Polypeptides. Part XX.[1] The Synthesis of Some Diastereoisomeric Poly-(γ-Benzyl Glutamate)s

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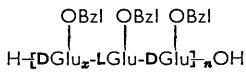
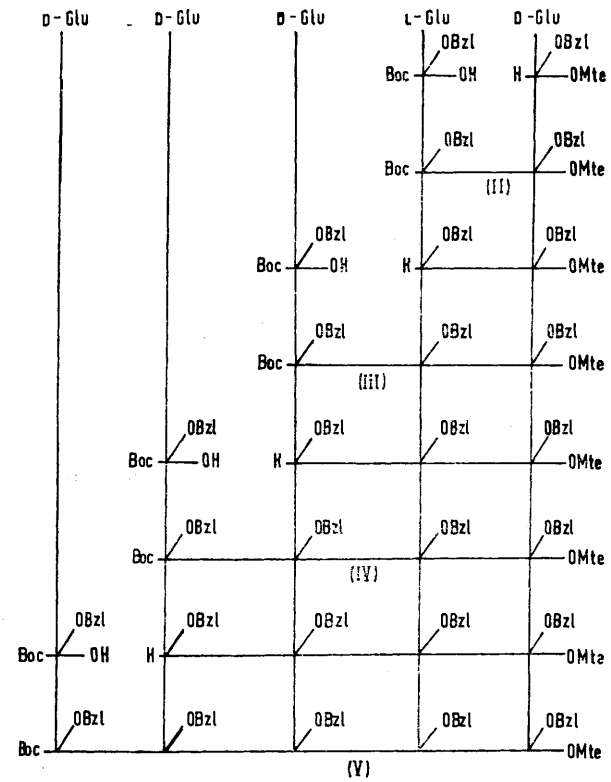
Four sequential diastereoisomeric poly-(γ-benzyl glutamate)s have been prepared by polymerisation of the corresponding oligopeptide *N*-succinimidyl esters, which were synthesised by conventional methods. The polypeptides had molecular weights (M_n) between 15,000 and 45,000.

Some years ago Marlborough and Rydon [2,3] synthesised a series of sequential diastereoisomeric poly-(γ-t-butyl glutamate)s in order to study their conformations in solution.[2,4] These polypeptides were prepared by polymerisation of the appropriate oligopeptides by means of dicyclohexylcarbodi-imide; this reaction is accompanied by substantial racemisation of the *C*-terminal residue, which complicates the interpretation of conformational studies. It has since been shown[5] that the polymerisation of oligopeptide *N*-succinimidyl esters is accompanied by little such racemisation and it therefore seemed desirable to apply this method to the preparation of another series of diastereoisomeric polyglutamic esters. Since there is a wealth of information on the conformation of poly-(γ-benzyl L-glutamate), we decided to synthesise the poly-benzyl esters (I; $x = 0$—3) rather than the t-butyl compounds; this work is described here. Our studies on the conformation of one member of the series (I; $x = 0$) have already been published; [4] similar work on the other compounds will be described elsewhere.



(I) †

The synthesis of the required fully protected oligopeptides is set out in the Scheme. The t-butoxycarbonyl group was used throughout for *N*-protection and all the couplings were carried out with dicyclohexylcarbodi-imide. Transpeptidation during the removal of the carboxy-protecting group from the *C*-terminal residue was avoided by protection as the 2-methylthioethyl



Scheme Synthesis of fully protected oligopeptides

† Abbreviations for amino-acids, *etc.*, are those recommended in I.U.P.A.C. Information Bulletin No. 26. Mte = $CH_2{\cdot}CH_2{\cdot}SMe$; Mse = $CH_2{\cdot}CH_2{\cdot}SO_2{\cdot}CH_3$; Su = $-N{\cdot}CO{\cdot}[CH_2]_2{\cdot}CO$.

[1] Part XIX, Akhtar Ali, P. M. Hardy, and H. N. Rydon, *J.C.S. Perkin I*, 1972, 1070.

