Polypeptides. Part XVIII.[1] Syntheses of Poly-(β-aspartic acid) and Poly-(γ-glutamic acid) and their Benzyl Esters

By **P. M. Hardy, J. C. Haylock,** and **H. N. Rydon,*** Department of Chemistry, The University, Exeter EX4 4QD

Poly-(α-benzyl L-aspartate) has been prepared by the polymerisation of α-benzyl β-*N*-succinimidyl L-aspartate and converted into poly-(β-L-aspartic acid) by hydrogenolysis; treatment of the poly(benzyl ester) with hydrogen bromide in acetic acid leads to a different polymer containing a substantial proportion of aspartimide residues. The analogous route cannot be used for the preparation of poly-(α-benzyl L-glutamate), which was best prepared by the polymerisation of αα'-dibenzyl γ-*N*-succinimidyl γ-L-glutamyl-L-glutamate; poly-(γ-L-glutamic acid) was obtained from poly-(α-benzyl L-glutamate) by the action of hydrogen bromide in acetic acid. The poly-acids had molecular weights of about 11,000 and no racemisation accompanied the polymerisations.

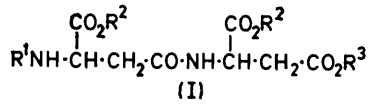
SOME years ago one of us [2] studied the optical rotatory dispersion of poly-(γ-D-glutamic acid) from *Bacillus anthracis* and concluded that it possessed an ordered, helical structure in solution in the un-ionised state. It seemed of interest to extend this study to the lower homologue, poly-(β-L-aspartic acid), and also to a synthetic specimen of poly-(γ-L-glutamic acid). Furthermore, since the side-chain carboxy-groups were thought to play a major part in stabilising the ordered structure of poly-(γ-glutamic acid), it was also desirable to investigate the conformations of the benzyl esters of the two poly-acids. The present paper describes the preparation of these polymers: the study of their conformations in solution will be the subject of a later paper.[3]

The synthesis, and some physical properties, of poly-(β-L-aspartic acid) were described by Kovács *et al.*;[4,5] the properties of this material were markedly different from those of our product and the reasons for this are discussed below. Owing no doubt to its immunological interest, much more work has been done on the synthesis of poly-(γ-glutamic acid). The D-compound was first synthesised by Waley [6] and this compound and several of its diastereoisomerides have since been synthesised by Bruckner and Kovács and their colleagues.[7-11] The molecular weights of most of these products were probably rather low,[8,9] whereas we hoped to obtain products with molecular weights in the region of 10,000.

Our initial attempts to synthesise poly-(β-L-aspartic acid) and its esters were all based on the polymerisation of β-dipeptides (I; $R^1 = R^3 = H$) with dicyclohexylcarbodi-imide. In all, three combinations of the protecting groups were investigated, the requisite fully protected dipeptides being prepared by dicyclocarbodiimide coupling of the appropriate carboxy- and amino-components. In the first series the synthesis was

[1] Part XVII, P. M. Hardy, H. N. Rydon, and R. C. Thompson, *J.C.S. Perkin I*, 1972, 5.
[2] H. N. Rydon, *J. Chem. Soc.*, 1964, 1328.
[3] P. M. Hardy, J. C. Haylock, and H. N. Rydon, in preparation.
[4] J. Kovács, R. Ballina, R. L. Rodin, D. Balasubramanian, and J. Applequist, *J. Amer. Chem. Soc.*, 1965, **87**, 119.
[5] J. Kovács and R. L. Rodin, *J. Org. Chem.*, 1968, **33**, 2418.
[6] S. G. Waley, *J. Chem. Soc.*, 1955, 517.
[7] V. Bruckner, M. Kajtár, J. Kovács, H. Nagy, and J. Wein. *Tetrahedron*, 1958, **2**, 211.
[8] V. Bruckner and M. Kajtár, *Acta Chim. Acad. Sci. Hung.*, 1959, **21**, 417.
[9] M. Kajtár and V. Bruckner, *Acta Chim. Acad. Sci. Hung.*, 1969, **62**, 191.
[10] M. Hollósi, M. Kajtár, and V. Bruckner, *Acta Chim. Acad. Sci. Hung.*, 1969, **62**, 305.
[11] J. Kovács, G. N. Schmit, and B. J. Johnson, *Canad. J. Chem.*, 1969, **47**, 3670.

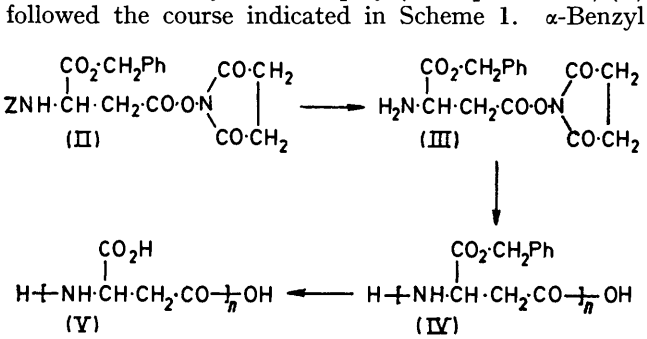
thwarted by the total failure to remove the methyl ester group by saponification from compound (I;



(I)

$R^1 = Z$, $R^2 = Bu^t$, $R^3 = Me$) * or its hydrogenolysis product (I; $R^1 = H$, $R^2 = Bu^t$, $R^3 = Me$) without simultaneous attack on the t-butyl ester group; similar anomalous alkali-sensitivity of t-butyl esters of aspartic acid derivatives has been reported by others [12,13] and it is probable that the difficulty arises owing to ready imide formation.[12] In the second series of experiments this difficulty was circumvented by using the t-butyl ester group to protect the α-carboxy-groups and the benzyl ester to protect the β-carboxy-group; hydrogenolysis of the fully protected dipeptide (I; $R^1 = Z$, $R^2 = Bu^t$, $R^3 = CH_2Ph$) gave a good yield of the di-t-butyl ester (I; $R^1 = R^3 = H$, $R^2 = Bu^t$); however, attempted polymerisation with dicyclohexylcarbodi-imide, under a variety of conditions, gave products of relatively low molecular weight, containing negligible amounts of polymer and much *N*-acylurea. In the third approach, although treatment of the fully protected dipeptide (I; $R^1 = Z$, $R^2 = Me$, $R^3 = Bu^t$) with trifluoroacetic acid followed by hydrogenolysis of the product gave a good yield of the dimethyl ester (I; $R^1 = R^3 = H$, $R^2 = Me$), treatment of this with dicyclohexylcarbodi-imide in dimethylformamide gave a mixture of four products, the most highly polymerised of which had a molecular weight of only about 2000.

