## 1255. *Synthesis of Polypeptides with Known Repeating Sequence of Amino-acids Related to Anthrax Polypeptide. Synthesis of Poly-β-L- and -D-glutamyl-β-alanine through the Pentachlorophenyl Active Ester* *

By J. Kovacs and B. J. Johnson

The preparation of two polypeptides with known repeating sequence by the use of the active pentachlorophenyl ester is described. Selective hydrogenolytic removal of the *N*-benzyloxycarbonyl protecting group in acidic conditions from *N*-benzyloxycarbonyl-α-t-butyl-L- and -D-glutamyl-β-alanine pentachlorophenyl esters (III) gave α-t-butyl-L- and -D-glutamyl-β-alanine pentachlorophenylester hydrochlorides (VI). These active esters were polymerised in the presence of a tertiary base to give optically and structurally pure poly-γ-L- and -D-glutamyl-β-alanine (VIII) after the removal of the t-butyl groups. These polymers were found to have approximate molecular weights of 10,000. Optical rotatory dispersion seems to indicate a random structure for both the D- and the L-polymers.

The structure of native polyglutamic acid (anthrax and subtilis polypeptides) is poly-γ-D-glutamic acid.[1] This was proved by total synthesis of poly-γ-D-glutamic acid [2] which was found to be identical with native polyglutamic acid, including its serological reaction. The structural requirements for the serological reaction had to be established by the use of model polypeptides containing γ-glutamyl residues. It was assumed that the carboxyl groups played an important role in this reaction.[3] The first aim was to vary the distances between the carboxyl groups on the γ-D-glutamyl residues by the preparation of a series of polypeptides in which the γ-D-glutamyl residues are separated by [4] α, β, or γ, etc., amino-acid residues.

In this Paper the usefulness of the active pentachlorophenyl esters for the synthesis of

* This Paper is the third in a series; the first and second Papers are to be found in ref. 5.

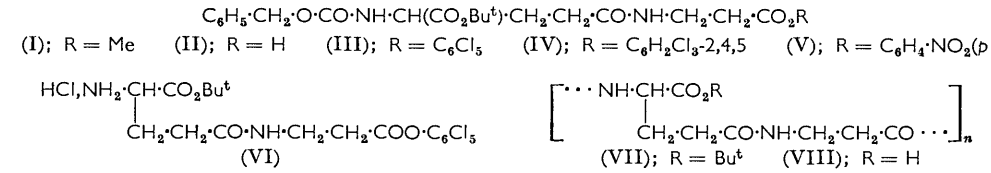
[1] J. Kovacs and V. Bruckner, *J.*, 1952, 4255; J. Kovacs, V. Bruckner, and K. Kovacs, *J.*, 1953, 145; V. Bruckner, J. Kovacs, and H. Nagy, *J.*, 1953, 148; V. Bruckner, J. Kovacs, and K. Kovacs, *J.*, 1953, 1512.

[2] V. Bruckner, M. Kajtar, J. Kovacs, H. Nagy, and J. Wein, *Tetrahedron*, 1958, **2**, 211.

[3] J. Kovacs, " Polyamino-acids, Polypeptides and Proteins," ed. M. A. Stahmann, University of Wisconsin Press, 1962, p. 32.

[4] J. Kovacs, U. R. Gatak, G. N. Schmit, F. Koide, J. W. Goodman, and D. E. Nitecki, International Symposium on the Chemistry of Natural Products, Kyoto, Japan, April 1964, Abstracts of Papers, 175

polypeptides with known repeating sequence [5] and of high molecuar weight is illustrated by the preparation of poly-γ-L- and -D-glutamyl-β-alanine (VIII).




In order to prepare α-t-butyl-glutamyl-β-alanine pentachlorophenyl ester hydrochloride (VI) which was required for the polymerisation, *N*-benzyloxycarbonyl-α-t-butylglutamic acid pentachlorophenyl ester [6] was coupled to the methyl ester of β-alanine.[7] The resulting *N*-benzyloxycarbonyl-α-t-butylglutamyl-β-alanine methyl ester (I) was hydrolysed with sodium hydroxide to give *N*-benzyloxycarbonyl-α-t-butylglutamyl-β-alanine (II) which was characterised as its dicyclohexylammonium salt. The oily free acid (II) gave one spot when up to 1130 γ was chromatographed on paper, indicating the presence of only one component. The free acid (II) was coupled to pentachlorophenol with dicyclohexylcarbodi-imide to give *N*-benzyloxycarbonyl-α-t-butylglutamyl-β-alanine pentachlorophenyl ester (III). By the same method both the *N*-benzyloxycarbonyl-α-t-butylglutamyl-β-alanine 2,4,5-trichlorophenyl ester (IV) and p-nitrophenyl ester (V) were made. The infrared spectra showed characteristic peaks at 5·62, 5·65, and 5·67 μ, respectively, for the pentachloro- (III), 2,4,5-trichloro- (IV), and *p*-nitro-phenyl (V) active esters. It is noteworthy that the wavelength at which these active esters absorb increases as their activity towards aminolysis decreases.*

