Sequential Polypeptides. Synthesis of Poly-(L-tyrosyl-L-glutamyl-L-tyrosyl-L-glutamyl), Poly-(L-glutamyl-L-tyrosyl-L-glutamyl), and Poly-(L-glutamyl-L-glutamyl-L-tyrosyl-L-glutamyl) by Use of Catechol Esters

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The racemization-free method of peptide coupling involving use of 2-hydroxyphenyl esters has been successfully applied to the synthesis of sequential polypeptides containing L-tyrosine and L-glutamic acid. The reactive hydroxy-group of these esters was protected by formation of a phenacyl ether; this led to easily crystallizable derivatives, and the protective group could be removed selectively with zinc dust in acetic acid. The benzyl group was used to protect side-chains of tyrosine and glutamic acid. It was removed by use of a saturated solution of hydrogen bromide in acetic acid; use of the equivalent hydrogen bromide–trifluoroacetic acid mixture leads to irreversible modification of tyrosine. Kinetic measurements have shown that rates of aminolysis are highly dependent on the concentration of triethylamine. Molecular weights of deprotected polypeptides were in most cases around 10,000.

THE conformations of polytyrosine and poly(glutamic acid) in dilute aqueous solution have recently been investigated extensively. The conformation of polytyrosine is, however, not clearly elucidated and results concerning the pH-induced transition are not yet consistent. The conformation of poly(glutamic acid) is better known and the phenomenon of aggregation in aqueous acidic medium now seems well understood.

Prior to the present work, Fasman [1] synthesized and studied some random copolymers of tyrosine and glutamic acid, and Noguchi, besides such copolymers,[2] prepared poly-(L-tyrosyl-L-glutamyl),[3] the only sequential polypeptide of tyrosine and glutamic acid described hitherto; it is however of low molecular weight and of doubtful optical purity. Ramachandran, Berger, and Katchalski [4a] later prepared and elucidated the conformational properties in aqueous solution of poly-(L-tyrosyl-L-alanyl-L-glutamyl). In addition, Schechter *et al.*[4b] have prepared oligo-(L-tyrosyl-L-alanyl-L-glutamyl) and compared the products with the former copolypeptide, with respect to their c.d. spectra.

Sequential polypeptides of tyrosine and glutamic acid should combine the inherent features of both aminoacyl residues. Glutamic acid would be expected to extend the range of solubility at lower pH values and confer to the copolymers some ability to aggregate, and interactions could well occur between the aromatic side-chains of tyrosyl residues. In addition, an enzyme-like hydrolytic activity might be induced by the presence of both aminoacyl residues arranged in a definite sequence. However, these problems cannot be resolved if polymeric materials do not possess high optical purity, since conformations of polypeptides are closely related to the optical integrity of the constituent aminoacyl residues.

The active ester method of synthesis in some cases induces racemization. Recent evidence shows that, with respect to racemization, use of *N*-succinimidyl esters [5] is a particularly satisfactory procedure. In the past few years, a new class of esters has been suggested, the reactivity of which involves an intramolecular general base catalysis rather than an electron-withdrawing effect of the activating group. Examples are esters of 8-hydroxyquinoline,[6] 1-hydroxypiperidine,[7,8] catechol,[9,10] and derivatives such as 3-acyloxy-2-hydroxy-*N*-ethylbenzamide.[11,12]

We have briefly reported [13] that use of catechol (2-hydroxyphenol) esters, first described by Jones and Young,[9] can lead to optically pure poly-(γ-benzyl-L-glutamate) (PBLG), as verified by comparison with a PBLG sample prepared by the *N*-carboxy-anhydride method. In addition, Cowell and Jones [10] have shown that poly(glycyl-L-prolyl-L-alanyl) prepared by use of catechol esters is optically pure.

In the present work, a γ-benzyl-L-glutamyl residue was always inserted as the *C*-terminal residue in monomer units. We then assumed that the optical purity of our sequential polypeptides would be comparable to that of PBLG, which was used as a model for racemization tests.

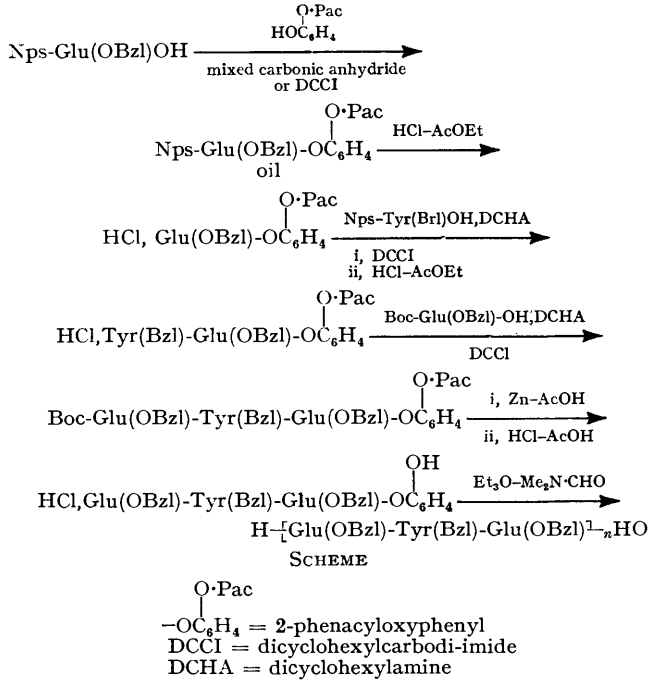
The use of catechol esters requires the temporary protection of the free hydroxy-group in order to avoid side reactions such as acylation or oxidation. Cowell and Jones [10] used the benzyl group for protection, which can be removed either by acidolysis with hydrogen bromide in acetic acid or by catalytic hydrogenolysis. Such conditions are not always compatible with the protection of side-chains, and we have investigated other more selective methods of protection of the