Our successful synthesis of poly-(β-L-aspartic acid) (V) followed the course indicated in Scheme 1. α-Benzyl



SCHEME 1

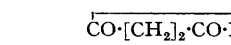
β-*N*-succinimidyl *N*-benzyloxycarbonyl-L-aspartate (II)† was prepared in good yield from α-benzyl *N*-benzyloxycarbonyl-L-aspartate and *N*-hydroxysuccinimide by the action of dicyclohexylcarbodi-imide; selective removal of the *N*-benzyloxycarbonyl group by treatment with 7% hydrogen bromide in a mixture of acetic and dichloroacetic acids [4] gave the hydrobromide of α-benzyl β-*N*-succinimidyl L-aspartate (III). Treatment of a 50% solution of this in dimethylformamide with 1 equiv. of triethylamine gave poly-(α-benzyl L-aspartate) (IV) in almost theoretical yield; after removal of small amounts of material of low molecular weight with G15 Sephadex, the polyester gave reasonably acceptable analytical figures, which suggested the retention of a little dimethylformamide. Complete acid hydrolysis showed that no racemisation accompanied the polymerisation. On the basis of the molecular weight of the derived poly-acid, this poly-ester had a molecular weight (M_n) of about 17,500. This is not greatly different from the molecular weight (10,000) reported by Kovács *et al.*[4] for poly-(α-benzyl L-aspartate) prepared by them by a very similar method using the pentachlorophenyl instead of the succinimidyl ester.

The conversion of the poly-ester (IV) into poly-(β-L-aspartic acid) (V) was not straightforward. Treatment at room temperature for 12—15 h with 45% hydrogen bromide in acetic acid, followed by dialysis against water and lyophilisation, gave a product (PAA-1) which we at first thought to be the required poly-acid. This material resembled the poly-(β-L-aspartic acid) prepared by Kovács *et al.*[4] in being laevorotatory in aqueous solution at all wavelengths from 280 to 600 nm; PAA-1 had $[\alpha]_{546}^{21.5}$ −20·2° as compared with −23° reported by Kovács for his product. In 0·1M-sodium hydroxide PAA-1 was dextrorotatory (from 300 to 600 nm), with $[\alpha]_{546}^{21.5}$ +30·3°; on neutralisation of the alkaline solution with 1 equiv. of M-hydrochloric acid, however, the original laevorotation was not restored and the solution remained dextrorotatory over the range 300—600 nm, with $[\alpha]_{546}^{21.5}$ +22·2°. Rapid titration with 0·02M-sodium hydroxide gave an acid equivalent of 152 [calc. for poly(aspartic acid), 115] for PAA-1, whereas back-titration of a solution in 0·1M-sodium hydroxide gave an acid equivalent of 126; the u.v. spectrum showed that there were no residual benzyl ester groups in PAA-1. We ascribe these anomalous findings to the formation of a substantial proportion (about 25%) of aspartimide residues [as in (VI)] during the treatment of the benzyl ester (IV) with hydrogen bromide (Scheme 2). In agreement with this interpretation, poly-(L-aspartimide) is laevorotatory,[14] and Noguchi *et al.*[15] obtained poly-(L-aspartimide) in 92% yield by treating poly-(β-benzyl L-aspartate) with hydrogen bromide in acetic acid.

Treatment of the aspartimide-containing polypeptide (VI) with alkali will generate a mixture of poly-(β-L-aspartic acid) (V) and copoly-(α,β-L-aspartic acid) (VII); although dextrorotatory the alkali-regenerated polypeptide has a lower rotatory power than pure poly-(β-L-aspartic acid) (see below) and is presumably such a copolymer, the lesser dextrorotation being due to the negative contribution of the α-L-aspartyl residues [poly-(α-L-aspartic acid) is laevorotatory in aqueous

* Here and elsewhere Z = $PhCH_2 \cdot O \cdot CO$.

† In this paper, *N*-succinimidyl refers to the group



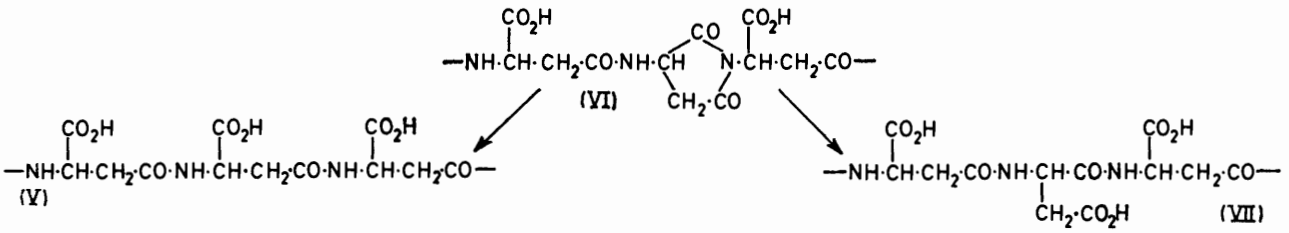
[12] R. W. Roeske, *J. Org. Chem.*, 1963, **28**, 1251.

[13] S. Bajusz, T. Lazar, and Z. Paulay, *Acta Chim. Acad. Sci. Hung.*, 1964, **41**, 329.

