Polypeptides. Part XVII.[1] The Synthesis of Some Sequential Polypeptides of γ-Benzyl D-Glutamate and L-Leucine †

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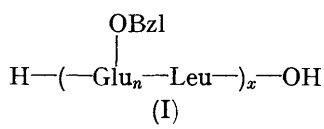
The succinimidyl esters of mono-, di-, tri-, and tetra-γ-benzyl-D-glutamyl-L-leucine (VII; n = 1—4) have been prepared from the corresponding acids (V; n = 1—4), which were synthesised by the dicyclohexylcarbodi-imide method; the t-butoxycarbonyl group was used for amino-group protection and the 2-methylthioethyl ester group for carboxy-protection. Polymerisation of these esters gave satisfactory yields of the corresponding sequential polypeptides (I; n = 1—4) with molecular weights of between 11,000 and 31,000. Little racemisation (1—2%) accompanies the polymerisations, and the method is recommended as a procedure of choice for the synthesis of sequential polypeptides.

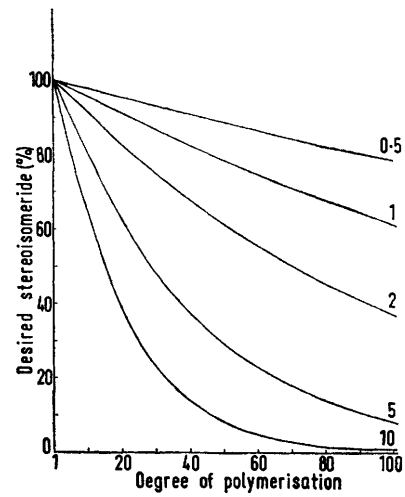
In the preceding paper the synthesis of a series of sequential polypeptides containing γ-t-butyl D- and L-glutamyl residues was described, these being residues which give rise to poly-(amino-acids) which adopt left-handed and right-handed α-helical conformations, respectively, in helicogenic solvents. The present paper is concerned with the synthesis of a further series of sequential polypeptides (I; n = 1—4) containing another pair of similarly related amino-acid residues, *viz.* γ-benzyl D-glutamyl and L-leucyl. L-Leucine was chosen in preference to other aliphatic amino-acids since poly-(L-leucine) forms a very stable α-helix [2] and its side-chain avoids the extremes of small size (as in alanine) and undue steric hindrance (as in valine). All the polymers contained more γ-



(I)

benzyl D-glutamyl than L-leucyl residues, since poly-(L-leucine) forms a considerably stronger α-helix than does poly-(γ-benzyl D-glutamate).[2]

A defect in our earlier work [1] was the considerable amount of racemisation (10—20%) undergone by the *C*-terminal residue of an oligopeptide during polymerisation by the dicyclohexylcarbodi-imide method. As one of us pointed out some time ago,[3] even a very small degree of racemisation can result in serious stereochemical inhomogeneity in the product of polymerisation of an oligopeptide. The amounts of polymers with 0, 1, 2 . . . inverted residues arising from racemisation of the *C*-terminal residue of the oligopeptide monomer are given by the 1st, 2nd, 3rd . . . terms of the binomial expansion of $(x + 1)^n$, where the fractional racemisation at each polymerisation step is $2/(x + 1)$ and the degree of polymerisation is $n + 1$. The fraction of the desired stereochemically homogeneous polymer in the product will be $x^n/(x + 1)^n$, the remainder being a mixture of 2^n diastereoisomerides which will be separable neither from one another nor from the desired product. The Figure illustrates the results of some calculations along these lines; clearly, for any reasonable degree of polymerisation, the amount of racemisation must be very small (not more than, say, 1—2%) if the polymer is to be sufficiently stereochemically homogeneous for its physical properties to be interpretable with any real confidence.



The effect of racemisation of the *C*-terminal residue on the amount of the derived stereochemically homogeneous polypeptide formed in the polymerisation of an oligopeptide. The number against each curve is the % racemisation at each polymerisation stage

Solely from the point of view of racemisation, the azide method would appear to be the method of choice for the polymerisation of oligopeptides. This method has, indeed, been used for the polymerisation of oligoglycines,[4] but the manifold possibilities for the formation of by-products,[5] easily removed from small peptides but not from macromolecules, make the method unattractive. In common with the majority of previous workers in this field, we concluded that the active ester method represents the best compromise for use in the polymerisation of oligopeptides. The method is highly selective, giving rise to few by-products,

† In this paper, *N*-succinimidyl refers to the radical $\overline{C(:O)\cdot[CH_2]_2\cdot C(:O)\cdot N}$-

[1] Part XVI, D. I. Marlborough and H. N. Rydon, preceding paper.

