Studies on Peptides. VIII. The Synthesis of α,γ-Glutamyl Peptides and the Cleavage Reactions of their γ-Glutamyl Peptide Linkages[1]

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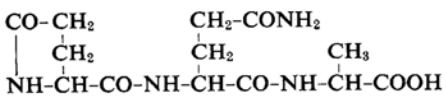
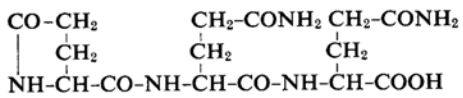
In previous papers[2,3], the structure of eisenine, tripeptide isolated from the brown marine alga *Eisenia bicyclis* Setchell[4,5], had been confirmed by synthesis. Eisenine was thus shown to be L-pyroglutamyl-L-glutaminyl-L-alanine (I). A similar tripeptide, fastigiatine, had been obtained by Fruton and his coworkers from the American marine alga *Pelvetia fastigiata*[6].



(I)



(II)

They suggested that fastigiatine is L-pyroglutamyl-L-glutaminyl-L-glutamine (II)[6]. However, our synthetic results have shown that this structure can not explain the physical constants of natural fastigiatine[1,7]. Nevertheless, even if this structure is not correct, a pyrrolidone ring is certainly present in the molecule of fastigiatine. During the course of our investigations on these two naturally occurring peptides, eisenine and fastigiatine, the fact, that both contain a pyrrolidone ring, seemed to us to be of particular interest.

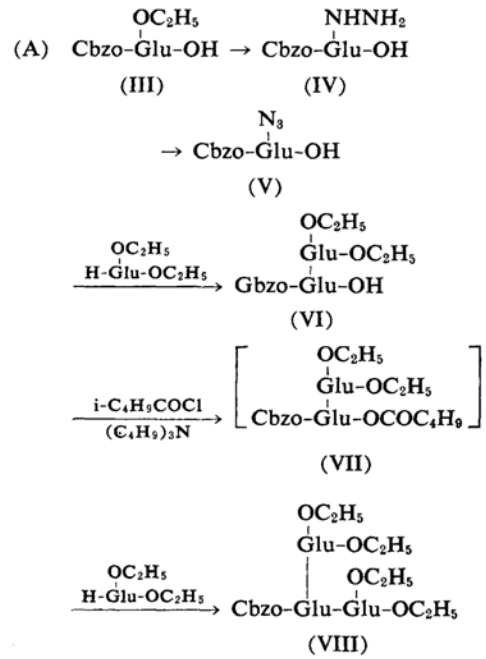
With regard to the mechanism of formation of such a pyrrolidone ring, two possibilities were considered. One of them is a biosynthetic process in which only the γ-glutamyl peptide linkage of an α,γ-glutamyl peptide existing in the living cell is cleft enzymatically thus forming the pyroglutamyl peptide. With regard to this mechanism, Woodward and Reihart[8] reported that γ-glutamyl transpeptidase obtained from kidney, cleft selectively the γ-glutamyl peptide linkage in glutathione and that pyrrolidone carboxylic acid was produced. Connell and Hanes[9], in discussing this cleavage reaction, suggested that the γ-glutamyl peptide linkage had not been split by pure transpeptidase but by γ-glutamyl lactamase.

The second possibility is a non-enzymatic formation of the pyrrolidone ring. When a powder of the alga is heated in aqueous ethyl alcohol on a boiling water bath in order to extract the peptide, the original α,γ-glutamyl peptide or glutaminyl peptide may be converted to a pyroglutamyl peptide such as eisenine or fastigiatine. In general, the γ-glutamyl peptide bond is much more labile than the α-peptide bond. Ellfolk and Synge[10], as well as Hird and Springell[11], have pointed out that pyrrolidone carboxylic acid was produced from glutathione in a non-enzymatic reaction rather than an enzymatic one. Young and his coworkers[12,13] have observed a similar non-enzymatic reaction in the cases of several γ-glutamyl peptides. Glutamine or glutaminyl peptides are also easily cyclized to pyrrolidone carboxylic acid or to a pyroglutamyl peptide respectively by merely heating their aqueous solutions[7,14,15]. Although the non-enymatic formation of pyrrolidone carboxylic acid from a γ-glutamyl peptide or of a pyroglutamyl peptide from a glutaminyl peptide has been studied, it is not certain whether a pyroglutamyl peptide can be produced by cleavage of α,γ-glutamyl peptide. In the present investigation several α,γ-glutamyl peptides were synthesized and their selective cleavage reactions were studied.

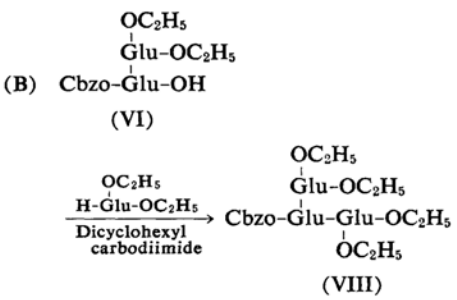
A few methods for the synthesis of α,γ-glutamyl peptides were investigated in a case of the tetraethyl ester of carbobenzyloxy-α,γ-L-glutamyl di-L-glutamic acid. The first method (A) was based on the following reactions.

1) For the previous publication see T. Shiba and S. Imai, *J. Chem. Soc. Japan, Pure Chem. Sec.* (*Nippon Kagaku Zasshi*), **80**, 497 (1959).
2) T. Kaneko, T. Shiba, W. Watarai, S. Imai, T. Shimada and K. Ueno, *Chem. & Ind.*, **1957**, 986.
3) T. Shiba, *J. Chem. Soc. Japan Pure Chem. Sec.* (*Nippon Kagaku Zasshi*), **80**, 492 (1959).
4) T. Ohira, *J. Agr. Chem. Soc. Japan* (*Nippon Nogei-Kagaku Kaishi*), **15**, 370 (1939).
5) T. Ohira, ibid., **16**, 293 (1940).
6) C. A. Dekker, D. Stone and J. S. Fruton, *J. Biol. Chem.*, **181**, 719 (1949).
7) T. Shiba, S. Imai and T. Kaneko, This Bulletin, **31**, 244 (1958).
8) G. E. Woodward and F. C. Reinhart, *J. Biol. Chem.*, **145**, 471 (1942).
9) G. E. Connell and C. S. Hanes, *Nature*, **177**, 377 (1956).
10) N. Ellfolk and R. L. Synge, *Biochem. J.*, **59**, 523 (1955).
11) F. J. R. Hird and P. H. Springell, ibid., **56**, 417 (1954).
12) W. J. LeQuesne and G. T. Young, *J. Chem. Soc.*, **1952**, 594.
13) D. A. Rowlands and G. T. Young, ibid., **1952**, 3937.
14) J. Melville, *Biochem. J.*, **29**, 179 (1935).
15) K. Narita, *Biochim. et Biophys. Acta*, **30**, 352 (1958).

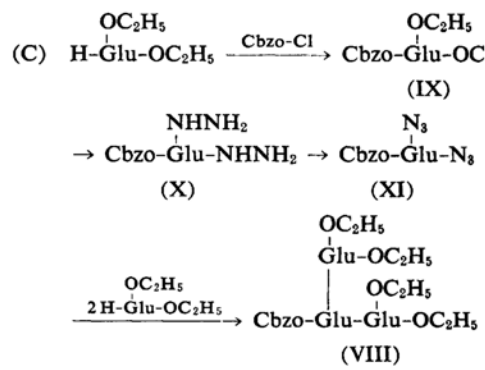
Carbobenzyloxy-γ-L-glutamyl azide (V) obtained from γ-ethyl carbobenzyloxy-L-glutamate (III) via the hydrazide (IV), was condensed with diethyl-L-glutamate to give the carbobenzyloxy-γ-dipeptide diethyl ester (VI). This diester VI was then combined with diethyl-L-glutamate via its mixed anhydride VII with isovaleric acid to obtain tetraethyl carbobenzyloxy-α, γ-L-glutamyl di-L-glutamate (VIII).