[14] A. J. Adler, G. D. Fasman, and E. R. Blout, *J. Amer. Chem. Soc.*, 1963, **85**, 90.

[15] J. Noguchi, T. Saito, and M. Asai, *Nippon Kagaku Zasshi*, 1960, **81**, 620 (*Chem. Abs.*, 1961, **55**, 6359b).

solution [16]]. In view of its laevorotation we believe that the poly-(β-L-aspartic acid) prepared by Kovács *et al.*,[4] like our PAA-1, contains a substantial proportion of aspartimide residues. This would also account for the anomalous titration curve recorded by Kovács *et al.*,[4] which indicates the presence of two different types of carboxy-group (pK_a 2·8 and 4·2); these could well be the



SCHEME 2

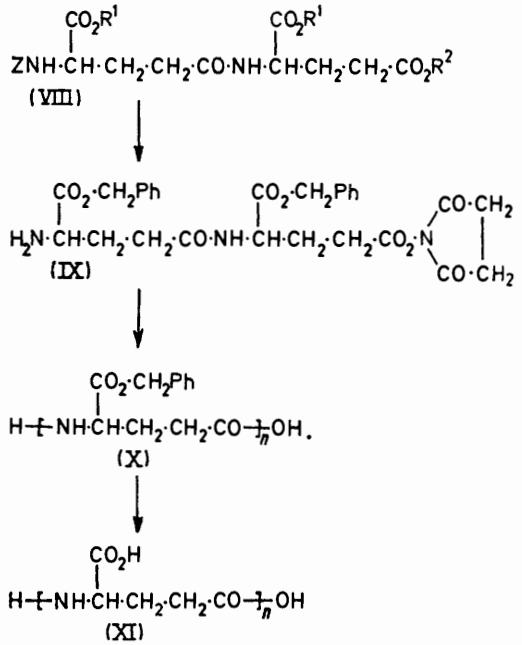
α- and β-side-chain carboxy-groups in the co-polymer (VII) formed on treatment of the original material with alkali.

Treatment of our poly-(α-benzyl L-aspartate) with sodium methoxide in chloroform, under conditions shown not to cause imide formation with the poly-β-ester,[14] gave a poly-(β-L-aspartic acid) (PAA-2) free from residual benzyl ester groups and with a satisfactory acid equivalent (126). However, the optical rotation of the complete acid hydrolysate showed the hydrolysis of the benzyl ester groups to have been accompanied by very extensive racemisation (*ca.* 90%) and the product was useless for our purpose.

A satisfactory preparation of poly-(β-L-aspartic acid) (V) was eventually achieved by catalytic hydrogenolysis of the polyester (IV) under pressure in dimethylformamide. The dialysed and lyophilised product (PAA-3), obtained in 80% yield, gave an elemental analysis and acid equivalent (130 by direct and 127 by back titration) which showed the presence of 0·7 molecule of water per aspartic acid residue; the u.v. spectrum showed the presence of about 3% of unreduced benzyl ester groups which could not be removed. This material was dextrorotatory in both water, $[\alpha]_{546}^{21\cdot5}$ +27·5°, and 0·1M-sodium hydroxide, $[\alpha]_{546}^{21\cdot5}$ +38·9°, and neutralisation of the alkaline solution gave a solution with the original optical rotation. The weight average molecular weight, M_w, determined by gel-filtration on G75 Sephadex, was 11,600, and the number average molecular weight, M_n, 9600. We believe this to be the first reported preparation of homogeneous poly-(β-L-aspartic acid) free from aspartimide or α-aspartyl residues.

As Kajtár and Bruckner [9] have pointed out, the polymerisation of a γ-active ester of an α-ester of glutamic acid is more likely to give a pyroglutamate than a polymer; in accordance with this prediction, treatment of the hydrobromide of α-benzyl γ-*N*-succinimidyl L-glutamate with triethylamine in dimethylformamide gave only benzyl L-pyroglutamate.

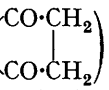
Since we had, in earlier work,[17] successfully prepared t-butyl poly-α-glutamates by the action of dicyclohexylcarbodi-imide on γγ'-di-t-butyl α-glutamyl-glutamates, we attempted to apply the same procedure to αα'-di-t-butyl γ-L-glutamyl-L-glutamate but obtained only materials of low molecular weight, as in the aspartic acid series. We therefore turned to the use of *N*-succinimidyl esters (Scheme 3). One variation of this scheme failed, owing to insufficient selectivity towards alkali of α-t-butyl and γ-methyl ester groups, saponification of the protected dipeptide (VIII; R^1 = Bu^t, R^2 = Me) giving a complex mixture of products. In the successful variation, the α-carboxy-groups were protected as their benzyl esters and the γ-carboxy-group as its



SCHEME 3

t-butyl ester. α-Benzyl *N*-benzyloxycarbonyl-L-glutamate [18] and α-benzyl γ-t-butyl L-glutamate [12] were coupled by means of dicyclohexylcarbodi-imide in dichloromethane to give a good yield of the fully protected dipeptide (VIII; R^1 = CH_2Ph, R^2 = Bu^t), from which the t-butyl group was smoothly removed by brief treatment with trifluoroacetic acid at room temperature to

[16] A. L. Jacobson, *Biopolymers*, 1965, **3**, 249.
[17] D. I. Marlborough and H. N. Rydon, *J.C.S. Perkin I*, 1972, 1.
[18] E. Klieger and H. Gibian, *Annalen*, 1962, **655**, 195.

give an almost quantitative yield of (VIII; $R^1 = CH_2Ph$, $R^2 = H$). This was converted into the *N*-succinimidyl ester (VIII; $R^1 = CH_2Ph$, $R^2 = N$ ) by treatment with *N*-hydroxysuccinimide and dicyclohexylcarbodi-imide in dioxan;[2] selective removal of the *N*-benzyloxycarbonyl group from this was effected in good yield by the action of 15% hydrogen bromide in acetic acid to give the α-benzyl γ-*N*-succinimidyl ester (IX) as its hydrobromide.

