755. *Amino-acids and Peptides. Part IX.* γ-L-Glutamyl-L-alanine, -L-valine, and -L-leucine.*

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The above-named dipeptides have been synthesised by the γ-azide route, and their autohydrolysis has been investigated by partition chromatography.

The discovery by Hanes, Hird, and Isherwood (*Nature*, 1950, **166**, 288; *Biochem. J.*, 1952, **51**, 25) of the enzymic transpeptidation of γ-glutamyl-peptides has lent added interest to the chemical investigation of these compounds. The enzymic reaction has indeed certain similarities to the transamidation which has been observed on heating aqueous solutions of γ-glutamyl-peptides, resulting in the formation of 5-ketopyrrolidine-2-carboxylic acid with consequent hydrolysis of the peptide bond (Le Quesne and Young, *J.*, 1952, 594); the latter reaction is the intramolecular counterpart of the intermolecular enzymic transpeptidation.

Continuing our study of such compounds, we have synthesised the γ-L-glutamyl derivatives of L-alanine, L-valine, and L-leucine. We have used the γ-azide route developed by Hegedüs (*Helv. Chim. Acta*, 1948, **31**, 737), by ourselves (*Nature*, 1949, **163**, 604; Part II, *J.*, 1950, 1959), and by Šorm and Rudinger (*Coll. Czech. Chem. Comm.*, 1950, **15**, 491).

Some difficulty has been reported (*e.g.*, Coleman, *J.*, 1950, 3223) with larger-scale preparations of γ-ethyl L-glutamate hydrochloride; we have obtained consistently good yields by increasing the proportion of ethanol and keeping the temperature below 30° throughout the experiment. L-Alanine ethyl ester hydrochloride, first prepared by Curtius and Koch (*J. pr. Chem.*, 1888, [ii], **38**, 487) with melting point 64—68°, has been made by Synge's low-temperature esterification procedure (*Biochem. J.*, 1948, **42**, 99), giving a material melting at 76°. We have also obtained L-valine methyl ester hydrochloride with melting point and optical rotation considerably higher than previously reported (Synge, *loc. cit.*). It will, however, be noted that our γ-L-glutamyl-L-valine has no observable rotation in aqueous and in acid solution; nevertheless, hydrolysis gave a solution with a rotation greater than would be possible if either one of the component amino-acids were racemised and, in view of the optical purity of the starting materials and the known route of synthesis, we believe our product to be as named.

γ-L-Glutamyl-L-alanine undergoes autohydrolysis in the normal manner, yielding alanine but little glutamic acid. We have taken advantage of the favourable differences in R_F values in partition chromatography of the products, to show that the rate of formation of alanine is closely paralleled by the formation of 5-ketopyrrolidine-2-carboxylic acid. The valine and leucine analogues, as would be expected, are much more resistant to hydrolysis.

Hamilton (*J. Biol. Chem.*, 1945, **158**, 375) has shown that the rate of ring closure of glutamine is greater in a phosphate buffer than in an acetate buffer of the same pH. By means of paper partition chromatography, we have followed the progress of the autohydrolysis of γ-L-glutamyl-glycine and -L-alanine in acetate and phosphate buffers; visual comparison of the intensities of the spots corresponding in position to glycine and alanine respectively suggests that these amino-acids may be formed a little more rapidly in the presence of phosphate, but quantitative determinations are required to confirm this conclusion.

Experimental

M. p.s are uncorrected. Combustion analyses are by Drs. Weiler and Strauss.

γ-Ethyl L-Glutamate Hydrochloride (*Modified Procedure*).—L-Glutamic acid (50 g.) was suspended in ethanol (1 l.), and dry hydrogen chloride passed in, the temperature being kept below 30°. After 45 minutes the clear solution was evaporated to dryness under reduced pressure and the product reprecipitated from ethanol by ether, giving γ-ethyl L-glutamate hydrochloride, m. p. 132—135° (59·4 g., 82%).

* Part VIII, *J.*, 1952, 1574.

