ON PROTEOLYTIC ENZYMES

VI. ON THE SPECIFICITY OF PAPAIN*

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Knowledge is rather meager regarding the specificity of proteinases, the group of enzymes which effect the hydrolysis of genuine proteins. It is only known that during their action acidic and basic groups are formed in equivalent amounts (2). The simplest explanation for this observation is that the proteinases split peptide linkages in the proteins. However, the known peptide-splitting enzymes cannot hydrolyze proteins and, furthermore, no synthetic peptides were found to be attacked by the proteinases. As long as the riddle of the action of the proteinases remains unsolved, the problem of the proteins themselves can scarcely be cleared up.

In the experiments reported in the present paper, a number of synthetic substrates for papain are described. The finding of synthetic substrates and the possibility of changing their structure almost at will permit a systematic study of papain specificity.

In proteins there are present basic and acidic side chains but nearly no free α-amino or α-carboxyl groups. Therefore, peptides were synthesized which contained no free α-amino group or α-carboxyl but which bore a β- or γ-carboxyl in the side chain, such as carbobenzoxyglycylglutamylglycine ethyl ester (I), carbobenzoxyglycylisoglutamine (II), and carbobenzoxyglycylasparagylglycine ethyl ester (III). All three tripeptides were split by papain-hydrocyanic acid, (I) and (II) being attacked more rapidly

* For Paper V of this series see (1).

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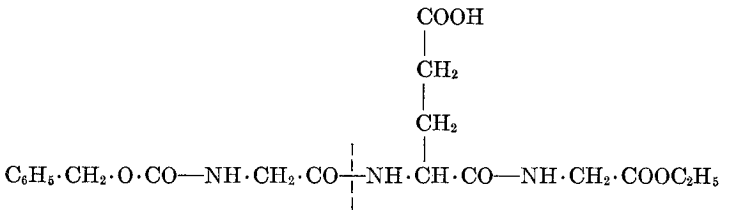
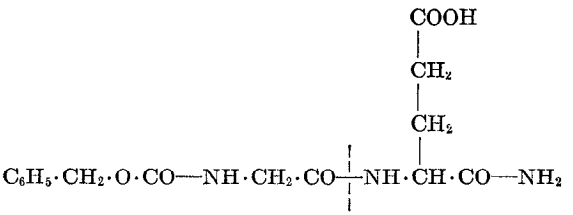
than (III). The tetrapeptide benzoyldiglycylglutamylglycine ethyl ester (IV) was split even more quickly (Table I). When the



(I)



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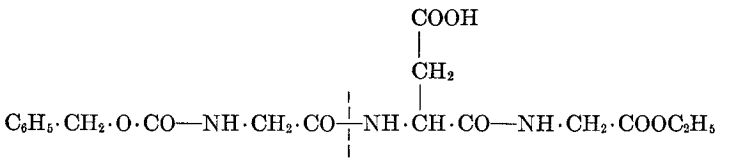

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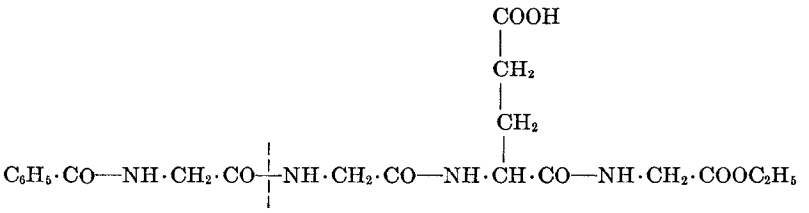


(IV)

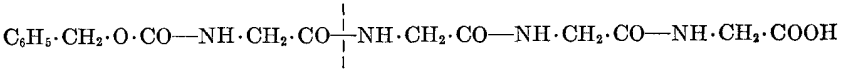
hydrolytic products resulting from these four compounds were isolated, it was found that carbobenzoxyglycine (or hippuric acid) was obtained in all cases. The site of hydrolysis was therefore not

determined by the glutamic acid portion but rather by the end acylamino group. It was then found that the free carboxyl of the side chain is not necessary for papain action. Furthermore, the surprising observation was made that simple compounds such as carbobenzoxytetraglycine (V), carbobenzoxytriglycine (VI), carbobenzoxydiglycine (VII), and even hippurylamide (VIII) were split by papain, and in the last mentioned case quite rapidly. In addition, splitting was observed for benzoyldiglycyl-*l*-leucylglycine (IX) and carbobenzoxytriglycyl-*l*-leucylglycine (X) (Table II). In all of the above examples an acid amide linkage is hydrolyzed, namely the one next to the end acylamino group. The

TABLE I

Hydrolysis of Peptides of Glutamic and Aspartic Acids

Substrate	Time	Hydrolysis		Isolation of products
		Titration	Van Slyke	
	hrs.	*per cent*	*per cent*	
Carbobenzoxyglycyl-*l*-glutamylglycine	5	64	63	Carbobenzoxyglycine, 50% yield
	23	91	92	
Carbobenzoxyglycyl-*l*-glutamylglycine ethyl ester	2		26	Carbobenzoxyglycine, 80% yield
	7½		50	
	23½		90	
Carbobenzoxyglycyl-*l*-isoglutamine	2½	24	27	Carbobenzoxyglycine, 84% yield
	5	65	68	
	18	100		
Benzoyldiglycyl-*l*-glutamylglycine ethyl ester	2½		62	Hippuric acid, 65% yield
	6		97	
Carbobenzoxyglycyl-*l*-asparagylglycine ethyl ester	23½		32	Carbobenzoxyglycine, 65% yield
	48		52	
	120		90	
Benzoyl-*l*-isoglutamine	2½	64		Benzoyl-*l*-glutamic acid, 75% yield
	26	90		
Carbobenzoxy-*l*-glutamylglycine ethyl ester	20		25	Carbobenzoxy-*l*-glutamic acid, 83% yield
	73		65	
	166		90	
Glycyl-*l*-glutamylglycine	6	2		
" ethyl ester	24		6	
Glycyl-*l*-glutamylglycine	4	0	0	
Glycyl-*l*-glutamic acid diketopiperazine	2	0	0	
	25	0	1	