[1] G. Fasman, K. Norland, and A. Pesce, *Biopolymers*, 1964, **1**, 325; G. Fasman, E. Bodenheimer, and C. Lindblow, *Biochemistry*, 1964, **3**, 1665.
[2] J. Noguchi and H. Yamamoto, *J. Biochem.*, 1969, **65**, 123.
[3] H. Yamamoto and J. Noguchi, *J. Biochem.*, 1970, **67**, 103.
[4] (*a*) J. Ramachandran, A. Berger, and E. Katchalski, *Biopolymers*, 1971, **10**, 1829; (*b*) B. Schlechter, I. Schlechter, J. Ramachandran, A. Conway-Jacobs, and M. Sela, *European J. Biochem.*, 1971, **20**, 301.
[5] P. M. Hardy, H. N. Rydon, and R. C. Thompson, *J.C.S. Perkin I*, 1972, **5**; P. M. Hardy, H. N. Rydon, and H. T. Storey, *ibid.*, p. 1523.
[6] H. D. Jakubke and A. Voigt, *Chem. Ber.*, 1966, **99**, 2419, 2949; H. D. Kakubke, A. Voigt, and S. Burkhardt, *ibid.*, 1967, **100**, 2367.
[7] F. Weygand, W. König, E. Nintz, D. Hoffman, P. Huber, N. M. Khan, and W. Prinz, *Z. Naturforsch.*, 1966, **21***b*, 325.
[8] B. O. Handford, J. H. Jones, and G. T. Young, *J. Chem. Soc.* (*C*), 1965, **6814**; J. H. Jones, B. Liberek, and G. T. Young, *ibid.*, 1967, 2371; J. H. Jones and G. T. Young, *ibid.*, 1968, 53.
[9] J. H. Jones and G. T. Young, *J. Chem. Soc.* (*C*), 1968, 436.
[10] R. D. Cowell and J. H. Jones, *J. Chem. Soc.* (*C*), 1971, 1082.
[11] D. S. Kemp and S. W. Chien, *J. Amer. Chem. Soc.*, 1967, **89**, 2743.
[12] D. S. Kemp, in 'Peptides 1971,' ed. H. Nesvadba, North Holland Publishing Co., Amsterdam, in the press.
[13] Y. Trudelle, *Chem. Comm.*, 1971, 639.

hydroxy-group. We recently suggested [13] the phenacyl ether system, since it can easily and selectively be removed by mild hydrogenolysis with zinc dust in aqueous or anhydrous acetic acid.[14] Nevertheless, the terminal amino-group of the repeating monomer unit must be kept protected when cleaving the phenacyl ether; otherwise, a complex mixture contaminated with zinc is obtained, which seems difficult to resolve. The t-butoxycarbonyl group was adequate for this purpose.

Some difficulties were encountered in the protection of the tyrosine side-chain. Previous investigations [15] had shown that the *O*-benzyloxycarbonyl group was attacked by the monoester of catechol or by catechol itself, leading to the formation of phenylene carbonate, which is highly reactive towards free amino-groups. It was also observed that polycondensation of a monomer unit bearing a benzyloxycarbonyl group on the side-chain of the tyrosyl residue was interrupted before conversion was complete. Consequently, the *O*-benzyl group was preferred.

As an illustration, the synthesis of a tripeptide monomer unit is given in the Scheme.



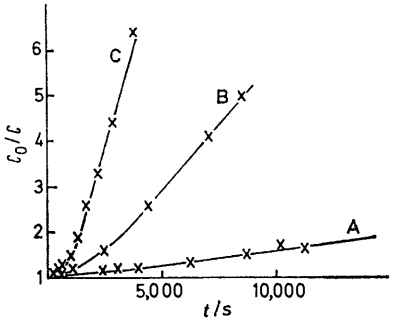
SCHEME

$-OC_6H_4$ = 2-phenacyloxyphenyl
DCCI = dicyclohexylcarbodi-imide
DCHA = dicyclohexylamine

Other investigations were connected with several factors related to the polycondensation step. As shown in the Figure, addition of triethylamine strongly increases the rate of polycondensation in benzene. This effect, already observed by Kemp [12] for 3-acyloxy-2-hydroxy-*N*-ethylbenzamides in dimethylformamide, is probably attributable to the ionization of the hydroxy-group of catechol, which favours intramolecular general base catalysis. Furthermore, a tertiary base is necessary to obtain high conversion yields.

[14] J. B. Hendrickson and C. Kandall, *Tetrahedron Letters*, 1970, **5**, 343.

Additional observations deal with the influence of the monomer concentration on the polycondensation reaction. As shown in the Table, the largest polymers are obtained from concentrated solutions, but molecular weights are never higher than 25,000 (for 1M-monomer); this seems to constitute the main shortcoming of the catechol ester method.



