Studies of the Synthesis of Peptides Containing C-Terminal Glutamine. II. The Synthesis and Use of α-p-Nitrobenzyl γ-Methyl L-Glutamate

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In a previous paper,[1] it has been described how the usual synthetic methods of peptides containing *C*-terminal glutamine are attended with many difficulties, and how the introduction of a xanthyl group into the γ-amide nitrogen is effective in reducing these troubles. The present study is concerned with another approach to the same problem using α-*p*-nitrobenzyl γ-methyl glutamate as an intermediate. It is known that the *p*-nitrobenzyl ester derivative of an amino acid[2] is easily crystallizable; morever, its ester bond is quite stable to dry hydrogen bromide in hydrous organic solvents. The cleavage of the *p*-nitrobenzyl ester linkage is effected not only by means of alkaline hydrolysis but also by catalytic hydrogenolysis. Since the methyl ester linkage is stable to hydrogen bromide or hydrogenolysis, glutamine peptide can be prepared from the carbobenzoxy peptide of the glutamate by catalytic hydrogenation, followed by ammonolysis.

The starting material, *N*-carbobenzoxy-(α-*p*-nitrobenzyl, γ-methyl)glutamate (I), was prepared in a good yield from *N*-carbobenzoxy-(γ-methyl)glutamate and *p*-nitrobenzyl bromide in the presence of triethylamine. Substance I was treated with hydrogen bromide in glacial acetic acid to give α-*p*-nitrobenzyl γ-methyl glutamate hydrobromide (II). The hydrobromide II was stable and easily crystallizable. First, carbobenzoxy-glycine was coupled with this compound I to form peptide under several conditions. In the case of a mixed anhydride with isobutyloxycarbonic acid or an active ester with *p*-nitrophenol, the product was not pure, and purification was very difficult. It was finally found that dicyclohexylcarbodiimide was the most effective reagent in this case. The urea-derivative formed and the unreacted starting materials were separated easily, and carbobenzoxy-glycyl-(α-*p*-nitrobenzyl, γ-methyl)-glutamate (III) was obtained as crystals. There was no difficulty in obtaining glycyl-(γ-methyl)-glutamate, which was converted to glycyl-glutamine as expected (Fig. 1). The optical purity of the glycyl-L-glutamine was checked after hydrolysis with 6 N hydrochloric acid, and no difference was observed between the

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[1] S. Akabori, S. Sakakibara and Y. Shimonishi, This Bulletin, **34**, 739 (1961); **35**, 1966 (1962).

[2] H. Schwarz and K. Arakawa, *J. Am. Chem. Soc.*, **81**, 5691 (1959).

optical rotation of the hydrolyzate and that of the artificial mixture of the same components. When this synthetic procedure was applied to several carbobenzoxy amino acids, the following depeptides were synthesized: L-alanyl-L-glutamine, L-valyl-L-glutamine, L-leucyl-L-glutamine, L-prolyl-L-glutamine, and L-phenylalanyl-L-glutamine.

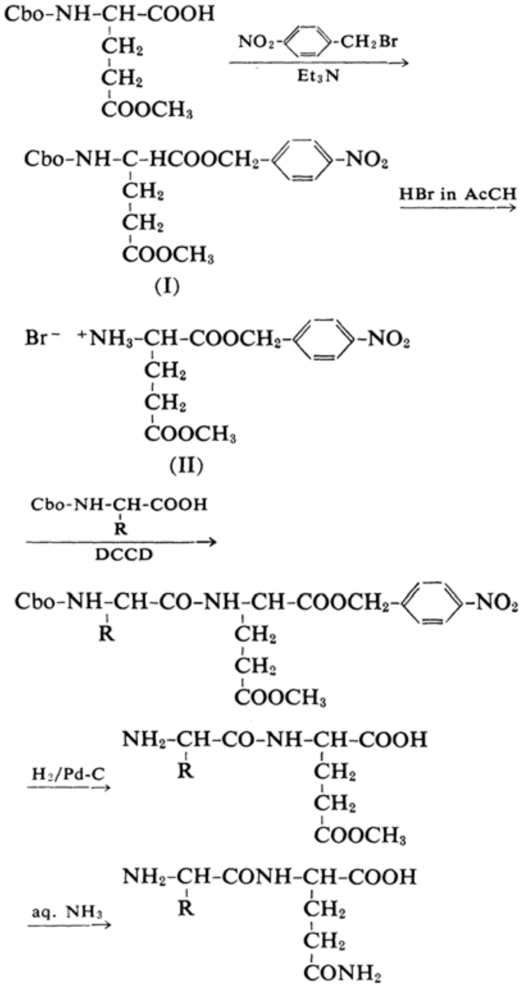

Fig. 1. Synthetic procedure.

