Polypeptides with a Known Repeating Sequence of Amino-acids. Synthesis of Poly-(α- and γ-L-glutamyl-L-alanylglycine)

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The preparation of two polypeptides with a known repeating sequence of amino-acids by the use of active pentachlorophenyl esters is described. Selective hydrogenolytic removal of the *N*-benzyloxycarbonyl protecting group under acidic conditions from *N*-benzyloxycarbonyl-γ-t-butyl and -α-t-butyl-L-glutamyl-L-alanylglycine pentachlorophenyl esters (III and IX) give γ-t-butyl and α-t-butyl-L-glutamyl-L-alanylglycine pentachlorophenyl ester hydrochlorides (IV and X). These active esters were polymerised on preformed monomers at a relatively high dilution to give optically pure poly-(α- and γ-L-glutamyl-L-alanylglycine) (VI and XII). These polymers had weight average molecular weights of 10,000 and 13,500 and number average molecular weights of 6700 and 8000. Optical rotatory dispersion studies at physiological pH indicate a random structure for both polymers.

LINEAR copolymers consisting of almost equimolecular amounts of glutamic acid and alanine are effective antigens in rabbits.[1] However, the sequence of the amino-acids in these copolymers was random, and it was decided to investigate the physical and serological properties of polypeptides of a known repeating sequence containing glutamyl and alanyl residues.

The synthesis of polypeptides [2-6] with a known sequence of amino-acids requires the preparation of polymerising units, the *C*-terminal ends of which have usually been activated. However it has been reported [7] that during the preparation of these units racemisation of the *C*-terminal amino-acid may occur. In order to avoid this racemisation, the activating group of the polymerising units to be described was carried by a glycyl residue.

In this Paper syntheses of the two isomeric poly-α- and γ-L-glutamyl-L-alanylglycine)s are reported.

N-Benzyloxycarbonyl-γ-t-butyl-L-glutamic acid pentachlorophenyl ester [2] was coupled to L-alanylglycine ethyl ester hydrobromide to give *N*-benzyloxycarbonyl-γ-t-butyl-L-glutamyl-L-alanylglycine ethyl ester (I). This was hydrolysed with potassium hydroxide in aqueous acetone to give *N*-benzyloxycarbonyl-γ-t-butyl-L-glutamyl-L-alanylglycine (II) as a solid. The free acid was coupled to pentachlorophenol with dicyclohexylcarbdiimide [8] to give *N*-benzyloxycarbonyl-γ-t-butyl-L-glutamyl-L-alanylglycine pentachlorophenyl ester (III). *N*-Benzyloxycarbonyl-α-t-butyl-L-glutamic acid pentachlorophenyl ester [9] was also coupled to L-alanylglycine ethyl ester hydrobromide to give *N*-benzyloxycarbonyl-α-t-butyl-γ-L-glutamyl-L-alanylglycine ethyl ester (VII). By the same methods as for the α-isomers, *N*-benzyloxycarbonyl-α-t-butyl-γ-L-glutamyl-L-alanylglycine (VIII) and *N*-benzyloxycarbonyl-α-t-butyl-γ-L-glutamyl-L-alanylglycine pentachlorophenyl ester (IX) were prepared.

The pentachlorophenyl activated esters were preferred

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[2] J. Kovacs and A. Kapoor, *J. Amer. Chem. Soc.*, 1965, **87**, 118; J. Kovacs, R. Giannotti, and A. Kapoor, *ibid.*, 1966, **88**, 2282.
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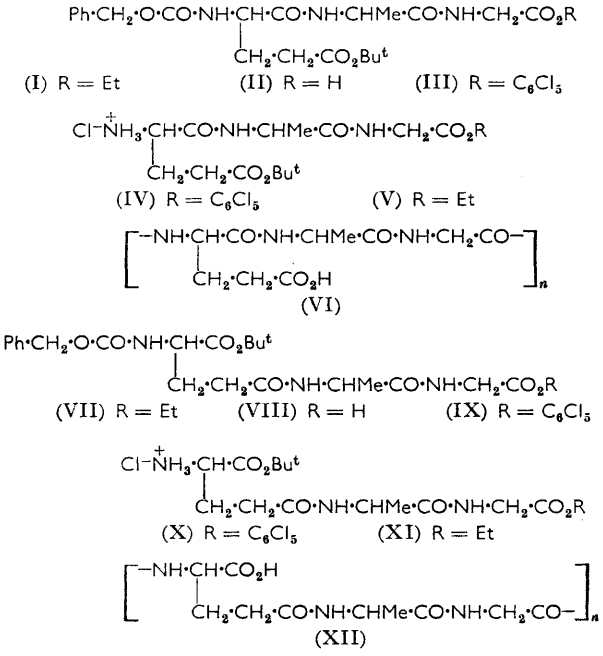
[6] F. H. S. Stewart, *Austral. J. Chem.*, 1965, **18**, 887.
[7] Delos F. Detar and R. Silverstein, *J. Amer. Chem. Soc.*, 1966, **88**, 1027; cf. ref. 2.
[8] J. C. Sheehan and G. P. Hess, *J. Amer. Chem. Soc.*, 1955, **77**, 1067.
[9] G. N. Schmidt, M.S. Thesis, St. John's University, New York, 1964.