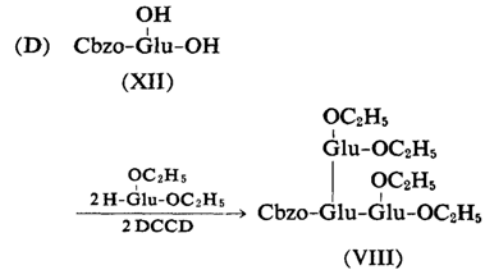
In the second method B, diethyl carbobenzyloxy-γ-L-glutamyl-L-glutamate (VI), synthesized as in method A, was coupled with diethyl-L-glutamate by means of dicyclohexyl carbodiimide. Method B gives a better yield of VIII than method A. However, both methods have the disadvantage of giving poor yields of the intermediate VI from the azide V and of requiring many synthetic steps.



Booth and his collaborators had synthesized the same carbobenzyloxy tetraester (VIII)[16]. Their method was based on the preparation of diethyl carbobenzyloxy-α-glutamyl glutamate, followed by its conversion into VIII via the acid chloride method. Their synthesis suffers from similar defects as methods A and B. Later, they succeeded in condensing one mole of *p*-nitrobenzoyl-α, γ-glutamyl diazide with two moles of diethyl glutamate, thus forming in a single reaction tetraethyl *p*-nitrobenzoyl-α, γ-glutamyl diglutamate[17]. In our third method C this diazide method was applied to the carbobenzyloxy derivative. Diethyl carbobenzyloxy-L-glutamate (IX) was obtained in good yield by the direct carbobenzyloxylation of diethyl L-glutamate. This diethyl ester (IX) was then converted into the dihydrazide (X,) and then to the diazide (XI), which was combined with two moles of diethyl L-glutamate to form tetraethyl carbobenzyloxy-α, γ-L-glutamyl di-L-glutamate (VIII). Method C was superior to methods A and B, because the synthetic path became shorter and an overall yield of 48% in reactions X to VIII was obtained.



In our last method D, carbobenzyloxy-L-glutamic acid was directly coupled with two moles of diethyl L-glutamate using two moles of dicyclohexyl carbodiimide. The starting material for this reaction is easily available, only one step is required in order to obtain the desired product and an excellent yield (88%) is obtained. Therefore, this method is superior to the other methods.



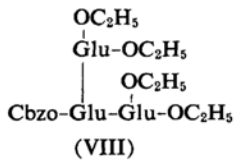
The carbobenzyloxy tetraester obtained through such methods, was difficult to crystal-

16) J. H. Mowat et al., *J. Am. Chem. Soc.*, **70**, 1096 (1948).

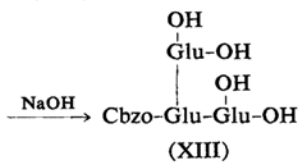
17) J. H. Mowat et al., ibid., **71**, 2308 (1949).

lize, but was obtained as a gelatinous precipitate upon addition of petroleum ether to its solution in ethyl acetate. Several samples of VIII showed varying melting points due to the occlusion of solvent, but their infrared spectra were all identical.

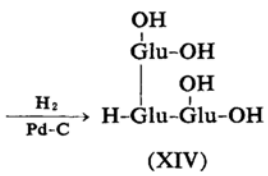
Carbobenzyloxy tetraester (VIII) prepared according to method D, was then hydrolyzed to carbobenzyloxy-α,γ-L-glutamyl di-L-glutamic acid (XIII), which was obtained as a syrup that did not crystallize. Compound XIII was hydrogenated to α,γ-L-glutamyl di-L-glutamic



(VIII)



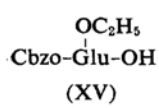
(XIII)



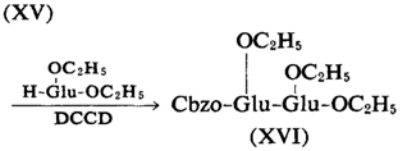
(XIV)

acid (XIV), which had a tendency to decompose during evaporation in vacuo and therefore was lyophilized.

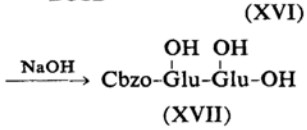
As preliminary experiment for our method D, γ-ethyl carbobenzyloxy-L-glutamate (XV) was coupled with diethyl L-glutamate using dicyclohexyl carbodiimide. Triethyl carbobenzyloxy-α-L-glutamyl-L-glutamate (XVI) was thus obtained in a good yield. Compound XVI was then hydrolyzed to carbobenzyloxy-α-L-glutamyl-L-glutamic acid (XVII), which was hydrogenated to α-L-glutamyl-L-glutamic acid (XVIII).



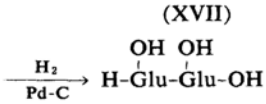
(XV)



(XVI)



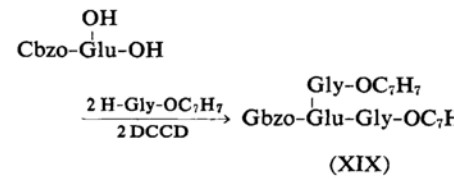
(XVII)



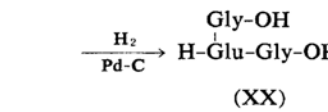
(XVIII)

For the purpose of preparing α-glutamyl dipeptides, the method of Bergmann and Zervas[18], in which carbobenzyloxy glutamic anhydride is coupled with an amino acid ester, has hitherto mostly employed. The reaction products, however, always contained a small amount of γ-peptide as a by-product, which must be separated from the α-isomer by means of successive extractions or a countercurrent distribution. Therefore, the anhydride method is not only very tedious, but also disadvantageous as far as its yield is concerned. In contrast, method D is believed to be a better synthetic approach to α-glutamyl peptides, since these defects are eliminated.

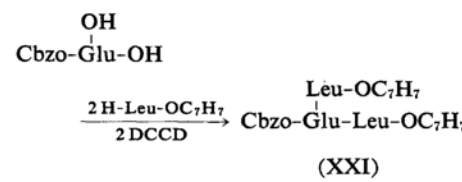
Subsequently, α,γ-L-glutamyl diglycine (XX), α,γ-L-glutamyl di-L-leucine (XXII) and α,γ-L-glutamyl di-Laspartic acid (XXIV) were also synthesized according to method D except that the amino acid benzyl esters were used in place of the ethyl esters. The benzyl esters of carbobenzyloxy-α,γ-glutamyl petides (XIX, XXI, XXIII) were hydrogenated to the α,γ-glutamyl peptides (XX, XXII, XXIV) in one step. The hydrolysis step required for ethyl esters was thus eliminated.



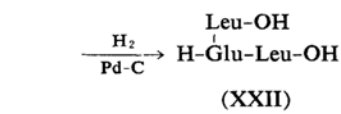
(XIX)



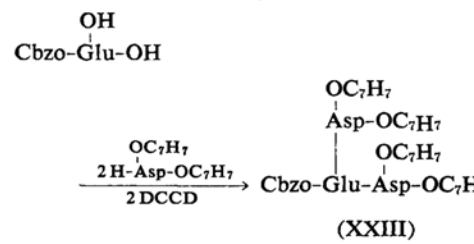
(XX)



(XXI)

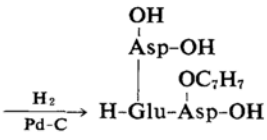


(XXII)



(XXIII)

18) M. Bergmann and L. Zervas, *Ber.*, **65**, 1192 (1932).