Experimental*

***N*-Carbobenzoxy-(α-*p*-nitrobenzyl, γ-methyl)-L-glutamate (I).**—A mixture of carbobenzoxy-(γ-methyl)L-glutamate (59.0 g., 0.2 mol.), *p*-nitrobenzyl bromide (54.0 g., 0.25 mol.) and triethylamine (36.0 ml., 0.25 mol.) in ethyl acetate (800 ml.) was refluxed for 5 hr. The triethylamine hydrobromide which precipitated was removed by filtration, and the filtrate was washed with 0.2 N hydrochloric acid, 5% aqueous sodium bicarbonate, and water, dried over anhydrous magnesium sulfate, and concentrated under reduced pressure to a residue, which was then triturated with petroleum benzine. The crude material (80.0 g.) was recrystallized as needles from ethyl acetate-petroleum benzine; yield, 70 g. (80%); m. p. 67～69°C; $[\alpha]_D^{28}$ $-15.2°$ (*c* 3.21, methanol).

Found: C, 58.51; H, 5.22; N, 6.37. Calcd. for $C_{21}H_{22}O_8N_2$: C, 58.60; H, 5.15; N, 6.51%.

α-*p*-Nitrobenzyl γ-Methyl L-Glutamate Hydrobromide (II).—This compound was prepared by Ben-Ishai's method.3) Compound I (43.0 g., 0.1 mol.) was treated with 33% hydrogen bromide in glacial acetic acid (61.5 g., 0.25 mol.). During the course of the reaction, the hydrobromide of the decarbobenzoxylated product deposited as a crystalline mass. After this mixture had stood for an hour at room temperature, ether (1000 ml.) was added and the precipitates were filtered, washed with a sufficient amount of ether, and dried over sodium hydroxide pellets in vacuo. The crude material (37.0 g.) was recrystallized as fine needles from methanol-ether; yield, 31.0 g. (83%); m. p. 156°C; $[\alpha]_D^{27}$ $+9.6°$ (*c* 5.02, methanol).

Found: C, 41.50; H, 4.59; N, 7.17; Br, 20.86. Calcd. for $C_{13}H_{17}O_6N_2Br$: C, 41.39; H, 4.54; N, 7.43; Br, 21.19%.

Carbobenzoxy-glycyl-(α-*p*-nitrobenzyl, γ-methyl)-L-glutamate (III).—A solution of II (22.6 g., 0.06 mol.) and triethylamine (8.2 ml., 0.06 mol.) in anhydrous chloroform (100 ml.) was cooled to $-15°C$. Solutions of carbobenzoxy-glycine (10.5 g., 0.05 mol.) in ethyl acetate (70 ml.) and of dicyclohexylcarbodiimide (10.3 g., 0.05 mol.) in ethyl acetate (30 ml.) were stirred into the above solution at $-15°C$, and the mixture was allowed to stand overnight at the same temperature. Dicyclohexyl urea and triethylamine hydrobromide were filtered off and washed with ethyl acetate. The combined filtrate and washings were washed with 5% aqueous sodium bicarbonate, 0.5 N hydrochloric acid, and a small amount of water, dried over anhydrous magnesium sulfate, and concentrated to dryness under reduced pressure. A residual oil was crystallized from ethyl acetate-petroleum benzine; wt., 22.5 g.; m. p. 105～106°C. The product was recrystallized from ethyl acetate-petroleum benzine; yield, 21.3 g. (87%); m. p. 106～108°C; $[\alpha]_D^{16}$ $-15.1°$ (*c* 5.11, DMF**).

Found: C, 56.61; H, 5.23; N, 8.58. Calcd. for $C_{23}H_{25}O_9N_3$: C, 56.67; H, 5.17; N, 8.62%.