because these activated esters invariably give higher-melting derivatives [2,10] and usually less purification problems than others.

The polymerising unit for the α-polymer, γ-t-butyl-L-glutamyl-L-alanylglycine pentachlorophenyl ester hydrochloride (IV), was prepared by hydrogenation of



the *N*-benzyloxycarbonyl-γ-t-butyl-L-glutamyl-L-alanylglycine pentachlorophenyl ester (III) with 10% palladium–carbon catalyst in the presence of one equivalent of hydrogen chloride in ethanol. The t-butyl ester was stable under these conditions. A similar procedure was used to prepare α-t-butyl-γ-L-glutamyl-L-alanylglycine pentachlorophenyl ester hydrochloride (X), the polymerising unit for the γ-polymer.

The normal method for preparing these polymers employs a high concentration of the polymerising unit in the presence of an organic base. These conditions minimise cyclisation of the polymerising unit and favour linear intermolecular polymerisation. However, these conditions could also favour the early precipitation of the polypeptide from solution. Polypeptides prepared by this method have also shown discrepancies between end-group assays and molecular weights obtained by other methods.[5,11] This deficiency of free amino-groups may be due to the formation of very large cyclic polypeptides; the growing polypeptide chain always carries the activating moiety.

To circumvent these possible defects of the normal method of preparing polypeptides, a preformed monomer was used, with the *C*-terminal end blocked so that it could act as a nucleus for growth of the polymer chain. For this purpose γ-t-butyl-L-glutamyl-L-alanylglycine ethyl ester hydrochloride (V) was prepared by hydrogenation of *N*-benzyloxycarbonyl-γ-t-butyl-L-glutamyl-L-alanylglycine ethyl ester (I) over 10% palladium–carbon, in the presence of one equivalent of hydrogen chloride.

A similar procedure was used to prepare α-t-butyl-γ-L-glutamyl-L-alanylglycine ethyl ester hydrochloride (XI) from *N*-benzyloxycarbonyl-α-t-butyl-γ-L-glutamyl-L-alanylglycine ethyl ester (VII), which was used as the nucleus for preparing the γ-polymer.

A relatively high dilution was used, so that the reaction mixture would not solidify, but could be easily stirred. Schwyzer *et al.*[12] have shown that tripeptide active esters undergo a doubling reaction to give cyclic hexapeptides. The usual concentration of reactants employed to form cyclic peptides is about 10 mmoles/l.[13] Thus it was felt justified to use conditions that would allow solution of the growing polymer, provided that the concentration of the polymerising unit was above 100 mmoles/l., thereby reducing the possibility of cyclic hexapeptide formation.

To this end, aliquot portions of γ-t-butyl-L-glutamyl-L-alanylglycine pentachlorophenyl ester hydrochloride (IV) were added to a solution of γ-t-butyl-L-glutamyl-L-alanylglycine ethyl ester hydrochloride (V) in dimethylformamide and dimethyl sulphoxide in the presence of triethylamine, to give the crude protected polymer. The action of 90% trifluoroacetic acid on this protected polymer yielded, after extensive dialysis, poly-(α-L-glutamyl-L-alanylglycine) (VI).

A similar method was used to form poly-γ-L-glutamyl-L-alanylglycine (XII) by the use of α-t-butyl-γ-L-glutamyl-L-alanylglycine ethyl ester hydrochloride (XI) and α-t-butyl-γ-L-glutamyl-L-alanylglycine pentachlorophenyl ester hydrochloride (X) as the polymerising unit. It was considered improbable that $\alpha \rightleftharpoons \gamma$ transpeptidation of the glutamyl residues had occurred during the removal of the t-butyl ester groups; Kovacs *et al.*[2,10] have shown conclusively that this does not occur with the use of 90% trifluoroacetic acid. Titration of poly(glutamylalanylglycine) with sodium hydroxide showed that all the t-butyl groups had been removed (*e.g.*, α-isomer: Equiv. 264. Calc. 266).