Addition of 1 equiv. of triethylamine to a 50% solution of the hydrobromide of (IX) in dimethylformamide liberated the free ester, which readily polymerised to give an almost quantitative yield of poly-(α-benzyl L-glutamate) (X). As expected, complete acid hydrolysis showed that no appreciable racemisation had accompanied the polymerisation. The poly-ester gave a satisfactory elemental analysis; its molecular weight, M_n based on that of the derived poly-acid (see below) was about 17,500.

Poly-(γ-L-glutamic acid) (XI) was prepared from its benzyl ester (X) in two ways. Catalytic hydrogenolysis in hexafluoroacetone sesquihydrate gave a good yield of the poly-acid which, however, was found, by its u.v. spectrum and equivalent, to contain about 8% of residual benzyl ester groups; Hollósi *et al.*[10] found 15—20% of such groups in material prepared by hydrogenolysis in a mixture of hexamethylphosphoramide and dimethylformamide. In contrast to our experience with poly-(α-benzyl aspartate), the benzyl ester groups were smoothly removed from poly-(α-benzyl L-glutamate) (X), without any indication of imide formation, by treatment with 45% hydrogen bromide in acetic acid and this proved to be the best method for the preparation of poly-(γ-L-glutamic acid). The product, so obtained in 85% yield after dialysis against water and lyophilisation, gave analytical figures for a hemihydrate (0·5 molecule water per glutamyl residue) and was shown to be stereochemically homogeneous by determination of the rotatory power of its complete acid hydrolysate.

Our synthetic poly-(γ-L-glutamic acid) had $[\alpha]_D^{21.5}$ −27·3° (1% aqueous solution), corrected for water and ash on the basis of its elemental analysis and acid equivalent; this is in satisfactory agreement with the (similarly corrected) values reported for synthetic preparations by Bruckner *et al.*,[7] $[\alpha]_D^{20}$ −25·4°, and Kovács *et al.*, $[\alpha]_D^{23}$ −24·3° (and +25·1° for the D-enantiomer).

The values recorded [2,6,19-22] for the optical rotatory power, $[\alpha]_D$, corrected for water and ash, of poly-(D-glutamic acid) from bacterial sources range from +22° to +32°, and our value of −27·3° for the synthetic L-enantiomer lies within this range. Since it is uncertain to what extent the variations in the optical rotatory power of the natural product are real and to what extent they are due to uncertainties in the corrections, we regard the agreement between the optical rotatory power of our synthetic material and that of the natural product as satisfactory.

Our synthetic material had weight average molecular weight (M_w) by gel filtration, 11,700 and number average molecular weight (M_n) 10,200; this is lower than that of the bacterial product,[23] but higher than that claimed for previous synthetic preparations.

EXPERIMENTAL

The purity of all intermediate and end-products was confirmed by paper-chromatography (Whatman no. 1 paper) or t.l.c. (Kieselgel G), whenever possible in two solvent systems. Compounds with free α-amino-groups were revealed with 0·2% ninhydrin in n-butanol or acetone at 100°; *N*-acylated compounds were revealed by chlorine–starch–iodide.[24]

Organic solutions were dried over magnesium sulphate; evaporations and concentrations were carried out under reduced pressure. Light petroleum was the fraction b.p. 60—80°. Optical rotations were measured with a Bendix-NPL Polarimeter model 143C (path-length 1 cm).

Synthesis of Poly-(β-L-aspartic acid)

Preparation of Monomers.—α-Benzyl L-aspartate, free from the β-isomer, was best prepared by the method of Kovács *et al.*,[25] and converted into its *N*-benzyloxycarbonyl derivative by the procedure of Bryant *et al.*[26] To the latter compound (12·5 g, 35 mmol) and *N*-hydroxysuccinimide (4·0 g, 35 mmol) in dioxan (120 ml), dicyclohexylcarbodiimide (7·2 g, 35 mmol) was added, and the solution was kept overnight at room temperature. The precipitated dicyclohexylurea was then filtered off and the filtrate evaporated. Dissolution of the residue in ethyl acetate, followed by washing with M-hydrochloric acid, saturated sodium hydrogen carbonate, and water, drying, and evaporation, gave an oil which crystallised on trituration with light petroleum. Recrystallisation from ethyl acetate–light petroleum gave *α-benzyl β-N-succinimidyl N-benzyloxycarbonyl-L-aspartate* (II) (11·0 g, 69%), m.p. 84°, $[\alpha]_D^{25}$ +25·0° (*c* 2·0 in $CHCl_3$) (Found: C, 60·2; H, 4·7; N, 6·4. $C_{23}H_{22}N_2O_8$ requires C, 60·8; H, 4·9; N, 6·2%). This ester (1·0 g, 2·2 mmol) was kept for 15 min at room temperature in a 7% solution of hydrogen bromide in acetic acid–dichloroacetic acid (1 : 6; 10 ml). Addition of anhydrous ether, followed by three precipitations from trifluoroacetic acid with ether, gave *α-benzyl β-N-succinimidyl L-aspartate* (III) as the *hydrobromide* (0·77 g, 85%), m.p. 156—157°, $[\alpha]_D^{25.5}$ −5·8° (*c* 2·0 in H_2O) (Found: C, 44·9; H, 7·0; N, 4·2. $C_{15}H_{17}BrN_2O_6$ requires C, 44·4; H, 6·9; N, 4·0%).