(XXIV)

The hydrogenolysis of the benzylesters (XIX, XXI, XXIII) was carried out in open vessels after the addition of palladium-charcoal (10%). In the case of peptides of glycine XIX and of leucine XXI, this hydrogenolysis took place readily, while tetrabenzyl carbobenzyloxy-α,γ-glutamyl diaspartate (XXIII) was difficult to hydrogenate, probably owing to its steric structure; the hydrogenated product XXIV could not be obtained in a pure state.

For the purpose of the preparation of α,γ-L-glutamyl di-L-lysine by a similar synthetic method, ε-carbobenzyloxy-L-lysine methyl ester hydrochloride (XXV) and ε-carbobenzyloxy-L-lysine benzyl ester hydrochloride (XXVI) were synthesized. The former (XXV) had been prepared from ε-carbobenzyloxy-α-carboxyl-L-lysine anhydride and methyl alcoholic hydrochloric acid by Bergmann and his coworkers[19]. Our advantageous method or preparation of ε-carbobenzyloxy lysine ester hydrochloride was based on the esterification of ε-carbobenzyloxy-L-lysine obtained from copper lysinate and carbobenzyloxy chloride[20]. ε-Carbobenzyloxy-L-lysine was esterified with thionyl chloride in methyl alcohol for benzyl alcohol to yield the methyl ester hydrochloride (XXV) and the benzyl ester hydrochloride (XXVI) respectively without decomposition of the carbobenzyloxy group. The condensation reactions of these lysine esters with carbobenzyloxy glutamic acid in the presence of dicyclohexyl carbodiimide were not successful. Complex mixtures were obtained in all cases. This failure may be due to the molecular structures of XXV and XXVI. However, these cabobenzyloxylysine ester hydrochlorides (XXV and XXVI) may be useful intermediates in the syntheses of other lysine peptides.

$$\text{Cbzo-NH(CH}_2)_4\text{CH(NH}_2\cdot\text{HCl)COOCH}_3$$

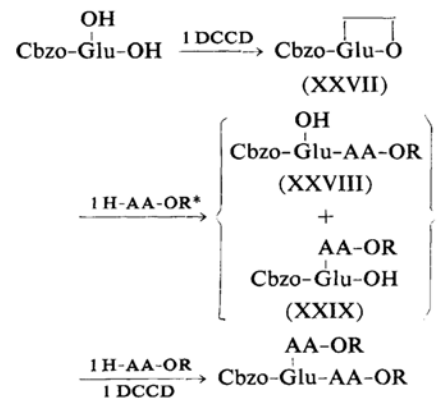
(XXV)

$$\text{Cbzo-NH(CH}_2)_4\text{(NH}_2\cdot\text{HCl)COOCH}_2\text{C}_6\text{H}_5$$

(XXVI)

In the course of all condensation reactions in the presence of cyclohexyl carbodiimide, pure dicyclohexyl urea always separated in crystalline form from the reaction mixture. In order to use it repeatedly for other reactions, dicyclohexyl carbodiimide was recovered from dicyclohexyl urea in fair yield according to the method of Amiard and Heymes[21].

Concerning the mechanism of the condensation reaction in the presence of dicyclohexyl carbodiimide, we supposed at first that two moles of amino acid ester would combine simultaneously with one mole of carbobenzyloxy glutamic acid in the presence of two moles of dicyclohexyl carbodiimide. However, when solutions of one mole of carbobenzyloxy-glutamic acid and of two moles of dicyclohexyl carbodiimide were mixed before the addition of any amino acid ester, it was observed that nearly one mole of dicyclohexyl urea separated out immediately. When two moles of amino acid ester were then added to the filtrate from this urea derivative, the expected α,γ-glutamyl peptide was obtained in good yield and another mole of dicyclohexyl urea was formed at the same time. Therefore, the reactioncan be better explained by a three step mechanism as follows. In the first step, one mole of carbobenzyloxy glutamic acid reacts with one mole of dicyclohexyl carbodiimide to form carbobenzyloxy-glutamic anhydride (XXVII). In the second step, the anhydride ring is opened by one mole of amino acid ester according to the same mechanism as in the method of Bergmann and Zervas[18] with the formation of a mixture of α- and γ-dipeptide derivatives XXVIII and XXIX. In the third step, these condense with another mole of amino acid ester by the action of one mole of dicyclohexyl carbodiimide to give a single product XXX.



(XXVII)

(XXVIII) + (XXIX)

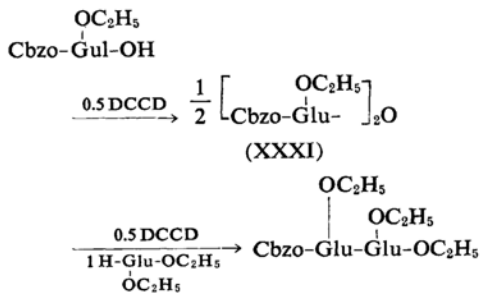
(XXX)

In fact, when one mole of carbobenzyloxy-L-glutamic acid and one mole of dicyclohexyl carbodiimide were mixed, about 0.9 mol. of

19) M. Bergmann, L. Zervas and W. F. Ross, *J. Biol. Chem.*, 111, 245 (1935).
20) A. Neuberger and F. Sanger., *Biochem. J.*, 37, 515 (1943).
21) G. Amiard and R. Heymes, *Bull. soc. chim. France*, 1956, 1360.
* -AA-=amino acid residue, -NH-CH(R)-CO-.

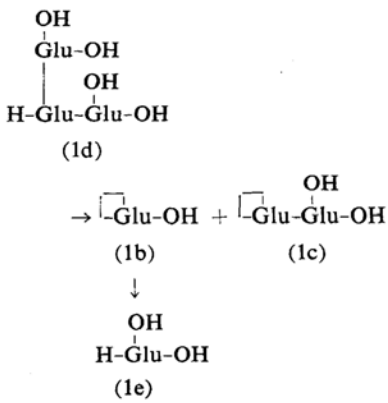
dicyclohexyl urea was always obtained as a precipitate; hence it appears that carbobenzyloxy glutamic anhydride (XXVII) must be formed, but all efforts to crystallize it from the reaction mixture were unsuccessful. Although the formation of the anhydride (XXVII) could not be directly proven, the above mentioned mechanism could be demonstrated indirectly by the following experiment. When one mole of pure carbobenzyloxy-L-glutamic anhydride (XXVII), prepared from carbobenzyloy glutamic acid and acetic anhydride by the usual method, was reacted with two moles of glycine benzyl ester by means of one mole of dicyclohexyl carbodiimide, dibenzyl-carbobenzyloxy-α, γ-L-glutamyl diglycinate (XIX) was obtained in very good yield (92%). This supports the above described three-step mechanism.

When the synthesis of α-glutamyl glutamic acid (XVIII) was carried out in a stepwise fashion, i.e. when γ-ethyl carbobenzyloxy-L-glutamate was mixed with a solution of dicyclohexyl carbodiimide, before diethyl L-glutamate was added, a precipitate of dicyclohexyl urea was also observed. It is assumed, that in this case one mole of the carbobenzyloxy-γ-ester and a half mole of dicyclohexyl carbodiimide form an intermolecular anhydride XXXI, which then reacts with one mole of diethyl glutamate by means of a half mole of dicyclohexyl carbodiimide in a similar manner as the α,γ-glutamyl peptide diester (XXX) is assumed to be formed from carbobenzyloxy-glutamic anhydride (XXVII).