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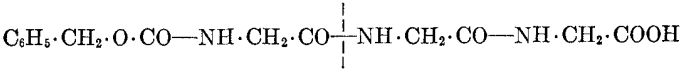

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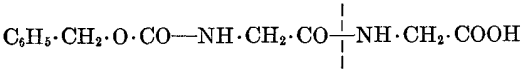

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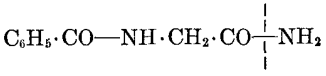

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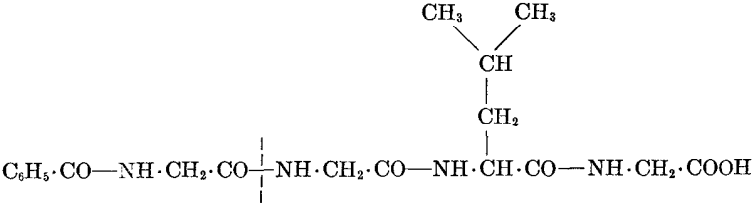

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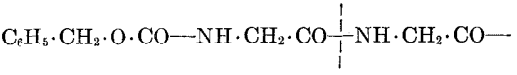
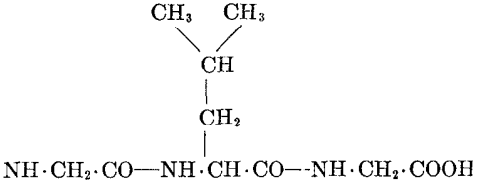




(X)

place of splitting is indicated in the formulæ by means of a dotted line. In all cases at least one of the split-products was isolated

TABLE II

Hydrolysis of Peptides of Glycine and Leucine

Substrate	Time	Hydrolysis		Isolation of products
		Titration	Van Slyke	
	hrs.	*per cent*	*per cent*	
Hippurylamide	4	59		Hippuric acid, 95% yield
	24	92		
Carbobenzoxyglycylglycine	24		25	Carbobenzoxyglycine,
	43		43	40% yield
	91		73	
Carbobenzoxytriglycine	24	50	48	Carbobenzoxyglycine,
	48	72	73	95%; glycylglycine, 40%
	72	83	85	yield
Carbobenzoxytetraglycine	24		85	Carbobenzoxyglycine,
	46		100	77%; triglycine, 52% yield
Carbobenzoxyglycylsarcosyldiglycine	46	4	3	
Benzoylglycyl-*l*-leucylglycine	4	90	90	Hippuric acid, 100%; *l*-
	24	100	100	leucylglycine, 45% yield
Benzoylglycyl-*d*-leucylglycine	19	12	12	Hippuric acid, 77% yield
	43	35	33	
	68	53	52	
	92	93	95	
Benzoyldiglycyl-*l*-leucylglycine	22	90		Hippuric acid, 75% yield
	43	100		
Carbobenzoxytriglycyl-*l*-leucylglycine*	25	93	101	Carbobenzoxyglycine, 75% yield; carbobenzoxytriglycine isolated
Carbobenzoxy-*l*-leucylglycylglycine	5	47	50	Carbobenzoxy-*l*-leucyl-
	18	87	90	glycine, 82%; glycine,
	42	95	97	72% yield
Carbobenzoxy-*d*-leucylglycylglycine	5	0	0	
	24	0	0	
Benzoyl-*dl*-leucylglycylglycine	8½	60	55	Carbobenzoxyglycine,
	25	92	92	60% yield

* Did not go into solution at start of reaction.

either directly or in the form of simple derivatives. In several cases both products were isolated.

It will be noted that all the substrates mentioned above have an acylglycyl group. We have investigated several specificity requirements of the enzyme for this class of papain substrates. The most striking fact is that papain requires two acid amide linkages. One of them is split, and the other is evidently necessary as a point of attachment for the enzyme. In contrast with the known peptidases (dipeptidase, aminopeptidase, and carboxypeptidase), papain does not require a free α-carboxyl or free amino group near the peptide linkage. In fact, a neighboring free amino group inhibits papain action. Carbobenzoxyglycylglutamylglycine ester and carbobenzoxyglycylglutamylglycine are hydrolyzed to about 50 per cent in 6 hours; glycylglutamylglycine is not attacked in that time.

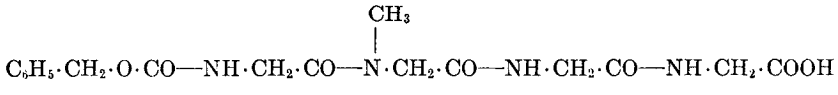
It was mentioned before that carbobenzoxytetraglycine is split by papain into carbobenzoxyglycine and triglycine. The analogous carbobenzoxyglycylsarcosyldiglycine (XI) is, however, not attacked. Thus, when the peptide hydrogen is substituted by a methyl group in the peptide linkage to be split, the bond becomes resistant to hydrolysis.[1]

In order to investigate the influence of spatial configuration on papain activity, benzoylglycyl-*l*-leucylglycine (XII) and the corresponding *d*-peptide were compared. In 5 hours the *l*-peptide was split quantitatively into hippuric acid and *l*-leucylglycine, both of which were isolated. The *d*-peptide was also split by papain to yield similar products but at a much slower rate. After 5 hours only 10 per cent splitting was observed, and 4 days were required to complete the hydrolysis. Here is a case of relative spatial specificity; it cannot as yet be stated whether this finding is to be attributed to steric hindrance or to a difference in the affinity of the enzyme for the *d* and *l* forms of the peptide.