A mixture of β-methyl *N*-benzyloxycarbonyl-L-aspartate [27] (42·2 g, 150 mmol), dichloromethane (255 ml), conc. sulphuric acid (1·5 ml), and liquid isobutene (180 ml), prepared at 0°, was kept at room temperature for 4 days in a pressure bottle. The bottle was then cooled in ice and

[19] V. Bruckner and M. K. Oskolas, *Acta Chim. Phys. Univ. Szeged.*, 1943, **1**, 144.
[20] H. Edelhoch and R. E. Lippoldt, *Biochim. Biophys. Acta*, 1960, **45**, 205.
[21] G. Ivanovics and V. Bruckner, *Z. Immunitäts.*, 1937, **90**, 304.
[22] G. Ivanovics and L. Erdös, *Z. Immunitäts.*, 1937, **90**, 5.
[23] W. E. Hanby and H. N. Rydon, *Biochem. J.*, 1946, **40**, 297.
[24] H. N. Rydon and P. W. G. Smith, *Nature*, 1952, **169**, 922.
[25] J. Kovács, H. N. Kovács, and R. Ballina, *J. Amer. Chem. Soc.*, 1963, **85**, 1839.
[26] P. M. Bryant, R. H. Moore, P. J. Pimlott, and G. T. Young, *J. Chem. Soc.*, 1959, 3868.
[27] H. Schwarz, F. M. Bumpus, and I. H. Page, *J. Amer. Chem. Soc.*, 1957, **79**, 5697.

opened, and the homogeneous solution washed twice with saturated sodium hydrogen carbonate and twice with water, dried, and evaporated. The resulting oily α-t-butyl β-methyl *N*-benzyloxycarbonyl-L-aspartate (47 g), which could not be induced to crystallise, was hydrogenated over 5% palladised charcoal (2·5 g) in 95% t-butyl alcohol (375 ml) at room temp. and 4 atm; after 3 h more catalyst (1·25 g) was added and the hydrogenation was continued for a further 3 h. The mixture was then filtered through kieselguhr and the filtrate evaporated. The residue was dissolved in anhydrous ether and ethereal hydrogen chloride added to give an apparent pH of 4. Recrystallisation from ethyl acetate–ether of the precipitate which formed on cooling and scratching gave *α-t-butyl β-methyl L-aspartate hydrochloride* (27·5 g, 83%), m.p. 144—146°, $[\alpha]_D^{21}$ +6·0° (*c* 2·2 in MeOH) (Found: C, 45·2; H, 7·7; N, 5·1. $C_9H_{18}ClNO_4$ requires C, 45·1; H, 7·5; N, 5·8%).

A further batch of α-t-butyl β-methyl *N*-benzyloxycarbonyl-L-aspartate (20·1 g, 60 mmol) was dissolved in acetone (150 ml) and shaken for 1 h at room temperature with M-sodium hydroxide (60 ml). Exactly 1 equiv. of citric acid (10% aqueous solution) was added and the acetone was removed by evaporation. The oil which separated was extracted into ethyl acetate and the extract was dried and evaporated. Dicyclohexylamine (13 ml) was added to the residue dissolved in anhydrous ether, and the mixture was shaken overnight. Recrystallisation of the precipitated solid from ethyl acetate–light petroleum gave *dicyclohexylammonium α-t-butyl N-benzyloxycarbonyl-L-aspartate* (26 g, 91%), m.p. 102—104°, $[\alpha]_D^{20}$ −5·0° (*c* 2·0 in 95% AcOH) (Found: C, 67·1; H, 9·3; N, 5·5. $C_{28}H_{44}N_2O_6$ requires C, 66·6; H, 8·8; N, 5·6%).

This salt (5·0 g, 10 mmol) and α-t-butyl β-methyl L-aspartate hydrochloride (2·4 g, 10 mmol) were separately dissolved in dichloromethane (25 ml). The solutions were mixed and shaken for 2 h, and the precipitated dicyclohexylammonium chloride was filtered off. Dicyclohexylcarbodi-imide (2·1 g, 10 mmol) was added to the filtrate and the mixture was kept at room temperature for 24 h. Dicyclohexylurea was then filtered off and the filtrate washed with saturated sodium hydrogen carbonate, M-hydrochloric acid, and water, dried, and evaporated. The residue was taken up in a little acetone; the solution was kept at 0° overnight, filtered, and evaporated. Trituration of the residue with light petroleum, followed by recrystallisation from ethyl acetate–light petroleum, gave *αα′-di-t-butyl β-methyl N-benzyloxycarbonyl-β-L-aspartyl-L-aspartate* (I; R^1 = Z, R^2 = Bu^t, R^3 = Me) (3·75 g, 75%), m.p. 89—90°, $[\alpha]_D^{21}$ +36·0° (*c* 2·2 in $CHCl_3$) (Found: C, 59·2; H, 7·0; N, 5·4. $C_{25}H_{36}N_2O_9$ requires C, 59·0; H, 7·1; N, 5·5%). A similar coupling reaction using α-t-butyl β-benzyl L-aspartate hydrochloride [12] in place of the corresponding methyl ester gave *αα′-di-t-butyl β-benzyl N-benzyloxycarbonyl-β-L-aspartyl-L-aspartate* (I; R^1 = Z, R^2 = Bu^t, R^3 = CH_2Ph) (72%), m.p. 69—70% (from ethyl acetate–light petroleum), $[\alpha]_D^{22}$ +17·0° (*c* 2·0 in 95% AcOH) (Found: C, 63·1; H, 7·0; N, 4·9. $C_{31}H_{40}N_2O_9$ requires C, 63·7; H, 6·9; N, 4·8%); hydrogenation of this compound (3·6 g) over palladised charcoal (0·5 g) in 90% t-butyl alcohol (80 ml) at 1 atm for 8 h, followed by filtration, evaporation, and recrystallisation from ethyl acetate–ether gave *αα′-di-t-butyl β-L-aspartyl-L-aspartate* (I; R^1 = R^3 = H, R^2 = Bu^t) (1·95 g, 88%), m.p. 179—180°, $[\alpha]_D^{22}$ +24·4° (*c* 4·0 in AcOH) (Found: C, 52·8; H, 7·9; N, 7·8. $C_{16}H_{28}N_2O_7$ requires C, 53·3; H, 7·8; N, 7·8%).