[2] D. I. Marlborough and H. N. Rydon in 'Some Newer Physical Methods in Structural Chemistry,' ed. R. Bonnett and J. G. Davis, United Trade Press, London, 1967, p. 211.

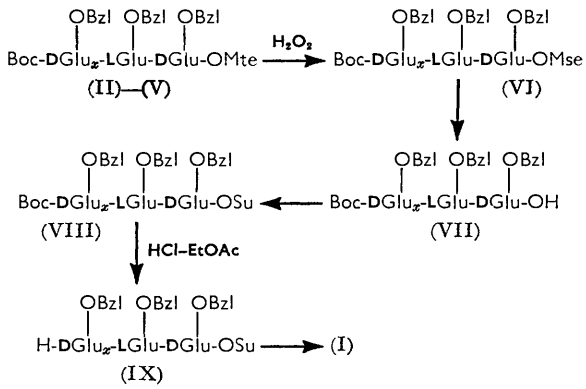
[3] D. I. Marlborough and H. N. Rydon, *J.C.S. Perkin I*, 1972, 1.

[4] P. M. Hardy, J. C. Haylock, D. I. Marlborough, H. N. Rydon, H. T. Storey, and R. C. Thompson, *Macromolecules*, 1971, **4**, 435.

[5] P. M. Hardy, H. N. Rydon, and R. C. Thompson, *J.C.S. Perkin I*, 1972, 5.

ester;[6] the ester grouping was eventually removed by oxidation to the 2-methylsulphonylethyl ester and fission at pH 10—10·5 at room temperature.[5,7] The yields throughout were satisfactory, but the fully protected oligopeptides (II)—(V), like other peptide *N*-t-butoxycarbonyl derivatives and 2-methylthioethyl esters, were reluctant to crystallise; only the two smaller peptides were obtained in crystalline form.

As in the earlier work[5] the fully protected oligopeptide *N*-succinimidyl esters (VIII) were prepared from the methylthioethyl esters by way of the corresponding acids (VII), which were coupled with *N*-hydroxysuccinimide by use of dicyclohexylcarbodi-imide; the yields were generally satisfactory throughout and the intermediate acids (VII) and fully protected succinimidyl esters (VIII) were crystalline solids.



Preliminary experiments having shown that the hydrochlorides of the succinimidyl esters (IX) were too unstable for satisfactory purification, no attempt was made to isolate them. Instead, they were suspended in sufficient chloroform to give 2M-solutions of the free esters (IX), and treated with 1 equiv. of triethylamine; the polymerisations were allowed to proceed at room temperature for 6 days, more solvent being added after 4 days. Chloroform was chosen as polymerisation solvent since preliminary experiments with the two smallest oligopeptides (IX; $x = 0$ and 1) had shown that this gave more polymer and less cyclic material than the other solvents investigated (light petroleum, benzene, tetrahydrofuran, dimethylformamide, and dimethyl sulphoxide).

The crude polymerisation products were freed from material of low molecular weight (largely cyclic) by Soxhlet extraction with methanol. In the case of the polydipeptide (I; $x = 0$) it was necessary to supplement this by fractional precipitation from chloroform with methanol. As in earlier work[1,3,5] the molecular weights, M_w and M_n, of the polymers were determined by gel-filtration on Sephadex G150, after conversion into the water-soluble polyglutamic acids by treatment with hydrogen bromide in acetic acid. The values recorded in the Table are for the poly-esters and have been calculated on the assumption that no degradation accompanied the debenzylation process. In three cases (I; $x = 0$, 2, and 3) this was confirmed by direct determination of M_n for the poly-esters by osmotic pressure

Molecular weights of sequential polypeptides

Polypeptide	Gel filtration M_w	M_n	M_w/M_n	n	Osmotic pressure M_n
H-[LGlu(OBzl)-DGlu(OBzl)]$_n$OH	28,300	23,400	1·21	53	21,000
H-[DGlu(OBzl)-LGlu(OBzl)-DGlu(OBzl)]$_n$OH	37,200	28,300	1·32	43	45,500
H-[DGlu$_2$(OBzl)-LGlu(OBzl)-DGlu(OBzl)]$_n$OH	18,400	15,500	1·19	18	15,000
H-[DGlu$_3$(OBzl)-LGlu(OBzl)-DGlu(OBzl)]$_n$OH	22,900	18,400	1·25	17	18,500

measurement; in the case of the polytripeptide (I; $x = 1$) osmotic pressure measurement gave a much higher value and it must be presumed that in this case treatment with hydrogen bromide causes considerable degradation, although it is not clear why this should be.