As reported previously [5] the pentachlorophenyl ester gives higher melting derivatives than the commonly used activated esters. In this case the m. p.s were 151, 96, and 56—57° for the pentachloro- (III), 2,4,5-trichloro- (IV), and *p*-nitro-phenyl (V) esters, respectively. As in other cases [5] the pentachlorophenyl ester affords easier purification.

The optical purity of *N*-benzyloxycarbonyl-α-t-butylglutamyl-β-alanine pentachlorophenyl ester hydrochloride was established to be 97 ± 3% by total hydrolysis in trifluoroacetic acid–6N-hydrochloric acid; the optical rotation of the hydrolysate was compared with that of a control which was run at the same time.

Hydrogenation of *N*-benzyloxycarbonyl-α-t-butylglutamyl-β-alanine pentachlorophenyl ester hydrochloride using 10% Pd–C catalyst in the presence of one equivalent of hydrogen chloride in methanol gave, in good yield, *N*-benzyloxycarbonyl-α-t-butylglutamyl-β-alanine pentachlorophenyl ester hydrochloride (VI).

In order to prepare a polymer with a high molecular weight, it is necessary for the dipeptide hydrochloride (VI) to be as pure as possible. Thus *N*-benzyloxycarbonyl-α-t-butylglutamyl-β-alanine pentachlorophenyl ester hydrochloride (VI) was repeatedly recrystallised. Polymerisation at room temperature, in dimethylformamide and excess of triethylamine was effected in the highest possible concentration to minimise the possibility of cyclisation. The resulting poly-α-t-butyl-γ-glutamyl-β-alanine (VII) showed no absorption bands characteristic for the pentachlorophenyl ester in either the ultraviolet or in the infrared spectrum.

* In addition to our semiquantitative observation of the activities of these esters, Pless and Boissonnas [8] and also Stich and Leemann [9] measured the half-time of reaction of *N*-benzyloxycarbonylphenylalanine pentachlorophenyl ester with benzylamine in dioxan–water and dioxan, respectively.

[5] J. Kovacs and A. L. Kapoor, *J. Amer. Chem. Soc.*, 1965, **87**, 118; J. Kovacs, R. Ballina, R. L. Rodin, D. Balasubramanian, and J. Applequist, *ibid.*, p. 119; J. Kovacs, H. Nagy Kovacs, J. K. Chakrabarti, and A. L. Kapoor, *Experientia*, 1965, **21**, 20.

[6] G. N. Schmit, M. S. Thesis, St. John's University, 1964.

[7] F. Lengfeld and J. Stieglitz, *Amer. Chem. J.*, 1893, **15**, 509.

[8] J. Pless and R. A. Boissonnas, *Helv. Chim. Acta*, 1963, **46**, 1609.

[9] K. Stich and H. G. Leemann, *Helv. Chim. Acta*, 1963, **46**, 1887.

The t-butyl groups were removed from poly-α-t-butyl-γ-glutamyl-β-alanine (VII) by using 90% trifluoroacetic acid to give poly-γ-D- and -L-glutamyl-β-alanine (VIII). It was dialysed to remove small peptides and lyophilised. Up to 300 γ of the polymer was chromatographed on paper, using n-butanol–water–acetic acid (4 : 1 : 1) as the solvent system; only one faint spot ($R_F$ 0·0) was observed, on developing with ninhydrin, indicating the absence of small peptides. As a control up to 3000 γ of polyglutamic acid, obtained from *Bacillus subtilis* was run simultaneously; this also gave one spot ($R_F$ 0·0) upon development with ninhydrin. Electrophoresis of 3000 γ of poly-γ-D- and -L-glutamyl-β-alanine (VIII) at pH 8·5, gave one band upon development with copper sulphate and potassium ferrocyanide.

