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Immunochemical Studies on the Poly-γ-D-glutamyl Capsule of *Bacillus anthracis*. II. The Synthesis of Eight Dipeptides and Four Tripeptides of Glutamic Acid*

Danute E. Nitecki and Joel W. Goodman

ABSTRACT: Eight dipeptides and four branched tripeptides of D- and L-glutamic acid were synthesized to be used as hapten inhibitors in the homologous immune system of capsular poly-γ-D-glutamic acid isolated from *Bacillus anthracis*. *N*-Carbobenzoxy and benzyl esters were used as protective groups in the synthesis, and the coupling was achieved with a dicyclohexylcarbodiimide reagent. Protective groups were removed by catalytic hydrogenolysis in one step. The purity of the starting materials and products in each synthetic step was ascertained by thin layer chromatography on silica gel, paper chromatography, and high voltage electrophoresis. The free peptides and their hydrolysates reacted quantitatively with ninhydrin and 2,4-dinitrobenzene-1-sulfonic acid reagents and the peptide hydrolysates showed the expected specific optical rotations. Diastereomeric α-dipeptides and tripeptides were shown to be separable by paper chromatography in several solvents and by high voltage electrophoresis.

Information about the extent of the areas of antigen molecules involved in combining with antibodies (determinant groups) as well as the contributions of various structural features to specificity can be obtained by hapten inhibition of the precipitin reaction.

In this investigation, the antigen studied was a capsular polypeptide isolated from a strain of *Bacillus anthracis*, to which rabbit antisera have been obtained. The polypeptide was shown to be essentially pure poly-γ-D-glutamic acid (Goodman and Nitecki, 1966), an antigen of relatively simple structure. The serological specificity of its reaction with rabbit antisera and hapten inhibition of the homologous immune system using amino acids and synthetic di- and tripeptides of glutamic acid were investigated. The results of serological investigation are presented in another publication (Goodman and Nitecki, 1966).

For this purpose eight dipeptides and four tripeptides of glutamic acid were synthesized. There are four possible α-dipeptides (**1–4**)[1] and four possible γ-dipeptides (**5–8**). Four of the dipeptides have been synthesized before by a different method (Sachs and Brand, 1953a).

In addition, four branched tripeptides have been synthesized in which one glutamic acid moiety is substituted in both α and γ positions by glutamic acid units (**9–12**). In two of these, the central α,γ-substituted glutamic acid is of opposite configuration to the other two glutamic acid moieties (**9** and **10**); the other two

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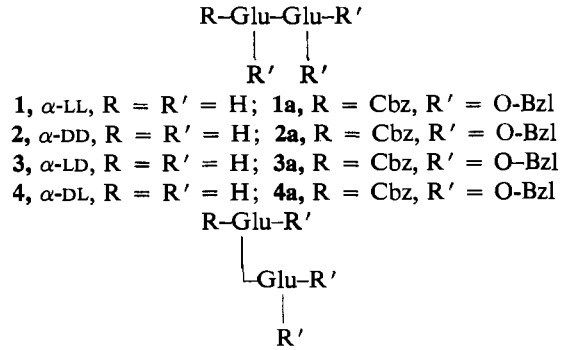
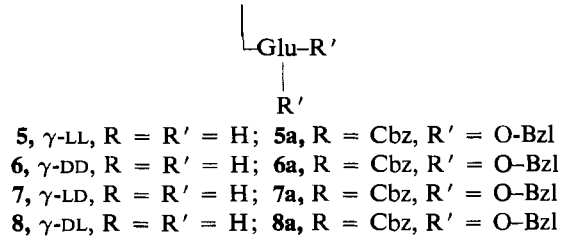
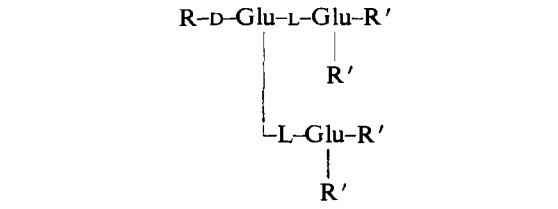
[1] The abbreviations used are described by Young (1962).



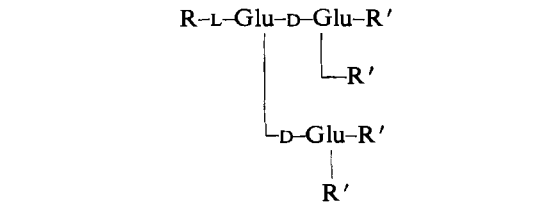
1, α-LL, R = R′ = H; **1a,** R = Cbz, R′ = O-Bzl
2, α-DD, R = R′ = H; **2a,** R = Cbz, R′ = O-Bzl
3, α-LD, R = R′ = H; **3a,** R = Cbz, R′ = O-Bzl
4, α-DL, R = R′ = H; **4a,** R = Cbz, R′ = O-Bzl



5, γ-LL, R = R′ = H; **5a,** R = Cbz, R′ = O-Bzl
6, γ-DD, R = R′ = H; **6a,** R = Cbz, R′ = O-Bzl
7, γ-LD, R = R′ = H; **7a,** R = Cbz, R′ = O-Bzl
8, γ-DL, R = R′ = H; **8a,** R = Cbz, R′ = O-Bzl



9, α,γ-DLL, R = R′ = H; **9a,** R = Cbz, R′ = O-Bzl



10, α,γ-LDD, R = R′ = H; **10a,** R = Cbz, R′ = O-Bzl
11, α,γ-LLL, R = R′ = H; **11a,** R = Cbz, R′ = O-Bzl
12, α,γ-DDD, R = R′ = H; **12a,** R = Cbz, R′ = O-Bzl

tripeptides contain all three glutamic acid units of identical configuration (**11** and **12**).