Since we had hardly sufficient material for conformational studies, and since it had been shown in other series[1,5] that the succinimidyl ester method of polymerisation caused negligible racemisation, we carried out no direct racemisation tests. It is reasonable to suppose that the random coil form of the polydipeptide (I; $x = 0$) will have a zero or vanishingly small optical rotatory power. Paradoxically, therefore, the optical rotatory power of this compound, in this conformation, will be a direct measure of its degree of racemisation. Measurements on solutions of (I; $x = 0$) in trifluoroacetic acid showed $[\alpha]$ to be 2% of that of poly-(γ-benzyl L-glutamate) in the same solvent, over the wavelength range 270—590 nm; we conclude, therefore, that this compound is 2% racemised. This method is not applicable to the other polypeptides, but there is no reason to suppose that the racemisation accompanying their formation is substantially different.

EXPERIMENTAL

The purity of all compounds was confirmed by t.l.c. on Kieselgel G, usually in two solvent systems. Compounds with free amino-groups were located by spraying with 0·3% ninhydrin in n-butanol and heating at 100° for 10 min, and *N*-acyl compounds by the chlorine–starch–iodide method.[8]

Organic solutions were dried over magnesium sulphate and evaporated or concentrated under reduced pressure in a rotary evaporator at the lowest practicable temperature. Light petroleum was the fraction of b.p. 60—80°. Optical rotations were measured with a Bendix-NPL Polarimeter, model 143 (path length 1 or 2 cm), except for those of the polymers, which were measured on a Bendix–Ericsson

[6] M. J. S. A. Amaral, G. C. Barrett, H. N. Rydon, and J. E. Willett, *J. Chem. Soc.* (*C*), 1966, 807.

[7] P. M. Hardy, H. N. Rydon, and R. C. Thompson, *Tetrahedron Letters*, 1968, 2525.

[8] H. N. Rydon and P. W. G. Smith, *Nature*, 1952, **169**, 922.

Polarmatic Recording Spectropolarimeter (path length 1 mm).

Synthesis of Oligopeptides

γ-Benzyl *N*-t-butoxycarbonyl-D- and -L-glutamates were prepared by the following simplified procedure.

γ-Benzyl D- or L-glutamate [9] (49·4 g, 0·21 mol) and t-butyl 2,4,5-trichlorophenyl carbonate [10] (68·0 g, 0·23 mol) were stirred for 4 days at room temperature in a mixture of dimethylformamide (400 ml), water (60 ml), and triethylamine (80 ml). The mixture was then evaporated and the residue treated with water (500 ml) and ether (500 ml). The mixture was shaken vigorously while the pH was adjusted to 8·5—9 with 2M-sodium hydroxide. The ether layer was separated and the aqueous layer extracted with ether (2 × 500 ml). The aqueous residue was then brought to pH 2 with M-hydrochloric acid and the product extracted with ether (3 × 500 ml); these extracts were combined, washed with saturated brine (2 × 500 ml), dried, and evaporated. Trituration of the residue with light petroleum and recrystallisation from ether–light petroleum at 0° gave the product (65 g, 92%), m.p. 65—66°, $[\alpha]_D^{25}$ ±13·4° (*c* 6·0 in $CHCl_3$) (Found: C, 60·5; H, 6·7; N, 4·3. Calc. for $C_{17}H_{23}NO_6$: C, 60·5; H, 6·9; N, 4·2%) (lit.,[4] m.p. 65—66°, $[\alpha]_D^{25}$ −13·2° for the D-isomer).