Coupling by the procedure described above, of dicyclohexylammonium α-methyl *N*-benzyloxycarbonyl-L-aspartate [28] and α-methyl β-t-butyl L-aspartate hydrochloride [28] gave *αα′-dimethyl β-t-butyl N-benzyloxycarbonyl-β-L-aspartyl-L-aspartate* (I; R^1 = Z, R^2 = Me, R^3 = Bu^t) (87%), m.p. 109—110° (from ethyl acetate–light petroleum), $[\alpha]_D^{22}$ +41·8° (*c* 2·3 in $CHCl_3$) (Found: C, 56·9; H, 6·6; N, 6·0. $C_{22}H_{30}N_2O_9$ requires C, 56·7; H, 6·5; N, 6·0%). This compound (2·4 g) was dissolved in trifluoroacetic acid (5 ml) and the solution kept at room temperature for 3 h, after which it was evaporated to dryness. The residue was washed well with ether, dried *in vacuo* and hydrogenated at 1 atm and room temp. for 2 h over 5% palladised charcoal (1 g) in 90% t-butyl alcohol (20 ml). Filtration and lyophilisation gave *αα′-dimethyl β-L-aspartyl-L-aspartate* (I; R^1 = R^3 = H, R^2 = Me) (1·6 g, 78%), m.p. 161—162°, $[\alpha]_D^{21}$ +56·1° (*c* 1·3 in MeOH) (Found: C, 43·1; H, 6·1; N, 10·1. $C_{10}H_{16}N_2O_7$ requires C, 43·5; H, 5·8; N, 10·1%).

Polymerisation.—Triethylamine (0·7 ml, 5 mmol) was added to a solution of α-benzyl β-*N*-succinimidyl L-aspartate hydrobromide (2·03 g, 5 mmol) in anhydrous dimethylformamide (2 ml). After shaking overnight, water was added and the precipitated polymer was collected by centrifugation, washed well with water, ethanol, and ether, and dried in a vacuum desiccator (P_2O_5). The product (1·04 g, 100%) was dissolved in dimethylformamide (70 ml) and dry G15 Sephadex was added in amount sufficient to absorb most of the liquid. Filtration with suction and evaporation of the filtrate gave a glass, which was triturated with anhydrous ether; the resulting poly-(α-benzyl L-aspartate) (IV) (0·75 g, 75%) had $[\alpha]_D^{33·5}$ −23·4° (*c* 1·16 in $CHCl_3$) [Found: C, 62·9; H, 5·3; N, 7·3. Calc. for $(C_{11}H_{11}NO_3)_n$: C, 64·4; H, 5·4; N, 6·8%].

This poly-ester (1·0 g) was shaken for 15 h at room temperature with 45% hydrogen bromide in acetic acid (20 ml). A large volume of anhydrous ether was then added and the precipitate was collected by centrifugation and washed well with ether. The product (0·59 g, 100%) was dissolved in water (50 ml) and dialysed against distilled water (500 ml) for 24 h. Lyophilisation of the dialysis residue gave poly-(β-L-aspartic acid) (PAA-1) (400 mg, 68%).

The poly-ester (213 mg, 1·1 mmol) was dissolved in chloroform (20 ml) and treated with 0·38M-sodium methoxide in methanol–benzene (1 : 3; 3·70 ml). After shaking for 2 h at room temperature, the solution was poured into anhydrous ether (100 ml). The precipitate was collected by centrifugation, dissolved in water (20 ml) and dialysed against distilled water (200 ml) for 15 h. Lyophilisation gave sodium poly-(β-L-aspartate) (84 mg, 63%). This was again dissolved in water (2 ml) and the solution passed through a column of Dowex-1 and washed through with a little more water; lyophilisation of the eluate gave poly-(β-L-aspartic acid) (PAA-2) (60 mg, 45%).

Poly-(α-benzyl L-aspartate) (620 mg) was dissolved in dimethylformamide (150 ml) and hydrogenated over 5% palladised charcoal for 3 days at 5 atm and room temp., the catalyst (500 mg) being changed three times during the course of hydrogenation. Filtration, evaporation, dissolution in water (40 ml), dialysis against distilled water (400 ml) for 24 h, and lyophilisation gave *poly-(β-L-aspartic acid)* (V) (PAA-3) (280 mg, 81%) [Found: C, 38·2; H, 4·9; N,

[28] E. Wünsch and A. Zwick, *Z. physiol. Chem.*, 1963, **333**, 108.

10·5. $(C_4H_5NO_3,0·7H_2O)_n$ requires C, 37·9; H, 5·0; N, 11·0%].

Synthesis of Poly-(γ-L-glutamic acid)

Preparation of Monomers.—The following were prepared from the starting materials indicated by coupling with dicyclohexylcarbodi-imide in dichloromethane by the method described for the analogous aspartic acid dipeptides. *αα'-Di-t-butyl γ-benzyl N-benzyloxycarbonyl-γ-L-glutamyl-L glutamate* (VIII; $R^1 = Bu^t$, $R^2 = CH_2Ph$), 50% yield from α-t-butyl γ-benzyl L-glutamate hydrochloride [12] and dicyclohexylammonium α-t-butyl *N*-benzyloxycarbonyl-L-glutamate,[18] m.p. 84—85° (from ethyl acetate–light petroleum), $[\alpha]_D^{20}$ +19·2° (*c* 1·0 in $CHCl_3$) (Found: C, 64·3; H, 7·4; N, 4·5. $C_{33}H_{44}N_2O_9$ requires C, 64·7; H, 7·2; N, 4·6%). αα'-Di-t-butyl γ-methyl *N*-benzyloxycarbonyl-γ-L-glutamyl-L-glutamate (VIII; $R^1 = Bu^t$, $R^2 = Me$), 85% yield from α-t-butyl γ-methyl L-glutamate hydrochloride (see below) and dicyclohexylammonium α-t-butyl *N*-benzyloxycarbonyl-L-glutamate,[18] m.p. 79—80° (from ethyl acetate–light petroleum), $[\alpha]_D^{23}$ −29·2° (*c* 2·0 in EtOH) {lit.,[11] m.p. 71—72°, $[\alpha]_D^{24}$ +9·9° (in CH_2Cl_2)} (Found: C, 60·6; H, 7·0; N, 5·2. Calc. for $C_{27}H_{40}N_2O_9$: C, 60·4; H, 7·5; N, 5·2%). *αα'-Dibenzyl γ-t-butyl N-benzyloxycarbonyl-γ-L-glutamyl-L-glutamate* (VIII; $R^1 = CH_2Ph$, $R^2 = Bu^t$), 82% yield from α-benzyl γ-t-butyl L-glutamate hydrochloride [12] and dicyclohexylammonium α-benzyl *N*-benzyloxycarbonyl-L-glutamate,[18] m.p. 78—80° (from ethyl acetate–light petroleum), $[\alpha]_D^{25}$ −24·0° (*c* 2·1 in MeOH) (Found: C, 66·9; H, 6·5; N, 4·3. $C_{36}H_{42}N_2O_9$ requires C, 66·9; H, 6·6; N, 4·3%).