All of these peptides were synthesized by dicyclohexylcarbodiimide coupling, using appropriately blocked glutamic acid derivatives and maintaining slightly acid conditions during the coupling reaction (Buzas *et al.*, 1965). The carbobenzoxy group was used for the protection of the amino group and carboxylic acid groups were protected by benzyl esters. All these blocking groups were removed in a single step by catalytic hydrogenolysis. For the synthesis of α-dipeptides, the appropriate carbobenzoxyglutamic acid γ-benzyl ester (Hanby *et al.*, 1950), freed from its dicyclohexylamine salt (Klieger *et al.*, 1961), and dibenzyl glutamate, liberated from its *p*-toluenesulfonic acid salt (Bose and Strube, 1963), were employed. The protected α-dipeptides were found to be gelatinous solids and could not be purified effectively by crystallization; they were subjected to purification by countercurrent distribution. For the synthesis of γ-dipeptides, *N*-carbobenzoxyglutamic acid α-benzyl ester (Sachs and Brand, 1953b) and free dibenzyl glutamate of the appropriate configuration were coupled. The protected γ-dipeptides were easily purified by crystallization. The tripeptides were obtained by combining the appropriate carbobenzoxyglutamic acid (Bergman and Zervas, 1932; Goldschmidt and Jutz, 1953) with 2 equiv of dibenzyl glutamate of the same or opposite configuration. The resulting *N*-carbobenzoxy tripeptide tetrabenzyl esters could be effectively purified by crystallization. The purity of all glutamic acid derivatives and peptides after each synthetic step was ascertained by thin layer chromatography on silica gel, high-voltage electrophoresis, and paper chromatography.

Within each group of the protected peptides, *i.e.*, γ-dipeptides, α-dipeptides, or tripeptides, the solubility in organic solvents decreased markedly in mixed configuration diastereomers as compared with isomers of uniform configuration. For example, the protected α-LL dipeptide (**1a**) was considerably more soluble in all solvents tried than the diastereomeric α-DL (**4a**).

The protecting groups were removed by catalytic hydrogenolysis. The free peptides were found to be hygroscopic solids which retained some water even after prolonged drying *in vacuo*. All peptides reacted quantitatively with ninhydrin and with 2,4,6,-trinitrobenzene-1-sulfonic acid (Satake *et al.*, 1960) reagents. Upon complete hydrolysis the peptides yielded the expected amounts of glutamic acid of appropriate optical activity. Dilute solutions of the peptides in water and in acetic acid, when stored in the refrigerator for several months, were found to deteriorate only slowly; the major component, as shown by high voltage electrophoresis after three months of storage, was still the original peptide. An estimate of the degree of decomposition indicates the following order of decreasing stability: tripeptides > α-dipeptides > γ-dipeptides.

In the course of this investigation it was found possible to separate some free diasteriomeric peptides by high-voltage electrophoresis and by paper chromatography, which agrees with earlier observations that unprotected LL and DD dipeptides move faster on paper than LD and DL diastereomers (Kienhuis and Verweij, 1964; Sokolowska and Biernat, 1964; Taschner *et al.*, 1963; Wieland and Bende, 1964). In the present instance, it was possible to separate diastereomeric α-dipeptides and tripeptides but not γ-dipeptides using either technique. It is difficult to interpret these observations as this behavior must be grossly dependent on the actual conformation of the molecule in solution, about which there is no information. A forthcoming investigation of the pK_a and isoelectric point differences between the various diastereomeric dipeptides and tripeptides may provide an explanation.

Experimental Section[2]

Thin Layer Chromatography. Silica gel G was used; the coated plates were air dried for at least 18 hr. The solvent systems for thin layer chromatography are

[2] The melting points are uncorrected. Rotations were determined in a O.C. Rudolph and Sons Model 80 Polarimeter and measured in a 2-dm tube with a 3-mm bore. Microanalyses were performed by the Microchemical Laboratory, University of California, Berkeley, California.

TABLE I: R_F Values of Glutamic Acid Derivatives and Protected Peptides on Silica Gel.

Solvent	Glu	Glu-O-Bzl | O-Bzl[a]	Cbz-Glu	Glu | O-Bzl	Cbz–Glu Glu-O-Bzl	Cbz–Glu | O–Bzl	Cbz-Glu-O-Bzl	Protected α-Dipeptides[b]	Protected γ-Dipeptides[c]	Protected α,γ-Tripeptides[d]
S–1	0.10	0.82	0.33	0.53	0.58	0.75	0.72			
S–2	0.10	0.79	0.75	0.38	0.45	0.87	0.91	0.95	0.95	0.95
S–3	0	0.74	0.11	0.33	0.21	0.64	0.55	0.90	0.94	0.85
S–4	0	0.85	0.23	0.20	0.15	0.90	0.87	—	—	—
S–5	0	0.94	0.90	0.39	0.48	0.95	0.95	—	—	—
S–6	0	0.73	0.37	—	0.32	0.70	0.70	0.95	—	0.91
S–7	0	0.29	0.79	0.07	0.08	0.86	0.87	—	—	—
S–11	0	0.07	0.23	0	0	0.85	0.83	0.75	0.75	0.75
S–12	0	0.16	0.10	0	0	0.35	0.47	0.43	0.50	0.45
S–13	0	0.04	0.30	0	0	0.67	0.66	0.87	0.80	0.72

