, one of which is responsible for the combination with

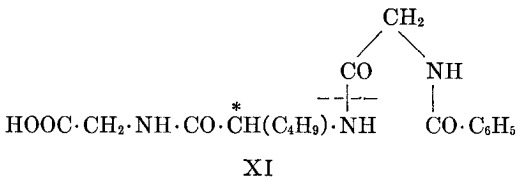
[3] To explain what is meant by large atomic groups, it might be recalled that with dipeptidase a methyl group in the prohibited position markedly slows down the catalysis, but that an isobutyl group prevents any splitting whatever in the case of dipeptidase (8) as well as of Papain Peptidase I. From these facts, it is concluded that the necessary approach must be within a distance of several Ångström units.

the substrate, while the other performs the splitting. We extend the theory of von Euler and Josephson in replacing the two affinity concept by a polyaffinity relationship, and assume a fixed relative position for the active groups in the enzyme. This extension makes it possible to explain antipodal specificity. As far as we know, this is the first case in which the specificity of a group of enzymes can be clarified with the aid of a simple hypothesis. It is possible that the antipodal specificity of the serological reactions discovered by Landsteiner and van der Scheer (10) may be explained with the aid of similar considerations (polyaffinity and spatial fixity of active groups in the antibodies).

The discussion of antipodal specificity has concerned itself until now with the asymmetry of those carbon atoms which lie between the active groups of the substrate; *i.e.*, within the binding plane of the substrate. For the substrates of Papain Peptidase I, there is involved only a single carbon atom of this kind; namely, the one lying between the two essential adjacent peptide bonds. It should be pointed out that two other cases of a different type of antipodal specificity have been observed for Papain Peptidase I. The substrates involved are the antipodes of carbobenzoxyleucylglycylglycine (X) and benzoylglycylleucylglycine (XI).



X



XI

In both formulæ, the peptide bond which is split is designated by a broken line and the two peptide bonds which determine the binding plane of the substrate are drawn as in all the previous formulæ of this type. It is evident that in both substrates the asymmetric carbon atoms lie outside the binding planes of the respective sub-

strates. However, as was reported earlier (1), there occurs antipodal specificity. Perhaps one should conclude from these examples that during the combination with the enzyme not only is the binding plane forced into a definite spatial position with respect to papain peptidase, but neighboring parts of the substrate molecule are fixed as well.

EXPERIMENTAL

Carbobenzoxyglutamylglycylglycine

Carbobenzoxyglutamylglycylglycine Ethyl Ester—To 7.4 gm. of carbobenzoxyglutamylglycine ethyl ester (1) in 10 cc. of absolute alcohol there were added 3 cc. of hydrazine hydrate, and the reaction mixture was left at room temperature for 24 hours. On addition of anhydrous ether, a syrupy precipitate resulted. After being washed several times with ether, the syrup was dissolved in 100 cc. of ice-cold water, covered with ether-ethyl acetate (1:1), and treated, at 0° with shaking, with 40 cc. of cold 5 N hydrochloric acid and an aqueous solution of 1.7 gm. of sodium nitrite added in several portions within 3 to 4 minutes. After washing the ether-ethyl acetate layer with water until the acid reaction with Congo red had disappeared, the solution was passed through a dry filter, dried briefly over Na_2SO_4, and mixed with an ethereal solution of glycine ethyl ester (prepared from 15 gm. of hydrochloride). The mixture was evaporated under diminished pressure, leaving a syrup which was heated for 3 hours at 30°, then for 5 minutes at 80°, dissolved in cold water, and acidified with 5 N hydrochloric acid to Congo red. The syrup which was precipitated crystallized on standing. On recrystallization from hot alcohol, followed by recrystallization from ethyl acetate, there were obtained needles with a melting point of 139–140°. Yield, 2.8 gm.

$C_{19}H_{25}O_8N_3$ (423.2). Calculated, N 9.9; found, N 10.0 (Kjeldahl)

The same substance is obtained by coupling carbobenzoxyglutamyl anhydride with glycylglycine ethyl ester in ethyl acetate solution. There are formed two isomers (α- and γ-peptides); the isolation of the required α-peptide in a pure state is difficult, giving only a small yield.

Carbobenzoxyglutamylglycylglycine—2.8 gm. of the above ester were dissolved in 15 cc. of NaOH, and after 30 minutes acidified

to Congo red with 5 N hydrochloric acid. The reaction mixture was concentrated at 40°, under diminished pressure, to about 4 cc. and the carbobenzoxytripeptide extracted with a large volume of ethyl acetate. The ethyl acetate solution was washed with 2 cc. of water, evaporated down, and the residue dissolved in dry acetone. The acetone solution was evaporated, leaving a syrup which when treated with a small volume of dry ethyl acetate at 0° yielded 1.6 gm. of small plates which melted at 142°.