Carbobenzoxy-L-alanyl-(α-*p*-nitrobenzyl, γ-methyl)L-glutamate (IV).—To a solution of II (22.6 g., 0.06 mol.) and triethylamine (8.2 ml., 0.06 mol.) in anhydrous chloroform (100 ml.), a solution of carbobenzoxy-L-alanine (11.2 g., 0.05 mol.) in ethyl acetate (70 ml.) was added at $-15°C$. Then dicyclohexylcarbodiimide (10.3 g., 0.05 mol.) in ethyl acetate (30 ml.), which had been cooled to $-15°C$, was added with swirling. The mixture was kept overnight at $-15°C$ and then treated as described above. The product was recrystallized from ethyl acetat-petroleum benzine as colorless needles; wt. 21.0 g. (87%); m. p. 132～138°C; $[\alpha]_D^{16}$ $-11.9°$ (*c* 5.48, DMF).

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* All melting points are uncorrected.

3) D. Ben-Ishai and A. Berger, *J. Org. Chem.*, 17, 1564 (1952).

** DMF: dimethylformamide

Found: C, 57.26; H, 5.54; N, 8.25. Calcd. for $C_{24}H_{27}O_9N_3$: C, 57.48; H, 5.43; N, 8.38%.

Carbobenzoxy-L-valyl-(α-*p*-nitrobenzyl, γ-methyl)-L-glutamate (V). — To a solution of II (17.4 g., 0.046 mol.) and triethylamine (6.3 ml., 0.046 mol.) in anhydrous chloroform (120 ml.), which had been cooled to −15°C, carbobenzoxy-L-valine (9.7 g., 0.039 mol.) in ethyl acetate (50 ml.) was added. Then dicyclohexylcarbodiimide (8.0 g., 0.039 mol.) in ethyl acetate (50 ml.), which had been cooled to −15°C, was added. The mixture was kept at the same temperature overnight and then treated as described above. The crude product (17.7 g.; m. p. 133°C) was collected with ethyl acetate-petroleum benzine. Recrystallization of the crude material from ethyl acetate-petroleum benzine gave colorless needles (15.2 g., 75%; m. p. 138∼140°); $[\alpha]^{16}_{\mathrm{D}}$ −6.1°C (*c* 6.02, DMF).

Found: C, 59.02; H, 6.09; N, 7.92. Calcd. for $C_{26}H_{31}O_9N_3$; C, 58.97, H, 5.90; N, 7.94%.

Carbobenzoxy-L-leucyl-(α-*p*-nitrobenzyl, γ-methyl) L-Glutamate (VI). — Compound II (22.6 g., 0.06 mol.) and triethylamine (8.2 ml., 0.06 mol.) in an hydrous chloroform (100 ml.), carbobenzoxy-L-leucine (13.3 g., 0.05 mol.) in ethyl acetate (70 ml.), and dicyclohexylcarbodiimide (10.3 g., 0.05 mol.) in ethyl acetate (30 ml.) were treated by the procedure described above. The crude product which had been collected from ethyl acetate-petroleum benzine was recrystallized from ethyl acetate-petroleum benzine as colorless needles; yield 23.0 g. (85%); m. p. 109∼113°C; $[\alpha[^{18}_{\mathrm{D}}$ −14.8° (*c* 6.10, DMF).

Found: C, 59.35; H, 6.24; N, 7.97. Calcd. for $C_{27}H_{33}O_9N_3$: C, 59.66; H, 6.12; N, 7.73%.

Carbobenzoxy-L-prolyl-(α-*p*-nitrobenzyl, γ-methyl)L-glutamate (VII). — Carbobenzoxy-L-proline (12.5 g., 0.05 mol.) in ethyl acetate (70 ml.), II (22.6 g., 0.06 mol.) and triethylamine (8.2 ml., 0.06 mol. in anhydrous chloroform (100 ml.), and dicyclohexylcarbodiimide (10.3 g., 0.05 mol.) in ethyl acetate (30 ml.) were mixed at −15°C. The mixture was maintained overnight at the same temperature and treated as above. The crude material (25.0 g.) was collected with ethyl acetate-petroleum benzine and melted at 102∼106°C. Recrystalization of the crude product from ethyl acetate-petroleum benzine gave colorles needless; yield 24.0 g. (91%); m. p. 104∼107°C; $[\alpha]^{18}_{\mathrm{D}}$ −33.4° (*c* 5.92, DMF).