[a] As *p*-toluenesulfonate salt. [b] Compounds **1a, 2a, 3a, 4a.** [c] Compounds **5a, 6a, 7a, 8a.** [d] Compounds **9a, 10a, 11a,** and **12a.**

abbreviated as follows: S-1, *n*-BuOH–pyridine–H_2O (5:2:1); S-2, *n*-BuOH–AcOH–H_2O–CH_3OH (100:5:10:20); S-3, *n*-BuOH–H_2O (6:1); S-4, $CHCl_3$–CH_3OH (4:3); S-5, $CHCl_3$–CH_3OH–AcOH (70:30:5); S-6, *n*-BuOH–pyridine–AcOH (120:15:5); S-7, acetone–AcOH (9:1); S-9, dioxane–H_2O (9:1); S-11, $CHCl_3$–AcOH (9:1); S-12, xylene–pyridine–AcOH (100:15:5); S-13, isopropyl ether–$CHCl_3$–AcOH (6:3:1); S-18, *n*-BuOH–AcOH–H_2O (120:30:50); S-19, pyridine–AcOH–H_2O (50:30:15); S-20, *n*-BuOH–pyridine–AcOH–H_2O (95:50:30:30); S-21, methyl ethyl ketone–AcOH–H_2O (70:30:25); S-22 *i*-PrOH–AcOH–H_2O (8:2:1); S-23, ethyl acetate–pyridine–AcOH–H_2O (5:5:1:3).

Solvents S-1–S-6 were found to be especially effective for checking the purity of various derivatives of glutamic acid; S-3 and S-11–S-13 for protected di- and tripeptides (the outstanding solvent for this purpose was S-13); and S-18–S-23 for free di- and tripeptides with S-21 the oustanding solvent system for unblocked peptides. The R_F values are shown in Tables I and II. As no attempts were made to control the temperature, aging of the solvents, thickness of the layers, etc., the R_F values were difficult to reproduce and should only be considered as relative guide lines.

Ninhydrin was used for the detection of materials containing free amino group. For N-protected compounds, chlorination was employed on silica gel and on paper. Variations of this method have been described (Rydon and Smith, 1952; Reindel and Hoppe, 1953; Pan and Dutcher, 1956; Pataki, 1963; Mazur *et al.*, 1962), but many of them fail to give reproducible results. In our hands, modification of Pan and Dutcher's method as described here has given satisfactory reproducibility and required a minimum of equipment.

Thoroughly dried developed silica gel plates or paper chromatograms were sprayed with a freshly diluted

TABLE II: R_F Values of Free Peptides on Silica Gel.

Solvent	Glu	α-Dipeptides[a]	γ-Dipeptides[b]	Tripeptides[c]
S-18	0.21	0.25	0.14	0.17
S-19	0.92	0.94	0.97	
S-20	0.26	0.28	0.09	0.13
S-21	0.51	0.62	0.51	0.60
S-22	0.32	0.39	0.22	0.30
S-23	0.30	0.20	0.17	0.15

[a] Compounds **1–4.** [b] Compounds **5–8.** [c] Compounds **9–12.**

10–15% solution of commercial Clorox bleach in water and left to dry in a ventilated hood at room temperature for exactly 30 min, then sprayed with ethanol and after an additional 10 min sprayed with a 1:1 mixture of 1% potassium iodide and freshly prepared solution of *o*-toluidine in 10% acetic acid–water solvent. The deep blue spots fade quite rapidly.

This method detected reproducibly the following free amino acids on silica gel plates in a concentration of 0.5–1 μmole: Asp, Glu, Arg, Hypro, Pro, His, Lys, Try, Ser and Tyr; amino acids Leu, Thr, Gly, Ile, Ala, Phe, and Val gave variable results or failed to show up entirely. On paper only the presence of Asp, Hypro, His, Try, Pro, and Tyr of the above-mentioned amino acids could be reliably detected. No difficulties in detection were experienced with *N*-carbobenzoxyamino acids, a large number of *N*-*t*-butyloxycarbonyl[3] amino

[3] Commercial preparations.

TABLE III: R_F Values of Various Derivatives of Glutamic Acid and Free Peptides on Paper.

					Dipeptides				Tripeptides	
Solvent	Glu	Glu-O-Bzl | O-Bzl[a]	Glu-O-Bzl	Glu | O-Bzl	α-LL[b] and α-DD[b]	α-LD and α-DL[c]	γ-LL and γ-DD[d]	γ-LD and γ-DL[e]	α,γ-LLL and α,γ-DDD[f]	α,γ-DLL and α,γ-LDD[g]
S–1	0.04	0.86	0.60	0.47						
S–3	0	0.84	0.39	0.39	0	0	0	0	0	0
S–9	0	0.95	0	0.16						
S–18	0.31	0.89	0.70	0.72	0.36	0.31	0.29	0.29	0.33	0.33
S–19	0.43	0.92	0.81	0.76	0.60	0.44	0.39	0.39	0.45	0.39
S–20	0.18				0.18	0.14	0.11	0.11	0.14	0.09
S–21	0.44				0.56	0.50	0.39	0.39	0.48	0.46
S–23	0.11				0.09	0.08	0.06	0.06	0.08	0.05
S–24	0.25				0.33	0.30	0.25	0.25	0.36	0.31

[a] As *p*-toluenesulfonate salt. [b] Compounds **1** and **2**. [c] Compounds **3** and **4**. [d] Compounds **5** and **6**. [e] Compounds **7** and **8**. [f] Compounds **11** and **12**. [g] Compounds **9** and **10**.

acids, or with our peptides.

