106. *Amino-acids and Peptides. Part VII.* The Autohydrolysis of Glutamyl-peptides.*

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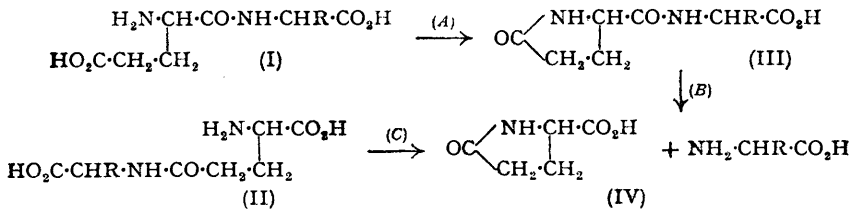
It was suggested in Part II (*J.*, 1950, 1959) that the autohydrolysis of α-glutamyl-peptides proceeds through the pyrrolidone derivative (equations *A* and *B*) whilst γ-glutamyl-peptides undergo an intramolecular substitution (equation *C*) resulting in fission of the peptide bond. Further evidence in support of these mechanisms is now presented.

In Part II (*J.*, 1950, 1959) a brief report was made of experiments in which aqueous solutions of α- (*e.g.*, I) and γ-L-glutamyl-peptides (*e.g.*, II) were allowed to undergo hydrolysis at 100°. Partition chromatography of the products after 24 hours showed the presence of appreciable amounts of glutamic acid in two cases only, those of α- and γ-L-glutamyl-L-glutamic acids. Hydrolysis with 0·5N-acid gave the expected products including glutamic acid in all cases, whilst a control experiment showed that under similar conditions a solution of glutamic acid still gave a strong colour with ninhydrin. We suggested that autohydrolysis of α- and γ-glutamyl-peptides is preceded or accompanied (respectively) by cyclisation to pyrrolidone derivatives, which do not react with ninhydrin, and we now offer further evidence consistent with these mechanisms.



Investigating first the case of α-glutamyl-peptides, we examined the products of autohydrolysis by means of paper partition chromatography in butanol, spraying the chromatogram with methyl-orange solution to detect 5-ketopyrrolidine-2-carboxylic acid (IV) and the intermediate 5-ketopyrrolidine-2-carboxyamido-acids (III). After 24 hours' heating at 100°, α-L-glutamyl-L-aspartic acid gave a chromatogram indicating the presence of the acid (IV), which was also isolated in small yield together with aspartic acid. From α-L-glutamylglycine both the acid (IV) and *N*-(5-ketopyrrolidine-2-carbonyl)glycine (III; R = H) were identified chromatographically, the hydrolysis stage being incomplete. From α-L-glutamyl-L-glutamic acid, *N*-(5-keto-L-pyrrolidine-2-carbonyl)-L-glutamic acid was isolated and identified with an authentic sample. The R_F value of this material in butanol is too close to that of 5-ketopyrrolidine-2-carboxylic acid to enable the latter to be distinguished with certainty, but a small amount of glutamic acid was isolated from the products and some hydrolysis had undoubtedly occurred. α-L-Glutamyl-L-tyrosine gave *N*-(5-keto-L-pyrrolidine-2-carbonyl)-L-tyrosine, identified on the chromatogram by comparison with a synthetic sample, but no 5-ketopyrrolidine-2-carboxylic acid. This agrees with the earlier observation that no tyrosine is formed, and here cyclisation appears to

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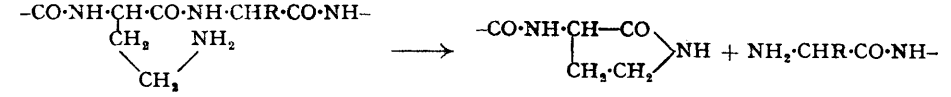
have occurred but not hydrolysis. α-L-Glutamyl-L-valine behaved similarly, in that neither the acid (IV) nor valine was found after 24 hours; the presence of *N*-(5-keto-L-pyrrolidine-2-carbonyl)-L-valine was inferred from the chromatogram, but in the absence of an authentic sample we were unable to confirm its identity.