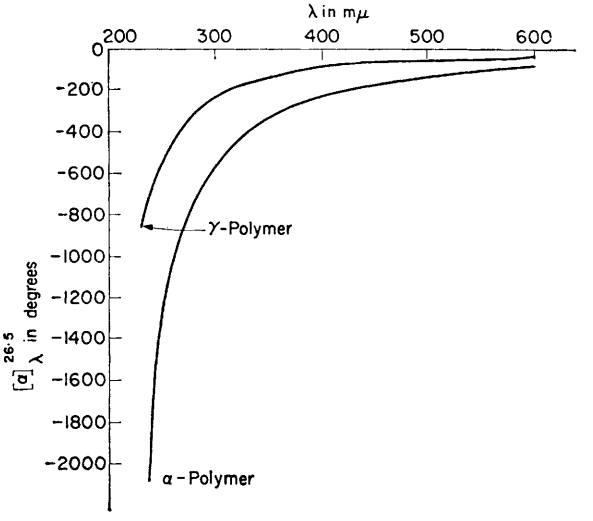
The weight average molecular weight was obtained by ultracentrifugation (see Experimental section).

No attempts were made to extrapolate to infinite dilution, and the values are therefore considered to be apparent molecular weights. The molecular weight of poly-(α-L-glutamyl-L-alanylglycine) was calculated to be approximately 10,000 and that of poly-(γ-L-glutamyl-L-alanylglycine), 13,500.

The number average molecular weight of these polymers was obtained by end-group analysis by Sanger's method.[14] This method is only applicable to synthetic

[10] J. Kovacs and B. J. Johnson, *J. Chem. Soc.*, 1965, 6777.
[11] B. J. Johnson, unpublished results.
[12] R. Schwyzer, J. P. Carrion, B. Gorup, H. Notting, and A. Tun-kyi, *Helv. Chim. Acta*, 1964, **47**, 441; R. Schwyzer and A. Tun-kyi, *ibid.*, 1962, **45**, 859; R. Schwyzer and P. Lieber, *ibid.*, 1958, **41**, 2168.
[13] E. Schroder and K. Lubke, 'The Peptides,' Academic Press, New York, 1965, p. 271.
[14] F. Sanger, *Biochem. J.*, 1945, **39**, 507.

polypeptides provided that they are linear and have no cyclic impurities, otherwise there would be a deficiency of amino-groups and high values of the molecular weight would be obtained. The α- and γ-polymers were converted to their respective DNP derivatives with a large excess of dinitrofluorobenzene. An apparent molecular weight was obtained by comparison of the absorption values at 350 mμ with those of known concentrations of DNP-glutamic acid to give molecular weights of 6100 and 8400 for the α- and γ-polymers, respectively. These values are only approximate because this method involves the comparison of the absorption of the *N*-terminal DNP-glutamyl residue of the polymer with that of DNP-glutamic acid. However, total hydrolysis * of these DNP-polymers afforded DNP-glutamic acid; comparison of its absorption with that of known concentrations of DNP-glutamic acid gave molecular weights of 6700 and 8000 for the α- and γ-polymers.



These values for the weight average molecular weight (M_w) and the number average molecular weight (M_n) are in good agreement for linear polymers with a heterogeneous distribution of molecular weights, where $M_w/M_n > 1$. This method of polymerisation at relatively high dilution has given linear polymers but in low yield. The tendency of α-tripeptide units to form cyclic hexapeptides discourages their use as polymerising units with this method, unless large quantities are available.

The structure of these polymers at physiological pH was of interest because of their design for antigenic purposes. Their optical purity was calculated to be nearly 100% by determining the optical activity of the total hydrolysate of each polymer and comparing it with that of a control. The optical rotatory dispersions of these polymers (α- and γ-) were measured at pH 7·3 ± 0·1. These results are shown in the Figure.