Paper chromatography was performed on Whatman No. 1 filter paper using a descending technique in the following solvent systems: S-1, S-3, S-9, S-18, S-19, S-20, S-21, S-23 (described above); S-24, *n*-BuOH–*i*-PrOH–H_2O–chloroacetic acid (65 ml:15 ml:20 ml: 3 g). The R_F values of various glutamic acid derivatives are shown in Table III.

In chromatographic separation of diastereomeric peptides equimolar mixtures of the diastereomers were investigated. Solvent systems in which the peptides moved very slowly were allowed to drip off the serrated edge of the paper; thus, chromatograms were allowed to run for 50–70 hr. The apparent solvent front was calculated from the known R_F value of glutamic acid; this, in turn, allowed calculation of apparent R_F values for diastereomeric peptides. The actual separations (in centimeters) between diastereomers as well as their apparent R_F values are shown in Table IV.

Paper Electrophoresis. The conditions for high-voltage paper electrophoresis on Whatman No. 1 were: solvents (Katz *et al.*, 1959), 10 ml of acetic acid and 1 ml of pyridine diluted to 200 ml with distilled water, pH 3.5–3.7; 10 ml of pyridine and 0.4 ml of acetic acid diluted to 200 ml with distilled water, pH 6.4–6.5; voltage, 35 v/cm of paper. Electrophoretic mobilities are reported as the ratio: distance the peptide moved/distance glutamic acid moved and abbreviated as E_G (Seu *et al.*, 1962). Negative signs indicate motion in the direction opposite to that of glutamic acid. The results are shown in Table V.

N-Carbobenzoxy-γ-benzyl-α-D-glutamyl-L-glutamic Acid Dibenzyl Ester (α-DL, **4a**). Free dibenzyl-L-glutamate (Bose and Strube, 1963) was prepared from its *p*-toluenesulfonic acid salt by suspending 34 g (67 mmoles) of the salt in a cold mixture in 60 ml of water and 170 ml of ether in a separatory funnel. A cold solution of 17 g of potassium carbonate in 90 ml of water was added to the above and the mixture was shaken vigorously until the solid disappeared. The ether layer was withdrawn and the aqueous layer was extracted four times with 170 ml of cold ether. The combined extracts were dried over anhydrous magnesium sulfate, two drops of concentrated hydrochloric acid were added, and the solvent was removed under reduced pressure until a thick clear colorless oil remained. This was dissolved in 100 ml of cold dioxane and used immediately.

N-Carbobenzoxy-γ-benzyl-D-glutamic acid dicyclohexylamine salt (Hanby *et al.*, 1950; Klieger *et al.*, 1961) was transformed to the free acid by adding 32 g (57 mmoles) of the salt to a separatory funnel containing 750 ml of 1 N hydrochloric acid and 2 l. of ethyl acetate and shaking vigorously. An emulsion containing some solid was formed between the two layers. The clear upper and lower layers were withdrawn and the emulsion treated again with 250 ml of 1 N hydrochloric acid and 500 ml of ethyl acetate. This process was repeated until the emulsion cleared up and the solid salt had completely dissolved. The combined ethyl acetate extracts were washed twice with 200 ml of water and dried over anhydrous magnesium sulfate and the solvent was removed under reduced pressure until a thick oil remained, which was dissolved in 100 ml of cold dioxane. This solution was combined with a cold dioxane solution containing freshly prepared free dibenzyl L-glutamate and a cold solution of 11.5 g (56 mmoles) of dicyclohexylcarbodiimide in 100 ml of dioxane. The reaction mixture was kept at 4° overnight, and the precipitated dicyclohexylurea (11 g, 90%) was collected by filtration and washed with copious amounts of ethyl acetate. The filtrate was diluted to 1.5 l. with ethyl

TABLE IV: Chromatographic Separation of Free Diastereomeric Peptides on Paper.[a]

Solvent	Developing Time (hr)	Glu R_F	α-Dipeptides[b] Apparent R_F α-DD	α-DL	Actual Separation on Chromatograms (cm)	Tripeptides[b] Apparent R_F α,γ-LLL	α,γ-DLL	Actual Separation on Chromatograms (cm)
S–18	24	0.31	0.36	0.31	3	0.33	0.33	No separation
S–19	16	0.43	0.60	0.44	7	0.45	0.39	No separation
S–20	72	0.18	0.18	0.14	10	0.14	0.09	7
S–21	9	0.44	0.56	0.50	3	0.48	0.46	No Separation
S–23	46	0.11	0.093	0.077	3.7	0.077	0.049	6
S–24	48	0.25	0.33	0.30	3	0.36	0.31	5

[a] Maximum path length available for migrating substance: 45 cm. [b] This behavior is identical with other pairs of α-dipeptide (or tripeptide) diastereomers.