The behaviour of synthetic samples of *N*-(5-keto-L-pyrrolidine-2-carbonyl)-glycine and -L-glutamic acid is consistent with the above interpretation; after 24 hours in water at 100°, the former gave the acid (IV) and glycine besides some unchanged material, whilst the latter appeared to be mainly unchanged. A crude sample of 5-keto-L-pyrrolidine-2-carbonyl-L-tyrosine gave no 5-ketopyrrolidine-2-carboxylic acid under these conditions. After similar heating of an aqueous solution of 5-ketopyrrolidine-2-carboxylic acid, both glutamic acid and unchanged material were detected chromatographically.

Partition chromatography of the products obtained on heating γ-L-glutamyl-glycine, -L-glutamic acid, and -L-aspartic acid for 24 hours at 100° showed the presence of the acid (IV) in each case, identity being confirmed by isolation in the first two experiments. From the second-named peptide glutamic acid also was isolated; this again suggests that the ring compound is a primary product of the hydrolysis and is not formed subsequently from glutamic acid. This is consistent with earlier observations that glutathione (Hopkins, *J. Biol. Chem.*, 1929, **84**, 314) and glutamine (Vickery, Pucher, Clark, Chibnall, and Westall, *Biochem. J.*, 1935, **29**, 2710) give 5-ketopyrrolidine-2-carboxylic acid when heated in aqueous solution. If mechanism (*C*) is correct, then hydrolysis under these conditions is in fact an intramolecular substitution, and the lability of γ-glutamyl-amide and -peptide bonds is due to the presence of the free α-amino-group in a position suitable for stable ring formation. This interpretation agrees with the known stability of the amide group in peptides such as L-leucyl-L-glutamine (Chibnall and Westall, *Biochem. J.*, 1932, **26**, 122; Melville, *ibid.*, 1935, **29**, 179) and of asparagine. It is tempting to suggest that under suitable conditions αγ-diaminobutyric acid may in analogous fashion form a source of instability in peptide chains:



Experimental

All m. p.s are uncorrected. Analyses are by Drs. Weiler and Strauss.

N-(5-*Keto*-L-*pyrrolidine*-2-*carbonyl*)-L-*glutamic Acid*.—Diethyl 5-keto-L-pyrrolidine-2-carbonyl-L-glutamate (0·3 g.; Angier *et al.*, *J. Amer. Chem. Soc.*, 1950, **72**, 74) in N-sodium hydroxide (2 ml.) was set aside for 2 hours. N-Hydrochloric acid (2 ml.) was added and the solution evaporated to dryness under reduced pressure. The residue was extracted with hot ethanol and the solution evaporated *in vacuo*; the remaining oil crystallised under ethyl acetate. The N-(5-*keto*-L-*pyrrolidine*-2-*carbonyl*)-L-*glutamic acid* (0·19 g., 77%) had m. p. 176—178°. A portion, recystallised from ethanol–ethyl acetate–ether, had m. p. 178—180° (Found: N, 10·6. $C_{10}H_{14}O_6N_2$ requires N, 10·8%).

N-(5-*Keto*-L-*pyrrolidine*-2-*carbonyl*)*glycine Ethyl Ester*.—5-Keto-L-pyrrolidine-2-carboxyhydrazide (1 g.; Angier *et al.*, *loc. cit.*) in water was converted into the azide in the usual way. The solution was then added portionwise, with shaking, to a mixture of glycine ethyl ester hydrochloride (1·5 g.), potassium hydrogen carbonate (4·8 g.), and water (7·5 ml.). The reaction mixture was shaken occasionally during the first hour. Next day the solution was saturated with potassium carbonate and extracted with ethyl acetate (3 × 20 ml.). The combined extracts were dried (Na_2SO_4) and evaporated to dryness under reduced pressure. The N-(5-*keto*-L-*pyrrolidine*-2-*carbonyl*)*glycine ethyl ester* (0·5 g., 33%) soon solidified and was transferred to the filter with a little ethyl acetate; it had m. p. 117—119°, unchanged by recrystallisation from ethyl acetate (Found: C, 50·6; H, 6·9; N, 12·9. $C_9H_{14}O_4N_2$ requires C, 50·5; H, 6·6; N, 13·1%).