An obvious extension of these experiments was the investigation of the behavior of papain towards the benzoyl (XIII) and carbobenzoxy (XIV) derivatives of leucylglycylglycine. With both compounds, observations were made which should have significance for the specificity of papain. The *d* form of the peptide is not split by papain. The *l* form is hydrolyzed to carbobenzoxy-*l*-leucylglycine and glycine. Other hydrolytic products could not

[1] Experiments of Dr. William F. Ross showed that carbobenzoxyglycylproline also is not attacked by papain.

be found. Thus, when the acyl derivatives of glycyl-*l*-leucylglycine and of *l*-leucylglycylglycine are compared, it becomes evident that when leucine is situated next to the acyl group the



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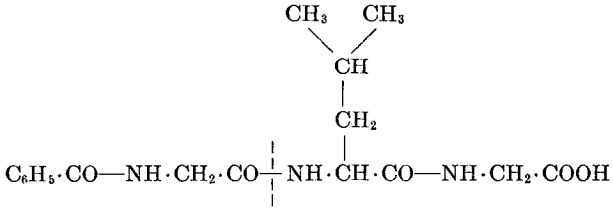

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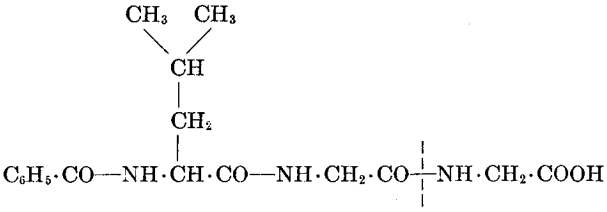

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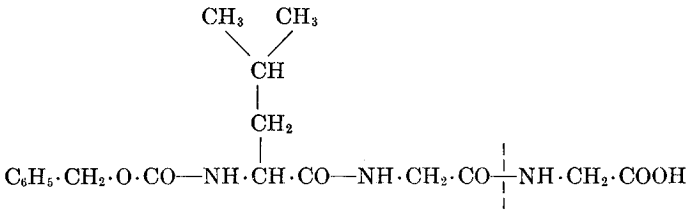

(XIV)

site of splitting is shifted one amino acid residue away from the acyl group. The presence of the isobutyl group produces in the *l* isomer a hydrolytic mechanism different from that found for the above described acylglycine peptides. The difference between the

d and *l* forms is here a case of absolute specificity. The influence of the asymmetry of the leucine residue is particularly significant, since the leucine does not participate in the peptide bond which is hydrolyzed. No example has hitherto been encountered in the chemistry of proteolytic enzymes where substitution of one aliphatic monoaminomonocarboxylic acid by another produced such a deep seated alteration in the mechanism of hydrolysis. One may well await eagerly the result of the introduction of other and more complex amino acids, as it affects the specificity of papain.

The fact that a large number of acylated peptides were found to be hydrolyzable by papain gives rise to the question: What connection is there between the splitting of these acyl peptides and the splitting of proteins? In order to answer this question it becomes necessary to reinvestigate the behavior of papain towards free polypeptides, although according to the literature synthetic peptides are resistant towards proteinases. To this end we are studying free polypeptides containing various amino acids.

It should be pointed out that it is not yet clear whether the crude enzyme preparation from *Carica papaya* represents an enzymatically homogeneous substance. It is certain, however, from earlier work (3) as well as from our experiments, that at pH 5 and after activation by hydrocyanic acid, papain showed no action on the known substrates of dipeptidase, aminopeptidase, and carboxypeptidase. The splittings described in this paper occur, furthermore, at positions which rule out the action of any of the above three enzymes. However, the possibility cannot be excluded that these observations are due to the action of only a part of the papain complex and that in addition to this part there are other proteolytic enzymes in the enzyme preparations employed by us. We have directed our attention to this problem and are investigating the well known inactivation of papain by oxidizing reagents and the reactivation by hydrocyanic acid in relation to the splitting of our synthetic substrates.

Correction

It was mentioned above that carbobenzoxyglycylglutamylglycine ester (I) is split by papain and that 80 per cent of carbobenzoxyglycine was isolated from the hydrolysate. In a preliminary note (4) we reported that (I) is also split by tryptic proteinase.

We observed an increase in carboxyl and none in amino nitrogen. Thus, it was concluded that with trypsin there occurs a splitting analogous to that found for papain, whereby the resulting dipeptide ester was transformed to a diketopiperazine at pH 8. In the isolation of the reaction products, performed in the meantime, no carbobenzoxyglycine could be isolated, showing that no tryptic hydrolysis of a peptide bond had occurred.

EXPERIMENTAL

Glycyl-l-Glutamyl-α-Glycine

Carbobenzoxy-l-Glutamyl-α-Glycine Ethyl Ester—To an anhydrous ether solution of glycine ethyl ester (prepared from 30 gm. of hydrochloride) there was added with cooling an ethyl acetate solution of 13 gm. of carbobenzoxy-*l*-glutamic acid anhydride (5). After 3 hours, the solution was washed with dilute HCl and evaporated under diminished pressure. The residue, after dissolving in ether, yields needles upon standing. The substance was recrystallized from hot ethyl acetate. M.p., 126°;[2] yield, 8.7 gm.