L-*Alanine Ethyl Ester Hydrochloride*.—L-Alanine (10 g.), dissolved in N-ethanolic hydrogen chloride (200 ml.), was set aside at room temperature for 24 hours. After evaporation to dryness at reduced pressure the process was twice repeated and the remaining syrup induced to crystallise by repeated evaporation with ethanol. Recrystallisation from ethanol–ether gave L-alanine ethyl ester hydrochloride (15 g., 88%) as deliquescent crystals, m. p. 76°, $[\alpha]_D^{19}$ +3·1° (*c*, 2·5 in water) (Found: C, 38·8; H, 7·8; N, 9·3; Cl, 23·3. Calc. for $C_5H_{12}O_2NCl$: C, 39·1; H, 7·9; N, 9·1; Cl, 23·1%).

Carbobenzyloxy-γ-L-glutamyl-L-alanine Ethyl Ester.—Carbobenzyloxy-γ-L-glutamylhydrazide (Part II, *loc. cit.*; 3·6 g.) was dissolved in water containing concentrated hydrochloric acid (4 ml.), chloroform (50 ml.) was added, and the mixture stirred at 0° while sodium nitrite (1·2 g.) in water (10 ml.) was gradually added. After being washed with water and dried (Na_2SO_4) for a few seconds, the chloroform solution of the azide was added to a solution of L-alanine ethyl ester (3 g.; liberated from the hydrochloride by the calculated amount of ammonia in chloroform) in chloroform (5 ml.) at 0°. The mixture was kept for several hours in ice and overnight at room temperature, washed with dilute hydrochloric acid and then water, dried (Na_2SO_4), and evaporated under reduced pressure. The product solidified under light petroleum and was crystallised from ethyl acetate–light petroleum, giving *carbobenzyloxy-γ-L-glutamyl-L-alanine ethyl ester*, m. p. 108—111° (2·2 g., 47%) raised by recrystallisation to 112—113° (Found: C, 57·0; H, 6·3; N, 7·4. $C_{18}H_{24}O_7N_2$ requires C, 56·8; H, 6·4; N, 7·4%).

Carbobenzyloxy-γ-L-glutamyl-L-alanine.—The above ester (1·7 g.) was dissolved in N-sodium hydroxide (15 ml.) and kept at room temperature for 2 hours. After acidification with 5N-hydrochloric acid the solution was extracted with ethyl acetate (4 × 30 ml.), and the combined extracts were washed with a little water, dried (Na_2SO_4), and evaporated under reduced pressure. The remaining *carbobenzyloxy-γ-L-glutamyl-L-alanine* (1·5 g., 95%) was recrystallised from acetone–ether; it then had m. p. 150—154° (Found: C, 54·9; H, 5·6; N, 8·2. $C_{16}H_{20}O_7N_2$ requires C, 54·6; H, 5·7; N, 8·0%).

γ-L-*Glutamyl-L-alanine*.—Carbobenzyloxy-γ-L-glutamyl-L-alanine (0·5 g.) in aqueous methanol (20 ml.) was hydrogenated in the normal manner in the presence of palladium black. The filtrate was evaporated to dryness and the product recrystallised from aqueous ethanol, giving *γ-L-glutamyl-L-alanine* (0·29 g., 94%), m. p. 185—187° (Found: C, 43·8; H, 6·6; N, 13·1. $C_8H_{14}O_5N_2$ requires C, 44·0; H, 6·5; N, 12·8%), $[\alpha]_D^{18}$ −22·1° (*c*, 5·0 in water); R_F: in *n*-butanol, 0·02; in phenol, 0·33.

L-*Valine Methyl Ester Hydrochloride*.—Esterification of L-valine (11·2 g.) was effected with N-methanolic hydrogen chloride as described by Synge (*loc. cit.*). After reprecipitation from methanol by ether, the hydrochloride (13·1 g., 85%) had m. p. 125—135°, raised to 161—162° by recrystallisation from acetone, $[\alpha]_D^{20}$ +15·6° (*c*, 3·8 in water) (Found: C, 43·0; H, 8·5; N, 8·8; Cl, 21·2. Calc. for $C_6H_{14}O_2NCl$: C, 42·7; H, 8·3; N, 8·3; Cl, 21·6%).