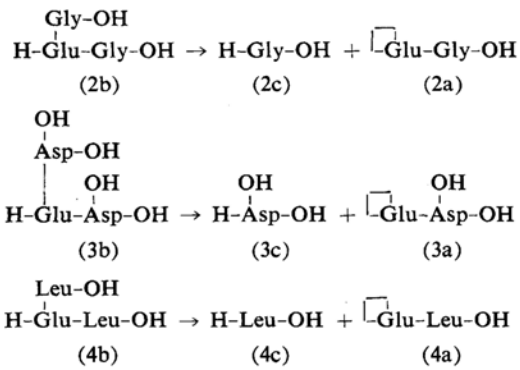


Muramatsu and Hagitani[22] have shown that formyl-β-alanine can also be converted to its bimolecular anhydride by means of dicyclohexyl carbodiimide. Only a small amount of other information on the formation of anhydrides by the reaction of dicyclohexyl carbodiimide with *N*-substituted-α-amino acids is available.

Finally, the cleavage of four α,γ-glutamyl peptides, as well as of α-glutamyl glutamic acid and of γ-glutamyl glutamic acid, caused by heating the aqueous solutions of these peptides, was investigated. The results are presented in Figs. 1—6. In the case of α,γ-L-glutamyl di-L-glutamic acid (1d), a small amount of pyrrolidone carboxylic acid (1b) was split off after only one week at 40°C. However, at 100°C both pyrrolidone carboxylic acid (1b) and glutamic acid (1e) began to appear already after one hour. The α,γ-peptide (1d) disappeared completely after nine hours, while a new spot 1c appeared after three hours and became gradually stronger on longer heating. It was not colored by ninhydrin, but colored by Bromothymol Blue, and is supposed to be pyroglutamyl glutamic acid. From the result of this experiment, it was concluded that α,γ-glutamyl diglutamic acid in aqueous solution was decomposed at 100°C to pyrrolidone carboxylic acid and or glutamic acid and pyroglutamyl glutamic acid by a selective cleavage of its γ-peptide linkage as shown below.

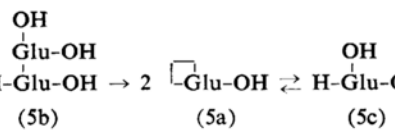


The cleavage of α,γ-L-glutamyl diglycine (Fig. 2), α,γ-L-glutamyl di-L-aspartic acid (Fig. 3), and α,γ-L-glutamyl di-L-leucine (Fig. 4) proceeded in a similar manner. The decompositions began after one hour at 100°C and were completed after ten to twenty hours. These cleavage reactions may be represented as follows.



22) I. Muramatsu and S. Hagitani, *J. Chem. Soc. Japan, Pure Chem. Sec. (Nippon Kagaku Zasshi)*, **80**, 1497 (1959).

In the course of the decomposition of γ-glytamyl glutamic acid (Fig. 5), the Bromothymol Blue positive spot (5a) of pyrrolidone carboxylic acid appeared before the ninhydrin spot of glutamic acid (5c). Since Bromothymol Blue is a less sensitive reagent than ninhydrin, this indicates that both glutamic acid residues of the dipeptide were first decomposed to pyrrolidone carboxylic acid, which was then converted to glutamic acid.



(5b) (5a) (5c)

On the other hand, α-L-glutamyl glutamic acid was rather stable and began to decompose after only twenty hours (Fig. 6).

From the results of this invesigation it may be concluded that α, γ-glutamyl peptides are subject to the selective cleavage of the γ-peptide bond on heating of the aqueous solutions, with the formation of a pyroglutamyl peptide and an amino acid. The rate of the decomposition was independent of the nature of the amino acid linked to the γ-glutamyl caboxyl group. On the basis of the cleavage reactions described above it is reasonable to assume that the pyroglutamyl peptides of marine algae were formed from glutaminyl peptides or from α, γ-glutamyl peptides in the course of the heating during their isolation.

Experimental

Diethyl Carbobenzyloxy-L-glutamate (IX).—A solution of 83 g. (0.35 mol.) of diethyl L-glutamate hydrochloride in 500 ml. of water was mixed with 44 ml. of an aqueous solution of 4 N sodium carbonate. To this solution of pH 7~8, 70 g. (0.41 mol.) of carbobenzyloxy chloride and 50 ml. of an aqueous solution of 4 N sodium carbonate were added. The reaction mixture was vigorously stirred at room temperature (25°C) for three hours. It was extracted twice with 100 ml. of ether. The combined extract was washed with aqueous sodium carbonate, dried with sodium sulfate, and then evaporated in vacuo to a syrupy residue, which crystallized after two weeks' standing at room temperature; yield 103 g. (89%). It was recrystallized from ethyl alcohol and water, needles, m. p. 39~42°C.

Found: C, 60.91; H, 6.73; N, 4.04. Calcd. for $C_{17}H_{23}O_6N$: C, 60.52; H, 6.87; N, 4.15%.

In order to confirm the optical purity of the product, 4.1 g. was dissolved in 80 ml. of 1 N sodium hydroxide solution at room temperature during two hours. Then, the solution was acidified with 5 N hydrochloric acid and extracted with ethyl acetate. The extract was shaken with an aqueous solution of 1 N sodium carbonate. The aqueous layer was separated, acidified and extracted with ethyl acetate. This extract was evaporated in vacuo to a residue, which was hydrogenated in 20 ml. of methyl alcohol and 20 ml. of water in an open vessel in the presence of 100 mg. of 5% palladium-charcoal. After filtration of the catalyst and evaporation of the filtrate a residue was obtained, which was crystallized from water; yield, 0.4 g. On recrystallization, 255 mg. of pure L-glutamic acid was obtained. The overall yield from carbobenzyloxy diester (IX) was 14 percent. $[\alpha]_D^{22}$ −31.8° (*c*, 2.74, 2 N HCl).

Found: C, 40.34; H, 6.17; H, 9.44. Calcd. for $C_5H_9O_4N$; C, 40.81; H, 6.17; N, 9.52%.

Carbobenzyloxy-L-glutamic Dihydrazide (X).—To a solution of 72 g. of diethyl carbobenzyloxy-L-glutamate (IX) in 150 ml. of 99% ethyl alcohol, 100 ml. of 90% hydrazine hydrate was added with stirring during forty-five minutes. The temperature of the reaction mixture was kept below 30°C. When a large amount of a solid product formed during the reaction, another 100 ml. of ethyl alcohol was added. Vigorous stirring was continued for one hour and a half. The white precipitate formed was filtered and then was dissolved in 5 N hydrochloric acid. A solution of sodium acetate was carefully added until a pH of 4 was reached. After standing overnight, 40 g. of fine needles were obtained; yield 60%, m. p. 183~184°C (decomp.). $[\alpha]_D^{21}$ −14.2° (*c*, 3.05, 2 N HCl). Recrystallization from hydrochloric acid and aqueous sodium acetate gave crystals of m. p. 203~204°C (dec.).

Found: C, 50.57; H, 5.91; N, 22.60. Calcd. for $C_{13}H_{19}O_4N_5$: C, 50.49; H, 6.15; N, 22.64%.