It has been assumed that the t-butyl group hinders transpeptidation in glutamyl peptides from occurring in an alkaline medium.[10] However, glutamyl peptides [11] and polypeptides [12] do undergo transpeptidation under conditions other than those which could be described as alkaline. Thus to see if any transpeptidation had occurred during the removal of the t-butyl group from the polymer (VII), *N*-benzyloxycarbonyl-α-t-butylglutamyl-β-alanine (II) was used as a model. The dipeptide (II) was treated with 90% trifluoroacetic acid under exactly the same conditions as those used on the poly-α-t-butyl-γ-glutamyl-β-alanine (VII), and, without any purification of the reaction mixture, other than the removal of the trifluoroacetic acid by reduced pressure. It was chromatographed on paper in a variety of solvent systems, all of which showed only one spot upon development with bromophenol blue.[13] If transpeptidation had occurred α-dipeptide would have been obtained in addition to the γ-dipeptide. The quantities (50—5000 γ) chromatographed allowed at least 1% of the dipeptide to be detected in the reaction mixture. Since only one spot was observed, it can be concluded that at least 99% of the reaction product is *N*-benzyloxycarbonyl-γ-glutamyl-β-alanine. Using this analogy we assumed that poly-γ-glutamyl-β-alanine (VIII) has practically all γ-peptide linkages.

Titration of poly-γ-glutamyl-β-alanine with sodium hydroxide showed that all of the t-butyl groups had been removed (*e.g.*, L-isomer, Equiv. 208; theory, 209).

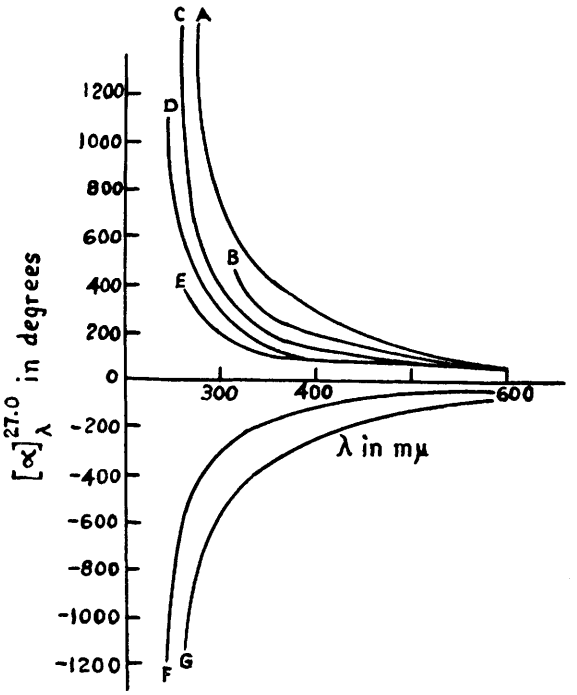
The viscosity was measured in dichloroacetic acid and 0·2M-sodium chloride at pH 7·1 giving intrinsic viscosities of 0·5 and 0·4 decilitre/g. for poly-γ-L-glutamyl-β-alanine (VIII), and 0·3 and 0·19 decilitre/g. for poly-γ-D-glutamyl-β-alanine (VIII).

The weight average molecular weight was obtained by ultracentrifugation* to give values for the sedimentation $S^{app}_{20w}$ 0·5 S and 0·6 S, as well as for diffusion $D^{app}_{20w}$ 14 × $10^{-7}$ cm.$^2$/sec. and 14·5 × $10^{-7}$ cm.$^2$/sec. for poly-γ-D-glutamyl-β-alanine (VIII) and poly-γ-L-glutamyl-β-alanine (VIII), respectively. From these values the molecular weight of approximately 10,000 was calculated for both polymers, a partial specific volume of 0·72 being assumed.

Rydon [14] has shown that native polyglutamic acid exists as a helical structure in aqueous solution. In order to see if poly-γ-glutamyl-β-alanine (VIII) had a similarly ordered structure, the optical rotatory dispersion of these polymers (L and D) (VIII) were measured in a variety of solvents and at different concentrations. Such results are shown in the Figure and Table. The dispersion data was fitted to the Drude equation, $[\alpha]_\gamma = k/(\lambda^2 - \lambda_c^2)$. The modified Drude plot [15] was used to find $\lambda_c$. From these result it can be concluded that

* Sedimentation velocity studies were kindly conducted by Dr. R. Heimer of the Seton Hall College of Medicine, Jersey City, New Jersey, using a Spinco model E centrifuge. Poly-γ-L-glutamyl-β-alanine (VIII) was run in 0·5M-phosphate buffer solution at pH 7·6 at a concentration of 1% and at a speed of 52,460 r.p.m. Poly-γ-D-glutamyl-β-alanine (VIII) was run in distilled water at a concentration of 0·5% and 1% at the same speed as that used for the L-isomer. Both polymers (L and D) sedimented under a single peak.