Carbobenzyloxy-γ-L-glutamyl-L-valine.—Carbobenzyloxy-γ-L-glutamylhydrazide (5 g.) was converted into the azide and coupled with L-valine methyl ester (4·4 g.) in chloroform as described for the alanine analogue. After the final evaporation of the chloroform, carbobenzyloxy-γ-L-glutamyl-L-valine methyl ester (4·3 g., 64%) remained as a syrup which could not be induced to crystallise; a portion (1·15 g.) was hydrolysed in the usual manner, giving *carbobenzyloxy-γ-L-glutamyl-L-valine* (0·95 g., 86%) as a syrup, which was dissolved in N-sodium carbonate and precipitated by acid; the oil crystallised after several months. Recrystallisation from ethyl acetate–light petroleum raised the m. p. from 148—155° to 153—156° (Found: C, 57·3; H, 6·5; N, 7·2. $C_{18}H_{24}O_7N_2$ requires C, 56·8; H, 6·4; N, 7·4%).

γ-L-*Glutamyl-L-valine*.—Carbobenzyloxy-γ-L-glutamyl-L-valine (0·34 g.) was hydrogenated in the normal manner. After reprecipitation from water with acetone the *γ-L-glutamyl-L-valine* (0·2 g., 90%) had m. p. 207° (Found: C, 48·3; H, 7·2; N, 11·7. $C_{10}H_{18}O_5N_2$ requires C, 48·7; H, 7·4; N, 11·4%), $[\alpha]_D^{19}$ 0·0° ± 0·5° (*c*, 2·4 in water); a similar value was observed in N-hydrochloric acid (*c*, 2·1). Hydrolysis gave a solution with a rotation higher than could be accounted for by L-glutamic acid or L-valine alone. R_F: in *n*-butanol, 0·01; in phenol, 0·45.

Carbobenzyloxy-γ-L-glutamyl-L-leucine.—Carbobenzyloxy-γ-L-glutamylhydrazide (5 g.) was converted into the azide and coupled with L-leucine methyl ester (11·5 g., from 14·8 g. of L-leucine methyl ester hydrochloride, m. p. 148—149°) in chloroform solution as above. Carbobenzyloxy-γ-L-glutamyl-L-leucine methyl ester (5·4 g., 78%) was obtained as a syrup. A portion (1·7 g.) was hydrolysed in the usual manner, giving *carbobenzyloxy-γ-L-glutamyl-L-leucine* (1·5 g., 91%) as a syrup which crystallised after precipitation from sodium carbonate solution and storage for several months; it had m. p. 83—85°. Recrystallisation from ethyl acetate–light petroleum gave a second crystalline form, m. p. 132—134° (Found: C, 57·9; H,

6·8; N, 6·8. $C_{19}H_{26}O_7N_2$ requires C, 57·9; H, 6·7; N, 7·1%). In the final crystallisation a second crop was obtained, m. p. 85—90°, which could be converted into the higher-melting form by seeding at the m. p.

γ-L-Glutamyl-L-leucine.—Carbobenzyloxy-γ-L-glutamyl-L-leucine (0·24 g.) was hydrogenated in the usual manner. After precipitation from water with acetone, the *γ-L-glutamyl-L-leucine* (0·12 g., 76%) had m. p. 185° (Found: C, 50·3; H, 7·7; N, 11·1. $C_{11}H_{20}O_5N_2$ requires C, 50·8; H, 7·7; N, 10·8%), $[\alpha]_D^{19}$ −13·5° (*c*, 2·3 in water); R_F: in *n*-butanol, 0·03; in phenol, 0·56.

Autohydrolysis of γ-L-Glutamyl-L-alanine.—Solutions (1%) of γ-L-glutamyl-L-alanine in water and in 0·5N-hydrochloric acid were heated in sealed tubes at 100°. At intervals tubes were removed and their contents examined by paper partition chromatography, phenol saturated with water being the mobile phase; controlled quantities of each solution were placed on the chromatogram by means of a graduated capillary tube. The chromatograms were developed in the normal manner with ninhydrin. The following relative intensities were evaluated visually:

Time (hrs.)	Aqueous solution			0·5N-Acid solution		
	Dipeptide	Glutamic acid	Alanine	Dipeptide	Glutamic acid	Alanine
0	+ + + +	—	—	+ + + +	—	—
1·5	+ + +	±	+	+ +	+ + +	+ + +
3	+ +	±	+ +	+	+ + +	+ + +
8	+	±	+ + +	−	+ + +	+ + + +
24	±	±	+ + + +	−	+ + +	+ + + +

Autohydrolysis of γ-L-Glutamyl-L-valine and -L-leucine.—Similar experiments with aqueous solutions (1%) of γ-L-glutamyl-L-valine and -L-leucine gave the following results:

Time (hrs.)	γ-L-Glutamyl-L-valine			γ-L-Glutamyl-L-leucine		
	Dipeptide	Glutamic acid	Valine	Dipeptide	Glutamic acid	Leucine
0	+ + + +	—	—	+ + + +	—	—
1	+ + +	—	±	+ + + +	—	±
4	+ + +	—	±	+ + +	—	±
10	+ + +	—	+	+ + +	—	+
24	+ + +	±	+ +	+ + +	±	+

After a total of 96 hours at 100° the intensities for the second amino-acid were: L-valine, + + + +; L-leucine, + + + +.

Effect of Buffer Solutions.—For γ-L-glutamyl-L-alanine and -glycine, hydrolyses were carried out in both acetate and phosphate buffers (pH 6·0; 0·05M).

Time (hrs.)	Acetate buffer			Phosphate buffer		
	γ-L-Glutamyl-L-alanine	Glutamic acid	Alanine	γ-L-Glutamyl-L-alanine	Glutamic acid	Alanine
0	+ + + +	—	—	+ + + +	—	—
1	+ + +	—	+	+ + + +	—	+
2	+ + +	—	+	+ + +	—	+ +
4	+ +	—	+ + +	+ +	±	+ + +
7	+	—	+ + +	+	±	+ + + +
12	+	—	+ + +	±	±	+ + + +
24	—	—	+ + + +	—	±	+ + + +
	γ-L-Glutamyl-glycine	Glutamic acid	Glycine	γ-L-Glutamyl-glycine	Glutamic acid	Glycine
0	+ + + +	?	—	+ + + +	?	—
0·5	+ + + +	?	±	+ + + +	?	+
1·5	+ + + +	?	±	+ + +	?	+ +
6	+ + +	?	+ + +	+ +	?	+ + + +
12	+ +	?	+ + + +	+	?	+ + + +
24	+	?	+ + + +	±	?	+ + + +

The poor separation of this dipeptide from glutamic acid makes the detection of the latter difficult.

Detection of 5-Ketopyrrolidine-2-caboxylic Acid during the Autohydrolysis of γ-L-Glutamyl-L-alanine.—By using more concentrated (10%) solutions of γ-L-glutamyl-L-alanine and employing butanol (acid-free and redistilled) saturated with water as the mobile phase, the progressive formation of 5-ketopyrrolidine-2-carboxylic acid during aqueous hydrolysis was observed by spraying the dried chromatogram with a solution of methyl-orange (saturated, in ethanol–ethyl

acetate, 1 : 1); in the results below, the figures in parentheses indicate R_F values. In parallel experiments in which the chromatograms were sprayed with methyl-red, differentiation between the acid and the dipeptide was less clear and the greater sensitivity of the indicator revealed considerable tailing; control experiments, however, indicate that this is a function of the concentration; the figures in the second table therefore represent the maximum R_F values of the spots observed and may be regarded as an indication of the concentration of the 5-keto-pyrrolidine-2-carboxylic acid.

	Methyl-orange		Methyl-red
Time (hrs.)	Dipeptide	5-Ketopyrrolidine-2-carboxylic acid	5-Ketopyrrolidine-2-carboxylic acid
0	+ + + (0·08)	—	—
0·5	+ + (0·08)	—	+ (0·25)
1	+ + (0·08)	—	+ (0·37)
1·5	+ (0·08)	—	+ (0·45)
3	± (0·08)	+ (0·5)	+ (0·53)
6	—	+ + (0·5)	+ (0·57)
12	—	+ + (0·5)	+ (0·60)
24	—	+ + + (0·5)	+ (0·64)

Comparison with the earlier table showing the formation of alanine under similar conditions confirms the suggestion that both products are formed simultaneously.

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