Tetraethyl Carbobenzyloxy-α, γ-L-glutamyl Di-L-glutamate (VIII). Method A.—Diethyl carbobenzyloxy-γ-L-glutamyl glutamate (VI) prepared from 6.0 g. (0.02 mol.) of carbobenzyloxy-γ-L-glutamyl hydrazide by the azide method[23], was dissolved in 25 ml. of toluene and 25 ml. of chloroform, and 3.7 g. (0.02 mol.) of tri-*n*-butylamine and 2.4 g. (0.02 mol.) of isovaleryl chloride were added with stirring to the solution kept below 5°C. Stirring was continued for two hours while the temperature of the reaction mixture was kept at 0°C. A solution of free diethyl glutamate prepared from 4.8 g. (0.02 mol.) of diethyl L-glytamate hydrochloride and 3.7 g. (0.02 mol.) of tri-*n*-butylamine, in 50 ml. of chloroform, was added dropwise below 0°C within ten minutes. The reaction mixture was stirred for three hours between 0° and −10°C, then permitted to stand at room temperature for a few hours. The reaction mixture was washed with water, 3% aqueous sodium bicarbonate, 0.5% hydrochloric acid and water, and dried with sodium sulfate. Upon addition of petroleum ether crystals formed, which were filtered and dried over phosphorus pentoxide in vacuo; yield, 5.9 g. (45% based on carbobenzyloxy-L-glutamyl-γ-hydrazide), m. p. 133~134°C (sintered at 103~104°C). The melting point could be raised up to 134~136°C* (sintered at 106~108°C) by

23) T. Shiba and S. Imai, *J. Chem. Soc. Japan, Pure Chem. Sec.* (*Nippon Kagaku Zasshi*), **80**, 176 (1959).

* J. H. Mowat, Y. Subborow et al. (*J. Am. Chem. Soc.*, 71, 2308 (1949)) have synthesized tetraethyl carbobenzyloxy-α, γ-L-glutamyl-L-glutamate from tetraethyl *p*-nitrobenzoyl-α, γ-L-glutamyl-L-glutamate by replacing the *p*-nitrobenzoyl group with a carbobenzyloxy group. Their sample had a melting point of 140.5~142.0°C (sintered at 105~107°C).

recrystallization from ethyl alcohol and water. $[\alpha]_D^{25}-15.9°$ (*c*, 3.01, 99% EtOH).

Found: C, 56.62; H, 7.16; N, 6.67. Calcd. for $C_{31}H_{45}O_{12}N_3$: C, 57.13; H, 6.96; N, 6.45%.

Method B.—To a solution of 5.7 g. (0.035 mol.) of diethyl glutamate hydrochloride in 50 ml. of tetrahydrofuran, 1.7 g. of triethylamine was added and the triethylamine hydrochloride formed was filtered off. To the solution of the free ester, 7.6 g. (0.0163 mol.) of diethyl carbobenzyloxy-γ-L-glutamyl-L-glutamate (VI) and 3.4 g. (0.0163 mol.) of dicyclohexyl carbodiimide in 50 ml. of tetrahydrofuran were added. After stirring for five hours, 0.4 ml. of glacial acetic acid was added and stirring was continued for another hour in order to decompose unreacted carbodiimide. After filtrations of the dicyclohexyl urea, which weighed 3.3 g. (89%) and melted at 228~229°C, the filtrate was evaporated in vacuo. The syrupy residue was dissolved in ethyl acetate, the solution was washed with dilute hydrochloric acid, aqueous potassium bicarbonate and water, dried with sodium sulfate and concentrated in vacuo. The gelatinous residue was recrystallized from ethyl acetate and petroleum ether to give 8.8 g. (83%) of a colorless powder, m. p. 124~127°C (sintered at 92~98°C).

Found: C, 56.85; H, 6.92; N, 6.69. Calcd. for $C_{31}H_{45}O_{12}N_3$: C, 57.13; H, 6.95; N, 6.45%.

Method C.—In a mixture of 50 ml. of water, 15 ml. of concentrated hydrochloric acid and 50 ml. of chloroform, 6.2 g. (0.02 mol.) of carbobenzyloxy-L-glutamic dihyrazide (X) was dissolved. After cooling to 0°C, 30 ml. of an aqueous solution of 5.5 g. (0.08 mol.) of sodium nitrite was added dropwise within ten minutes. The reaction temperature must not exceed 5°C. After stirring for one hour at 0°C, the aqueous layer was separated and extracted twice with 30 ml. of chloroform. The combined chloroform solution was washed twice with cold water and dried with sodium sulfate. A solution of diethyl L-glutamate was prepared by dissolving 24.0 g. (0.10 mol.) of diethyl L-glutamate hydrochloride and 20.5 g. (0.20 mol.) of potassium bicarbonate in 50 ml. of water and extracting this solution three times with 50 ml. of chloroform. The combined extract was washed with water and dried with sodium sulfate. The solution of diazide obtained above was added dropwise during five minutes to this solution, while the temperature was kept at −5°C. The reaction mixture was stirred for two hours between −10°C to 0°C, and then permitted to stand overnight at room temperature. The reaction mixture was washed with dilute hydrochloric acid and then with a small amount of water, dried with sodium sulfate and evaporated in vacuo. The syrupy residue was reprecipitated from ethyl acetate and petroleum ether. The gelatinous precipitate was filtered and crystallized from ethyl alcohol and water to yield crystals, which after drying for a day in vacuo at 65°C weighed 6.3 g. (48%), m. p. 129~131°C (sintered at 99~102°C).

Found: C, 56.88; H, 6.82; N, 6.76; Calcd. for $C_{31}H_{45}O_{12}N_3$: C, 57.13; H, 6.96; N, 6.45%.

Method D.—A solution of 5.6 g. (0.02 mol.) of carbobenzyloxy-L-glutamic acid (XII) in 25 ml. of tetrahydrofuran and a solution of 10.0 g. (0.048 mol.) of dicyclohexyl carbodiimide 25 ml. of tetrahydrofuran were mixed. White crystalline dicyclohexyl urea began to form after a few minutes. Without removing the precipitate, a solution of diethyl L-glutamate was added. This solution had been prepared by adding 6.0 g. (0.06 mol.) of triethylamines to a solution of 14.3 g. (0.06 mol.) of diethyl L-glutamate hydrochloride in 30 ml. of tetrahydrofuran and removing the triethylamine hydrochloride formed by filtration. After the addition of the diethyl L-glutamate, the reaction mixture was stirred for five hours and then allowed to stand overnight. In order to decompose unreacted carbodiimide, 0.5 ml. of glacial acetic acid was added to the reaction mixture and the precipitate of dicyclohexyl urea (8.3 g.) was filtered off. The filtrate was evaporated in vacuo and the residue was dissolved in 100 ml. of ethyl acetate. The solution was washed with dilute hydrochloric acid, aqueous potassium bicarbonate and water and then dried with sodium sulfate. Upon addition of petroleum ether a gelatinous precipitate was obtained, which was filtered and dried in vacuo over phosphorus pentoxide; yield 11.4 g. (88%), m. p. 85~91°C. A recrystaillzed sample melted at 118~120°C (sintered at 102~104°C).

Found: C, 56.89; H, 6.73; N, 6.93. Calcd. for $C_{31}H_{45}O_{12}N_3$: C, 57.13; H, 6.96; N, 6.45%.

α,γ-L-Glutamyl Di-L-glutamic Acid (XIV).—To a solution of 29.5 g. (0.045 mol.) of tetraethyl carbobenzyloxy-α,γ-L-glutamyl di-L-glutamate in 100 ml. of 1 N sodium hydroxide was added. After standing for one hour at room temperature the reaction mixture was neutralized with 2 N hydrochloric acid, then concentrated to about 100 ml. in vacuo, acidified to Thymol Blue and extracted five times with 100 ml. of ethyl acetate. The extract was shaken five times with aqueous sodium carbonate. The combined aqueous layers were neutralized, concentrated to 200 ml., acidified and extracted again six times with 100 ml. of ethyl acetate. The extracted was dried with sodium sulfate and evaporated in vacuo to 20 g. of a syrup, which did not crystallize; yield 82%.