From the enzymatic hydrolysis to 90 per cent of 915 mg. of this substance there were isolated 450 mg. of a product with a melting point of 110–112°; mixed melting point with carbobenzoxyglutamic acid, 112°.

l-Glutamyl-α-Glycine Ethyl Ester—8.2 gm. of the above substance were hydrogenated in the usual manner in alcohol and 2 cc. of glacial acetic acid. The residue, after evaporation of the solution, was repeatedly evaporated with alcohol. The spongy mass obtained upon precipitation with alcohol and ether was heated in absolute alcohol. Needles; yield, 4.1 gm.; m.p., 151°.

$C_9H_{16}O_5N_2$ (232.1). Calculated, N 12.1; found, N 12.0 (Kjeldahl)

Carbobenzoxyglycyl-l-Glutamyl-α-Glycine Ethyl Ester—To 4.5 gm. of the previous substance in an ice-cooled saturated aqueous solution of 2.5 gm. of sodium bicarbonate there were added, in three portions within 5 to 6 minutes, 5 gm. of carbobenzoxyglycyl chloride and 10 cc. of 2 N NaOH. On acidifying, a syrup was

[2] Grassmann and Schneider (6) report a melting point of 122° for this substance.

obtained which crystallized on standing in the ice box. Yield, 8.3 gm. The substance was recrystallized from hot alcohol. Needles; m.p., 169°.

$C_{19}H_{25}O_8N_3$.	Calculated.	C 53.9, H 5.9, N 9.9
423	Found.	" 54.0, " 5.9, " 10.1 (Dumas)

From the enzymatic hydrolysis to 90 per cent of 742 mg. of this substance there were isolated 260 mg. of a product with a melting point of 119°; mixed melting point with carbobenzoxyglycine, 119°.

Carbobenzoxyglycyl-l-Glutamyl-α-Glycine—0.7 gm. of the carbobenzoxytripeptide ester was dissolved in 4 cc. of N NaOH, allowed to stand ½ hour at room temperature, acidified with concentrated HCl, and the solution extracted three times with ethyl acetate. The extract was evaporated down and ether was added, yielding a solid mass which was recrystallized from alcohol-ether. Yield, 0.33 gm.; needles; m.p., 98–100°.

$C_{17}H_{21}O_8N_3$ (395.2). Calculated, N 10.6; found, N 10.5 (Kjeldahl)

From the enzymatic hydrolysis to 92 per cent of 264 mg. of this substance there were isolated 46 mg. of a product with a melting point of 119°; mixed melting point with carbobenzoxyglycine, 119°.

Glycyl-l-Glutamyl-α-Glycine—1.3 gm. of the previous substance were dissolved in 50 per cent methanol and 1 cc. of glacial acetic acid and hydrogenated in the usual manner. After the solution was evaporated, water was added and evaporated off several times. The residue was recrystallized from hot alcohol by adding water to dissolve the crystals and cooling slowly. Needles; yield, 0.8 gm.

$C_9H_{15}O_6N_3$.	Calculated.	C 41.4, H 5.8, N 16.1
261	Found.	" 41.3, " 5.9, " 16.0 (Dumas)

Glycylglutamic Acid Diketopiperazine

To 6.4 cc. of N lithium hydroxide solution saturated with CO_2 at 20° there were added 1.1 gm. of glutamyl-α-glycine ethyl ester. 4 hours later, after being acidified to a weak Congo red reaction, the solution was evaporated down and the residue washed with absolute alcohol. Needles; yield, 0.4 gm.; m.p., 240°. The substance gave no nitrogen by the Van Slyke procedure.

$C_7H_{10}O_4N_2$ (186). Calculated, N 15.0; found, N 14.9 (Kjeldahl)

Carbobenzoxyglycyl-l-Asparagyl-α-Glycine Ethyl Ester

Carbobenzoxy-l-Asparagyl-α-Glycine Ethyl Ester—12 gm. of carbobenzoxy-*l*-aspartic acid anhydride were added to an ice-cooled solution of glycine ethyl ester (prepared from 25 gm. of hydrochloride) in 150 cc. of anhydrous ethyl acetate. On standing at 20°, needles (13 gm.) separated out which were filtered off after 3 hours and heated in ethyl acetate. They were dissolved in hot water, and the solution acidified with concentrated HCl and extracted twice with ethyl acetate. After evaporation the residue was washed with ether. Needles; yield, 8.5 gm.; m.p., 105–107°. The substance was recrystallized several times from acetone, whereupon a constant melting point of 128°[3] was obtained, but the yield drops sharply.

$C_{16}H_{20}O_7N_2$ (352.2). Calculated, N 8.0; found, N 8.0 (Kjeldahl)

l-Asparagyl-α-Glycine Ethyl Ester—2 gm. of the above ester were dissolved in 50 per cent ethyl alcohol and 0.5 cc. of glacial acetic acid and hydrogenated in the usual manner. The residue (needles) obtained upon evaporating the solution was repeatedly treated with alcohol followed by evaporation. The crystals were transferred to the filter with alcohol. Yield, 1.1 gm.; m.p., 232°.