The dispersion data was fitted to the Drude equation: $[\alpha]_\lambda = a_c\lambda_c/(\lambda^2 - \lambda_c^2)$. The modified Drude plot [15] was used to find λ_c and a_c. The values obtained were (α-polymer) a_c — 583°; λ_c 207 mμ; and (γ-polymer) a_c — 211°, λ_c 212 mμ. Therefore the polymers had no helical structure under the conditions used.

The biological investigations will be reported in detail at a later date.

* Control experiments show that there is a loss of 5% of DNP-glutamic acid during this hydrolysis.

[15] J. T. Yang and P. Doty, *J. Amer. Chem. Soc.*, 1957, **79**, 761.

EXPERIMENTAL

Microanalyses were carried out by Dr. S. M. Nagy, Massachusetts Institute of Technology, Cambridge, Massachussetts. Melting points were taken with a Fisher-Johns apparatus. Optical rotations were taken with a Carl Zeiss precision polarimeter and the u.v. measurements with a Beckman DK2 spectrophotometer (1 cm. cells).

*N-Benzyloxycarbonyl-γ-t-butyl-*L*-glutamyl-*L*-alanylglycine Ethyl Ester* (I).—A mixture of L-alanylglycine ethyl ester hydrobromide (9·0 g., 0·0353 mole) and triethylamine (3·6 g., 1 equiv.) was added to *N*-benzyloxycarbonyl-γ-t-butyl-L-glutamic acid pentachlorophenyl ester (20 g., 0·0342 mole) in methylene chloride (250 ml.). The mixture was stirred for 24 hr., washed quickly with dilute hydrochloric acid (3 × 100 ml.), water (100 ml.), saturated sodium hydrogen carbonate solution (100 ml.), and water (2 × 100 ml.), and dried (Na_2SO_4). The solvent was removed under reduced pressure to give a solid, which was chromatographed on a silicic acid (pH 7·0) column. The pentachlorophenol was eluted with chloroform and the *tripeptide ethyl ester* (I), eluted with ethyl acetate, gave a crystalline solid (14 g., 81%), m. p. 125—126° (from ethyl acetate–hexane), $[\alpha]_D^{24}$ —13·7° (*c* 1·75 in ethyl acetate) (Found: C, 58·7; H, 7·3; N, 8·4. $C_{24}H_{35}N_3O_8$ requires C, 58·4; H, 7·15; N, 8·5%).

*N-Benzyloxycarbonyl-α-t-butyl-γ-*L*-glutamyl-*L*-alanylglycine Ethyl Ester* (VII).—A similar procedure was used to prepare this *γ-isomer* of compound (I) (80%), m. p. 126°, $[\alpha]_D$ —31·5° (*c* 1·625 in ethyl acetate), mixed m. p. with the α-isomer, 102—107° (Found: C, 58·6; H, 7·2; N, 8·5%).

*N-Benzyloxycarbonyl-γ-t-butyl-*L*-glutamyl-*L*-alanylglycine* (II).—Potassium hydroxide (0·57 g.) in water (10 ml.) was added to the tripeptide ethyl ester (I) (5·5 g., 0·0112 mole) in acetone (100 ml.). The mixture was stirred at room temperature for 45 min., evaporated under reduced pressure to a small volume, and then flooded with water (300 ml.). The alkaline solution was washed with ethyl acetate (2 × 200 ml.), acidified with dilute hydrochloric acid, and rapidly extracted with ethyl acetate (2 × 200 ml.). The ethyl acetate extracts were washed with water (2 × 150 ml.), dried (Na_2SO_4), and evaporated under reduced pressure to yield the solid *tripeptide acid* (4·8 g., 87%), m. p. 98—100° (from ethyl acetate–hexane), $[\alpha]_D^{25}$ —11·2° (*c* 1 in ethyl acetate) (Found: C, 56·9; H, 6·7; N, 9·05. $C_{22}H_{31}N_3O_8$ requires C, 56·75; H, 6·7; N, 9·0%).

*N-Benzyloxycarbonyl-α-t-butyl-γ-*L*-glutamyl-*L*-alanylglycine* (VIII).—The γ-isomer, prepared by the above method, gave white *crystals* (70%), m. p. 129—130° (from ethyl acetate–hexane), $[\alpha]_D^{24}$ —24·2° (*c* 1·55 in ethyl acetate) (Found: C, 56·5; H, 6·75; N, 8·9%).