A solution of 7.0 g. of syrupy carbobenzyloxy-α,γ-L-glutamyl di-L-glutamic acid in 100 ml. of methyl alcohol and 100 ml. of water, was hydrogenated by the usual method using 1.0 g. of 5% palladium-charcoal. After the catalyst was filtered off, the filtrate was concentrated in vacuo below 35°C to a volume of about 100 ml., and then lyophilized; yield 3.6 g. (96%), m. p. 121~123°C (dec.). $[\alpha]_D^{30}+1.4°$ (*c*, 2.55, 2 N HCl).

Found: C, 42.55; H, 6.27; N, 10.07. Calcd. for $C_{15}H_{23}O_{10}N_3 \cdot H_2O$: C, 42.55; H, 5.95; N, 9.93%.

Triethyl Carbobenzyloxy-α-L-glutamyl-L-glutamate (XVI).—To a solution of 5.8 g. (0.024 mol.) of diethyl-L-glutamate hydrochloride in 50 ml. of tetrahydrofuran, 1.6 g. of triethylamine was added. After removal of the triethylamine hydrochloride formed, the filtrate was poured to a mixture of 5.0 g. (0.016 mol.) of γ-ethyl carbobenzyloxy-L-glutamate (XV) and 3.6 g. (0.017 mol.) of dicyclohexyl carbodiimide in 50 ml. of tetrahydrofuran. The reaction mixture was stirred for three hours, and

then treated with 0.5 ml. of glacial acetic acid to decompose the excess of carbodiimide. The insoluble dicyclohexyl urea was removed by filtration and washed; Yield 3.2 g. (84%). The solvent was replaced by ethyl acetate and the solution was washed with dilute hydrochloric acid, aqueous potassium bicarbonate and water. After drying with sodium sulfate the solvent was evaporated in vacuo to a residue, which was crystallized from ethyl acetate and petroleum ether; yield 6.5 g. (81%), m. p. 60~64°C (sintered at 45~52°C).

Found: C, 58.72; H, 7.03; N, 5.83. Calcd. for $C_{24}H_{34}O_9N_2$: C, 58.29; H, 6.93; N, 5.67%.

Carbobenzyloxy-α-L-glutamyl-L-glutamic Acid (XVII).—To a solution of 6.2 g. (0.0125 mol.) of carbobenzyloxy triester (XVI) in 50 ml. of methyl alcohol, 50 ml. of 1 N sodium hydroxide was added. After standing at room temperature for two and a half hours a small amount of undissolved substance was removed by filtration and the filtrate was acidified with 2 N hydrochloric acid and extracted fou times with ethyl acetate. The dried extract was evaporated in vacuo to a syrup, which was dissolved in 100 ml. of hot water. On cooling, crystals formed which were recrystallized from aqueous ethyl alcohol; yield 2.8 g. (54%), m. p. 161~164°C (decomp.) (sinters at 139°C).

Found: C, 53.13; H, 5.92; N, 7.03. Calcd. for $C_{18}H_{22}O_9N_2$: C, 53.68; H, 5.40; N, 6.83%.

α-L-Glutamyl-L-glutamic Acid (XVIII).—A solution of 1.9 g. of carbobenzyloxy dipeptide XVII in 40 ml. of methyl alcohol and 20 ml. of water was hydrogenated using 5% palladium-charcoal. The catalyst was filtered off and washed with hot water. The filtrate and the washings were combined and evaporated in vacuo. The residue was crystallized from water and ethyl alcohol; yield 0.9 g. (61%), m.p. 178~179°C (decomp.). Its infrared spectrum was completely identical with that of α-L-glutamyl-L-glutamic acid prepared from carbobenzyloxy glutamic anhydride according to Bergmann's method.

Found: C, 43.30; H, 5.63; N, 9.99. Calcd. for $C_{10}H_{16}O_7N_2$: C, 43.48; H, 5.84; N, 10.14%.

Di-benzyl Carbobenzyloxy-α, γ-L-glutamyl Diglycinate (XIX). — Benzyl glycinate-*p*-toluenesulfonate was prepared according to the method of Izumiya and Makisumi[24]. To a solution of 15.0 g. (0.045 mol.) of this ester salt (m. p. 130~131°C) in 100 ml. of chloroform, 4.5 g. (0.045 mol.) of triethylamine was added. The mixture was poured into a solution of 5.6 g. (0.020 mol.) of carbobenzyloxy-L-glutamic acid in 100 ml. of tetrahydrofuran. Then a solution of 11.0 g. (0.053 mol.) of dicyclohexyl carbodiimide in 100 ml. of tetahydrofuran was added. After the reaction mixture was stirred at room temperature for seven hours, the cyclohexyl urea formed was filtered off; weight 4.4 g. (50%). A small amount of glacial acetic acid was added to the filtrate. After two hours' stirring the reaction mixture was evaporated in vacuo and the residue was dissolved in ethyl acetate. Undissolved dicyclohexyl urea was filtered off. Upon addition of petroleum ether to the filtrate a precipitate was obtained which was recrystallized from ethyl alcohol, and dried in vacuo over phosphorus pentoxide; yield 5.5 g. (48%), m. p. 146~149°C.

Found: C, 64.25; H, 5.76; N, 7.48. Calcd. for $C_{31}H_{33}O_8N_3$: C, 64.68; H, 5.78; N, 7.30%.

α, γ-L-Glutamyl Diglycine (XX). — A solution of 5.1 g. of dibenzyl ester XIX in 50 ml. of dioxane, 50 ml. of methyl alcohol and 50 ml. of water, was hydrogenated by the usual method using 1.0 g. of 5% palladum-charcoal. After the catalyst was filtered off, the filtrate was evaporated in vacuo and the residue was lyophilized; yield 1.6 g. (70%), m. p. 73~77°C (sintered at about 68°C). It was dried in vacuo over phosphorus pentoxide for an elementary analysis. pK'_{a_1} 2.70; pK'_{a_2} 3.69; pK'_{a_3} 8.02.

Found: C, 38.75; H, 6.03; N, 14.56; MW, 290. Calcd. for $C_9H_{15}O_6N_3 \cdot H_2O$: C, 38.71; H, 6.14; N, 15.05%, MW, 279.

The molecular weight was calculated from the neutralization equivalent as dibasic acid.

Diethyl Carbobenzyloxy-α, γ-L-glutamyl Di-L-leucinate (XXI).—A solution of 7.0 g. (0.025 mol.) of carbobenzyloxy-L-glutamic acid in 75 ml. of tetrahydrofuran and a solution of 3.4 g. (0.065 mol.) of dicyclohexyl carbodiimide in 75 ml. of tetrahydrofuran were mixed. Without removing the precipitate of dicyclohexyl urea which formed, a solution of benzyl L-leucinate was added. This solution had been prepared by adding 6.5 g. (0.065 mol.) of triethylamine to a suspension of 23.6 g. (0.06 mol.) of benzyl L-leucinate-*p*-toluenesulfonate[24] (m. p. 154~155°C) in 150 ml. of chloroform. After the addition of the benzyl L-leucinate, the reaction mixture was stirred for five hours. The precipitate of dicyclohexyl urea was removed by filtration and washed with tetrahydrofuran; weighte 8.3 g. (57%). After addition of a small amount of glacial acetic acid and filtration of a further precipitate (2.2 g.), the filtrate was evaporated to dryness in vacuo. The residue was dissolved in ethyl acetate and washed with water, dilute sodium bicarbonate, dilute hydrochloric acid and water. The solvent was removed and petroleum ether was added to the residue. After cooling, a gelatinous precipitate formed. It was recrystallized from ethyl alcohol and water, and dried in vacuo over phosphorus pentoxide; yield 10.9 g., m. p. 107~116°C (softened at 98°C). From the mother liquor, 3.1 g. of crystals were obtained; total yield 82%.