$C_8H_{14}O_5N_2$ (218.1). Calculated, N 12.8; found, N 12.7 (Kjeldahl)

Carbobenzoxyglycyl-l-Asparagyl-α-Glycine Ethyl Ester—2 gm. of the previous substance were dissolved in an ice-cooled solution of 1.3 gm. of K_2CO_3 in 10 cc. of water and immediately treated with 1.2 gm. of carbobenzoxyglycyl chloride and 0.65 gm. of K_2CO_3 (in 5 cc. of water). After 2 minutes 1.2 gm. of chloride and 0.65 gm. of carbonate were added again. The solution was extracted with ether, and the aqueous layer acidified, yielding a syrup which crystallized. Needles; yield, 2.5 gm. After recrystallization from alcohol, the melting point was 148°.

[3] Grassmann and Schneider (6) report a melting point of 113°. The acid obtained from the above ester by hydrolysis with 2 moles of NaOH for 20 minutes melted at 168°. After the material recrystallized from water, the melting point rose to 171°. Grassmann and Schneider report a melting point of 160° for the acid.

$C_{18}H_{23}O_8N_3$.	Calculated.	C 52.8, H 5.7, N 10.3
409.2	Found.	" 52.7, " 5.9, " 10.0

From the enzymatic hydrolysis to 90 per cent of 306 mg. of this substance there were isolated 82 mg. of a product with a melting point of 117°; mixed melting point with carbobenzoxyglycine, 119°.

Benzoyldiglycyl-l-Glutamylglycine Ethyl Ester

Glycyl-l-Glutamylglycine Ethyl Ester—3 gm. of the corresponding carbobenzoxy compound in aqueous alcohol and 0.7 cc. of glacial acetic acid were hydrogenated in the usual manner. The solution was evaporated down, yielding a residue which was dissolved in water, and the resulting solution evaporated down. Needles; yield, 1.7 gm.

$C_{11}H_{19}O_6N_3$ (289.2). Calculated, N 14.5; found, N 14.4 (Kjeldahl)

Benzoyldiglycyl-l-Glutamylglycine Ethyl Ester—0.6 gm. of the previous substance was dissolved in an ice-cooled solution of 0.2 gm. of soda in 5 cc. of water and immediately treated with 0.4 gm. of hippuryl chloride and 0.2 gm. of soda (in 5 cc. of water) in two portions. The precipitate obtained on acidifying was recrystallized twice from alcohol. Needles; m.p., 252°.

$C_{20}H_{26}O_8N_4$ (337.2). Calculated, N 12.5; found, N 12.4 (Kjeldahl)

From the enzymatic hydrolysis to 97 per cent of 328 mg. of this substance there were isolated 70 mg. of a product with a melting point of 189°; mixed melting point with hippuric acid, 189°.

Carbobenzoxyglycyl-l-Isoglutamine

1.5 gm. of *l*-isoglutamine in 15 cc. of ice-cooled water were treated during 15 minutes with 0.6 gm. of MgO and 2.1 gm. of carbobenzoxyglycyl chloride. After being acidified the solution was extracted with ethyl acetate. Crystals (prisms) were obtained on concentrating the ethyl acetate layer. A second crystallization was obtained by allowing the aqueous layer to stand at 0°. Prisms; yield, 1 gm.; m.p., 185°.

$C_{15}H_{19}O_6N_3$.	Calculated.	C 53.4, H 5.7, N 12.5
337.2	Found.	" 53.1, " 5.9, " 12.4 (Kjeldahl)

From the enzymatic hydrolysis to 100 per cent of 420 mg. of this substance there were isolated 210 mg. of a product with a melting point of 120°; mixed melting point with carbobenzoxyglycine, 120°.

Benzoyl-l-Isoglutamine

1.4 gm. of *l*-isoglutamine were dissolved in an ice-cooled solution of 1 gm. of soda in 15 cc. of water and immediately treated with 1.2 cc. of benzoyl chloride and 1 gm. of soda in two portions. After small amounts of benzamide were filtered off, the solution was acidified, yielding crystals which were recrystallized from hot water. Needles; yield, 1 gm.; m.p., 158°.

$C_{12}H_{14}O_4N_2$ (250.1). Calculated, N 11.2; found, N 11.2 (Kjeldahl)

From the enzymatic hydrolysis to 96 per cent of 600 mg. of this substance there were isolated 450 mg. of a product with a melting point of 133–135°

Benzoyl-*l*-glutamic acid. Calculated, N 5.6; found, N 5.5

Carbobenzoxytriglycine

5.7 gm. of glycine anhydride were dissolved in 50 cc. of N NaOH (requires ½ hour) and then treated at 0° with 11.3 gm. of carbobenzoxyglycyl chloride and 55 cc. of N NaOH in four portions. On acidifying, crystals were obtained which were recrystallized from water. Plates; yield, 11.6 gm.; m.p., 196°.

$C_{14}H_{17}O_6N_3$.	Calculated.	C 52.0, H 5.3, N 13.0
323.1	Found.	" 52.2, " 5.4, " 13.1 (Kjeldahl)

From the enzymatic hydrolysis to 85 per cent of 809 mg. of this substance there were isolated: (*a*) 380 mg. of a product with a melting point of 120°, mixed melting point with carbobenzoxyglycine, 120°; (*b*) on coupling with carbobenzoxy chloride, 215 mg. of a product with a melting point of 178°, mixed melting point with carbobenzoxydiglycine, 178°; (*c*) 73 mg. of unchanged carbobenzoxytriglycine with a melting point of 195°.

Carbobenzoxytetraglycine

1.6 gm. of triglycine in 7.2 cc. of N NaOH were treated at 0° with 1.6 gm. of carbobenzoxyglycyl chloride and 7.2 cc. of N NaOH. The crystals obtained on acidifying were recrystallized from a large volume of water. Yield, 1.7 gm.; m.p., 230°.