Kinetics of polycondensation of γ-benzyl-L-glutamyl-*O*-benzyl-L-tyrosyl-γ-benzyl-L-glutamic acid 2-hydroxyphenyl ester hydrochloride, in benzene. Temp. 37°, concentration in monomer 0·1M, c_0/c calculated from the intensity of the $\nu_{C=O}$ absorption band of the 2-hydroxyphenyl ester. Curve A 1 equiv. triethylamine; curve B 1·5 equiv.; curve C 2·5 equiv.

We met some difficulties when synthesizing the sequential alternating copolypeptide poly-(L-tyrosyl-L-glutamyl), since the dipeptide monomeric units *O*-benzyl-L-tyrosyl-γ-benzyl-L-glutamic acid 2-hydroxyphenyl ester hydrochloride and γ-benzyl-L-glutamyl-*O*-benzyl-L-tyrosine 2-hydroxyphenyl ester hydrochloride, both give dioxopiperazines almost quantitatively, whatever the concentration and the solvent. We were thus compelled to synthesize the tetrapeptide monomeric unit, which exhibits a poor ability to polycondense. No polycondensation occurs in chloroform or dimethyl sulphoxide, and the reaction is slow in benzene and little faster in dimethylformamide, which appeared to be a suitable solvent for all other monomers.

Monomer	Concn. (M)	Crude polymer yield (%) [b]	$[\eta]$ [c]	$\bar{M}_w$ [d]
(I)	5 [a]	81	16·6	18,000
	1	77	20	25,000
	0·1	Oligomers		
	0·01	0		
(II)	1	77·5	17·2	19,500
	0·1	34·4	11·3	7500
	0·01	0		

[a] When concentration exceeds 1M some lack of homogeneity may affect the development of polycondensation. [b] After precipitation with ethanol. [c] Measured in dichloroacetic acid at 25°; in ml g^{-1}. [d] Estimated from the viscometric relationship of Doty (J. C. Mitchell, A. E. Woodward, and P. Doty, *J. Amer. Chem. Soc.*, 1957, **79**, 3955) concerning poly-(γ-benzyl L-glutamate). Solvent dimethylformamide, tertiary amine triethylamine (2 equiv.), polycondensation for 7 days at room temperature. Monomer (I): γ-benzyl-L-glutamyl-γ-benzyl-L-glutamyl-*O*-benzyl-L-tyrosyl-γ-benzyl-L-glutamic acid 2-hydroxyphenyl ester hydrochloride. Monomer (II): γ-benzyl-L-glutamyl-*O*-benzyl-L-tyrosyl-γ-benzyl-L-glutamic acid 2-hydroxyphenyl ester hydrochloride.

[15] Y. Trudelle and G. Spach, in ' Peptides 1971,' ed. H. Nesvadba, North Holland Publishing Co., Amsterdam, in the press.

The removal of benzyl groups protecting the side-chains of glutamyl and tyrosyl residues proved more awkward than expected. As reported elsewhere,[16] the use of HBr–trifluoroacetic acid induces some irreversible modification of the tyrosyl residues, even when scavengers such as anisole or resorcinol are present. The HBr–acetic acid (6·5N) reagent is better, but treatment must be carried out for no longer than an hour at room temperature to avoid degradation of the polymer. Spectroscopic and viscometric studies show that this reagent allows complete removal of γ-benzyl groups without significant degradation, as verified on a sample of poly-(γ-benzyl glutamate). Likewise, *O*-benzyl groups of tyrosyl residues are completely removed as evidenced by u.v. spectra in alkaline solution. However, partial acetylation takes place during acidolysis, but this is easily corrected by dissolving the acetylated polymer in an alkaline medium.

EXPERIMENTAL

Optical rotations were determined with a Perkin-Elmer 141M polarimeter (1 dm cell) and i.r. spectra with a Perkin-Elmer 257 spectrophotometer or a Beckman IR 11 spectrophotometer (when high accuracy was needed). U.v. spectra were recorded on a Unicam SP 800A or a Perkin-Elmer 402 spectrophotometer. Viscosities were measured with an Ubbelohde viscometer (Cannon CUSMU size 75) and molecular weights with a Hewlett–Packard Osmometer 503; thin-wall Visking dialysis tubing was used for membranes. M.p.s were determined with a hot-plate Leitz microscope. T.l.c. was carried out on Eastman Chromatogram Sheet 6060 (silica gel) with ethyl acetate (A) or acetic acid butanol–water (3 : 20 : 7) (B) as solvent. All compounds were dried *in vacuo* (0·1 Torr), at 40° in most cases. Dimethylformamide used to carry out polycondensations was distilled four times, the last time from benzyloxycarbonylglycine *p*-nitrophenyl ester, and stored over molecular sieves (4 Å). Triethylamine was distilled twice, the first time from benzyloxycarbonylglycine *p*-nitrophenyl ester.