[10] R. Schwyzer and H. Kappeler, *Helv. Chim. Acta*, 1961, **44**, 1991; R. Schwyzer and H. Dietrich, *ibid.* p. 2003.
[11] J. Kovacs, K. Medzihradszky, and V. Bruckner, *Naturwiss.*, 1954, **41**, 450.
[12] V. Bruckner, J. Kovacs, and K. Medzihradzky, *Naturwiss.*, 1955, **42**, 96.
[13] H. J. Petrowitz and G. Pastuska, *J. Chromatog.*, 1962, **7**, 128.
[14] H. N. Rydon, *J.*, 1964, 1328.
[15] J. T. Yang and P. Doty, *J. Amer. Chem. Soc.*, 1957, **79**, 761.




in all of the solvents and at the concentrations used, the configuration of poly-γ-L- and-D-glutamyl-β-alanine is not helical.

The biological investigations will be reported later when other members of the series have been prepared.

| Polymer | Concentration (g./100 ml.) | Solvent | $\lambda_c$ (mμ) | Polymer | Concentration (g./100 ml.) | Solvent | $\lambda_c$ (mμ) |
|---|---|---|---|---|---|---|---|
| A | 0·10 | Water | 202 | F | 0·26 | Water | 223 |
| B | 1·0 | D.C.A.–$CHCl_3$ (1 : 3) | 228 | G | 0·11 | Water | 206 |
| | | | | H * | 0·53 | D.M.F. | 243 |
| C | 0·25 | Water | 215 | I * | 0·68 | Dioxan–water | 236 |
| D | 4·57 | Water | 237 | | | water (3 : 1) | |
| E | 1·085 | 0·2M-NaCl (pH 7·1) | 221 | | | | |

* Not shown in Figure. D.C.A. = Dichloroacetic acid. D.M.F. = Dimethylformamide.

## Experimental

Infrared spectra, where reported, were all performed in potassium bromide pellets. Optical rotations were taken using a Rudolph precision ultraviolet spectrometer.

*N-Benzyloxycarbonyl-α-t-butyl-L-glutamyl-β-alanine Methyl Ester* (I).—A mixture of β-alanine methyl ester hydrobromide [7] (7 g., 0·038 mole) and triethylamine (3·9 g., 1 equiv.) was added to *N*-benzyloxycarbonyl-α-t-butyl-L-glutamic acid pentachlorophenyl ester (21 g., 0·036 mole) in methylene dichloride (250 ml.). The mixture was stirred for 24 hr., then washed quickly with dilute hydrochloric acid (3 × 100 ml.) and then consecutively with water (100 ml.), saturated sodium hydrogen carbonate solution (100 ml.), and water (2 × 100 ml.). After being dried ($MgSO_4$), the solvent was removed under reduced pressure to give an oil, which was purified by chromatography on a silica-gel column. The pentachlorophenol was eluted with benzene–light petroleum (1 : 1) and the *dipeptide methyl ester* (I) was removed from the column with ethyl acetate as an oil which, crystallised from ethyl acetate–light petroleum (yield 12 g., 82%), had m. p. 44—45°. A sample was recrystallised three times, which raised the m. p. to 51°, $[\alpha]_D^{23.5}$ +1·4° (*c* 8·1 in methylene dichloride) $\lambda_{max.}$ 6·05 μ (amide I), 5·76 μ ($CO_2Bu^t$) and 5·7 μ ($CO_2{\cdot}CH_3$) (Found: C, 59·7; H, 7·03; N, 6·3. $C_{21}H_{30}N_2O_7$ requires C, 59·7; H, 7·15; N, 6·6%).

N-*Benzyloxycarbonyl*-α-*t-butyl*-D-*glutamyl*-β-*alanine Methyl Ester* (I).—A similar procedure was used to prepare this D-*isomer* of compound (I) (yield 72%) m. p. 46—47°. Three crystallisations from ethyl acetate–light petroleum raised the m. p. to 50—51°, $[\alpha]_D^{29.5}$ −1·3° (*c* 8·1 in methylene dichloride). The infrared spectrum was similar to that of the L-isomer (Found: C, 59·8; H, 7·0; N, 6·8%).