$C_{16}H_{20}O_7N_4$.	Calculated.	C 50.5, H 5.3, N 14.7
380.1	Found.	" 50.5, " 5.3, " 14.7 (Kjeldahl)

From the enzymatic hydrolysis to 100 per cent of 950 mg. of this substance there were isolated: (*a*) 390 mg. of a product with a

melting point of 118°, mixed melting point with carbobenzoxyglycine, 118°; (*b*) on coupling with benzylcarbonyl chloride, 390 mg. of a product with a melting point of 195°, mixed melting point with carbobenzoxytriglycine, 195°.

Carbobenzoxyglycylsarcosyldiglycine

To 4.1 gm. of sarcosyldiglycine in 20 cc. of N NaOH were added at 0° 4.2 gm. of carbobenzoxyglycyl chloride and 20 cc. of N NaOH in four portions. The syrup obtained on acidification was dissolved in a hot mixture of acetone and absolute alcohol, the solution filtered, and the filtrate evaporated, yielding a syrup which crystallized on standing. Yield, 4.2 gm. The substance was recrystallized from ethyl acetate by addition of methanol to dissolve, and cooling slowly. Needles; m.p., 127°.

$C_{17}H_{22}O_7N_4$.	Calculated.	C 51.7, H 5.6, N 14.2
394.1	Found.	" 51.7, " 5.8, " 14.0 (Kjeldahl)

l-Leucylglycine

Carbobenzoxy-l-Leucylglycine Ethyl Ester—To a solution of 2.9 gm. of *l*-leucylhydrazide in a mixture of 10 cc. of glacial acetic acid, 5 cc. of concentrated HCl, and 25 cc. of water, there was added at 0° 0.6 gm. of $NaNO_2$ in 5 cc. of water. After the resulting syrup was extracted with ether, the ether layer was successively washed with cold water, bicarbonate, and dilute HCl. The substance obtained upon evaporating off the ether was recrystallized from ethyl acetate-petroleum ether. Needles; yield, 1.5 gm.; m.p., 103–104°.

$C_{18}H_{28}N_2O_5$ (350.2). Calculated, N 8.5; found, N 8.3

Carbobenzoxy-l-Leucylglycine—18 gm. of the above ester in methanol solution were allowed to stand for 20 minutes with 1.1 moles of 2 N NaOH, and after the solution was acidified the methanol was evaporated off. The substance crystallized after the remaining solution was extracted with bicarbonate and acidified. Prisms; yield, 11 gm.; m.p., 115°.

$C_{16}H_{22}O_5N_2$.	Calculated.	C 59.6, H 6.9, N 8.7
322.2	Found.	" 59.4, " 7.0, " 8.5

Free Dipeptide—This was obtained in 75 per cent yield upon hydrogenation of the previous substance. It was transferred to

the filter with absolute alcohol after evaporation of the solvent. Needles.

$[\alpha]_D^{24} = +85.8°$ (c = 2.4 per cent in water)[4]

$C_8H_{16}O_3N_2$.	Calculated.	C 51.0, H 8.6, N 14.9
188.1	Found.	" 50.9, " 8.7, " 14.9

Glycyl-l-Leucylglycine

Carbobenzoxyglycyl-l-Leucylglycine Methyl Ester—4 gm. of *l*-leucylglycine were kept overnight at 0° in 40 cc. of methanol previously saturated with HCl gas. After the solvent was evaporated off, the resulting ester hydrochloride was converted to the ester in the usual manner, with ethyl acetate as the solvent. After the addition of 2.3 gm. of carbobenzoxyglycyl chloride, another 2.3 gm. of chloride and bicarbonate solution were added and the reaction mixture was shaken with cooling. After the solution was washed with dilute HCl and bicarbonate, the ethyl acetate was evaporated off. The substance was recrystallized from ethyl acetate-petroleum ether. Yield, 6.8 gm.; m.p., 131°.

$C_{19}H_{27}O_6N_3$ (393.2). Calculated, N 10.7; found, N 10.6 (Kjeldahl)

Free Tripeptide—2.8 gm. of the above ester were hydrolyzed in 15 minutes in methanol and 4.4 cc. of 2 N NaOH. After being acidified, the methanol was removed by evaporation, and the aqueous residue extracted with ethyl acetate. The ethyl acetate solution was extracted with bicarbonate, the aqueous layer acidified, and again extracted with ethyl acetate. The syrup obtained upon evaporation of the ethyl acetate solution was hydrogenated in methanol and 0.6 cc. of glacial acetic acid. Evaporation of the solution yielded needles which were transferred to the filter by means of absolute alcohol.

$[\alpha]_D^{24} = -41.2°$ (c = 2.5 per cent in water)

$C_{10}H_{19}O_4N_3$ (245.1). Calculated, N 17.1; found, N 16.9

Benzoylglycyl-l-Leucylglycine—To a solution of 1 gm. of the free tripeptide in 4.1 cc. of N NaOH there were added at 0° in several portions 0.5 cc. of benzoyl chloride and 4.2 cc. of N NaOH. On acidifying, a syrup resulted which crystallized on scratching. The material was heated in ether and recrystallized from water. Yield, 0.9 gm.; m.p., 186°.

[4] Fischer (7) gives $[\alpha]_D^{20} = +81.5$–$86°$.