Found: C, 59.27; H, 5.48; N, 8.15. Calcd. for $C_{26}H_{29}O_9N_3$: C, 59.19; H, 5.54; N, 7.97%.

Carbobenzoxyl-L-phenylalanyl-(α-*p*-nitrobenzyl, γ-methyl)L-glutamate (VIII). — Carbobenzoxy-L-phenylalanine (15.0 g., 0.05 mol.) in ethyl acetate (70 ml.), II (22.6 g., 0.06 mol.) and triethylamine (8.2 ml., 0.06 mol.) in anhydrous chloroform (100 ml.) and dicyclohexylcarbodiimide (10.3 g., 0.05 mol.) in ethyl acetate (30 ml.) were mixed at −15°C. The mixture was allowed to stand overnight at the same temperature. Ethyl acetate (100 ml.) was added to the reaction vessel, and the mixture was heated to boiling. The undissolved materials were removed by filtration and washed with a small amount of hot ethyl acetate. From the filtrate, a crystalline material was filtered and dried in a vacuum desiccator. The crude material (32.0 g., m. p. 147∼148.5°C) was recrystallized as colorless fine needles from ethyl acetate; yield 25.9 g. (87%); m. p. 151∼152°C; $[\alpha]^{18}_{\mathrm{D}}$ −13.4° (*c* 5.43, DMF).

Found: C, 62.18; H, 5.53; N, 7.47. Calcd. for $C_{30}H_{31}O_9N_3$: C, 62.38; H, 5.41; N, 7.28%.

Carbobenzoxy-(γ-methyl)-L-glutamyl-(α-*p*-nitrobenzyl, γ-methyl)L-glutamate (IX).—To a solution of carbobenzoxy-(γ-methyl)L-glutamate (11.2 g., 0.038 mol.) in ethyl acetate (50 ml.), II (17.2 g., 0.046 mol.) and triethylamine (6.2 ml., 0.046 mol.) in anhydrous chloroform (80 ml.) was added. To the mixture, which had been cooled to −15°C, was added a cooled solution of dicyclohexylcarbodiimide (7.85 g., 0.38 mol.) in ethyl acetate (30 ml.). After the treatment of the reaction mixture in the same manner as descrired in the synthesis of IV, the crude material (19.8 g.) was obtained and recrystallized from ethyl acetate as needles; yield 18.5 g. (85%); m. p. 97∼100°C; $[\alpha]^{18}_{\mathrm{D}}$ −9.7° (*c* 6.51, DMF).

Found: C, 56.87; H, 5.58; N, 7.35. Calcd. for $C_{27}H_{31}O_{11}N_3$: C, 56.54; H, 5.45; N, 7.33.

Glycyl-(γ-methyl)L-glutamate (X).—Compound III (15.0 g., 0.031 mol.) was suspended in a mixture of methanol (200 ml.) and water (100 ml.), and then hydrogenated over a 3% palladium-charcoal catalyst (1.5 g.) for 6 hr. The catalyst was then filtered off and washed with water. The filtrate was evaporated to dryness, and the residue was dissolved in a small amount of hot water. The insoluble material was removed by filtration. When acetone was added to the filtrate, a crystalline material (6.0 g.) was obtained. Recrystallization from water-acetone gave colorless needles; yield 5.5 g. (82%); m. p. 178∼179°C (decomp.); $[\alpha]^{19}_{\mathrm{D}}$ −3.5° (*c* 5.11, water).

Found: C, 44.14; H, 6.57; N, 12.59. Calcd. for $C_8H_{14}O_5N_2$: C, 44.03; H, 6.47; N, 12.84%.

L-Alanyl-(γ-methyl)L-glutamate (XI). — Compound IV (15.0 g., 0.03 mol.) and 3% palladium-charcoal (1.5 g.) were suspended in a mixture of methanol (200 ml.) and water (100 ml.) and hydrogenated until the evolution of carbon dioxide stopped. The catalyst was then filtered off and washed with a small amount of hot water. The filtrate and washing were combined and concentrated under reduced pressure. A crude product (6.5 g., m. p. 186∼187°C (decomp.)) was collected with acetone. Recrystallization of the crude material from water-acetone gave colorless needs; yield 6.2 g. (90%); m. p. 186°C (decomp.); $[\alpha]^{20}_{\mathrm{D}}$ +8.0° (*c* 5.72, water).