N-*Benzyloxycarbonyl*-α-*t-butyl*-L-*glutamyl*-β-*alanine* (II).—Sodium hydroxide (1 g., 0·025 mole) in water (10 ml.), was added to the dipeptide methyl ester (I) (10 g., 0·0237 mole) in methanol (200 ml.). The mixture was kept at room temperature for 1 hr., then poured into water (600 ml.), and washed with ethyl acetate (2 × 100 ml.). The aqueous layer was acidified with dilute hydrochloric acid and rapidly extracted with ethyl acetate (2 × 100 ml.). Removal of the ethyl acetate under vacuum yielded 8·5 g. (98%) of (II) as a viscous oil, $\lambda_{max.}$ 5·85 μ ($CO_2H$), which gave only one spot when up to 1130 γ was chromatographed on 3MM paper in n-butanol saturated with water and also in n-butanol–water–acetic acid (4 : 1 : 1).

N-*Benzyloxycarbonyl*-α-*t-butyl*-D-*glutamyl*-β-*alanine* (II).—This D-isomer (II) was prepared using the above method in 70% yield. The viscous oily free acid was characterised as the dicyclohexylamine salt. Addition of dicyclohexylamine (18·1 g., 0·1 mole) in ether (10 ml.) to the oil (32 g., 0·08 mole) dissolved in ether (50 ml.) gave a white crystalline material. This was filtered off to yield 26·5 g. (56%), m. p. 120—121°, of the N-*benzyloxycarbonyl*-α-*t-butyl*-D-*glutamyl*-β-*alanine dicyclohexylammonium salt.*

A sample, recrystallised from methanol–ether, had m. p. 122°, $[\alpha]_D^{25}$ +5·48° (*c* 5·19 in methanol) (Found: C, 65·0; H, 8·7; N, 7·4. $C_{32}H_{51}N_3O_7$ requires C, 65·15; H, 8·7; N, 7·1%).

N-*Benzyloxycarbonyl*-α-*t-butyl*-L-*glutamyl*-β-*alanine Pentachlorophenyl Ester* (III).—To a solution of the free acid (II) (8·5 g., 0·021 mole) in methylene dichloride (100 ml.) was added dicyclohexylcarbodi-imide (4·3 g., 0·021 mole). The mixture was stirred for 10 min., then pentachlorophenol (4·7 g., 0·0214 mole) was added. After stirring of the mixture for 24 hr. at room temperature, the excess of dicyclohexylcarbodi-imide was decomposed with glacial acetic acid (3 ml.). The precipitate was filtered off and the filtrate concentrated under vacuum to a semi-solid material which was triturated with ethyl acetate, and the insoluble urea was removed. This procedure was repeated twice. The filtrate was washed quickly with dilute hydrochloric acid (2 × 50 ml.) and then consecutively with water (50 ml.), saturated sodium hydrogen carbonate solution (50 ml.), and water (50 ml.). The solution was dried ($MgSO_4$) and the solvent removed under vacuum. The solid *ester*, crystallised from methanol (yield 10·1 g., 74%), had m. p. 149—150°. Two further recrystallisations from methanol raised the m. p. to 151°, $[\alpha]_{313}^{27}$ +14·0° (*c* 4·92 in chloroform), $\lambda_{max.}$ 5·62 μ ($CO_2C_6Cl_5$) (Found: C, 47·8; H, 4·2; N, 4·4. $C_{26}H_{27}Cl_5N_2O_7$ requires C, 47·55; N, 4·1; N, 4·3%).

N-*Benzyloxycarbonyl*-α-*t-butyl*-D-*glutamyl*-β-*alanine Pentachlorophenyl Ester* (III).—Prepared similarly to the L-isomer (yield 60%), this *ester* had m. p. 150—151°, $[\alpha]_{313}^{27}$ −14·1° (*c* 5·16 in chloroform). The infrared spectrum was superimposable on that of the L-isomer (Found: C, 47·55; H, 4·3; N, 4·05%).

*Optical Purity of* (III).—*N*-Benzyloxycarbonyl-α-t-butyl-L-glutamyl-β-alanine pentachlorophenyl ester (III) (0·1313 g., 0·0002 mole) was dissolved in trifluoroacetic acid (1 ml.) and 6N-hydrochloric acid (1 ml.) and then heated for 43 hr. at 110—115° in a sealed tube. Upon cooling, pentachlorophenol crystallised; it was filtered off and washed with trifluoroacetic acid. The filtrate was made up to a total volume of 3 ml. with the trifluoroacetic acid washings, $[\alpha]_D^{23.5}$ +25·7° (*c* 0·98 in 6N-HCl–trifluoroacetic acid, 1 : 2 v/v).