Found: C, 67.86; H, 7.11; N, 6.53. Calcd. for $C_{39}H_{49}O_8N_3$: C, 68.10; H, 7.18; N, 6.11%.

α, γ-L-Glutamyl Di-L-leucine (XXII).—A solution of 5.0 g. of dibenzyl ester XXI in 30 ml. of methyl alcohol, 80 ml. of dioxane and 30 ml. of water was hydrogenated using 1.0 g. of 5% palladium-charcoal. After filtration of the catalyst, the filtrate was concentrated in vacuo to a volume of about 65 ml. The remaining solvent was removed by lyophilization; yield 1.8 g. (67%), m. p. 137~141°C (softened at 134°C, pK'_{a_1} 2.81; pK'_{a_2} 3.75; pK'_{a_3} 8.07). The molecular weight was calculated from the neutralization equivalent as a dibasic acid.

Found: C, 52.65; H, 8.37; N, 10.13; MW, 464. Calcd. for $C_{17}H_{31}O_6N_3 \cdot H_2O$: C, 52.22; H, 8.51; N, 10.75%; MW, 391.

24) N. Izumiya and S. Makisumi, *J. Chem. Soc. Japan, Pure Chem. Sec.* (*Nippon Kagaku Zasshi*), **78**, 1768 (1957).

Tetrabenzyl Carbobenzyloxy-α, γ-L-glutamyl Di-L-aspartate (XXIII).—To a suspension of 31.0 g. (0.070 mol.) of dibenzyl-L-aspartate-*p*-toluenesulfonate (m. p. 152°C)[24] in 200 ml. of chloroform, 7.0 g. (0.070 mol.) of triethylamine was added to give a clear solution of the free ester. Solution of 7.0 g. (0.025 mol.) of carbobenzyloxy-L-glutamic acid in 100 ml. of tetrahydrofuran and of 14.5 g. (0.070 mol.) of dicyclohexyl carbodiimide in 100 ml. of tetrahydrofuran were added to the solution of the free ester with ice-cooling. The mixture was then stirred at room temperature for nine hours. A gelatinous precipitate was filtered off. This precipitate seemed to be a mixture of the expected tetrabenzyl ester and dicyclohexyl urea. Therefore, it was extracted with tetrahydrofuran and the extract was evaporated. The residue was dissolved in ethyl alcohol and tetrahydrofuran, and crystallized by adding water to the solution. Recrystallization from the same solvents gave 7.6 g. of crystals, m. p. 139~145°C. Some dicyclohexyl urea which had separated out from the mother liquor of the gelatinous precipitate was removed by filtration, and the filtrate was washed with acid, aqueous sodium bicarbonate and water. Upon addition of petroleum ether a precipitate formed which was recrystallized from a mixture of ethyl acetate, tetrahydrofuran and petroleum ether; weighted 4.5 g., m. p. 152~158°C (softened at 145°C). The total yield was 12.1 g. (56%).

Found: C, 67.11; H, 5.75; N, 4.91. Calcd. for $C_{49}H_{49}O_{12}N_3$: C, 67.51; H, 5.63; N, 4.88%.

α, γ-L-Glutamyl Di-L-aspartic Acid (XXIV).—A solution of 3.0 g. of compound XXIII in 30 ml. of methyl alcohol, 90 ml. of dioxane and 30 ml. of water, was hydrogenated using 0.7 g. of 5% palladium-charcoal. After filtration of the catalyst, the filtrate and the washings were combined, concentrated in vacuo to about 30 ml. and then lyophilized to a solid, which weighed 1.2 g., m. p. 58~63°C. Elementary analysis, however, indicated that this substance was a mixture of the expected compound XXIV and starting material XXIII. Therefore, 0.7 g. of this substance was again hydrogenated. A paper chromatogram of the reduction product showed only one ninhydrin-positive spot but elementary analysis showed that the substance was still not pure.

Methyl ε-Carbobenzyloxy-L-lysinate Hydrochloride (XXV).—To a suspension of 2.0 g. (0.072 mol.) of ε-carbobenzyloxy-L-lysine[20], m. p. 248~250°C (decomp.) in 15 ml. of methyl alcohol, 1.0 g. (0.084 mol.) of thionyl chloride was added dropwise at 0°C. The reaction mixture was permitted to stand one hour and a half in a water bath of 40°C and then twenty hours at 25°C. After evaporated to dryness in vacuo, water was added to a residue and evaporated again in vacuo. Addition of water followed by evaporation was repeated several times to remove the excess of thionyl chloride and hydrochloric acid. The residue was crystallized from 99% ethyl alcohol-ethyl acetate. Pure ester hydrochloride was obtained after recrystallization from the same solvent; weighted 2.0 g. (84%), m. p. 113~114°C (sintered at 111°C).

Found: C, 54.21; H, 6.80; N, 8.41; Cl, 10.99. Calcd. for $C_{15}H_{23}O_4N_2Cl$: C, 54.30; H, 7.24; N, 8.45; Cl, 10.71%.

Benzyl ε-Carbobenzyloxy-L-lysinate Hydrochloride (XXVI).—To a suspension of 1.7 g. (0.006 mol.) of ε-carbobenzyloxy-L-lysine[20] in 10 ml. of benzyl alcohol there was added dropwise at −10°C, 0.9 g. (0.0076 mol.) of thionyl chloride. The reaction mixture was permitted to stand at 40°C for four hours, and then at 29°C for twenty three hours. It was then concentrated in vacuo. Upon addition of ether, gelatinous precipitate formed, which was filtered off, washed with ether, and recrystallized from 99% ethyl alcohol-ether; weight 0.5 g., m. p. 131°C. A second crop of ester hydrochloride was obtained from the mother liquor by addition of more ether; weight 0.2 g. The total yield was 24%. A sample recrystallized from the same solvent for elementary analysis melted at 133~134°C.

Found: C, 61.34; H, 6.84; N, 6.98; Cl, 8.55. Calcd. for $C_{21}H_{27}O_4N_2Cl$: C, 61.98; H, 6.69, N, 6.89; Cl, 8.71%.

Recovery of Dicyclohexyl Carbodiimide from Dicyclohexylurea.—To 49.5 g. (0.22 mol.) of dicyclohexyl urea, 90 g. of pyridine and 43.9 g. (0.22 mol.) of *p*-toluenesulfonyl chloride were added. The reaction mixture was stirred for one and a half hours at 70~75°C, whereby a faintly yellow precipitate formed. After cooling, petroleum ether was added. The reaction mixture was filtered, and a small amount of triethylamine was added to the filtrate which was then boiled for one hour. A brown precipitate separated out and was filtered off. The filtrate was evaporated in vacuo to a syrup, which was distilled under reduced pressure. It solidified at room temperature; yield 31.8 g. (70%), b. p. 120~130°C/3 mmHg.

Cleavage Reactions of α, γ-Glutamyl Peptides in Hot Water.—Solutions of 200~300 mg. samples of α,γ-glutamyl diglutamic acid, α,γ-glutamyl diglycine, α,γ-glutamyl diaspartic acid, α,γ-glutamyl dileucine, γ-glutamyl glutamic acid and α-glutamyl glutamic acid in 20~30 ml. of distilled water were heated in a boiling water bath. An aliquot of each solution was chromatographed using *n*-butyl alcohol-acetic acid-water (4 : 1 : 2) as a developing solvent. The chromatograms were then sprayed first with bromothymol blue in 95% ethyl alcohol and then with ninhydrin in *n*-butyl alcohol. The original peptides were revealed by bromothymol blue as well as by ninhydrin except in the case of α,γ-glutamyl dileucine which showed coloration only with ninhydrin (Figs. 1—6).