2-Phenacyloxyphenol (O-*Phenacylcatechol*).—The method of Lazennec[17] was slightly modified as follows. Sodium (23 g, 1 mol) was added in portions under nitrogen to a cooled and stirred solution of catechol (110 g, 1 mol) in absolute ethanol (400 ml). When dissolution of sodium was complete, the mixture was allowed to warm to room temperature and a solution of phenacyl bromide (199 g, 1 mol) in absolute ethanol (800 ml) was added during 1 h. The mixture was then refluxed for 30 min, cooled, and filtered to remove sodium bromide. The filtrate was evaporated to dryness and the residue dissolved in acetone. The solution was poured into water, giving a yellow solid paste, which was recrystallized from benzene–hexane after decolourising with charcoal to give chromatographically pure 2-phenacyloxyphenol (58·5 g, 25·6%), m.p. 110—111°, R_F 0·62 (benzene–methanol–acetic acid, 45 : 8 : 3; spray reagents, Pauly and 2,4-dinitrophenylhydrazine), $\nu_{max.}$ ($CHCl_3$) 3560 and 1692 cm^{-1}, $\nu_{max.}$ (KBr) 3425 and 1685 cm^{-1}, $\lambda_{max.}$ ($CHCl_3$) 280sh, 275·5, and 242sh nm.

γ-Benzyl-L-glutamic Acid 2-Phenacyloxyphenyl Ester Hydrochloride.—Method A. 2-Nitrophenylsulphenyl-γ-benzyl-L-glutamic acid dicyclohexylamine salt (28·55 g, 50 mmol) was treated as usual to remove dicyclohexylamine. After removal of the solvent, the residue was dissolved in acetone (150 ml), the solution was cooled to 0°, and dicyclohexylcarbodi-imide (10·3 g, 50 mmol), 2-phenacyloxyphenol (11·4 g, 50 mmol), and pyridine (4 ml, 50 mmol) were added with stirring. After 16 h at 0°, the mixture was filtered and evaporated to give an oil. This was converted into the hydrochloride by dissolving in ethyl acetate and adding a solution of hydrogen chloride in ether (35·8 ml; 4·2N). After 10 min the precipitate was filtered off and washed with ethyl acetate and light petroleum. Recrystallization from acetone–ethanol gave the pure *ester hydrochloride* (3·37 g, 14%), m.p. 155—158°, $[\alpha]_{546}^{25}$ 7·67° (*c* 1·0 in $CHCl_3$), $\nu_{max.}$ ($CHCl_3$) 1772, 1735, and 1707 cm^{-1}, argentometric titration shows 99·7% Cl^- (Found: C, 63·75; H, 5·5; Cl, 7·2; N, 3·0; O, 19·5. $C_{26}H_{26}ClNO_6$ requires C, 64·5; H, 5·45; Cl, 7·3; N, 2·9; O, 19·85%).

Method B. The esterification was carried out with the same starting material as described by Jones and Young[9] for phthaloylglycine 2-benzyloxyphenyl ester. The purification procedure was modified as follows. After evaporation of dichloromethane, ethyl acetate was added and triethylamine hydrochloride was filtered off. The filtrate was extracted several times with brine and dried (Na_2SO_4). The 2-nitrophenylsulphenyl derivative was converted into the hydrochloride as in method A. Recrystallization from acetone–ethanol yielded the pure ester hydrochloride (7·65 g, 32%). Method B appears to give better yields than method A.

2-Nitrophenylsulphenyl-O-benzyl-L-tyrosyl-γ-benzyl-L-glutamic Acid 2-Phenacyloxyphenyl Ester.—Standard coupling procedure on 10 millimolar scale. Dicyclohexylcarbodiimide (2·06 g, 10 mmol) was added to a stirred suspension, cooled at −20°, of γ-benzyl-L-glutamic acid 2-phenacyloxyphenyl ester hydrochloride (4·83 g, 10 mmol) and 2-nitrophenylsulphenyl-*O*-benzyl-L-tyrosine dicyclohexylamine salt (6·05 g, 10 mmol) in chloroform (110 ml). After 1 h, the mixture was allowed to reach room temperature and stirred overnight. Chloroform was evaporated off and the residue was stirred with acetone. The insoluble matter was filtered off, the filtrate evaporated, and the residue (oily in this case) crystallized from absolute ethanol containing a little acetone, giving the pure *dipeptide ester* (8·08 g, 95%), m.p. 88—91°, $[\alpha]_{546}^{25}$ −30·3° (*c* 1·0 in $CHCl_3$), $\nu_{max.}$ ($CHCl_3$) 1764, 1730, and 1675 cm^{-1} (Found: C, 66·9; H, 5·3; N, 4·9; O, 19·05; S, 3·75. $C_{48}H_{43}NO_{10}S$ requires C, 67·5; H, 5·05; N, 4·9; O, 18·8; S, 3·75%).