Preparation of N-*Butoxycarbonylpeptide* 2-*Methylthioethyl Esters*.—The appropriate *N*-butoxycarbonyl-amino-acid or -peptide α-2-methylthioethyl ester (0·03 mol) was dissolved in ethyl acetate (300 ml) and treated with 3·5M-hydrogen chloride in ethyl acetate (300 ml). After 1 h at room temperature, the solution was evaporated and the clear oily residue was kept overnight in a vacuum desiccator over sodium hydroxide. This hydrochloride was dissolved in acetonitrile (50 ml). γ-Benzyl *N*-t-butoxycarbonylglutamate (10·1 g, 0·03 mol) and triethylamine (4·17 ml, 0·03 mol) were added and the mixture was kept overnight at room temperature. Acetic acid (0·1 ml) was added and the mixture was stirred for 15 min, filtered, and evaporated. The oily residue was taken up in acetone (10 ml) and kept overnight at 4°. The solution was filtered (kieselguhr) and evaporated. The residue was dissolved in ethyl acetate (200 ml), washed successively with M-hydrochloric acid (3 × 150 ml), saturated brine (150 ml), saturated sodium hydrogen carbonate (3 × 150 ml), and saturated brine (150 ml), dried, and evaporated. The residual oil was triturated with light petroleum or di-isopropyl ether and the solid was recrystallised. The following were prepared in this way: *di-γ-benzyl* α-2-*methylthioethyl* N-t-*butoxycarbonyl*-L-*glutamyl*-D-*glutamate* (II) (77%), m.p. 59—61° (from di-isopropyl ether containing 2% of acetone), $[\alpha]_D^{23}$ −8·3° (*c* 5·7 in $CHCl_3$) (Found: C, 61·3; H, 6·6; N, 4·6. $C_{32}H_{42}N_2O_9S$ requires C, 61·0; H, 6·7; N, 4·4%); *tri-γ-benzyl* α-2-*methylthioethyl* N-t-*butoxycarbonyl*-D-*glutamyl*-L-*glutamyl*-D-*glutamate* (III) (67%), m.p. 61·5—63·5° (from ether at 0°), $[\alpha]_D^{23}$ −4·3° (*c* 5·7 in $CHCl_3$) (Found: C, 62·5; H, 6·7; N, 4·9. $C_{44}H_{55}N_3O_{12}S$ requires C, 62·2; H, 6·5; N, 4·9%).

Tetra-γ-benzyl α-2-methylthioethyl *N*-t-butoxycarbonyl-di-D-glutamyl-L-glutamyl-D-glutamate (IV) and penta-γ-benzyl α-2-methylthioethyl *N*-t-butoxycarbonyltri-D-glutamyl-L-glutamyl-D-glutamate (V), obtained in almost theoretical yield, were oils which could not be induced to crystallise.

Preparation of N-*Butoxycarbonylpeptide* 2-*Methylsulphonylethyl Esters* (VI).—The 2-methylthioethyl ester (0·02 mol), in AnalaR acetone (200 ml), was treated with aqueous 0·3M-ammonium molybdate (8·6 ml) and hydrogen peroxide (30% w/v; 50 ml). After 2 h at room temperature, the acetone was evaporated off at room temperature, water (75 ml) was added, and the mixture was extracted with chloroform (3 × 100 ml). The combined extracts were washed with brine (3 × 150 ml), dried, and evaporated.

Tri-γ-benzyl α-2-*methylsulphonylethyl* N-t-*butoxycarbonyl*-D-*glutamyl*-L-*glutamyl*-D-*glutamate* (VI; *x* = 1), so obtained in 90% yield, had m.p. 48—49°, $[\alpha]_D^{19}$ −7·5° (*c* 4·9 in $CHCl_3$) (Found: C, 59·9; H, 6·4; N, 4·7. $C_{44}H_{55}N_3O_{14}S$ requires C, 59·9; H, 6·3; N, 4·8%); the other three esters, obtained in 75—95% yield, could not be crystallised and were used directly in the next stage.