$[\alpha]_D^{24} = -35.8°$ (c = 2.7 per cent in water + 1 equivalent of NaOH)

$C_{17}H_{23}O_5N_3$.	Calculated.	C 58.4, H 6.6, N 12.0
349.2	Found.	" 58.5, " 6.8, " 12.0 (Kjeldahl)

From the enzymatic hydrolysis to 100 per cent of 524 mg. of this substance there were isolated: (*a*) 225 mg. of a product with a melting point of 186°; mixed melting point with hippuric acid, 186°; (*b*) on coupling with benzylcarbonyl chloride, 219 mg. of a product with a melting point of 115°, mixed melting point with carbobenzoxy-*l*-leucylglycine, 115°.

Carbobenzoxy-*l*-leucylglycine. Calculated, N 8.7; found, N 8.7 (Kjeldahl)

Benzoylglycyl-d-Leucylglycine

Carbobenzoxyglycyl-d-Leucylglycine Methyl Ester—This was prepared from 2 gm. of *d*-leucylglycine (Hoffmann-La Roche) in the same manner as the *l* isomer. Yield, 2 gm.; m.p., 131°.

$C_{19}H_{27}O_6N_3$ (393.2). Calculated, N 10.7; found, N 10.7 (Kjeldahl)

Benzoylglycyl-d-Leucylglycine Methyl Ester—1.3 gm. of the previous substance were dissolved in methanol containing 2 moles of HCl gas, and hydrogenated. The solution was evaporated, the residue dissolved in 3 cc. of water, overlaid with ethyl acetate, and then 1 cc. of benzoyl chloride and bicarbonate solution added at 0° with shaking. The ethyl acetate solution was washed as usual and concentrated down. On addition of ether 1 gm. of needles was obtained, having a melting point of 180°.

$C_{18}H_{25}O_5N_3$ (363.2). Calculated, N 11.6; found, N 11.5 (Kjeldahl)

Benzoyltripeptide—The above ester was saponified in methanol and 1.1 moles of N NaOH in 15 minutes. After being acidified, the methanol was evaporated off, and the product recrystallized from water. Needles; m.p., 186°.

$[\alpha]_D^{24} = +35.2°$ (c = 2.7 per cent in water + 1 equivalent of NaOH)

$C_{17}H_{23}O_5N_3$.	Calculated.	N 12.0
349.2	Found.	" 11.7 (Dumas)
		" 12.0 (Kjeldahl)

From the enzymatic hydrolysis to 96 per cent of 437 mg. of this substance there were isolated 132 mg. of a product which on

recrystallization melted at 185°; mixed melting point with hippuric acid, 186°.

Diglycyl-l-Leucylglycine

To a solution of 2.4 gm. of glycyl-*l*-leucylglycine in 10 cc. of N NaOH there were added at 0° in four portions 2.3 gm. of carbobenzoxyglycyl chloride and 10 cc. of N NaOH. The syrup obtained on acidifying the solution was taken up in ethyl acetate, and the extract washed with water and evaporated down. On treatment with acetone, 2.7 gm. of prisms were obtained (m.p., 118–119°) which were immediately hydrogenated in aqueous methanol and 0.25 cc. of glacial acetic acid. Upon repeated evaporation from water, crystals were obtained which were recrystallized from alcohol-water. Rosettes; yield, 1.5 gm.

$[\alpha]_D^{24} = -43.2°$ (c = 2.5 per cent in water)

$C_{12}H_{22}O_5N_2$.	Calculated.	C 47.7, H 7.3, N 18.5
302.1	Found.	" 47.6, " 7.4, " 18.4 (Kjeldahl)

Benzoyltetrapeptide—This was prepared from the free peptide in the same manner as benzoylglycyl-*l*-leucylglycine. Yield, 70 per cent; m.p., 195°.

$C_{19}H_{26}O_6N_4$ (406.2). Calculated, N 13.8; found, N 13.7 (Kjeldahl)

From the enzymatic hydrolysis to 98 per cent of 280 mg. of this substance there were isolated 85 mg. of a product with a melting point of 186°; mixed melting point with hippuric acid, 186°.

Triglycyl-l-Leucylglycine

Carbobenzoxypentapeptide—To a solution of 1.4 gm. of diglycyl-*l*-leucylglycine in 4.6 cc. of N NaOH there were added in three portions at 0° 1 gm. of carbobenzoxyglycyl chloride and 4.7 cc. of N NaOH. The precipitate obtained on acidification was recrystallized from 50 per cent methanol. Yield, 2.2 gm.; m.p., 225°.

$C_{22}H_{31}O_8N_5$.	Calculated.	C 53.5, H 6.3, N 14.2
493.3	Found.	" 53.6, " 6.5, " 14.1 (Kjeldahl)

From the enzymatic hydrolysis to 100 per cent of 690 mg. of this substance there were isolated: (*a*) 200 mg. of a product with a melting point of 120°, mixed melting point with carbobenzoxy-

glycine, 120°; (*b*) 50 mg. of a product with a melting point of 193–194°, mixed melting point with carbobenzoxytriglycine, 194°.

Free Pentapeptide—1.5 gm. of carbobenzoxy compound were suspended in 50 per cent methanol and 0.2 cc. of glacial acetic acid and hydrogenated as usual. After repeated addition and evaporation of water the substance was recrystallized by adding water to the alcoholic suspension to dissolve the crystals and the solution cooled slowly. Needles; yield, 0.8 gm.