O-Benzyl-L-tyrosyl-γ-benzyl-L-glutamic Acid 2-Phenacyloxyphenyl Ester Hydrochloride.—A solution of hydrogen chloride in ether (6·3 ml; 4·2N) was added with stirring to a solution of the preceding dipeptide (7·7 g, 8·8 mmol) in ethyl acetate (50 ml). After 10 min the precipitate was filtered off, washed with acetone and light petroleum, and dried. The crude compound was dissolved in dimethylformamide (30 ml) and precipitated with dry ether to give the pure *dipeptide ester hydrochloride* (6·3 g, 97%), m.p. 162—165° (decomp.), $[\alpha]_{546}^{25}$ −27·5° (*c* 1·0 in $Me_2N{\cdot}CHO$), argentometric titration shows 100% Cl^-, $\nu_{max.}$ (KBr) 1750, 1730, 1698, and 1662 cm^{-1} (Found: C, 67·75; H, 5·8; Cl, 4·65; N, 3·8. $C_{42}H_{41}ClN_2O_8$ requires C, 68·4; H, 5·55; Cl, 4·8; N, 3·8%).

t-Butoxycarbonyl-γ-benzyl-L-glutamyl-O-benzyl-L-tyrosyl-γ-benzyl-L-glutamic Acid 2-Phenacyloxyphenyl Ester.—This

[16] Y. Trudelle and G. Spach, *Tetrahedron Letters*, 1972, 3475.
[17] I. Lazennec, *Bull. Soc. chim. France*, 1903, 502.

was prepared from t-butoxycarbonyl-γ-benzyl-L-glutamic acid dicyclohexylamine salt and from the preceding dipeptide on a 4·07 mmol scale by the standard coupling procedure. After evaporation of acetone the solid residue was recrystallized from absolute ethanol giving the pure *tripeptide ester* (3·54 g, 85·5%), m.p. 137—140°, $[\alpha]_{546}^{25}$ −44·6° (*c* 1·0 in $CHCl_3$), $\nu_{max.}$ ($CHCl_3$) 1766, 1728, 1702, and 1670 cm^{-1} (Found: C, 69·35; H, 5·95; N, 4·25; O, 20·35. $C_{59}H_{61}N_3O_{13}$ requires C, 69·5; H, 6·0; N, 4·1; O, 20·4%).

t-Butoxycarbonyl-γ-benzyl-L-glutamyl-O-benzyl-L-tyrosyl-γ-benzyl-L-glutamic Acid 2-Hydroxyphenyl Ester.—Standard procedure for removal of phenacyl groups on 10 millimolar scale. Zinc dust (13·15 g, 200 mmol) was added over 10 min to a stirred solution of the phenacyl derivative (10 mmol) in aqueous acetic acid (90%; 118 ml). Stirring was continued for a further 20 min, the insoluble matter was filtered off, and the filtrate was evaporated to dryness. The oily residue was extracted with chloroform and zinc acetate filtered off. Evaporation of chloroform left an oil which was triturated with light petroleum until it crystallized. Treatment of the preceding tripeptide (2·8 mmol) in this way and recrystallization of the crude solid from cyclohexane–ethyl acetate gave the chromatographically pure *tripeptide* (2·2 g, 87·5%), m.p. 134—135°, $[\alpha]_{546}^{25}$ −30·1° (*c* 1·0 in $CHCl_3$), R_F 0·56 (A), 0·69 (B), $\nu_{max.}$ ($CHCl_3$) 1766, 1728, and 1690br cm^{-1} (Found: C, 67·8; H, 6·35; N, 4·75. $C_{51}H_{55}N_3O_{12}$ requires 67·9; H, 6·1; N, 4·65%).

γ-Benzyl-L-glutamyl-O-benzyl-L-tyrosyl-γ-benzyl-L-glutamic Acid 2-Hydroxyphenyl Ester Hydrochloride.—The preceding tripeptide (2·1 g, 3·33 mmol) was dissolved with stirring in a solution of hydrogen chloride in acetic acid (24 ml; 1·1N). After 15 min the acetic acid was evaporated off leaving an oil, which was reprecipitated from benzene with dry ether to give, after thorough washing with dry ether, the pure *tripeptide ester hydrochloride* (1·9 g, 98%), m.p. 150—153° (decomp.), $[\alpha]_{546}^{25}$ −9·4° (*c* 1·0 in $Me_2N{\cdot}CHO$), argentometric titration shows 99·6% Cl^-, $\nu_{max.}$ (KBr) 1760, 1735, and 1660 cm^{-1} (Found: C, 65·35; H, 6·1; Cl, 4·2; N, 5·4; O, 19·2. $C_{46}H_{48}ClN_3O_{10}$ requires C, 65·9; H, 5·8; Cl, 4·25; N, 5·0; O, 19·1%).

2-Nitrophenylsulphenyl-γ-benzyl-L-glutamyl-O-benzyl-L-tyrosyl-γ-benzyl-L-glutamic Acid 2-Phenacyloxyphenyl Ester.—This was prepared from *O*-benzyl-L-tyrosyl-γ-benzyl-L-glutamic acid 2-phenacyloxyphenyl ester hydrochloride and 2-nitrophenylsulphenyl-γ-benzyl-L-glutamic acid dicyclohexylamine salt on a 5·4 mmol scale, by the standard coupling procedure. After evaporation of acetone the solid residue was recrystallized from absolute ethanol containing a little acetone giving the pure *tripeptide ester* (5·25 g, 90%), m.p. 113—114°, $[\alpha]_{546}^{25}$ −63·5° (*c* 1·0 in $CHCl_3$), $\nu_{max.}$ ($CHCl_3$) 1770, 1734, 1702sh, and 1670 cm^{-1} (Found: C, 67·1; H, 5·2; N, 5·05; O, 19·35; S, 2·95. $C_{60}H_{56}N_4O_{13}S$ requires C, 67·1; H, 5·2; N, 5·2; O, 19·4; S, 3·0%).