Found: C, 46.37; H, 6.95; N, 12.03. Calcd. for $C_9H_{15}O_5N_2$: C, 46.54; H, 6.94; N, 12.06%.

L-Valyl-(γ-methyl)L-glutamate (XII).—Compound V (12.8 g.) and a 3% palladium-charcoal catalyst (1.5 g.) in a mixture of methanol (250 ml.) and water (80 ml.) were treated in the same manner as has been described above. Recrystallization of the crude material from water-acetone gave fine colorless needles; yield 4.3 g. (68%); m. p. 195∼195.5°C; $[\alpha]^{20}_{\mathrm{D}}$ +25.9° (*c* 6.07, water). L-Valyl-(γ-methyl)L-glutamate was submitted to elementary analysis after being dried over phosphorus pentoxide in vacuo at 110°C. The results of the analysis were identical with the calculated values for the ester plus one and half moles of water, as is shown by the analytical data.

Found: C, 46.31; H, 7.99; N, 9.86. Calcd. for $C_{11}H_{20}O_5N_2\cdot 3/2H_2O$: C, 46.00; H, 8.08; N, 9.76%.

L-Leucyl-(γ-methyl)L-glutamate (XIII). — Compound **VI** (15.0 g., 0.028 mol.) and a 3% palladium-charcoal catalyst (1.5 g.) in a mixture of methanol (200 ml.) and water (100 ml.) were treated in the same way as has been described above. The crude product (6.6 g.) was recrystallized from water-acetone as colorless needles; yield 5.3 g. (70%); m. p. 196~197°C (melted and decomposed with coloring); $[\alpha]_D^{20}$ +27.2° (*c* 5.62, water).

Found: C, 52.45; H, 8.06; N, 10.52. Calcd. for $C_{12}H_{22}O_5N_2$; C, 52.54: H, 8.08; N, 10.21%.

L-Prolyl(γ-methyl)L-glutamate (XIV). — Compound VII (15.0 g., 0.029 mol.) and a 3% palladium-charcoal catalyst (1.5 g.) were suspended in a mixture of methanol (200 ml.) and water (200 ml.) and treated in the same way. The crude product (5.1 g.) was obtained and recrystallized from water as colorless long needles; yield 4.8 g. (66%); m. p. 208°C (decomp.); $[\alpha]_D^{20}$ −38.5° (*c* 6.02, water).

Found: C, 47.82; H, 7.30; N, 10.14. Calcd. for $C_{11}H_{18}O_5N_2\cdot H_2O$: C, 47.81; H, 7.17; N, 10.29%.

L-Phenylalanyl-(γ-methyl)L-glutamate (XV). — Compound VIII (15.0 g., 0.026 mol.) was suspended in a mixture of methanol (200 ml.) and water (50 ml.) and catalytically hydrogenated in the presence of 3% palladium-charcoal (1.5 g.). The crude material (8.0 g.) was recrystallized from water-acetone as prisms; yield 6.1 g. (77%); m. p. 228~232°C (decomp.); $[\alpha]_D^{20}$ +27.1° (*c* 6.09, water).

Found: C, 57.90; H, 6.72; N, 9.07. Calcd. for $C_{15}H_{20}O_5N_2$: C, 58.43; H, 6.54; N, 9.09%.

γ-Methyl-L-glutamyl-(γ-methyl)L-glutamate (XVI).—Compound IX (11.5 g., 0.02 mol.) and a 3% palladium-charcoal catalyst (1 g.) in a mixture of methanol (130 ml.) and water (60) ml. were treated in the manner described above. The crude product was recrystallized from water-acetone as colorless needles; yield 3.2 g. (53%); m. p. 153~154°C (decomp.); $[\alpha]_D^{20}$ +32.3° (*c* 5.75, water).

Found: C, 47.26; H, 6.51; N, 9.09. Calcd. for $C_{12}H_{20}O_7N_2$: C, 47.36; H, 6.63; N, 9.21%.

L-Glycyl-L-glutamine (XVII). — Compound X (2.0 g., 0.092 mol.) was dissolved in concentrated aqueous ammonia (100 ml.) The solution was then allowed to stand for 24 hr. at room temperature (about 15°C) and then concentrated to a syrup under reduced pressure at room temperature. After being flushed once with a small amount of water, the residue was crystallized from water-ethanol. The crude product (1.8 g.) was recrystallized as color less needles from water-ethanol; yield 1.8 g. (90%); m. p. 203~204°C (decomp.); $[\alpha]_D^{21}$ −1.6° (*c* 3.85, water); $[\alpha]_D^{20}$ −7.0° (*c* 3.29, N HCl).