[2] See G. D. Fasman, ' Poly-α-amino-acids,' Dekker, New York, 1967, ch. 11.

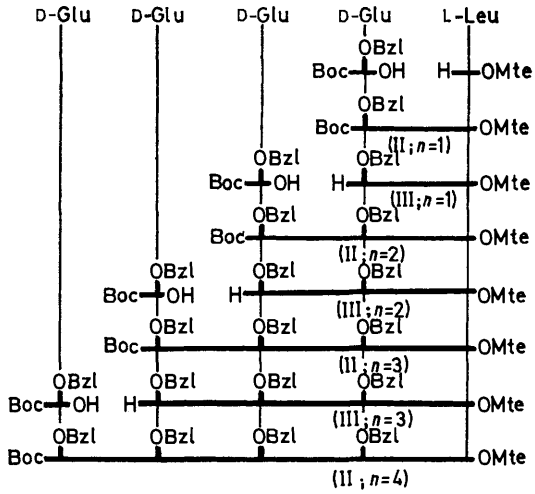
[3] H. N. Rydon, Abstracts for Chemical Society Anniversary Meeting, Exeter, 1967, A10.

[4] M. Z. Magee and K. Hofmann, *J. Amer. Chem. Soc.*, 1949, **71**, 1515; H. N. Rydon and P. W. G. Smith, *J. Chem. Soc.*, 1955, 2542.

[5] *Cf.* J. Rudinger, *Pure and Appl. Chem.*, 1963, **7**, 335.

and the esters themselves can be readily purified prior to polymerisation, a very desirable feature if high molecular weight products are to be obtained.[6] On the other hand the method can lead to extensive racemisation, as is known to be the case with the esters most widely used hitherto for the polymerisation of oligopeptides, *viz.* the *p*-nitrophenyl [6,7] and pentachlorophenyl [8] compounds. Of the other active esters available when we started work, we chose the succinimidyl compounds, since it had been shown that both their preparation from peptides [9] and their use for peptide synthesis [10] was accompanied by only a very slight degree of racemisation; the use of these esters for the polymerisation of oligopeptides had not previously been reported, although Segal and Traub [11] have since used the method for the preparation of some collagen models. Since our work began, several new active esters have been found to bring about racemisation-free peptide couplings,[12] and some of these may prove to be highly satisfactory alternatives to succinimidyl esters for the synthesis of sequential polypeptides.[13]

The required oligopeptides (II; $n = 1$—4) were synthesised in stepwise fashion with L-leucine at the *C*-terminus (Scheme 1) * in order to minimise the



SCHEME 1

number of synthetic intermediates. The reactivity of the succinimidyl ester group precludes its use for carboxy-protection throughout the synthesis; we used the 2-methylthioethyl ester group [14] for carboxy-protection, owing to its easy removal under alkaline conditions too mild for there to be any danger of transpeptidation involving the glutamyl side-chains, and found it wholly satisfactory for the purpose. The t-butoxycarbonyl group was used for amino-group protection; we found t-butyl 2,4,5-trichlorophenyl carbonate [15] much better than t-butyl azidoformate [16,17] for the introduction of this group into γ-benzyl glutamate. The couplings were all carried out with dicyclohexylcarbodi-imide. The hydrochlorides of the intermediates (III; $n = 1$—3), obtained from the *N*-t-butoxycarbonyl derivatives (II; $n = 1$—3) by the action of hydrogen chloride in ethyl acetate, could not be crystallised, but further coupling of the oily products with γ-benzyl *N*-t-butoxycarbonyl-D-glutamate in the presence of triethylamine gave the fully protected dipeptides (II; $n = 2$—4) in crystalline form.