*N-Benzyloxycarbonyl-γ-t-butyl-*L*-glutamyl-*L*-alanylglycine Pentachlorophenyl Ester* (III).—To a solution of the tripeptide free acid (II) (3 g., 0·0065 mole) and pentachlorophenol (1·72 g., 0·0065 mole) in methylene chloride (70 ml.) was added dicyclohexylcarbodi-imide (1·46 g., 0·007 mole). The mixture was stirred at room temperature for 24 hr. and the precipitate was filtered off, [$\nu_{max.}$ 1775 cm.$^{-1}$ (penta-

chlorophenyl ester)]. It was extracted into boiling methanol (300 ml. fractions); the final fractions when cooled yielded the *tripeptide pentachlorophenyl ester* (3·1 g., 67%), m. p. 174—175° (Found: C, 47·05; H, 4·2; N, 5·75. $C_{28}H_{30}Cl_5N_3O_8$ requires C, 47·1; H, 4·2; N, 5·9%).

N-*Benzyloxycarbonyl-α-t-butyl-γ-*L*-glutamyl-*L*-alanylglycine Pentachlorophenyl Ester* (IX).—Prepared like the α-isomer (66%), this *ester* had m. p. 193° (Found: C, 47·0; H, 4·3; N, 5·8%).

*γ-t-Butyl-*L*-glutamyl-*L*-alanylglycine Pentachlorophenyl Ester Hydrochloride* (IV).—Palladium–charcoal (10%; 0·5 g.) was suspended in glacial acetic acid (30 ml.) and absolute ethanol containing 0·1 g. hydrogen chloride per ml. (1·02 ml.; 1 equiv.) and hydrogenated at atmospheric pressure for 10 min. A solution of the tripeptide pentachlorophenyl ester (III) (2·0 g., 0·0028 mole) in glacial acetic acid–ethanol (40 : 60; 150 ml.) was added, and the hydrogenation was continued until no further evolution of carbon dioxide occurred. The catalyst was filtered off and the filtrate was evaporated under reduced pressure to a small volume. Addition of dry ether precipitated the *hydrochloride* (1·0 g., 58%), m. p. 196° (from ethanol–ether), $[\alpha]_D^{24}$ −21° (*c* 2·1 in methanol) (Found: C, 39·1; H, 4·3; N, 6·9. $C_{20}H_{25}Cl_6N_3O_6$ requires C, 39·0; H, 4·1; N, 6·8%).

*α-t-Butyl-γ-*L*-glutamyl-*L*-alanylglycine Pentachlorophenyl Ester Hydrochloride* (X).—A similar procedure was used to prepare the *γ-isomer* of compound (IV) (75%), $[\alpha]_D^{24}$ −24° (*c* 1·5 in methanol), m. p. 141° (Found: C, 38·8; H, 4·2; N, 6·6%).

*γ-t-Butyl-*L*-glutamyl-*L*-alanylglycine Ethyl Ester Hydrochloride* (V).—Palladium–charcoal (10%; 0·5 g.) was suspended in glacial acetic acid (4 ml.) and absolute ethanol (20 ml.) containing hydrogen chloride (0·185 g., 1 equiv.) and hydrogenated for 10 min. A solution of the tripeptide (I) (2·5 g., 0·0051 mole) in absolute ethanol (100 ml.) was added and hydrogenation was continued until there was no further evolution of carbon dioxide. The catalyst was filtered off and the filtrate evaporated under reduced pressure to give an oil which was triturated with dry ether to give the *tripeptide hydrochloride* (1·9 g., 93%), m. p. 155° (from ethanol–ether), $[\alpha]_D^{24}$ −16·2° (*c* 1·85 in methanol) (Found: C, 48·6; H, 7·5; N, 10·5. $C_{16}H_{30}ClN_3O_6$ requires C, 48·5; H, 7·6; N, 10·6%).

*α-t-Butyl-γ-*L*-glutamyl-*L*-alanylglycine Ethyl Ester Hydrochloride* (XI).—This *compound* was prepared in a similar way to (V) (83%), m. p. 98—100°, $[\alpha]_D^{24}$ −21·2° (*c* 1·65 in MeOH) (Found: C, 48·12; H, 7·6; N, 10·5%).