In Fig. 1, the spot 1d is the original peptide XIV. After heating the solution for one hour at 100°C, pyrrolidone carboxylic acid (1b) and glutamic acid (1e) were formed. A spot (1c) is supposed to be pyroglutamyl glutamic acid because it was not colored by ninhydrin but only by bromothymol blue. The original peptide (1d) disappeared completely after seven hours. An unknown spot (1a) appeared after twenty-one hours' heating. In another experiment in which the solution was heated at 40°C, the original peptide (1d) was hardly decomposed at all even after one week (Fig. 1).

Fig. 2 shows that the peptide XX (2b) was

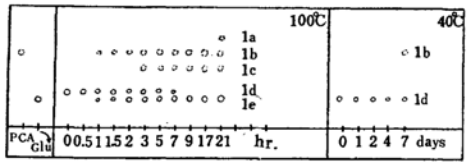


Fig. 1. The cleavage reaction of α,γ-L-glutamyl di-L-glutamic acid.

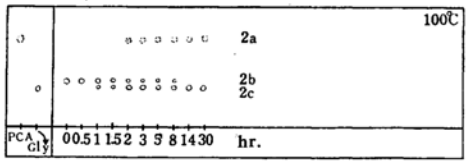


Fig. 2. The cleavage reaction of α,γ-L-glutamyl diglycine.

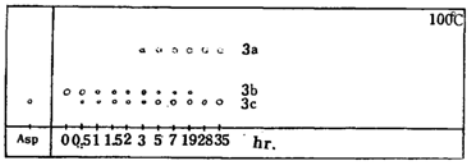


Fig. 3. The cleavage reaction of α,γ-L-glutamyl di-L-aspartic acid.

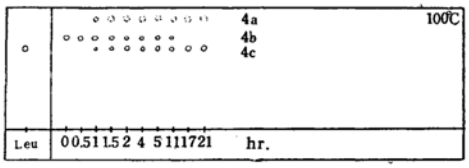


Fig. 4. The cleavage reaction of α,γ-L-glutamyl di-L-leucine.

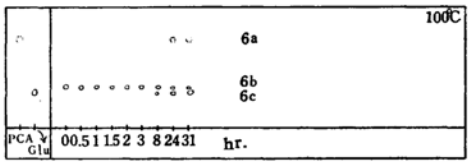


Fig. 5. The cleavage reaction of γ-L-glutamyl-L-glutamic acid.

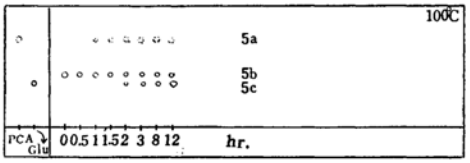


Fig. 6. The cleavage reaction of α-L-glutamyl-L-glutamic acid.

○: Ninhydrin coloration, ◎: Bromothymol blue coloration, PCA: Pyrrolidone carboxylic acid.

completely decomposed after eight hours, while glycine (2c) and pyroglutamyl glycine (2a) were formed. Spot 2a was an acidic substance like spot 1c without a free amino group and its structure was shown by the following experiment to be pyroglutamyl glycine. The peptide XX was refluxed with water for eight hours, and then Dowex 50 (H form) was added to the solution until a pH of 1.94 was reached. After filtration the filtrate was evaporated in vacuo to a syrup, which was shown by paper chromatography to be identical with 2a. This syrupy substance was hydrolyzed by 6 N hydrochloric acid at 100°C for eight hours. A two-dimensional paper chromatogram of the hydrolyzate showed two ninhydrin-positive spots of glutamic acid and glycine. Therefore, spot 2a must be due to pyroglutamyl glycine.

Fig. 3 shows the decomposition of the peptide XXIV. It was completely decomposed after twenty eight hours, while aspartic acid (3c) and pyroglutamyl aspartic acid (3a) began to appear after half an hour and three hours respectively.

The cleavage of the peptide XXII is shown in Fig. 4. A spot of the original peptide (4b) disappeared after seventeen hours, while pyroglutamyl leucine (4a) and leucine (4c) appeared.

In Fig. 5, spots 5a, 5b and 5c correspond to pyrrolidone carboxylic acid, original γ-glutamyl glutamic acid and glutamic acid, respectively. Pyrrolidone carboxylic acid (5a) appeared before glutamic acid (5c).

Fig. 6 shows that in contrast to the peptides in Figs. 1—5 α-glutamyl glutamic acid (6b) could not be completely decomposed even after thirty-one hours, and that glutamic acid (6c) and pyroglutamic acid (6a) appeared only after weight and twenty-eight hours respectively.

Reaction between Carbobenzyloxy Glutamic Acid and Dicyclohexyl Carbodiimide.—A solution of 3.7 g. (0.018 mol.) of dicyclohexyl carbodiimide in 30 ml. of tetrahydrofuran was added to a solution of 5.0 g. (0.018 mol.) of carbobenzyloxy-L-glutamic acid in 30 ml. of tetrahydrofuran. A precipitate of dicyclohexyl urea separated out immediately. After stirring at room temperature (20°C) for four hours the dicyclohexyl urea was filtered off; weight 3.8 g., m. p. 228~229.5°C. The filtrate was evaporated to dryness in vacuo and the residue was dissolved in ethyl acetate. A small amount (0.2 g.) of undissolved dicyclohexyl urea was filtered off again. The total weight of dicyclohexyl urea obtained in the two crops was 4.0 g. It corresponds almost to the theoretical amount expected from the dehydration reaction. The filtrate was again evaporated to a syrup which could not be crystallized.

Synthesis of Dibenzyl Carbobenzyloxy-α,γ-L-glutamyl Diglycinate from Carbobenzyloxy-L-glutamic Anhydride.—A solution of 2.6 g. (0.010 mol.) of carbobenzyloxy-L-glutamic anhydride[23], synthesized from carbobenzyloxy-L-glutamic acid and acetic anhydride, in 50 ml. of tetrahydrofuran was prepared. Solutions of 7.8 g. (0.023 mol.) of benzyl glycinate-*p*-toluenesulfonate[24] and 4.3 g. (0.023 mol.) of tri-*n*-butylamine in 100 ml. of tetrahydrofuran and of 2.3 g. (0.011 mol.) of dicyclohexyl carbodiimide in 10 ml. of tetrahydrofuran were then

added at room temperature. The reaction mixture was stirred for six hours and the crystal of dicyclohexyl urea was filtered off; weight 1.4 g. (56%), m. p. 228~230°C. While the filtrate was concentrated, a large amount of a crystalline material separated out, which was collected by filtration, suspended in 0.25 N aqueous sodium carbonate, filtered and washed carefully with water; yield 5.3 g. (92%), m. p. 145~149°C. This crystalline material was suspended in 50 ml. of 2 N hydrochloric acid, filtered, washed with water and then recrystallized from ethyl alcohol to give 4.2 g. of pure crystals, m. p. 152~154°C (sintered at 148°C). The infrared spectrum was identical with the one of the ester (XIX) prepared directly from carbobenzyloxy glutamic acid.

Found: C, 64.70; H, 5.84; N, 7.31. Calcd. for $C_{31}H_{33}O_8N_3$: C, 64.68; H, 5.78; H, 7.30%.

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