γ-Benzyl-L-glutamyl-O-benzyl-L-tyrosyl-γ-benzyl-L-glutamic Acid 2-Phenacyloxyphenyl Ester Hydrochloride.—A solution of hydrogen chloride in ether (3·8 ml; 4·2N) was added with stirring to a solution of the preceding tripeptide (5·75 g, 5·36 mmol) in ethyl acetate (300 ml). After 10 min, the mixture was concentrated and the precipitate filtered off. After drying, the crude compound was reprecipitated from dimethylformamide with dry ether giving the pure *tripeptide ester hydrochloride* (4·7 g, 80%), m.p. 161—164°, $[\alpha]_{546}^{25}$ −12·05° (*c* 1·0 in $Me_2N{\cdot}CHO$), $\nu_{max.}$ (KBr) 1758, 1732, 1694, and 1639 cm^{-1} (Found: C, 67·8; H, 5·9; Cl, 3·7; N, 4·25; O, 18·45. $C_{54}H_{54}ClN_3O_{11}$ requires C, 67·8; H, 5·65; Cl, 3·7; N, 4·4; O, 18·45%).

t-Butoxycarbonyl-γ-benzyl-L-glutamyl-γ-benzyl-L-glutamyl-O-benzyl-L-tyrosyl-γ-benzyl-L-glutamic Acid 2-Phenacyloxyphenyl Ester.—This was prepared from t-butoxycarbonyl-γ-benzyl-L-glutamic acid dicyclohexylamine salt and the preceding tripeptide on a 4·7 mmol scale by the standard coupling procedure. After evaporation of acetone, the solid residue was recrystallized from absolute ethanol giving the pure *tetrapeptide ester* (5·22 g, 90%), m.p. 138—139°, $[\alpha]_{546}^{25}$ −46·5° (*c* 1·0 in $CHCl_3$), $\nu_{max.}$ ($CHCl_3$) 1757, 1730, 1701sh, and 1675 cm^{-1} (Found: C, 68·65; H, 6·15; N, 4·4; O, 20·65. $C_{71}H_{74}N_4O_{16}$ requires C, 68·8; H, 6·0; N, 4·5; O, 20·7%).

t-Butoxycarbonyl-γ-benzyl-L-glutamyl-γ-benzyl-L-glutamyl-O-benzyl-L-tyrosyl-γ-benzyl-L-glutamic Acid 2-Hydroxyphenyl Ester.—This was prepared from the preceding tetrapeptide on a 4 mmol scale by the standard procedure for removal of phenacyl groups. The crude solid was recrystallized, after decolourising with charcoal, from from cyclohexane–acetone, giving the chromatographically pure *tetrapeptide ester* (3·85 g, 87%), m.p. 153—154°, $[\alpha]_{546}^{25}$ −45·8° (*c* 1·0 in $CHCl_3$), R_F 0·52 (A), 0·73 (B), $\nu_{max.}$ ($CHCl_3$) 1761sh, 1726, and 1678br cm^{-1} (Found: C, 67·25; H, 6·15; N, 5·05; O, 21·55. $C_{63}H_{68}N_4O_{15}$ requires C, 67·4; H, 6·05; N, 5·0; O, 21·45%).

γ-Benzyl-L-glutamyl-γ-benzyl-L-glutamyl-O-benzyl-L-tyrosyl-γ-benzyl-L-glutamic Acid 2-Hydroxyphenyl Ester Hydrochloride.—The preceding tetrapeptide (3·5 g, 3·12 mmol) was dissolved with stirring in a solution of hydrogen chloride in acetic acid (35 ml; 1·1N). After 15 min, acetic acid was evaporated off and the oily residue dissolved in benzene. The solution was briefly refluxed on charcoal and poured carefully into a large volume of dry ether, giving the *tetrapeptide ester hydrochloride* as crystals (2·7 g, 82%), m.p. 78—80°, $[\alpha]_{546}^{25}$ −15·15° (*c* 1·0 in $CHCl_3$), argentometric titration shows 99% Cl^-, $\nu_{max.}$ ($CHCl_3$) 1760sh, 1730, and 1668br cm^{-1} (Found: C, 65·1; H, 5·95; Cl, 3·3; N, 5·2; O, 20·15. $C_{58}H_{61}ClN_4O_{13}$ requires C, 65·9; H, 5·8; Cl, 3·35; N, 5·3; O, 19·7%).

t-Butoxycarbonyl-O-benzyl-L-tyrosyl-γ-benzyl-L-glutamyl-O-benzyl-L-tyrosyl-γ-benzyl-L-glutamic Acid 2-Phenacyloxyphenyl Ester.—This was prepared from t-butoxycarbonyl-*O*-benzyl-L-tyrosine and from γ-benzyl-L-glutamyl-*O*-benzyl-L-tyrosyl-γ-benzyl-L-glutamic acid 2-phenacyloxyphenyl ester hydrochloride on a 6·3 mmol scale. The standard coupling procedure was slightly modified as follows. First, triethylamine (0·875 ml, 6·3 mmol) was added to retain the hydrogen chloride. Second, after removal of chloroform, the solid residue was stirred with a large volume of hot acetone; the mixture was cooled to room temperature and filtered. The acetone was evaporated off and the crude product recrystallized from absolute ethanol (105 ml), acetone (135 ml), and chloroform (17 ml) giving, upon storage at −15° overnight, the pure *tetrapeptide ester* (6·55 g, 81·5%), m.p. 172—173°, $[\alpha]_{546}^{25}$ −43° (*c* 1·0 in $CHCl_3$), $\nu_{max.}$ ($CHCl_3$) 1768, 1725, 1695sh, and 1675 cm^{-1} (Found: C, 70·75; H, 6·15; N, 4·45; O, 18·65. $C_{75}H_{76}N_4O_{15}$ requires C, 70·75; H, 6·05; N, 4·4; O, 18·85%).