The tri-ester (VIII; $R^1 = Bu^t$, $R^2 = CH_2Ph$) (2·04 g) was hydrogenated over 5% palladised charcoal in methanol (30 ml) for 4 h at 1 atm. and room temp. Filtration, evaporation, and recrystallisation from methanol–ether gave *αα'-di-t-butyl γ-L-glutamyl-L-glutamate* (1·2 g, 95%), m.p. 127—128°, $[\alpha]_D^{20}$ −25·7° (*c* 0·7 in $Me_2N{\cdot}CHO$) (Found: C, 55·2; H, 8·2; N, 7·0. $C_{18}H_{32}N_2O_7$ requires C, 55·7; H, 8·3; N, 7·2%).

The tri-ester (VIII; $R^1 = CH_2Ph$, $R^2 = Bu^t$) (20·0 g, 31 mmol) was kept at room temperature in trifluoroacetic acid (40 ml) for 1 h. Evaporation, at room temperature, followed by addition of anhydrous ether and recrystallisation of the precipitated solid from acetone–ether, gave *αα'-dibenzyl N-benzyloxycarbonyl-γ-L-glutamyl-L-glutamate* (VIII; $R^1 = CH_2Ph$, $R^2 = H$) (16·3 g, 90%), m.p. 148—149°, $[\alpha]_D^{25}$ −22·7° (*c* 2·2 in $Me_2N{\cdot}CHO$) (Found: C, 65·0; H, 5·8; N, 4·7. $C_{32}H_{34}N_2O_9$ requires C, 65·1; H, 5·8; N, 4·7%). Dicyclohexylcarbodi-imide (2·06 g, 10 mmol) was added to a solution of this compound (5·96 g, 10 mmol) in dioxan (40 ml), followed, after 10 min at room temperature, by *N*-hydroxysuccinimide (1·15 g, 10 mmol). Next day, the precipitated dicyclohexylurea was filtered off and the filtrate evaporated. The residue was dissolved in ethyl acetate and washed with M-hydrochloric acid, saturated sodium hydrogen carbonate, and water. Evaporation of the dried solution, trituration of the residue with light petroleum, and recrystallisation from ethyl acetate gave *αα'-dibenzyl γ-N-succinimidyl N-benzyloxycarbonyl-γ-L-glutamyl-L-glutamate* (VIII; $R^1 = CH_2Ph$, $R^2 =$ $\overline{CO{\cdot}[CH_2]_2{\cdot}CO{\cdot}N}$–) (2·6 g, 53%), m.p. 124—125°, $[\alpha]_D^{25}$ −21·7° (*c* 1·8 in $Me_2N{\cdot}CHO$) (Found: C, 62·9; H, 5·1; N, 6·1. $C_{36}H_{37}N_3O_{11}$ requires C, 62·9; H, 5·4; N, 6·1%).

[29] E. Taschner, A. Chimiak, B. Bator, and T. Sokolowska, *Annalen*, 1961, **646**, 134.

This ester (2·45 g, 5 mmol) was kept for 20 min at 0—5° with 15% hydrogen bromide in acetic acid (9 ml). Precipitation with anhydrous ether and recrystallisation of the precipitate from propan-2-ol gave *αα'-dibenzyl γ-N-succinimidyl γ-L-glutamyl-L-glutamate* (IX) as its *hydrobromide* (1·9 g, 86%), m.p. 129—131°, $[\alpha]_D^{25}$ −6·0° (*c* 5·7 in $Me_2N{\cdot}CHO$) (Found: C, 52·9; H, 5·0; N, 6·6. $C_{28}H_{32}BrN_3O_9$ requires C, 52·9; H, 5·0; N, 6·6%).

Dicyclohexylcarbodi-imide (8·8 g, 42 mmol) was added to α-benzyl *N*-benzyloxycarbonyl-L-glutamate [18] (14·5 g, 39 mmol) and *N*-hydroxysuccinimide (5·1 g, 42 mmol) dissolved in dioxan (16 ml). After 18 h at room temperature, the precipitated dicyclohexylurea was filtered off. Evaporation of the filtrate, trituration of the residue with light petroleum, and recrystallisation from ethyl acetate–light petroleum gave *α-benzyl γ-N-succinimidyl N-benzyloxycarbonyl-L-glutamate* (14·6 g, 80%), m.p. 103—104°, $[\alpha]_D^{25}$ +4·1° (*c* 4·0 in $CHCl_3$) (Found: C, 61·5; H, 5·1; N, 6·0. $C_{24}H_{24}N_2O_8$ requires C, 61·5; H, 5·2; N, 6·0%). This ester (2·0 g, 4·3 mmol) was kept for 15 min at 0—5° with 7% hydrogen bromide in acetic acid–dichloroacetic acid (1 : 6; 30 ml); precipitation with anhydrous ether followed by recrystallisation from trifluoroacetic acid–ether gave *α-benzyl γ-N-succinimidyl L-glutamate hydrobromide* (0·93 g, 52%), m.p. 135—136°, $[\alpha]_D^{25}$ −11·1° (*c* 6·3 in 10% AcOH) (Found: C, 46·3; H, 4·6; N, 6·7. $C_{16}H_{19}BrN_2O_6$ requires C, 46·3; H, 4·9; N, 6·7%).