N-(5-*Keto*-L-*pyrrolidine*-2-*carbonyl*)*glycine*.—5-Keto-L-pyrrolidine-2-carbonylglycine ethyl ester (0·24 g.) in N-sodium hydroxide (1·20 ml.) was set aside for 2 hours. The product, N-(5-*keto*-L-*pyrrolidine*-2-*carbonyl*)*glycine* (0·17 g., 81%), was isolated as described above for the glutamic acid analogue; it had m. p. 157—162°. It was recrystallised from ethanol–ether;

the second (main) fraction had m. p. 162—166° (Found : C, 45·4; H, 5·2; N, 14·6. $C_7H_{10}O_4N_2$ requires C, 45·2; H, 5·4; N, 15·0%).

N-(5-*Keto*-L-*pyrrolidine*-2-*carbonyl*)-L-*tyrosine Ethyl Ester*.—5-Keto-L-pyrrolidine-2-carboxyhydrazide (1 g.) in water was converted into the azide and added to a mixture of L-tyrosine ethyl ester (1.8 g.), potassium hydrogen carbonate (4 g.), ethyl acetate (10 ml.), and dioxan (10 ml.). The reaction mixture was stirred for 2 hours and then left overnight. The aqueous layer was separated and extracted twice with ethyl acetate; the combined ethyl acetate layers were washed with dilute hydrochloric acid and then water, dried (Na_2SO_4), and evaporated under reduced pressure. N-(5-*Keto*-L-*pyrrolidine*-2-*carbonyl*)-L-*tyrosine ethyl ester*, recrystallised from ethyl acetate (yield, 0·35 g.; 16%), had m. p. 132—135°, raised to 133—135° by a further crystallisation (Found : C, 59·8; H, 6·2; N, 8·4. $C_{16}H_{20}O_5N_2$ requires C, 60·0; H, 6·3; N, 8·7%). Hydrolysis gave crude *N*-(5-keto-L-pyrrolidine-2-carbonyl)-L-tyrosine which was difficult to purify and was used in the chromatographic studies below.

Effect of Heating Aqueous Solutions of Pyrrolidone Derivatives.—Aqueous solutions (*ca.* 5%) were kept at 100° for 24 hours and samples then examined by paper partition chromatography using (*a*) phenol saturated with water in an atmosphere containing ammonia, followed by a ninhydrin spray, and (*b*) *n*-butanol saturated with water, acidic products being detected by spraying the paper with a saturated solution of methyl-orange in ethanol–ethyl acetate (1 : 1). In the table, under " (*b*) " are given the R_F values of the spots observed.

	(*a*)			(*b*)	
	Glutamic acid	Glycine	Tyrosine	Before heating	After heating
5-Keto-L-pyrrolidine-2-carboxylic acid ...	+++	—	—	0·46	0·46
5-Keto-L-pyrrolidine-2-carbonyl-L-glutamic acid	+	—	—	0·40	0·43
5-Keto-L-pyrrolidine-2-carbonylglycine ...	+	++	—	0·30	0·28 0·44
5-Keto-L-pyrrolidine-2-carbonyl-L-tyrosine	—	—	—	0·66	0·67

Detection of Pyrrolidone Derivatives formed by the Action of Heat on Aqueous Solutions of Glutamyl-peptides.—Aqueous solutions of glutamyl-peptides (approx. 3—5%) were kept at 100° for 24 hours. The products were examined by paper chromotography as above, using butanol and methyl-orange. The R_F values of the products were :