Poly-(α-L*-glutamyl-*L*-alanylglycine*) (VI).—To a solution of γ-t-butyl-L-glutamyl-L-alanylglycine ethyl ester hydrochloride (V) (0·026 g., 0·000065 mole) in dimethylformamide (3 ml.) and triethylamine (0·66 g., 0·0063 mole) was added γ-t-butyl-L-glutamyl-L-alanylglycine pentachlorophenyl ester hydrochloride (IV) (0·3632 g., 0·00059 mole), such that the overall concentration was 159 mmoles/litre. The solution was stirred at room temperature for 12 hr. The infrared spectrum of the mixture showed the disappearance of the pentachlorophenyl ester peak. Further additions of the tripeptide pentachlorophenyl ester (IV) were made to the mixture at 12 hr. intervals, and, to maintain a solution which could be stirred, aliquot portions of solvent were added; *i.e.*, 0·5 g. of (IV) and 1 ml. of dimethylformamide (*c* 162 mmoles/l.); 0·5 g. of (IV) (*c* 162 mmoles/l.); 0·5 g. of (IV) and 1 ml. of dimethyl sulphoxide (*c* 135 mmoles/l.); 0·5 g. of (IV) and 0·5 ml. of dimethyl sulphoxide (*c* 116 mmoles/l.); 0·68 g. of (IV) and 2 ml. of dimethyl sulphoxide (*c* 122 mmoles/l.). Finally 5 ml. of dimethyl sulphoxide was added and the syrupy solution was stirred for another 8 hr.; total quantity of pentachlorophenyl ester hydrochloride (IV) 3·043 g., total polymerising period 92 hr., total volume of dimethylformamide–dimethyl sulphoxide (5 : 9) 14 ml. The solution was diluted with ether (3 × 200 ml.), and the triturated polymer was extracted with hot ethanol (100 ml.), collected by centrifugation, and washed with ether (200 ml.), water (2 × 150 ml.), and ether (3 × 200 ml.) to give the t-butyl polymer (1·3 g.). The infrared spectrum showed no pentachlorophenyl ester band. The polymer was dissolved with difficulty in 90% trifluoroacetic acid (50 ml.), left at room temperature for 50 min., and then precipitated with ether (400 ml.), washed with ether (4 × 200 ml.), and air-dried, to give the *free polymer* (0·8 g.). The polymer was dissolved in water at pH 7. This solution was dialysed in a cellophane bag against distilled water [(5 × 1 l.) for 1 hr. and then (4 × 4 l.) for 12 hr. periods]. The pH of the polymer solution was adjusted to 2·5 and dialysis against distilled water (6 × 4 l.) continued with water changes at 12 hr. intervals, to yield, after lyophilisation, 0·22 g. of product (Found: C, 44·8; H, 6·3; N, 16·1%; Equiv. 264. $C_{10}H_{15}N_3O_5$,0·5H_2O requires C, 45·1; H, 6·1; N, 15·8%); Equiv. 266).

Poly-(γ-L*-glutamyl-*L*-alanylglycine*) (XII).—To a solution of α-t-butyl-L-glutamyl-L-alanylglycine ethyl ester hydrochloride (XI) (0·01 g., 2·5 × 10^{-7} mole) in dimethyl sulphoxide (1 ml.) and triethylamine (0·22 ml. 0·00174 mole) was added α-t-butyl-L-glutamyl-L-alanylglycine pentachlorophenyl ester hydrochloride (X) (0·4 g., 0·000649 mole), so that the overall concentration was 532 mmoles/l. The mixture was stirred at room temperature for 12 hr. More tripeptide activated ester (X) and solvent were added at 12 hr. intervals as follows: 0·4 g. of (X) and 1 ml. of dimethyl sulphoxide (*c* 294 mmoles/l.); 0·4 g. of (X) and 0·21 ml. of triethylamine (*c* 268 mmoles/l.); 0·4 g. of (X) and 0·1 ml. of triethylamine (*c* 258 mmoles/l.); 0·2 g. of (X) and 0·1 ml. of triethylamine (*c* 124 mmoles/l.). Total amount of tripeptide pentachlorophenyl ester used, 1·8 g. (0·00292 mole), over a polymerising period of 60 hr. The solution remained viscous throughout the reaction period but never solidified. The polymer was triturated with ether (300 ml.), and washed with ether (2 × 200 ml.), methanol (2 × 200 ml.), ether (2 × 200 ml.), water (2 × 200 ml.), and ether (4 × 200 ml.), to yield the protected polymer (0·5 g.). This polymer was dissolved in 90% trifluoroacetic acid (50 ml.), left at room temperature for 50 min., and then precipitated with ether (400 ml.), washed with ether (2 × 200 ml.), and air dried, to give the *free polymer*. This polymer was dissolved in water and dialysed against distilled water (8 × 4 l.), with water changes at 12 hr. intervals, to yield, after lyophilisation 0·18 g. of product (Found: C, 44·8; H, 6·1; N, 15·5%; Equiv. 263).