Preparation of Benzyl N-*t-Butoxycarbonylpolyglutamyl-glutamates* (VII).—The α-2-methylsulphonylethyl ester (20 mmol) was dissolved in a mixture of AnalaR acetone (200 ml) and water (100 ml). The apparent pH (glass electrode) was brought to 10·5 and kept between 10·0 and 10·5 by dropwise addition of aqueous 0·5M-sodium hydroxide, added by means of an autotitrator. After the theoretical amount of alkali had been added (12—15 h), the pH was brought to 7 with M-hydrochloric acid and the acetone evaporated off. The residue was taken up in cold M-hydrochloric acid (500 ml) and extracted twice with ethyl acetate. The combined extracts were washed with brine, dried, and evaporated. The following were prepared in this way.

Di-γ-benzyl N-t-*butoxycarbonyl*-L-*glutamyl*-D-*glutamate* (VII; *x* = 0). The crude product was decolourised by boiling in methanol (55 ml) with charcoal. Filtration and evaporation, followed by trituration with di-isopropyl ether and two recrystallisations from ethyl acetate–light petroleum, gave the pure *peptide* (6·75 g, 60%), m.p. 89—90° $[\alpha]_D^{23}$ −10·8° (*c* 4·7 in $CHCl_3$) (Found: C, 63·0; H, 6·7; N, 5·4. $C_{29}H_{36}N_2O_9$ requires C, 62·6; H, 6·5; N, 5·0%).

Tri-γ-benzyl N-t-*butoxycarbonyl*-D-*glutamyl*-L-*glutamyl*-D-*glutamate* (VII; *x* = 1) obtained in 91% yield by trituration of the crude oily product with di-isopropyl ether, could not be recrystallised but was chromatographically pure; m.p. 51—53°, $[\alpha]_D^{21}$ −13·0° (*c* 4·9 in $CHCl_3$) (Found: C, 63·5; H, 6·4; N, 5·6. $C_{41}H_{49}N_3O_{12}$ requires C, 63·5; H, 6·4; N, 5·4%).

Tetra-γ-benzyl N-*t-butoxycarbonyldi*-D-*glutamyl*-L-*glutamyl*-D-*glutamate* (VII; *x* = 2). The crude product (9·6 g, 78%), shown by t.l.c. to contain some starting material, was dissolved in ethyl acetate (60 ml) and treated with dicyclohexylamine (2·1 g). The precipitated oil solidified on trituration with ether; recrystallisation from ethyl acetate–light petroleum gave the pure salt, m.p. 85—86°. This was suspended in ethyl acetate (400 ml) and shaken twice with M-hydrochloric acid (400 ml). The organic layer was washed thrice with brine (400 ml), dried, and evaporated. Trituration with light petroleum gave the pure *peptide* (8·15 g, 66%), m.p. 99—101°, $[\alpha]_D^{24}$ +13·8° (*c* 5·1 in $CHCl_3$) (Found: C, 63·6; H, 6·2; N, 6·1. $C_{53}H_{62}N_4O_{15}$ requires C, 64·0; H, 6·3; N, 5·6%).

Penta-γ-benzyl N-*t-butoxycarbonyltri*-D-*glutamyl*-L-*glutamyl*-D-*glutamate* (VII; *x* = 3), obtained in 21% yield by trituration of the crude product with diethyl ether–di-isopropyl ether followed by recrystallisation from ethyl acetate–di-isopropyl ether, had m.p. 138—139° (Found: C, 64·7; H, 6·3; N, 5·8%. $C_{65}H_{75}N_5O_{18}$ requires C, 64·3; H, 6·2; N, 5·8%).

[9] S. Guttmann and R. A. Boissonas, *Helv. Chim. Acta*, 1958, **41**, 1852.

[10] W. Broadbent, J. S. Morley, and B. E. Stone, *J. Chem. Soc.* (*C*), 1967, 2632.