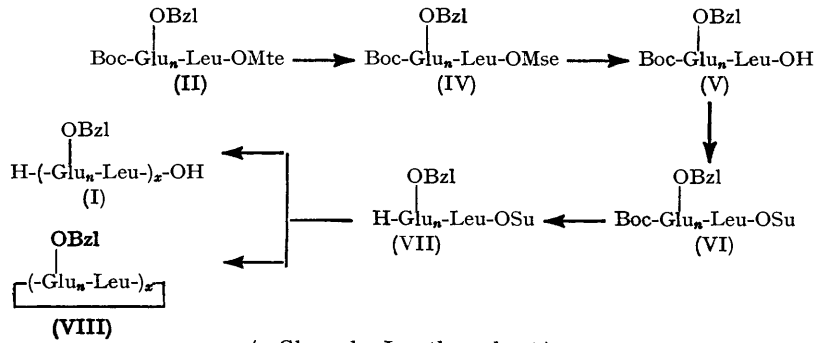
The synthetic plan now required conversion of the 2-methylthioethyl esters (II) into the succinimidyl esters (VII); this was accomplished as in Scheme 2. Removal of the 2-methylthioethyl ester group from the protected peptides (II) by the action of alkali on the methiodides [14] gave only poor yields of the carboxylic acids (V), mainly owing to side-reactions in the preparation of the sulphonium salts; an alternative method [18] involving sensitising the ester to alkali by conversion into the 2-methylsulphonylethyl ester (IV) proved satisfactory. The oxidations of the 2-methylthioethyl esters to the methylsulphonyl compounds was brought about in high yield with hydrogen peroxide in aqueous acetone, in the presence of ammonium molybdate as catalyst. Although, as has been pointed out,[19] the use of hydrogen peroxide in an organic solvent is potentially hazardous, we have had no untoward experiences in such oxidations; of course, we do not heat the reaction mixtures and we finally remove the acetone with a rotary evaporator from a tepid water-bath.

The *N*-t-butoxycarbonyl peptides (V) were obtained in 75—95% yields and were converted into the corresponding succinimidyl esters (VI) by condensation with *N*-hydroxysuccinimide in the presence of dicyclohexylcarbodi-imide in dichloromethane; these conditions have been shown to give optically pure products from *N*-protected peptide acids.[9] We found it advan-

* In this Scheme and elsewhere the abbreviations for amino-acid residues and protecting groups are those recommended by I.U.P.A.C., Information Bulletin No. 26. Mte = $CH_2{\cdot}CH_2{\cdot}SMe$; Mse = $CH_2{\cdot}CH_2{\cdot}SO_2{\cdot}Me$.

[6] D. F. De Tar, in 'Peptides: Proc. Eighth European Peptide Symp.,' 1967, p. 125.
[7] F. H. C. Stewart, *Austral. J. Chem.*, 1965, **18**, 887; 1966, **19**, 1503, 2373, and later papers.
[8] J. Kovacs and B. J. Johnson, *J. Chem. Soc.*, 1965, 6777; J. Kovacs, R. Gianotti, and A. Kapoor, *J. Amer. Chem. Soc.*, 1966, **88**, 2282; B. J. Johnson, *J. Chem. Soc.* (*C*), 1967, 1638; 1968, 3008; 1969, 1412; J. Kovacs, L. Kisfaludy, and M. Q. Ceprini, *J. Amer. Chem. Soc.*, 1967, **89**, 183.
[9] F. Weygand and U. Ragnarsson, *Z. Naturforsch.*, 1966, **21b**, 1141.
[10] G. W. Anderson, F. M. Callahan, and J. E. Zimmermann, *J. Amer. Chem. Soc.*, 1967, **89**, 178, 715; N. Izumiya and M. Muraoka, *ibid.*, 1969, **91**, 2391; D. S. Kemp, Z. Bernstein, and J. Rebek, *ibid.*, 1970, **92**, 4756.
[11] D. M. Segal and W. Traub, *J. Mol. Biol.*, 1969, **43**, 487; D. M. Segal, *ibid.*, p. 497.
[12] Review: H. D. Law, *Ann. Reports* (*B*), 1967, **64**, 463.
[13] *Cf.* J. H. Jones, *Chem. Comm.*, 1969, 1436; R. D. Cowell and J. M. Jones, *J. Chem. Soc.* (*C*), 1971, 1082.
[14] M. J. S. A. Amaral, G. C. Barrett, H. N. Rydon, and J. E. Willett, *J. Chem. Soc.* (*C*), 1966, 807.
[15] W. Broadbent, J. S. Morley, and B. E. Stone, *J. Chem. Soc.* (*C*), 1967, 2632.
[16] E. Schröder and E. Klieger, *Annalen*, 1964, **673**, 196.
[17] C. H. Li, B. Gorup, D. Chung, and J. Ramachandran, *J. Org. Chem.*, 1963, **28**, 181.
[18] P. M. Hardy, H. N. Rydon, and R. C. Thompson, *Tetrahedron Letters*, 1968, 2525.
[19] C. J. Stirling, *Chem. in Brit.*, 1969, **5**, 36.