t-Butoxycarbonyl-O-benzyl-L-tyrosyl-γ-benzyl-L-glutamyl-O-benzyl-L-tyrosyl-γ-benzyl-L-glutamic Acid 2-Hydroxyphenyl Ester.—This was prepared from the preceding tetrapeptide on a 5·1 mmol scale by the standard pro-

cedure for removal of phenacyl groups and with double the quantity of aqueous acetic acid. Evaporation of chloroform left a crystalline residue which was washed with light petroleum and recrystallized from cyclohexane–acetone. At this stage, the tetrapeptide was found to contain a base-titratable impurity which was removed by precipitating the product from acetone with dilute hydrochloric acid (0·02N). The chromatographically pure *tetrapeptide ester* then obtained (4·98 g, 84·5%) had m.p. 178—180°, $[\alpha]_{546}^{25}$ −49·6° (*c* 1·0 in $CHCl_3$), R_F 0·55 (A), 0·73 (B), $\nu_{max.}$ ($CHCl_3$) 1761sh, 1722, and 1678br cm^{-1} (Found: C, 69·65; H, 6·2; N, 5·1; O, 19·5. $C_{67}H_{70}N_4O_{14}$ requires C, 69·65; H, 6·1; N, 4·85; O, 19·4%).

O-Benzyl-L-tyrosyl-γ-benzyl-L-glutamyl-O-benzyl-L-tyrosyl-γ-benzyl-L-glutamic Acid 2-Hydroxyphenyl Ester Hydrochloride.—The preceding tetrapeptide (4·73 g, 4·34 mmol) was dissolved in acetic acid (10 ml) by slight warming. To the solution, cooled to room temperature, a solution of hydrogen chloride in acetic acid (40 ml; 1·3N) was added. After 15 min the solvent was evaporated off and the solid residue dissolved in acetone. After decolourising with charcoal, the solution was poured into a large volume of dry ether, giving the pure *tetrapeptide ester hydrochloride* (3·87 g, 90%), m.p. 98—102°, $[\alpha]_{546}^{25}$ −37·5° (*c* 1·0 in $CHCl_3$), argentometric titration shows 99·2% Cl^-, $\nu_{max.}$ (film from $CHCl_3$) 1760sh, 1735, and 1645 cm^{-1} (Found: C, 67·85; H, 5·85; Cl, 3·15; N, 5·15; O, 17·45. $C_{62}H_{63}ClN_4O_{12}$ requires C, 68·2; H, 5·85; Cl, 3·25; N, 5·15; O, 17·6%). This compound seems to exist in another crystalline form, weakly soluble in chloroform.

Poly-(O-benzyl-L-tyrosyl-γ-benzyl-L-glutamyl-O-benzyl-L-tyrosyl-γ-benzyl-L-glutamyl), Poly-(γ-benzyl-L-glutamyl-O-benzyl-L-tyrosyl-γ-benzyl-L-glutamyl), and Poly-(γ-benzyl-L-glutamyl-γ-benzyl-L-glutamyl-O-benzyl-L-tyrosyl-γ-benzyl-L-glutamyl).—The three polymers were prepared by the same standard procedure. The peptide ester hydrochloride (1 mmol) was triturated with dimethylformamide (0·2 ml) and triethylamine (0·028 ml, 2 mmol). After 24 h more dimethylformamide (0·2 ml) was added to improve the homogeneity of the paste. After a further 72 h, the mixture, which had stiffened to a violet gel, was diluted with a large volume of absolute ethanol. The precipitate was washed repeatedly with absolute ethanol by centrifugation, dried, and extracted exhaustively with absolute ethanol for 8 days in a hot Soxhlet extractor. Drying *in vacuo* at 40° for 24 h gave *poly Tyr(Bzl)-Glu(OBzl)-Tyr(Bzl)-Glu(OBzl)* (66%), $\nu_{max.}$ (film from $CHCl_3$) 3282, 1738, 1659, 1628w, and 1548 cm^{-1}; *poly Glu(OBzl)-Tyr(Bzl)-Glu(OBzl)* (68%), $\nu_{max.}$ (film from $CHCl_3$) 3288, 1738, 1655, and 1550 cm^{-1}; or *poly Glu(OBzl)-Glu(OBzl)-Tyr(Bzl)-Glu(OBzl)* (72%), $\nu_{max.}$ (film from $CHCl_3$) 3288, 1738, 1655, and 1548 cm^{-1}.