Preparation of Benzyl Succinimidyl N-Butoxycarbonyl-polyglutamylglutamates (VIII).—The benzyl ester (10 mmol) was dissolved in dichloromethane (25 ml) and the solution cooled to −5°. Dicyclohexylcarbodi-imide (2·3 g, 11 mmol) was added and the mixture was stirred at −5° for 10 min, after which *N*-hydroxysuccinimide (2·3 g, 20 mmol) was added. The mixture was stirred overnight at room temperature; acetic acid (0·05 ml) was then added and stirring was continued for a further 15 min. The mixture was filtered (kieselguhr) and the filtrate made up to 200 ml with dichloromethane. The solution was washed with brine (3 × 150 ml), dried, and evaporated. The residue was kept overnight at −10° in acetone (30 ml), and again filtered and evaporated. The following were prepared in this manner: *di-γ-benzyl* α-N-*succinimidyl* N-*t-butoxycarbonyl*-L-*glutamyl*-D-*glutamate* (VIII; $x = 0$) (76%), m.p. 112—113° (from ethyl acetate–light petroleum), $[\alpha]_D^{24}$ +5·9° (*c* 4·4 in $CHCl_3$) (Found: C, 61·1; H, 6·2; N, 6·7. $C_{33}H_{39}N_3O_{11}$ requires C, 60·6; H, 6·0; N, 6·4%); *tri-γ-benzyl* α-N-*succinimidyl* N-*t-butoxycarbonyl*-D-*glutamyl*-L-*glutamyl*-D-*glutamate* (VIII; $x = 1$) (70%), m.p. 116—118° (from ethyl acetate–di-isopropyl ether), $[\alpha]_D^{20}$ −0·9° (*c* 4·3 in $CHCl_3$) (Found: C, 61·8; H, 6·0; N, 6·6. $C_{45}H_{52}N_4O_{14}$ requires C, 61·9; H, 6·0; N, 6·4%); *tetra-γ-benzyl* α-N-*succinimidyl* N-*t-butoxycarbonyldi*-D-*glutamyl*-L-*glutamyl*-D-*glutamate* (VIII; $x = 2$) (89%), m.p. 38—40° (by trituration with light petroleum) (Found: C, 62·7; H, 6·1; N, 6·4. $C_{57}H_{65}N_5O_{17}$ requires C, 62·7; H, 6·0; N, 6·4%); *penta-γ-benzyl* α-N-*succinimidyl* N-*t-butoxycarbonyltri*-D-*glutamyl*-L-*glutamate* (VIII; $x = 3$) (94%), m.p. 88—90° (by trituration with ether) (Found: C, 63·5; H, 6·2; N, 6·8. $C_{69}H_{78}N_6O_{20}$ requires C, 63·2; H, 6·0; N, 6·4%).

Preparation and Properties of Polymers

Poly-(γ-benzyl-L-*glutamyl-γ-benzyl*-D-*glutamate)* (I; $x = 0$).—Di-γ-benzyl α-*N*-succinimidyl *N*-t-butoxycarbonyl-L-glutamyl-D-glutamate (7·01 g, 10·7 mmol) in ethyl acetate (100 ml) was treated with 3·5M-hydrogen chloride in ethyl acetate (100 ml). After 1 h at room temperature the solution was evaporated and the residue dissolved in ethyl acetate (30 ml). Addition of light petroleum (100 ml), centrifugation, and washing the solid with ether (3 × 100 ml) by centrifugation, gave the hydrochloride of the succinimidyl ester (IX; $x = 0$) (5·82 g, 92%), which was dried overnight in a vacuum desiccator (NaOH). Triethylamine (1·38 ml, 9·9 mmol) was added to this hydrochloride (9·9 mmol) in chloroform (5·25 ml) and the mixture was stirred with a spatula for a few min. After 4 days in a stoppered tube at room temperature, chloroform (25 ml) was added, followed by a further 25 ml after 2 more days. The chloroform solution was then washed with water (4 × 100 ml), dried, and evaporated. After being dried (P_2O_5) in a vacuum desiccator, the crude polymer (3·46 g, 79%) was extracted (Soxhlet) with methanol (125 ml) for 24 h and redried (P_2O_5); yield 1·85 g (42%).