The following procedure for the preparation of α-t-butyl γ-methyl L-glutamate was much more convenient, for large scale working, than that of Taschner *et al.*[29] γ-Methyl *N*-benzyloxycarbonyl-L-glutamate [30] (92 g, 310 mmol) was kept for 4 days at room temperature in a pressure bottle with a mixture, prepared at 0°, of liquid isobutene (320 ml), dichloromethane (215 ml), and conc. sulphuric acid (3·1 ml). The bottle was cooled to 0° and opened, and the contents were washed twice with saturated sodium hydrogen carbonate and once with water, dried, and evaporated. The resulting uncrystallisable oil (105 g) was hydrogenated in 95% t-butyl alcohol (750 ml) over 5% palladised charcoal (12 g) at room temp. and 4 atm. The mixture was filtered and the filtrate evaporated to dryness. The residual oil was dissolved in anhydrous ether and ethereal hydrogen chloride was added to bring the apparent pH to 4. Recrystallisation from ethyl acetate of the solid which slowly separated gave the diester hydrochloride (54 g, 75%), m.p. 132—133°, $[\alpha]_D^{21}$ +20·9° (*c* 2·0 in EtOH) (lit.,[26] m.p. 133—133·5°, $[\alpha]_D^{20}$ +21·7°).

Polymerisation.—Triethylamine (1·0 ml, 5·9 mmol) was added to a solution of αα'-dibenzyl γ-*N*-succinimidyl γ-L-glutamyl-L-glutamate hydrobromide (2·61 g, 5·9 mmol) in dimethylformamide (3 ml) and the mixture was shaken overnight at room temperature. Water was then added to precipitate the polymer, which was collected by centrifugation, washed well with water, ethanol, and ether, and dried *in vacuo* (P_2O_5). The product (1·74 g, 97%) was dissolved in dimethylformamide (100 ml); dry G15-Sephadex was then added in amount sufficient to absorb most of the liquid. The suspension was then filtered with suction and the filtrate evaporated to dryness, at room temperature, to give *poly-(γ-benzyl L-glutamate)* (X) as a glassy solid, which was washed by trituration with ether and dried; $[\alpha]_D^{33·5}$ −53·0° (*c* 1·2 in Me_2SO) [Found: C, 6·49; H, 5·9; N, 6·6. $(C_{12}H_{13}NO_3)_n$ requires C, 6·57; H, 5·9; N, 6·0%].

[30] W. E. Hanby, S. G. Waley, and J. Watson, *J. Chem. Soc.*, 1950, 3239.

This poly-ester (1·0 g, 4·5 mmol) was dissolved in 45% hydrogen bromide in acetic acid (20 ml) and the solution kept at room temperature for 15 h. An excess of anhydrous ether was then added and the precipitate collected by centrifugation, washed well with ether, dissolved in water (50 ml), and dialysed for 24 h against distilled water (500 ml). The dialysis residue was lyophilised to give poly-(γ-L-glutamic acid) (0·50 g, 85%), $[\alpha]_D^{21\cdot5}$ −27·3° (*c* 1·05 in H_2O), +20·1° (*c* 0·9 in H_2O with 1 equiv. NaOH) [Found: C, 43·7; H, 5·4; N, 10·3%; equiv. by titration, 141. Calc. for $(C_5H_7NO_3,0{\cdot}5H_2O)_n$: C, 43·5; H, 5·8; N, 10·1%; equiv., 138].

Molecular Weight Determinations

Aqueous solutions of the polymers were applied to a column of G75-Sephadex (45 × 1·5 cm). Elution was carried out with water, 1 ml fractions being collected. The optical density of each fraction at 215 nm was measured and plotted against the elution volume (V_e). The column was calibrated with blue dextran (for V_0), chymotrypsin, and ribonuclease; since it is now known that calibration with proteins gives falsely high values for poly-(amino-acid) molecular weights,[1] the calibration curve was corrected on the assumption that the relationship [31] between the protein and poly-(amino-acid) calibrations was the same for G-75 as for G-150 Sephadex; the resulting molecular weights are, therefore, necessarily only approximate, although we do not think they are far from the true values. The elution curve relating optical density to V_e was transformed into one relating optical density to molecular weight, from which the weight average molecular weight, M_w, was calculated; the number average molecular weight, M_n, was similarly calculated from a curve relating optical density divided by molecular weight to molecular weight.

The results are as follows:

Polymer	M_w	M_n	M_w/M_n
Poly-(β-L-aspartic acid)	11,600	9600	1·21
Poly-(γ-L-glutamic acid)	11,700	10,200	1·15

Racemisation Tests

The polymer was heated in a sealed tube at 100° with 6M-hydrochloric acid for 24 h. The optical rotation of the hydrolysate was then measured, after removal of benzyl chloride by extraction with ether if necessary, and compared with that of a similarly treated solution of the appropriate amino-acid or ester. The results were as follows:

Polymer	$[\alpha]_D$ Hydrolysate	$[\alpha]_D$ Control	Racemisation (%)
Poly-(α-benzyl L-aspartate)	+22·0°	+21·9°	0
Poly-(β-L-aspartic acid) (PAA-2)	+2·9	+24·7	88
Poly-(β-L-aspartic acid) (PAA-3)	+24·7	+24·8	0
Poly-(α-benzyl L-glutamate)	+26·6	+26·8	1
Poly-(γ-L-glutamic acid)	+26·2	+26·6	2

We thank the S.R.C. for a Research Studentship (to J. C. H.).

[1/1699 *Received*, 17*th September*, 1971]

[31] R. C. Thompson, Ph.D. Thesis, University of Exeter, 1968.