α-L-Glutamylglycine	0·31, 0·48	γ-L-Glutamylglycine	0·52
α-L-Glutamyl-L-glutamic acid	0·44	γ-L-Glutamyl-L-glutamic acid	0·52
α-L-Glutamyl-L-aspartic acid	0·07, 0·50	γ-L-Glutamyl-L-aspartic acid	0·08, 0·47
α-L-Glutamyl-L-tyrosine	0·69		
α-L-Glutamyl-L-valine	0·71		

Autohydrolysis of α-L-*Glutamyl*-L-*aspartic Acid*.—α-L-Glutamyl-L-aspartic acid (200 mg.) in water (10 ml.) was kept at 100° for 24 hours. The solution was then evaporated to dryness under reduced pressure, and the residue extracted with ethanol and collected (103 mg.). Paper partition chromatography with phenol saturated with water showed the presence of much aspartic acid and some glutamic acid in the residue.

The filtrate was evaporated *in vacuo* and the residue extracted with boiling ethyl acetate (4 × 8 mls.). A syrup remained which did not crystallise. Light petroleum was added to the combined extracts which were left at 0° for some hours and then filtered. The product (10 mg.; m. p. 140—153°) recrystallised from ethyl acetate–light petroleum, whereafter it had m. p. 147—155° (5 mg.), unaltered on admixture with 5-keto-L-pyrrolidine-2-carboxylic acid (Found : C, 46·4; H, 5·4. Calc. for $C_5H_7O_3N$: C, 46·5; H, 5·5%).

Autohydrolysis of α-L-*Glutamyl*-L-*glutamic Acid*.—α-L-Glutamyl-L-glutamic acid (175 mg.) in water (10 ml.) was kept at 100° for 24 hours, then evaporated to dryness under reduced pressure. The residue was extracted several times with boiling ethyl acetate (80 ml. in all), whereafter removal of the solvent left a residue (5 mg.) of m. p. 120—134°. The residue from the extraction was dissolved in hot ethanol, leaving a solid (4 mg.) shown by paper chromatography to contain glutamic acid and a trace of dipeptide.

The ethanolic solution was evaporated under reduced pressure and the residue extracted with boiling ethyl acetate, giving a solid (39 mg., 24%), m. p. 169—179°, raised to 177—180° by recrystallisation from ethanol–ether. The mixed m. p. with 5-keto-L-pyrrolidine-2-carbonyl-L-glutamic acid was 175—178°. The R_F value was 0·42 (5-keto-L-pyrrolidine-2-carbonyl-L-

glutamic acid, 0·42; 5-keto-L-pyrrolidine-2-carboxylic acid, 0·50; both on the same chromatogram).

Autohydrolysis of γ-L-Glutamylglycine.—γ-L-Glutamylglycine (200 mg.) in water (10 ml.) was kept at 100° for 24 hours, then evaporated to dryness under reduced pressure and extracted with ethanol. The residue (62 mg.) showed by paper chromatography the presence of much glycine and some dipeptide or glutamic acid. The filtrate was evaporated to dryness under reduced pressure and the residue extracted several times with boiling ethyl acetate. The combined extracts gave on evaporation 5-keto-L-pyrrolidine-2-carboxylic acid (52 mg.), m. p. 149—155° alone or mixed with the authentic compound, R_F 0·51.

Autohydrolysis of γ-L-Glutamyl-L-glutamic Acid.—γ-L-Glutamyl-L-glutamic acid (200 mg.) in water (10 ml.) was kept at 100° for 24 hours, then evaporated to dryness under reduced pressure. The remaining oil was extracted repeatedly with hot ethyl acetate, to which, after some vacuum-evaporation, was added light petroleum. After storage overnight at 0°, filtration gave 5-keto-L-pyrrolidine-2-carboxylic acid (50 mg.), m. p. 150—156°, unaltered on admixture with the authentic compound, R_F 0·50. The residue from the extractions was stirred with ethanol leaving a white solid (30 mg.). Paper chromatography showed the presence of much glutamic acid and some dipeptide.

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