TABLE V: Electrophoretic E_G Values of Derivatives of Glutamic Acid and Free Peptides on Paper.

pH	Glu	Glu-O-Bzl	Glu(O-Bzl)	Dipeptides α-LL and α-DD[a]	α-LD and α-DL[b]	γ-LL and γ-DD[c]	Tripeptides γ-LD and γ-DL[d]	α,γ-LLL and α,γ-DDD[e]	α,γ-DLL and α,γ-LDD[f]
3.5–3.7	+1.0	−6.0	−0.7	−2.1	−0.5	+5.6	+5.6	+4.4	+3.8
6.4–6.5	+1.0	0	0	+1.22	+1.22	+1.22	+1.22	+1.34	+1.34

[a] Compounds **1** and **2**. [b] Compounds **3** and **4**. [c] Compounds **5** and **6**. [d] Compounds **7** and **8**. [e] Compounds **10** and **12**. [f] Compounds **9** and **10**.

acetate and washed five times with 300 ml of cold 0.5 N hydrochloric acid, three times with cold 4% sodium bicarbonate solution, and twice with 300 ml of cold water while keeping the volume of the organic layer constant by adding ethyl acetate. The organic layer was dried over anhydrous magnesium sulfate and the solvent removed under reduced pressure until approximately 150 ml remained. The solution was stored at 4° overnight; a yellowish gelatinous solid was collected by filtration and air dried. The yield of crude peptide was 28 g (74%), mp 85–90°. Thin layer chromatography showed several impurities corresponding to both starting materials and dicyclohexylurea.

This peptide could be reprecipitated from methanol, ethanol, carbon tetrachloride, ethyl acetate, toluene, and benzene, but in all these solvents it precipitated as a clear gel which retained large amounts of solvent upon filtration and thus very little purification was achieved. This behavior was also true of all the other α-peptides (α-LD, α-LL, α-DD).

Therefore, all protected α-dipeptides were purified by countercurrent extraction using an apparatus[4] consisting of 30-transfer tubes of 40/40 ml volume with a solvent system of methanol–carbon tetrachloride–chloroform–water (10:10:5:4) (Schwarz and Arakawa, 1959). The peptides remained in the lower layer of carbon tetrachloride–chloroform; about 100 transfers of the upper phase by a single withdrawal technique were found to remove all impurities. The tubes containing pure dipeptide (checked by thin layer chromatography) were combined, the solvent was removed, and the solid was recrystallized once from methanol. About 6–8 g of peptide could be purified by one run and the average recovery of pure peptide was about 80%.

In one case (α-LL) the methanolic gel obtained after countercurrent purification solidified upon standing at room temperature for 7 days into a coarse granular solid which was easily filtered. This material had a higher melting point (103–106°) than the α-DD isomer (94–95°). This did not occur with other α-dipeptides.

N-Carbobenzoxy-γ-benzyl-α-D-glutamyl-L-glutamic acid dibenzyl ester (α-DL, **4a**) mp 90–92°, $[\alpha]^{25-27}D$ +1.0° (*c* 4, acetic acid), −9.4° (*c* 8, dimethylformamide).

Anal. Calcd for $C_{39}H_{40}N_2O_9$: C, 68.82; H, 5.92; N, 4.12. Found: C, 68.66; H, 5.81; N, 4.16.

N-Carbobenzoxy-γ-benzyl-α-L-glutamyl-D-glutamic

[4] Manufactured by H. O. Post Scientific Instrument Co.

acid dibenzyl ester (α-LD, **3a**) was obtained as described above. Crude yield was 80%, mp 85–90°. After countercurrent purification the yield was 56%, mp 90–91°, $[\alpha]^{25-27}D$ −0.8° (*c* 4, acetic acid), +9.7° (*c* 8, dimethylformamide); lit. (Sachs and Brand, 1953a) mp 91–92°, $[\alpha]^{23}D$ −0.5 (*c* 2, acetic acid).

N-Carbobenzoxy-γ-benzyl-α-D-glutamyl-D-glutamic acid dibenzyl ester (α-DD, **2a**) was obtained by the same method in 78% crude yield; after countercurrent purification the yield was 58%, mp 94–95°, $[\alpha]^{25-27}D$ +10.4° (*c* 4, acetic acid), +10.6° (*c* 8, dimethylformamide).

Anal. Calcd for $C_{39}H_{40}N_2O_9$: C, 68.82; H, 5.92; N, 4.12. Found: C, 68.64; H, 5.66; N, 4.42.

N-Carbobenzoxy-γ-benzyl-α-L-glutamyl-L-glutamic acid dibenzyl ester (α-LL, **1a**) was obtained as described above in 76% crude yield. After countercurrent purification, mp 103–106°, $[\alpha]^{25-27}D$ −10.4° (*c* 4, acetic acid), −10.6 (*c* 8, dimethylformamide); lit. (Sachs and Brand, 1953a) mp 104–105°, $[\alpha]^{23}D$ −10.4° (*c* 2, acetic acid).

N-Carbobenzoxy-α-benzyl-γ-D-glutamyl-D-glutamic Acid Dibenzyl Ester (γ-DD, **6a**). A cold solution of 18.5 g (50 mmoles) of carbobenzoxy-α-benzyl-D-glutamic acid, prepared the same way as the L isomer (Sachs and Brand, 1953b), in 100 ml of dioxane, was combined with a cold dioxane solution of free dibenzyl D-glutamate, freshly prepared (as described above) from 30 g (60 mmoles) of its *p*-toluenesulfonate salt, and with a cold solution of 10.3 g (50 mmoles) of dicyclohexylcarbodiimide in 100 ml of dioxane. A precipitate appeared within a few minutes after mixing; the reaction solution was kept at 4° overnight. Precipitated dicyclohexylurea (9 g, 86%) was removed by filtration, and washed with ethyl acetate, and the combined filtrates were diluted to 1.5 l. with ethyl acetate. This solution was processed as described above for the synthesis of the α-peptides. After removal of the solvent under reduced pressure a waxy pale yellow solid remained which was dissolved in 200 ml of warm methanol, decolorized with Norit, and yielded upon cooling 23.5 g (66%) of crude γ-dipeptide. The crude material was purified by several recrystallizations from methanol until it was shown to be pure by thin layer chromatography. Impure fractions could be recovered from various methanolic mother liquors by the addition of water, bringing the yield up to 80% of the theoretical.