Optical Purity of Poly-(α-L*-glutamyl-*L*-alanylglycine*).—Poly-(α-L-glutamyl-L-alanylglycine) (VI) (0·03 g., 0·1138 mmole) was dissolved in 6N-hydrochloric acid (2 ml.) and heated under reflux at 100—110° for 24 hr. The solution was evaporated to dryness and the residue dissolved in 6N-hydrochloric acid so that the final volume was 2 ml., $[\alpha]_D^{25}$ +20·74° (calculated on the basis of the expected amounts of L-glutamic acid and L-alanine).

A control of L-glutamic acid (0·0168 g.), L-alanine (0·0102 g.), glycine (0·0086 g.), and 6N-hydrochloric acid (2 ml.) was heated simultaneously with and under the same conditions as those used for the polymer (VI). After 24 hr. the solution

was evaporated to dryness and made up to 2 ml. with 6N-hydrochloric acid, $[\alpha]_D^{25}$ +20·93°, to give an optical purity of 99·1 ± 2%.

Optical Purity of Poly-(γ-L-glutamyl-L-alanylglycine) (XII).—Determined as for the α-polymer, this was 98·7 ± 2%.

Number Average Molecular Weight (DNP Method).—To a solution of dialysed poly-γ-L-glutamyl-L-alanylglycine (XII) (0·01 g.), sodium hydrogen carbonate (0·017 g.), and 0·025M-borax (0·5 ml.) was added dinitrofluorobenzene (0·035 g.) in ethanol (2 ml.). The mixture was kept at 40° for 2 hr. and then evaporated under reduced pressure to dryness. The residue was dissolved in water (10 ml.) and extracted with ether (3 × 20 ml.). The aqueous layer was acidified with 3N-hydrochloric acid (15 ml.) and extracted with ether (3 × 25 ml.). The acid solution was made up to 50 ml. with 3N-hydrochloric acid. The absorbance of this solution was 0·34 at 350 mμ, equivalent to 0·37 mg. of DNP-glutamic acid, indicating an apparent molecular weight of 8400. A more accurate determination of the molecular weight was obtained by measuring the amount of DNP-glutamic acid released upon hydrolysis. The acidic solution of the DNP-polymer (48 ml.) was evaporated to dryness and dissolved in a 10% solution of 60% perchloric acid in glacial acetic acid (2 ml.) in a test tube, the end of which was drawn out to a fine capillary. The solution was warmed at 100—110° for 4 hr., cooled, diluted to 10 ml. with water, and extracted with ether (3 × 25 ml.). This solution was evaporated to dryness and made up to 25 ml. with ether. Chromatography on Whatman No. 3 MM paper (n-butanol–acetic acid–water; 4 : 1 : 1) showed a single spot identical with that from DNP-glutamic acid. The absorbance of this solution was 0·655 at 350 mμ, equivalent to 0·385 mg. of DNP-glutamic acid, and indicating a molecular weight of 8000.

The number average molecular weight of the poly-α-L-glutamyl-L-alanylglycine, determined in a similar manner, was 6700.

Weight Average Molecular Weight.—The weight average molecular weight was obtained from the sedimentation velocity studies carried out on a Spinco model E centrifuge. Both polymers, (VI) and (XII), were run in 0·1M-sodium chloride solution at pH 7·3, at a concentration of 0·5% and at a speed of 59,780 r.p.m. Each polymer sedimented under a single peak (which broadened gradually during 2 hr.) to give values for sedimentation S_{20w}^{app} = 0·6s and 0·75s; for diffusion D_{20w}^{app} = 12·7 × 10^{-7} and 11·0 × 10^{-7} cm.²/sec. for polymers (VI) and (XII), respectively. From these values molecular weights were calculated to be 10,000 for polymer (VI) and 13,500 for (XII) (assumed partial specific volume 0·75).

Optical Rotatory Dispersion Studies.—The optical rotatory dispersion studies were conducted with a Cary 60 recording spectropolarimeter (0·5 and 1·0 cm. cells) at 26·5° with concentrations of 0·3—0·6 g. of polymers (VI) and (XV) per 100 ml. of water; the pH values of these solutions were corrected to 7·3 ± 0·1. Refractive index corrections were not made.

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