A control consisting of benzyl alcohol (0·0216 g., 0·0002 mole), pentachlorophenol (0·0533 g., 0·0002 mole), β-alanine (0·0178 g., 0·0002 mole) and L-glutamic acid (0·0294 g., 0·0002 mole), trifluoroacetic (1 ml.) and 6N-hydrochloric acid (1 ml.) was heated in a sealed tube, under the same conditions. After heating for 43 hr. the solution was cooled and the pentachlorophenol crystallised out; it was filtered off, washed with trifluoroacetic acid and the filtrate was made up to a total volume of 3 ml. with the trifluoroacetic acid washings, $[\alpha]_D^{23.5}$ +26·5° (*c* 0·98 in 6N-HCl-trifluoroacetic acid, 1 : 2 by volume). This indicated that the optical purity of (III) was 97 ± 3%. (The major error results from dilution.) The optical purity of the D-isomer of (III), determined exactly as for the L-isomer, was 98 ± 3%.

N-*Benzyloxycarbonyl*-α-*t-butyl*-D-*glutamyl*-β-*alanine* 2,4,5-*Trichlorophenyl Ester* (IV).—To the free acid (II) (1 g., 0·00245 mole) in methylene dichloride (20 ml.) was added dicyclohexylcarbodi-imide (0·6 g., 0·00292 mole) and stirred for 10 min., then 2,4,5-trichlorophenol (0·5 g., 0·0025 mole) was added and the mixture stirred overnight. Glacial acetic acid (0·2 ml.) was

introduced and the mixture kept for a further 10 min. The preciptated urea was filtered off, and the filtrate was evaporated under reduced pressure to give an oil. It was triturated with ethyl acetate and the urea filtered off. The filtrate was washed with saturated sodium hydrogen carbonate solution, and water, and dried ($MgSO_4$). The filtrate was concentrated to small volume and chromatographed on a column of silica-gel with benzene as eluent, which removed the 2,4,5-trichlorophenol. The eluent was changed to ethyl acetate and an oil was obtained which, crystallised from ethyl acetate–light petroleum, had m. p. 81–83°, 0·9 g. (63%). A sample was recrystallised three times from absolute ethanol to raise the m. p. to 96°, $\lambda_{max.}$ 5·65 μ, ($CO_2$·$C_6H_2$·$Cl_3$-2,4,5) (Found: C, 53·0; H, 4·8; N, 4·9. $C_{26}H_{29}Cl_3N_2O_7$ requires C, 53·1; H, 5·0; N, 4·8%).

*N-Benzyloxycarbonyl-α-t-butyl-*D*-glutamyl-β-alanine* p-*Nitrophenyl Ester* (V).—This, made in the same way as the dipeptide 2,4,5-trichlorophenyl ester (IV), had m. p. 56—57° (47%), $\lambda_{max.}$ 5·67 μ ($CO_2$·$C_6H_4$·$NO_2$-*p*) (Found: C, 58·7; H, 6·1; N, 8·2. $C_{26}H_{31}N_3O_9$ requires C, 58·9; H, 5·9; N, 7·9%).

*α-t-Butyl-*L*-glutamyl-β-alanine Pentachlorophenyl Ester Hydrochloride* (VI).—Palladium–charcoal catalyst (10%, 1 g.) suspended in glacial acetic acid (10 ml.) and methanol (20 ml.) was hydrogenated at atmospheric pressure until no further uptake of hydrogen had occurred. Dry methanol containing 0·194 g. of hydrogen chloride per ml. (2·4 ml., 0·013 mole) was added to the hydrogenated catalyst, and the hydrogenation was continued for another 5 min. A slurry of the dipeptide pentachlorophenyl ester (III) (7·2 g., 0·011 mole) in methanol (50 ml.) was added to the mixture and hydrogenated. When no further uptake of hydrogen had occurred (205 ml. in 40 min.), the catalyst and any unchanged material were filtered off, and the filtrate was evaporated under reduced pressure to small volume. Addition of ether precipitated the hydrochloride, 5·22 g. (85%), m. p. 147—148°. It was recrystallised to constant m. p. 159—160° from methanol–ether (Found: C, 38·55; H, 3·8; N, 5·0; $Cl^-$, 6·5. $C_{18}H_{22}Cl_6N_2O_5$ requires C, 38·7; H, 4·0; N, 5·0; $Cl^-$, 6·5%).