Poly-(L-tyrosyl-L-glutamyl-L-tyrosyl-L-glutamyl).—To a solution of the protected polytetrapeptide (0·236 g, 1·0 mmol) in dichloromethane (2·9 ml) were added anisole (2·76 ml, 50 mmol per tyrosyl residue) and a saturated solution of hydrogen bromide in acetic acid (41·5 ml). After 1 h, the clear solution was concentrated to *ca.* 5 ml; the polymer was precipitated with ether, filtered off, washed with ether, and dried. The crude partly acetylated polymer was dissolved in barium hydroxide (0·37N). After 15 min the deacetylated polymer was precipitated with dilute hydrochloric acid, washed repeatedly with water by centrifugation, dissolved in sodium hydroxide (0·1N), and dialysed exhaustively for 1 week against phosphate buffer (1/15M, pH 7), to prevent precipitation which occurs slowly below pH 6. The solution was finally deionized by dialysing against pure water. The contents of the dialysis tube were filtered and concentrated, and the polymer was precipitated with dilute hydrochloric acid, thoroughly washed by centrifugation with water, suspended in a few ml of water and lyophilized. Drying at 40° *in vacuo* (0·02 Torr) for 24 h, gave the purified *polymer* (0·038 g, 26%), $[\alpha]_{546}^{25}$ † −55·1° [*c* 0·1 in NaOH (0·2N), NaCl 0·1M)], $\nu_{max.}$ ±2 cm^{-1} (film from Me_2SO) 3284, 1718br, 1659, 1627, and 1548 cm^{-1} (Found: C, 53·15; H, 6·05; N, 9·1. $C_{28}H_{32}N_4O_{10},1·5H_2O$ requires C, 52·7; H, 5·95; N, 8·8%), $\lambda_{max.}$ [*c* 0·25 mg ml^{-1} in NaOH (0·2N), NaCl (0·1M); cell 1 mm] 293·5 (ε 2180 l mol^{-1} cm^{-1}) and 242·5 (ε 10,000) (ε calculated per tyrosyl residue in dehydrated polymer).

Poly-(L-glutamyl-L-tyrosyl-L-glutamyl).—This polymer was prepared from the benzylated polytripeptide on a 1 mmol scale by the procedure just described. Dialysis was carried out against pure water only. The purified *polymer* (0·119 g, 85%) showed $[\alpha]_{546}^{25}$ † −57·2° [*c* 0·1 in NaOH (0·2N), NaCl (0·1M)], $\nu_{max.}$ ±2 cm^{-1} (film from Me_2SO) 3286, 1716br, 1657, and 1551 cm^{-1} (Found: C, 50·65; H, 5·95; N, 9·4. $C_{19}H_{23}N_3O_8,1·5H_2O$ requires C, 50·9; H, 5·8; N, 9·4%), $\lambda_{max.}$ [*c* 0·66 mg ml^{-1} in NaOH (0·2N), NaCl (0·1M); cell 1 mm] 294 (ε 2280 l mol^{-1} cm^{-1}) and 243 nm (ε 10,580) (ε calculated per tyrosyl residue in dehydrated polymer).

Poly-(L-glutamyl-L-glutamyl-L-tyrosyl-L-glutamyl).— This polymer was prepared from the benzylated polytetrapeptide on a 1 mmol scale as described for the preceding one. The purified *polymer* (0·122 g, 88%) showed $[\alpha]_{546}^{25}$ † −74·5° [*c* 0·1 in NaOH (0·2N), NaCl (0·1M)], $\nu_{max.}$ ±2 cm^{-1} (film from Me_2SO) 3286, 1717, 1657, and 1549 cm^{-1} (Found: C, 49·4; H, 5·9; N, 9·75. $C_{24}H_{30}N_4O_{11},2H_2O$ requires C, 49·2; H, 5·8; N, 9·6%), $\lambda_{max.}$ [*c* 0·45 mg ml^{-1} in NaOH (0·2N), NaCl (0·1M); cell 1 mm] 293·5 (ε 2255 l mol^{-1} cm^{-1}) and 242·5 nm (ε 10,950) (ε calculated per tyrosyl residue in dehydrated polymer).

Estimation of the Molecular Weights of Deprotected Polymers.—Molecular weights were measured at 25°. Solvents were Sorensen buffers: phosphate buffer (1/15M) or glycine buffer (0·1M), containing 0·005% of Tween 80 as surfactant. Osmotic pressure was extrapolated to zero concentration using three solutions for each polymer *i.e.* 2, 1, and 0·66 g l^{-1}. Membranes were made by the following procedure: after exhaustive dialysis of the polymer, a 47 mm disc was cut out from the wet tubing (thin-wall Visking dialysis tubing 18/32) and equilibrated in the appropriate buffer before setting on the osmometer. Because equilibria were attained very slowly (30 min at least), recording was necessary to detect the correct steady state. The following data were obtained: poly-(L-tyrosyl-L-glutamyl-L-tyrosyl-L-glutamyl), pH 7·06, M_n 22,600; pH 7·06 (after a long storage of the solution) M_n 74,000; pH 10, M_n 15,700; such values seem to indicate the occurrence of an aggregation phenomenon. Poly-(L-glutamyl-L-tyrosyl-L-glutamyl), pH 7·4, M_n 10,400. Poly-(L-glutamyl-L-glutamyl-L-tyrosyl-L-glutamyl), pH 7·4, M_n 8050.

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† Concentrations calculated from optical density at 293·5—294 nm.