Found: C, 37.95; H, 6.98; N, 19.52. Calcd. for $C_7H_{13}O_4N_3\cdot H_2O$: C, 38.00; H, 6.84; N, 19.00%.

L-Alanyl-L-glutamine (XVIII). — Compound XI (3.5 g., 0.015 mol.) was dissolved in concentrated aqueous ammonia (150 ml.) and treated in the manner described above. The crude product (3.0 g.) was collected with water-ethanol and recrystallized from the same solvents as needles; yield 2.6 g. (74%); m. p. 215~216°C (decomp.); $[\alpha]_D^{24}$ −3.0° (*c* 3.31, N HCl). A sample for elementary analysis was dried over phosphorus pentoxide in vacuo for 20 hr. at 110°C.

Found: C, 44.50; H, 7.06; N, 18.85. Calcd. for $C_8H_{15}O_4N_3$: C, 44.23; H, 6.96; N, 19.35%.

L-Valyl-L-glutamine (XIX).—Compound XII (3.6 g., 0.014 mol.) in concentrated aqueous ammonia (140 ml.) was treated in the manner described above. The crude product (2.5 g.) was obtained and crystallized from water as needles; yield 2.2 g. (65%); m. p. 218~219°C (decomp.); $[\alpha]_D^{20}$ +15.8° (*c* 3.08, N HCl).

Found: C, 49.12; H, 7.78; N, 17.20. Calcd. for $C_{10}H_{19}O_4N_3$: C, 48.96; H, 7.81; N, 17.13%.

L-Leucyl-L-glutamine (XX).—Compound XIII (4.1 g., 0.015 mol.) was treated in the same manner described above. The crude product (3.5 g.) was obtained and recrystallized from water as colorless needles; yield 3.2 g. (84%); m. p. 236~237°C (decomp.); $[\alpha]_D^{19}$ +14.2° (*c* 3.57, N HCl).

Found: C, 50.68; H, 8.13; N, 16.10. Calcd. for $C_{11}H_{21}O_4N_3$: C, 50.95; H, 8.16; N, 16.21%.

L-Prolyl-L-glutamine (XXI). — Compound XIV (2.6 g., 0.01 mol.) in concentrated aqueous ammonia (100 ml.) was treated in the manner described above. Recrystallization of the crude product from water gave colorless needles; yield 2.0 g. (77%); m. p. 211~212.5°C (decomp.); $[\alpha]_D^{21}$ −52.2° (*c* 3.91, N HCl).

Found: C, 45.93; H, 7.31; N, 16.19. Calcd. for $C_{10}H_{17}O_4N_3\cdot H_2O$: C, 45.97; H, 7.33; N, 16.08%.

L-Phenylalanyl-L-glutamine (XXII).—Compound XV (3.1 g., 0.01 mol.) in concentrated aqueous ammonia (100 ml.) was treated in the manner described above. The crude product (2.7 g.) was obtained and recrystallized from water as needles. The first crop of crystallization was 1.8 g. and the second crop was 0.8 g.; yield 2.6 g. (87%); m. p. 213~214°C (decomp.); $[\alpha]_D^{19}$ +17.3° (*c* 3.42, N HCl).

Found: C, 57.04; H, 6.42; N, 14.49. Calcd. for $C_{14}H_{19}O_4N_3$: C, 57.32; H, 6.53; N, 14.33%.

The Hydrolysis of Glycyl-L-glutamine Monohydrate. — Glycyl-L-glutamine monohydrate (104 mg.) was placed in a volumetric flask (3.04 ml.) filled with 6 N hydrochloric acid, and the solution was refluxed for 5 hr. After the hydrolysis, the volume of the solution was adjusted to exactly 3.04 ml. by adding 6 N hydrochloric acid, and the optical rotation of the solution was determined; $[\alpha]_D^{19}$ +31.3°. The optical rotation of a solution containing L-glutamic acid (68.1 mg.), glycine (34.7 mg.) and ammonium chloride (24.7 mg.) gave $[\alpha]_D^{19}$ +31.4°.

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