The final yield of pure carbobenzoxy-α-benzyl-γ-D-glutamyl-D-glutamic acid dibenzyl ester (γ-DD, **6a**) was 18.5 g (54%), mp 137–139°, $[\alpha]^{25-27}D$ +4.2° (*c* 4, acetic acid), +17.8 (*c* 8, dimethylformamide).

Anal. Calcd for $C_{39}H_{40}N_2O_9$: C, 68.82; H, 5.92; N, 4.12. Found: C, 68.80; H, 5.98; N, 4.32.

N-Carbobenzoxy-α-benzyl-γ-L-glutamyl-L-glutamic acid dibenzyl ester (γ-LL, **5a**) was prepared as described above. The yield after several recrystallizations was 41%, mp 136°; $[\alpha]^{25-27}D$ −4.1° (*c* 4, acetic acid), −17.7° (*c* 8, dimethylformamide); lit. (Weygand and Hunger, 1962) mp 134–137°, $[\alpha]^{26}D$ −5.1° (*c* 2, acetic acid).

N-Carbobenzoxy-α-benzyl-γ-L-glutamyl-D-glutamic acid dibenzyl ester (γ-LD, **7a**) was synthesized as described for the γ-DD isomer. The crude product was obtained in 56% yield. After several recrystallizations the yield of pure peptide was 33%, mp 127–128°, $[\alpha]^{25-27}D$ +3.9° (*c* 4, acetic acid), +4.0° (*c* 8, dimethylformamide); lit. (Sachs and Brand, 1953a) mp 129–131°, $[\alpha]^{23}D$ +3.3° (*c* 2, acetic acid).

N-Carbobenzoxy-α-benzyl-γ-D-glutamyl-L-glutamic acid dibenzyl ester (γ-DL, **8a**) was obtained as described above in a yield of 62% for the crude material and 54% after purification by recrystallization; mp 126–128°, $[\alpha]^{25-27}D$ −3.9° (*c* 4, acetic acid), −3.9° (*c* 8, dimethylformamide).

Anal. Calcd for $C_{39}H_{40}N_2O_9$: C, 68.82; H, 5.92; N, 4.12. Found: C, 68.78; H, 6.12; N, 4.06.

N-Carbobenzoxy-α,γ-bis(D-glutamic acid dibenzyl ester)-D-glutamic Acid. (α,γ-DDD, **12a**). Solution of 11.2 g (40 mmoles) of carbobenzoxy-D-glutamic acid (Bergman and Zervas, 1932; Goldschmidt and Jutz, 1953) in 100 ml of dioxane was mixed with a dioxane solution of dibenzyl D-glutamate, freed from 50 g (100 mmoles) of toluenesulfonate salt as described above. To this mixture a cold solution of 16.5 g (80 mmoles) of dicyclohexylcarbodiimide in 100 ml of dioxane was added; a precipitate appeared almost immediately. The solution was kept overnight at 4°; dicyclohexylurea was collected by filtration; the reaction solution was diluted to 1.5 l. with ethyl acetate and processed in the same manner as described for the synthesis of the α-dipeptides. After washing, the solvent was removed under reduced pressure to a volume of approximately 250 ml and the solution was kept in the refrigerator for 2 days. A gelatinous precipitate was collected by filtration and triturated with petroleum ether several times. The yield of crude granular product, air dried, was 22 g (61%). The product was recrystallized four times from 200 to 300 ml of methanol until the tripeptide was shown to be pure by silica gel thin layer chromatography in several solvent systems. The yield of pure carbobenzoxy tripeptide tetrabenzyl ester was 15 g (42%), mp 119–122°, $[\alpha]^{25-27}D$ +8.3° (*c* 4, acetic acid), +14.0° (*c* 4, dimethylformamide).

Anal. Calcd for $C_{51}H_{53}N_3O_{12}$: C, 68.08; H, 5.94; N, 4.67. Found: C, 67.61; H, 5.78; N, 5.09.

N-Carbobenzoxy-α,γ-bis(L-glutamic acid dibenzyl ester)-L-glutamic acid (α,γ-LLL, **11a**) was obtained pure in the same manner in 41% yield mp, 118–120°, $[\alpha]^{25-27}D$ −8.1° (*c* 4, acetic acid), −13.8° (*c* 4, dimethylformamide).

Anal. Calcd for $C_{51}H_{53}N_3O_{12}$: C, 68.08; H, 5.94; N, 4.67. Found: C, 67.98; H, 5.71; N, 4.95.

N-Carbobenzoxy-α,γ-bis(D-glutamic acid dibenzyl ester)-L-glutamic acid (α,γ-DLL, **9a**), as well as its enantiomorph (α,γ-LDD, **10a**), was much less soluble in organic solvents than the diastereomeric tripeptides α,γ-LLL (11a) and α,γ-DDD (**12a**). The amount of methanol required to dissolve 26 g of crude α,γ-DLL (**9a**), obtained in 65% yield, was 600 ml and this volume increased with each recrystallization as the peptide was getting purer (as revealed by thin layer chromatography on silica gel). The fourth recrystallization required 1.5 l. of methanol to yield 12 g (30%) of pure tripeptide, mp 150–153°, $[\alpha]^{25-27}D$ −16.7° (*c* 4, dimethylformamide).

Anal. Calcd $C_{51}H_{53}N_3O_{12}$: C, 68.08; H, 5.94; N, 4.67.