$[\alpha]_D^{21} = -28.4°$ (c = 2.5 per cent in water)

$C_{14}H_{25}O_6N_5$.	Calculated.	C 46.8, H 7.0, N 19.5
359.1	Found.	" 46.7, " 7.1, " 19.3 (Kjeldahl)

Carbobenzoxy-l-Leucylglycylglycine

Carbobenzoxy-l-Leucyldiglycine Ethyl Ester—An ethereal solution of carbobenzoxyleucylazide (from 12 gm. of hydrazide) was added to an ethyl acetate solution of diglycine ethyl ester (from 10 gm. of hydrochloride). The ether was evaporated off and after standing several hours at 50°, the ethyl acetate solution was treated with gaseous HCl. 5 gm. of unchanged ester were recovered as the hydrochloride. After the filtrate was washed with water and bicarbonate, it was evaporated down and to the residue ether was added. Needles; yield, 6 gm.; m.p., 105°.

$C_{20}H_{29}O_6N_3$.	Calculated.	C 58.9, H 7.2, N 10.3
407.2	Found.	" 59.1, " 7.4, " 10.3 (Kjeldahl)

Carbobenzoxytripeptide—The saponification proceeded in the same manner as for carbobenzoxyglycylleucylglycine. Needles; yield, 75 per cent; m.p., 144°.

$[\alpha]_D^{22} = -12.8°$ (c = 4.9 per cent in alcohol)

$C_{18}H_{25}O_6N_3$.	Calculated.	C 57.0, H 6.6, N 11.1
379.2	Found.	" 57.0, " 6.9, " 10.9 (Kjeldahl)

From the enzymatic hydrolysis of 950 mg. of this substance to 96 per cent there were isolated: (*a*) 600 mg. of a product with a melting point of 116°, mixed melting point with carbobenzoxy-*l*-leucylglycine, 116°; (*b*) on coupling with carbobenzoxy chloride 440 mg. of a product with a melting point of 120°, mixed melting point with carbobenzoxyglycine, 120°. For the product of (*a*) 8.7 per cent N was found; calculated for carbobenzoxy-*l*-leucylglycine, N 8.7.

Carbobenzoxy-d-Leucylglycylglycine

To a solution of 1.2 gm. of *d*-leucylglycylglycine (Hoffmann-La Roche) in 5 cc. of N NaOH there were added at 0° 0.9 gm. of carbobenzoxy chloride and 7 cc. of N NaOH. After acidification and extraction with ethyl acetate, the carbobenzoxy compound was taken up in bicarbonate and the solution acidified. When recrystallized from methanol-water the yield was 0.6 gm.; m.p., 144°.

$[\alpha]_D^{22} = +12.3°$ (c = 4.9 per cent in alcohol)

$C_{18}H_{25}O_6N_3$.	Calculated.	C 57.0, H 6.6, N 11.1
379.2	Found.	" 56.9, " 6.9, " 11.0 (Kjeldahl)

Benzoyl-dl-Leucylglycylglycine

1.5 gm. of *dl*-leucyldiglycine in 20 cc. of water containing 4 gm. of $KHCO_3$ were treated with 1.4 gm. of benzoyl chloride in the usual manner. Several hours after acidifying, the resulting precipitate was filtered off, dried, heated in acetone, and recrystallized from water. Needles; yield, 1.1 gm.; m.p., 168°.

$C_{17}H_{23}O_5N_3$.	Calculated.	C 58.4, H 6.6, N 12.0
349.2	Found.	" 58.4, " 6.8, " 12.0 (Kjeldahl)

From the enzymatic hydrolysis of 500 mg. of this substance to 95 per cent (calculated for the *l* form) there were isolated, after extraction with ethyl acetate and carbobenzoxylation of the aqueous layer, 96 mg. of a product with a melting point of 120° after recrystallization; mixed melting point with carbobenzoxyglycine, 120°.

Carbobenzoxyglycylglycine—This was prepared according to Bergmann and Zervas (5).

From the enzymatic hydrolysis to 75 per cent of 1.330 gm. of this substance there were isolated 400 mg. of a product with a melting point of 120°; mixed melting point with carbobenzoxyglycine, 120°.

Hippurylamide—This was prepared according to Fischer (8).

From the enzymatic hydrolysis to 92 per cent of 267 mg. of this substance there were isolated 220 mg. of a product with a melting point of 187°; mixed melting point with hippuric acid, 186°.

Isolation of Split-Products

Upon completion of the enzymatic hydrolysis, the solution was concentrated under diminished pressure to 3 to 4 cc. and then

concentrated HCl added to Congo red acidity. The products, carbobenzoxyglycine or hippuric acid, crystallized out and were identified. In other cases, the acid solution was extracted with ethyl acetate and the split-product obtained by evaporation and precipitation with ether. In those cases where the second split-product was isolated as well, the filtrate of the above crystals was made slightly alkaline with concentrated NaOH and treated in the usual manner with an amount of benzylcarbonyl chloride slightly larger than that calculated from the extent of splitting. After the material was acidified, the resulting carbobenzoxy derivatives were compared with synthetic preparations.

Enzymatic Studies

The enzyme solutions employed in these experiments were made up in the concentrations and activated with 3 per cent HCN as recommended by Grassmann (9) with a papain preparation obtained by evaporation of the juice of *Carica papaya*. In several cases (*e.g.*, hippurylamide, carbobenzoxytriglycine) we repeated the enzymatic hydrolysis using the commercial papain, Merck, and obtained comparable results.

The concentration of substrate was in all cases 0.5 mM and each cc. of the reaction mixture contained 0.2 cc. of enzyme solution and 0.1 cc. of 0.2 M citrate buffer of pH 5.0. The temperature in all cases was 40°. The extent of hydrolysis was determined by titration of the carboxyl groups according to Grassmann and Heyde (10) and by amino nitrogen estimations in the micro-Van Slyke apparatus.

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