*α-t-Butyl-*D*-glutamyl-β-alanine Pentachlorophenyl Ester Hydrochloride* (VI).—Prepared similarly to the L-isomer in 59% yield, this *salt* had m. p. 160° after three recrystallisations (Found: C, 38·6; H, 3·9; N, 5·1; $Cl^-$, 6·4%).

*Poly-α-t-butyl-γ-*L*-glutamyl-β-alanine* (VII).—To a suspension of analyticaly pure α-t-butyl-L-glutamyl-β-alanine pentachlorophenyl ester hydrochloride (VI) (4·42 g., 0·00791 mole) suspended in dimethylformamide (4 ml.) was added triethylamine (2·5 ml., 0·0175 mole). The mixture was stirred at room temperature, but within 30 min. it had completely set to an unstirable mass. This was allowed to stand overnight and then triturated with ether (200 ml.), water (200 ml.), and ether (200 ml.). The polypeptide, so obtained, was dissolved by warming in methanol (40 ml.), filtered, and precipitated on addition of ether to the filtrate. The t-butyl polypeptide (VII) was collected by centrifugation (yield 1·78 g., 88%), $\lambda_{max.}$ 5·75 μ ($CO_2Bu^t$), 6·05 μ (CO–NH, amide I), and 6·45 μ (CO–NH, amide II); the pentachlorophenyl ester peak had completely disappeared. The ultraviolet spectrum showed no absorption bands between 305—360 mμ at a concentration of 0·6 g./100 ml. in methanol which would also indicate the absence of the pentachlorophenyl ester (Found: C, 56·1; H, 7·8; N, 10·8. $(C_{12}H_{20}N_2O_4)_\infty$ requires C, 56·2; H, 7·9; N, 10·9%).

*Poly-α-t-butyl-*D*-glutamyl-β-alanine* (VII).—Analytically pure α-t-butyl-D-glutamyl-β-alanine pentachlorophenyl ester hydrochloride (VI) was polymerised by the same procedure as for the L-isomer; D-peptide (III) (2·72 g.) in dimethylformamide (4 ml.) and triethylamine (1·2 ml.) yielding 0·89 g. (71·5%). The infrared spectrum was superimposable on that of the L-isomer. The ultraviolet spectrum (0·6 g./100 ml. of polypeptide in methanol) also showed no absorption peaks indicative of the pentachlorophenyl ester between 305 and 360 mμ (Found: C, 54·9; H, 8·1; N, 10·9%).

*Poly-γ-*L*-glutamyl-β-alanine* (VIII).—Poly-α-t-butyl-γ-L-glutamyl-β-alanine (VII) (1·78 g., 0·00695 mole) was dissolved in 90% trifluoroacetic acid (37 ml.) and kept at room temperature for 50 min. Ether (400 ml.) was added and the white precipitate was collected, washed with ether (2 × 200 ml.), and air dried to give 1·05 g. (52·5%) of the free polymer (VI). It was dissolved in distilled water (50 ml.) and dialysed by use of a cellophane bag (Visking Co., size 20 D.C.) against distilled water (5 × 50 ml.) for 47 hr., to yield after lyophilisation 0·78 g. (74%), $\lambda_{max.}$ 5·8 μ ($CO_2H$), 6·05 μ (CO–NH amide I), and 6·5 μ (CO–NH amide II) (Found: C, 46·1; H, 6·4; N, 13·75%; Equiv. 208. $(C_8H_{12}N_2O_4,0{\cdot}5H_2O)_\infty$ requires C, 45·9; H, 6·3; N, 13·4%; Equiv. 209).

In order to see if any small peptides were present after dialysis, the polymer (1000, 2000, and 3000 $\gamma$) was chromatographed on 3MM paper in n-butanol–water–acetic acid (4 : 1 : 1) from which only one spot was obtained using ninhydrin as the developing agent, thus indicating the absence of small peptides. Paper electrophoresis of poly-$\gamma$-L-glutamyl-β-alanine (VIII) (3000 $\gamma$) in M/15-sodium acetate, pH 8·5, at 12·9 V/cm. for 6 hr. also showed, after development with 2% copper sulphate then 5% potassium ferrocyanide, a single band located 34 cm. from the origin towards the anode.