TABLE VI: Free Peptides: Analytical Data, Specific Rotations, and Specific Rotations of Peptide Hydrolysates.

Compound	Formula	Carbon, % Calcd	Carbon, % Found	Hydrogen, % Calcd	Hydrogen, % Found	Nitrogen, % Calcd	Nitrogen, % Found	$[\alpha]_D$ (deg)[a]	$[\alpha]_D$ (deg)[b]	$[\alpha]_D$ (deg) of Hydrolysate[c]
γ-D-Glu	$C_{10}H_{16}N_2O_7$	43.48	43.49	5.84	5.74	10.14	9.99	−3.4		−29.6
└D-Glu (γ-DD, **6**)										
γ-L-Glu								+3.8[d]		+29.8
└L-Glu (γ-LL, **5**)										
γ-D-Glu	$C_{10}H_{16}N_2O_7$	43.48	43.20	5.84	5.50	10.14	10.30	−36.2	−19.4	0
└L-Glu (γ-DL, **8**)										
γ-L-Glu										
└D-Glu (γ-LD, **7**)								+36.2[e]	+19.8	0
α-D-Glu-D-Glu (α-DD, **2**)	$C_{10}H_{16}N_2O_7 \cdot 0.3H_2O$	42.64	42.70	5.94	5.81	9.95	10.00	−16.6		−28.4
α-L-Glu-L-Glu (α-LL, **1**)	$C_{10}H_{16}N_2O_7 \cdot 0.3H_2O$	42.64	42.76	5.94	6.01	9.95	10.49	+16.3[f]		+28.4
α-L-Glu-D-Glu (α-LD, **3**)								+56.2[g]	+23.0	0
α-D-Glu-L-Glu (α-DL, **4**)	$C_{10}H_{16}O_7N_2 \cdot 0.5H_2O$	42.11	41.86	6.01	5.89	9.82	9.84	−56.5		0
α,γ-L-Glu-L-Glu	$C_{15}H_{23}N_3O_{10} \cdot 0.3H_2O$	43.86	43.93	5.79	5.59	10.22	10.23	+4.3	+17.6	+29.2
└L-Glu (α,γ-LLL, **11**)										
α,γ-D-Glu-D-Glu	$C_{15}H_{23}N_3O_{10} \cdot 0.3H_2O$	43.86	43.81	5.79	5.67	10.22	10.25	−4.8	−18.3	−30.0
└D-Glu (α,γ-DDD, **12**)										
α,γ-L-Glu-D-Glu	$C_{15}H_{23}N_3O_{10} \cdot 0.3H_2O$	43.86	43.80	5.79	5.49	10.22	10.25	+49.0	+22.8	−30.6
└D-Glu (α,γ-LDD, **10**)										
α,γ-D-Glu-L-Glu										
└L-Glu (α,γ-DLL, **9**)	$C_{15}H_{23}N_3O_{10} \cdot 0.5H_2O$	43.49	43.42	5.84	5.63	10.14	10.20	−49.3	−21.4	+28.6

[a] At 25–27°; *c* 2, 0.5 N HCl. [b] At 25–27°; *c* 3–4, dimethylformamide. [c] In 6 N HCl. Dipeptides of DL and LD configuration yielded optically inactive solutions, as expected; for the other peptides specific rotations were calculated to yield values directly comparable with controls treated in the same manner. Controls: D-glutamic acid. −30.3 ± 1 (average of six determinations); L-glutamic acid, +29.9 ± 1 (average of six determinations). [d] Lit. (Sachs and Brand, 1953a) + 3.8 (*c* 1–2, 0.5 N HCl); lit. (Vitali *et al.*, 1965), +4.3 (*c* 2, 0.5 N HCl). [e] Lit. (Sachs and Brand, 1953a) +36.7 (*c* 1–2, 0.5 N HCl). [f] Lit. (Sachs and Brand, 1953a) + 18.2 (*c* 1–2, 0.5 N HCl);lit. (Vitali *et al.*, 1965) +16.6 (*c* 2, 0.5 N HCl). [g] Lit. (Sachs and Brand, 1953a) +56.4 (*c* 1–2, 0.5 N HCl).

Found: C, 67.81; H, 5.98; N, 4.68.

N-Carbobenzoxy-α,γ-bis(L-*glutamic acid dibenzyl ester*)-D-*glutamic acid* (α,γ-LDD, **10a**) was obtained as described above in 45% yield, mp 151–153°, $[\alpha]^{25-27}D$ +16.9° (*c* 4, dimethylformamide).

Anal. Calcd for $C_{51}H_{53}N_3O_{12}$: C, 68.08; H, 5.94; N, 4.67. Found: C, 68.25; H, 5.77; N, 4.49.

Hydrogenolysis. The protected peptides were dissolved in a mixture of methanol–acetic acid–water, 30 ml/mmole of peptide. The composition was varied in order to dissolve the peptide (in some instances the blocked peptide did not dissolve immediately, but only after 1–1.5 hr of hydrogenolysis). Ten per cent palladium on charcoal[5] was used as a catalyst in amounts of one-third of the weight of the peptide. The solution was stirred and hydrogen gas was passed slowly through at atmospheric pressure for 4–6 hr. The suspended catalyst was removed by suction filtration through a pad of Norit. The solvent was removed under reduced pressure, and the remaining free peptide was dissolved in water and lyophilized several times until a white, fluffy, easy to handle, amorphous solid was obtained. The yields were close to the theoretical.