To this polymer (1·1 g), dissolved in chloroform (55 ml), methanol (46·5 ml) was added, with stirring, during 45 min. Next day the solid was collected by centrifugation and washed with 1 : 1 chloroform–methanol (10 ml). The solid product was again precipitated from chloroform (30 ml) with methanol (32·2 ml), washed, and dried. The pure *polymer* (220 mg) so obtained had $[\alpha]_D^{24}$ +8·4° (*c* 3·2 in $CHCl_3$), −2·1° (*c* 2·7 in $CF_3{\cdot}CO_2H$) [Found: C, 65·9; H, 6·6; N, 6·4. $(C_{12}H_{13}NO_3)_n$ requires C, 65·7; H, 6·0; N, 6·4%].

Poly-(γ-benzyl-D-*glutamyl-γ-benzyl*-L-*glutamyl-γ-benzyl*-D-*glutamate)* (I; $x = 1$).—Tri-γ-benzyl α-*N*-succinimidyl D-glutamyl-L-glutamyl-D-glutamate (1·218 g, 1·4 mmol) in ethyl acetate (10 ml) was treated with 3·5M-hydrogen chloride in ethyl acetate (10 ml). After 1 h at room temperature light petroleum (50 ml) was added and the precipitated hydrochloride was collected by centrifugation, washed by trituration with ether (3 × 50 ml), and dried in a vacuum desiccator (NaOH). This hydrochloride (963 mg) was polymerised in chloroform (0·7 ml) as already described. The crude product was extracted (Soxhlet) with methanol (125 ml) for 30 h and dried (P_2O_5) in a vacuum desiccator. The pure *polypeptide* (419 mg, 54%) so obtained had $[\alpha]_D^{24}$ −37·5° (*c* 0·64 in $CHCl_3$), +12·5° (*c* 1·0 in $CF_3{\cdot}CO_2H$) (Found: C, 66·2; H, 6·1; N, 6·6%).

Poly[di-(γ-benzyl-D-*glutamyl)-γ-benzyl*-L-*glutamyl-γ-benzyl*-D-*glutamate]* (I; $x = 2$) (26%), $[\alpha]_D^{25}$ −27·1° (*c* 1·0 in $CHCl_3$), +10·8° (*c* 1·0 in $CF_3{\cdot}CO_2H$) (Found: C, 64·9; H, 6·2; N, 6·6), and *poly[tri-(γ-benzyl*-D-*glutamyl)-γ-benzyl*-L-*glutamyl-γ-benzyl*-D-*glutamate]* (I; $x = 3$) (58%) $[\alpha]_D^{25}$ −24·6° (*c* 0·65 in $CHCl_3$), +17·9° (*c* 0·91 in $CF_3{\cdot}CO_2H$) (Found: C, 65·9; H, 6·0; N, 6·4%), were prepared in similar fashion.

Molecular Weights.—(a) *By gel filtration.*—The procedure was that described previously;[5] the Sephadex G150 column (335 × 25 mm) was calibrated with poly-L-glutamic acid (M_n 37,000), copoly-(L-leucine, D-glutamic acid) (M_n 20,000), and poly-L-aspartic acid (M_n 12,500). Weight average molecular weights, M_w, were computed from plots of optical density against molecular weight, and number average molecular weights, M_n, from plots of optical density divided by molecular weight against molecular weight. The results, given in the Table, are for the poly-benzyl esters and were calculated from the experimental results for the poly-acids, on the assumption that no degradation took place during the debenzylation with hydrogen bromide in acetic acid.

(b) *By osmotic pressure.* Osmotic pressures of solutions (3—10 g l^{-1}) of the poly-benzyl esters in tetrachloroethane were measured at 37° with a Hewlett–Packard 501 High Speed Membrane Osmometer; the molecular weights were calculated from the osmotic pressures extrapolated to zero concentration.

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