$C_{17}H_{21}O_8N_3$ (395.2). Calculated, N 10.6; found, N 10.6 (Kjeldahl)

In the splitting of the carbobenzoxytripeptide by papain, more than one peptide linkage was hydrolyzed. A portion of the reaction mixture was worked up after 24 hours, at which time the increase in carboxyl represented about 1 mole. It was concentrated under diminished pressure, acidified, extracted with a large volume of ethyl acetate, the extract evaporated down, and the residue brought to crystallization with ether. Carbobenzoxyglutamylglycine was obtained in 55 per cent yield. M.p., 143°. The mixed melting point with a preparation made according to Grassmann and Schneider (11) showed no depression.

$C_{15}H_{18}O_7N_2$ (338.2). Calculated, N 8.3; found, N 8.3 (Kjeldahl)

l-Leucyl-l-Leucylglycine

Carbobenzoxy-l-Leucine—To the solution of 13.1 gm. of *l*-leucine in 50 cc. of 2 N NaOH there were added, at 0° with shaking, 17 gm. of carbobenzoxy chloride and 60 cc. of 2 N NaOH in several portions. The syrup obtained on acidifying was taken up in ether and the ethereal solution extracted with potassium bicarbonate solution. The bicarbonate solution was acidified, the carbobenzoxyleucine again taken up in ether, and the ether evaporated off. Yield, 20 gm. of syrup.

Carbobenzoxy-l-Leucyl-l-Leucylglycine Methyl Ester—6 gm. of carbobenzoxy-*l*-leucylglycine (1) were dissolved in methanol containing 1 mole of aqueous hydrochloric acid and hydrogenated in the presence of palladium, the solution was evaporated down, and the dipeptide esterified as usual at 0° with methanol-hydrogen chloride. The free ester was liberated with potassium carbonate and taken up in ethyl acetate.

7 gm. of syrupy carbobenzoxy-*l*-leucine (dried over P_2O_5) were dissolved in 30 cc. of dry ether and treated as usual with 7 gm. of PCl_5 at 0°. The reaction mixture was filtered, diluted with 70 cc. of ether, washed three times with ice-cold water in a separatory funnel, passed through a dry filter, dried quickly over Na_2SO_4, and added at 0° to the above solution of leucylglycine ester. The mixture was shaken with about 20 cc. of saturated potassium bicarbonate solution, the upper layer successively washed with hydrochloric acid, bicarbonate, and water, dried, and evaporated down. The resulting syrup was treated with ether-petroleum ether, yielding small prisms after standing overnight. The crystals were transferred to the filter with a small amount of ether. M.p., 108°. Yield, 3.6 to 4.0 gm.

$C_{23}H_{35}O_6N_3$ (449.3). Calculated, N 9.4; found, N 9.4 (Kjeldahl)

l-Leucyl-l-Leucylglycine—3 gm. of the above ester were dissolved in a mixture of methanol and 1.1 mole of 2 N NaOH. After 20 minutes the solution was acidified and the methanol evaporated off under diminished pressure. The resulting syrup was taken up in ethyl acetate-ether (1:1) and extracted with potassium bicarbonate. The bicarbonate extract was acidified and the substance again taken up in ethyl acetate, which was then evaporated off. The residue was dissolved in water-methanol containing 0.5 cc. of glacial acetic acid, and hydrogenated catalytically. The reaction mixture was evaporated down and the resulting crystals transferred to the filter with alcohol. Yield, 1.3 gm.

$C_{14}H_{27}O_4N_3$.	Calculated.	C 55.8, H 9.0, N 13.9
301.2	Found.	" 55.8, " 9.2, " 14.0 (Dumas)

Benzoyl-l-Leucyl-l-Leucylglycine—1 gm. of the tripeptide was suspended in a mixture of 10 cc. of half saturated potassium bicarbonate solution and treated at 0° with 0.6 cc. of benzoyl chloride in several portions over a period of 30 minutes. The syrup obtained on acidifying was treated with hot water and allowed to stand overnight. The substance was then dissolved in acetone, water added to incipient precipitation, and enough acetone added to dissolve. On standing at room temperature, 1.1 gm. of needles were obtained, which on recrystallization (as above) melted at 161°. The air-dried substance was analyzed.