The intrinsic viscosity was measured with the Cannon–Ubbelholde viscometer (100 K 352 and 75 K 618). The minimum time for flow was 112 sec., $[\eta]$ 0·5 dl./g. in dichloroacelic acid, and $[\eta]$ 0·42 dl./g. in 0·2M-NaCl at pH 7·1.

*Poly-γ-D-glutamyl-β-alanine* (VIII).—Prepared in a similar way to the L-isomer (yield 71·5%), the crude *polypeptide* (VIII) was dissolved in distilled water (45 ml.) and dialysed, using a cellophane bag (Visking Co., size 20 D.C.), against distilled water (5 × 540 ml.) for 64 hr., the lyophilised to yield 48·5%, $\lambda_{max.}$ 5·8 μ ($CO_2H$), 6·1 μ (CO–NH amide I), and 6·5 μ (CO–NH amide II) (Found: C, 45·4; H, 6·6; N, 13·2%; Equiv. 206).

Chromatography and electrophoresis were conducted under the same conditions as those used for the L-isomer showing only one spot in the systems used. However, electrophoresis of the D-polymer (VIII) gave a single band which moved 37 cm. from the origin towards the anode.

The intrinsic viscosity was found by using the Cannon–Ubbelholde viscometer (150 K 182 and 75 K 618). The minimum time for flow was 102 sec., $[\eta]$ 0·3 dl./g. in dichloroacetic acid and $[\eta]$ 0·19 dl./g. in 0·2M-NaCl at pH 7·3.

*Optical Purity of Poly-γ-D-glutamyl-β-alanine* (VIII).—Poly-γ-D-glutamyl-β-alanine (52·3 mg., 0·0002503 mole) was dissolved in 6N-hydrochloric acid (2 ml.) and heated under reflux at 110—115° for 48 hr. The solution was made to a total of 3 ml. with 6N-hydrochloric acid, $[\alpha]_D^{28}$ −30·85° (*c* 1·226 in 6N-HCl).

To a control consisting of L-glutamic acid (36·8 mg., 0·0002503 mole) and β-alanine (22·3 mg., 0·0002503 mole) was added 6N-hydrochloric acid (2 ml.) and heated simultaneously with and under the same conditions as those used for the D-polymer (VIII). After 48 hr. the solution was diluted to 3 ml. with 6N-hydrochloric acid, $[\alpha]_D^{28}$ +31·51° (*c* 1·226 in 6N-HCl), to give an optical purity of 97·9 ± 3%. The major error, ±2%, is due to dilution.

*Optical Purity of Poly-γ-L-glutamyl-β-alanine* (VIII).—Determined as for the D-isomer, it was 96·9 ± 3%.

*Transpeptidation Studies.*—*N*-Benzyloxycarbonyl-α-t-butyl-D-glutamyl-β-alanine (II) (0·4 g.) was dissolved in 90% trifluoroacetic acid (1 ml.) and left at room temperature for 50 min. The trifluoroacetic acid was removed under reduced pressure to yield an oil. This was chromatographed on 3MM paper in a variety of solvents and at concentrations ranging from 50 to 5000 $\gamma$ in each solvent system, such that 1% of a dipeptidedicarboxylic acid could be detected. The solvent systems used were n-butanol saturated with water (descending) $R_F$ 0·81; benzene–methanol (83 : 17) (ascending) $R_F$ 0·95; n-butanol saturated with water, phenol–water (2 dimensional, ascending), $R_F$ 0·85 and 0·87; and also n-butanol–water–acetic acid (4 : 1 : 1), phenol–water (2 dimensional, ascending), $R_F$ 0·8 and 0·86.

The developing agent used was Bromophenol Blue,[13] pH 3·4—4·0. In all of these solvent systems only one spot was observed in each case. Thus there is at least 99% $\gamma$-peptide linkages present. Using this analogy, it is concluded that the free polymer (VIII) also consists of at least 99% $\gamma$-peptide linkages.

*Optical Rotatory Dispersion.*—Optical rotations were measured with a Rudolph precision ultraviolet polarimeter, model 2005–340–8003, using a Xenon compact arc (model 614) and a mercury (type 5H Hanovia) quartz lamp as light sources. However, the Xenon lamp was used only when absolutely necessary owing to its very rapid fluctuations in light intensity. The usual wavelength range was 245—600 mμ for those solvents which were transparent down to the lower wavelength ranges, otherwise the optical rotation was started just above their cut-off points. The path length in all cases was 1 cm. The temperature was kept at 27·0 ± 0·2° unless indicated otherwise. The concentrations at which each o.r.d. run was made are shown in the Table.

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