Free Peptides. All free peptides were hygroscopic, soluble in water, and moderately soluble in acetone and methanol. For analysis, γ-di- and tripeptides were dried at 110° for 48 hr *in vacuo;* even so, the tripeptides retained 0.3–0.5 mole of water. Under these conditions α-dipeptides lost about 0.5 mole of water, probably *via* formation of pyroglutamic acid derivatives. Therefore, α-dipeptides were dried at 56° *in vacuo* for 24 hr and sent for analysis; they were found to retain 0.3–0.5 mole of water/mole of peptide. The analytical data are shown in Table VI.

Hydrolysis of Peptides. Portions of 25–50 mg of free peptides as well as varying amounts of L- and D-glutamic acid were each dissolved in 3 ml of 6 N hydrochloric acid and heated in sealed tubes in an autoclave for 4 hr at 110°. The volumes were adjusted to 5 ml with 6 N hydrochloric acid, the optical rotations determined, and the specific rotations calculated so that one could compare them directly with reference values for L- and D-glutamic acid. The results are shown in Table VI. The same solutions, after appropriate dilution and neutralization, were used for quantitative microanalytical amino group determinations by the ninhydrin and 2,4,6,-trinitrobenzene-1-sulfonic acid (TNBS)[6] methods.

Quantitative Ninhydrin and TNBS Determinations. Amino groups of all free peptides were quantitatively determined using microanalytical ninhydrin (Rosen, 1957) and 2,4,6-trinitrobenzene-1-sulfonic acid (TNBS) (Satake *et al.*, 1960) methods. The unhydrolyzed peptides reacted quantitatively with both reagents, as determined spectrophotometrically, using glutamic acid as standard. The procedures were repeated with peptide hydrolysates (described above) and in all cases the peptides yielded expected amounts of glutamic acid within experimental limits. Concentrations of peptides and standards used for ninhydrin and TNBS determinations were 0.1–0.4 μmole/ml; the reproducibility limits of the standards were ±5% for the ninhydrin reaction and ±3% for the TNBS reaction.

The TNBS method has the distinct advantage over the 2,4-dinitrofluorobenzene method of permitting the extent of the reaction to be directly determined spectroscopically without the necessity of isolating the product or removing the by-products.

[5] Product of Matheson Coleman and Bell.

[6] Abbreviations used: TNBS, 2,4,6-trinitrobenzene-1-sulfonic acid.

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Peptide–Protein Interaction as Studied by Gel Filtration*

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ABSTRACT: The gel-filtration method of Hummel and Dreyer has been used for the determination of the binding constants in the interaction of bovine serum albumin with L-tryptophan or tryptophan derivatives, including peptides. This method, as modified in the present work, appears to be at least as precise as dialysis equilibrium and, when automated, offers many advantages in speed, convenience, and flexibility. Evidence is offered for the attainment of equilibrium conditions in the operation of the gel-filtration method, and for its utility in the quantitative measurement of the competition of two small molecules for a protein binding site. The results of earlier dialysis–equilibrium studies on the binding of L-tryptophan and of acetyl-L-tryptophan by serum albumin, by McMenamy and his associates, have been largely confirmed in the present work. Data are also given for the primary association constants in the binding, to bovine serum albumin, of D-tryptophan, acetyl-L-tryptophanamide, acetyl-D-tryptophanamide, and several tryptophan-containing peptides. The available evidence suggests that serum albumin has a single strong binding site for L-tryptophan, and that the primary binding of tryptophan derivatives occurs at this site.

The specific interaction of peptides with proteins is a phenomenon of considerable biochemical importance. One of the most striking demonstrations of such interaction is the combination of the S-protein and S-peptide of ribonuclease to regenerate enzymic activity (Richards and Vithayathil, 1959). This discovery, together with the work of Smyth *et al.* (1963) in establishing a revised amino acid sequence for the S-peptide, has been followed by the work of Hofmann's group (Finn and Hofmann, 1965), who have examined the ability of various synthetic peptides to replace the intact S-peptide in the regeneration of ribonuclease activity. It has been suggested (Hofmann, 1962) that some peptide hormones may act *in vivo* by virtue of their ability to interact specifically with "receptor" proteins and, by analogy to the case of ribonuclease-S, to cause the generation of enzymic activity. Evidence has been adduced for the specific binding of the peptide hormones oxytocin and vasopressin by a protein fraction (neurophysin) of beef posterior pituitary (for a recent report, see Ginsburg and Ireland, 1964).

From a more general point of view, the study of the specific interaction of peptides with proteins has significance for several central problems of protein chemistry. Among these are (1) the contribution of specific interaction between parts of a long peptide chain so as to confer upon it a characteristic conformation (Schellman and Schellman, 1964); (2) the specific interaction between protein (and peptide) antigens and the antibody γ-globulins elicited upon their administration to suitable animals; (3) the interaction between enzymes that act at peptide bonds and their substrates, leading to specificity of enzymic catalysis. For all these problems, more systematic quantitative studies are needed, in which the binding of synthetic peptides of known structure to well-defined proteins has been examined.

In the specific interaction of peptides with proteins, at least four types of bonding may be envisaged: (1) electrostatic interaction between oppositely charged ions; (2) interaction between apolar side-chain groups; (3) hydrogen bonding either involving side-chain groups such as those of tyrosine and glutamic acid, or involving CO–NH groups; (4) interaction between CO–NH groups and aromatic structures (Robinson and Jencks, 1965). With multifunctional peptides, it may be expected that cooperative bonding through several groups of the peptide will occur, provided the stereochemical requirements for such multifunctional binding are met by the

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