$C_{21}H_{31}O_5N_3 \cdot \frac{1}{2}H_2O$.	Calculated.	C 60.9, H 7.9, N 10.1,	H_2O 2.2
414.2	Found.	" 60.9, " 7.6, " 10.1 (Dumas),	" 1.7

The splitting of the benzoyl tripeptide by papain exceeded the hydrolysis of one peptide linkage. As soon as the splitting corresponded to the splitting of one peptide bond, a portion of the solution was concentrated under diminished pressure, acidified with hydrochloric acid, and extracted with ether. The aqueous portion was then made alkaline, carbobenzoxylated, again acidified, and extracted with ether. On evaporating the ether, there was obtained carbobenzoxyleucylglycine in 67 per cent yield. M.p. and mixed m.p., 113°.

Benzoylglycylglycine Piperidide

Carbobenzoxyglycyl Piperidide—To an ice-cooled, aqueous piperidine solution (about 5 moles of amine) there was added, with shaking, 1 mole of carbobenzoxyglycyl chloride in portions. After a short interval, the piperidide separated out in a 60 per cent yield (calculated for the chloride). On recrystallization from ether-petroleum ether, there were obtained needles. M.p., 78°.

$C_{15}H_{20}O_3N_2$ (276.2). Calculated, N 10.2; found, N 10.5 (Dumas)

Benzoylglycylglycine Piperidide—Carbobenzoxyglycyl piperidide (1 mole) was dissolved in methanol and 2 moles of glacial acetic acid and hydrogenated catalytically. The solution was evaporated down, the residue dissolved in cold bicarbonate solution, and treated at 0° with 1 mole of hippuryl chloride in several portions. The chloride slowly went into solution while the piperidide separated out. Yield, 40 per cent. On recrystallization from aqueous bicarbonate solution and ethyl acetate there were obtained needles which melted at 134°.

$C_{16}H_{21}O_3N_3$ (303.2). Calculated, N 13.8; found, N 13.7 (Dumas)

Hippurylanilide—This substance was prepared from hippuryl chloride and aniline-water. M.p., 214°.

$C_{15}H_{14}O_2N_2$ (254.1). Calculated, N 11.0; found, N 10.8 (Kjeldahl)

Hippurylisoamylamide—This substance was prepared from hippuryl chloride and isoamylamine-water. M.p., 98°.

$C_{14}H_{20}O_2N_2$ (248.1). Calculated. N 11.3; found, N 11.0 (Kjeldahl)

Carbobenzoxy-l-Glutamyl Methylamide—4 gm. of carbobenzoxy-*l*-glutamyl anhydride were added within 5 minutes in portions to 7 cc. of ice-cooled 33 per cent aqueous methylamine solution. After 15 minutes, the reaction mixture was acidified, yielding a syrup which crystallized on scratching. The crystals (needles) were recrystallized from methanol-water. Yield, 2 gm. M.p., 178°.

$C_{14}H_{18}O_5N_2$ (294.1). Calculated, N 9.5; found, N 9.5 (Kjeldahl)

Carbobenzoxy-l-Glutamylisoamylamide—This substance was prepared in a similar manner as the methylamide. On recrystallization from methanol-water and ether, the melting point was 135°.

$C_{18}H_{26}O_5N_2$ (350.2). Calculated, N 8.0; found, N 8.3 (Dumas)

Diglycyl-l-Leucylglycine—This substance was prepared as described in (1).

Triglycyl-l-Leucylglycine—This substance was prepared as described in (1).

Benzoyl-l-Leucineamide—3 gm. of *l*-leucine ethyl ester hydrochloride were converted to the free ester in ether solution, which was then allowed to react with 2.2 cc. of benzoyl chloride in ethyl acetate and 16 cc. of a 10 per cent sodium carbonate solution. The ether layer was washed with water, bicarbonate, and dilute hydrochloric acid and evaporated down, yielding crystals which were allowed to react with gaseous ammonia in absolute alcohol for 3 days. The solution was then evaporated down, yielding a syrup which crystallized upon treatment with water. Upon recrystallization from ethyl acetate the substance melted at 187°. Yield, 2 gm.

$C_{13}H_{18}O_2N_2$. Calculated, C 66.7, H 7.6, N 11.8
238.2 Found. " 66.6, " 7.6, " 11.7 (Dumas)
$[\alpha]_D^{25} = -6.4°$ (2.5 per cent in alcohol)

From the enzymatic hydrolysis to 84 per cent of 120 mg. of this substance there were isolated, in the usual manner, 100 mg. of a product melting at 105°.

Benzoyl-d-Leucineamide—This substance was prepared in the same manner as the *l* form. M.p. 187°.

$C_{13}H_{18}O_2N_2$. Calculated. C 66.7, H 7.6, N 11.8
238.2 Found. " 66.5, " 7.8, " 12.0 (Dumas)
$[\alpha]_D^{25} = +6.4°$ (2.5 per cent in alcohol)

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