tageous to leave the mixture of carbodi-imide and *N*-protected peptide for 10 min at 0 °C before adding the hydroxysuccinimide; unless this is done appreciable amounts of the β-alanine derivative described by Gross and Bilk [20] are obtained and the yields of active ester are correspondingly diminished. The *N*-t-butoxycarbonyl group was removed from the succinimidyl esters (VI) by the action of a tenfold excess of hydrogen chloride in ethyl acetate, and the resulting hydrochlorides of the oligopeptides (VII) were purified, to constant optical rotation, by recrystallisation from mixed solvents, at room temperature to avoid the decomposition which occurs at elevated temperatures; the hydrochloride of the tripeptide (VII; $n = 2$) could



(D-Glu and L-Leu throughout.)

SCHEME 2

not be recrystallised, but the crude product gave a satisfactory elemental analysis and was used directly in the polymerisation. The overall yields of the hydrochlorides of the succinimidyl esters (VII) from the carboxylic acids (V) varied from 53—71%.

Small-scale polymerisations were carried out with all four oligopeptide succinimidyl esters (VII; $n = 1$—4), primarily to establish suitable conditions for preparative work. These reactions were carried out in a variety of solvents (light petroleum, benzene, dioxan, tetrahydrofuran, dimethylformamide, dimethyl sulphoxide) at concentrations between 0·05 and 3·3 mol kg^{-1}; the products were examined by t.l.c. and by gel filtration on LH 20 Sephadex, which separated them into polymer (I), dioxopiperazine (VIII; $x = 1$) and other cyclic peptides of intermediate molecular weight (VIII; $x > 1$). The detailed results are given in the Experimental section (Tables 3—6) but some comment is warranted here. Moderately good (40—50%) yields of polymer were obtained from the dipeptide (VII; $n = 1$) in all four solvents tried, and the yield of dioxopiperazine was surprisingly low (15—25%); the other products were the cyclic trimer (cyclohexapeptide) and pentamer (cyclodecapeptide), the latter in larger amount than the former. Polymerisation of the tripeptide (VII; $n = 2$) in five solvents, and without added solvent, gave rather poor yields of polymer, less in dimethylformamide and dimethyl sulphoxide than in hydrocarbons; large amounts of cyclic dimer (cyclohexapeptide) and smaller, but not negligible, amounts of cyclononapeptide were formed, but there was very little, if any, cyclotripeptide.[21] In accordance with expectation, more polymer was formed in the more concentrated solutions, although the effect was not very pronounced. The tetrapeptide (VII; $n = 3$) gave good yields of polymer (50—75%) in most solvents, together with substantial amounts of cyclic dimer (cyclo-octapeptide), which was the main product in dimethylformamide; little or no cyclotetrapeptide was formed.[22] The pentapeptide (VII; $n = 4$) gave 40—60% yields of polymer in the four solvents tried; the other major product was the cyclopentapeptide, there being little cyclic dimer (cyclodecapeptide); this is surprising in the light of Schwyzer's classical work on gramicidin-S,[23] but other cases similar to ours are on record.[24]

The conditions used, and the yields of polymer obtained in the large-scale preparative polymerisations are given in Table 1. The polymerisations were allowed to proceed for 4 days at room temperature, after which the reaction mixtures were diluted with chloroform and kept for another few days before work-up. The crude polymers were freed from material of low molecular weight by Soxhlet extraction with methanol; thin-layer

TABLE 1

Polymerisation of oligopeptides (VII)

n =	1	2
Solvent	Tetrahydrofuran	Light petroleum
Concn. (mol kg^{-1})	2·4	0·33
Yield of polymer (%)	51	34
n =	3	4
Solvent	Tetrahydrofuran	Benzene
Concn. (mol kg^{-1})	1·2	0·62
Yield of polymer (%)	56	57

[20] H. Gross and L. Bilk, in ' Peptides: Proc. Ninth European Peptide Symp.,' 1968, p. 156.

[21] *Cf.* R. Schwyzer, J. P. Carrion, B. Gorup, H. Nolting, and T.-K. Aung, *Helv. Chim. Acta*, 1964, **47**, 441.

[22] *Cf.* R. Schwyzer, B. Iselin, W. Rittel, and P. Sieber, *Helv. Chim. Acta*, 1956, **39**, 872.

[23] R. Schwyzer and P. Sieber, *Helv. Chim. Acta*, 1958, **41**, 2186.

[24] See M. Waki and N. Izumiya, *J. Amer. Chem. Soc.*, 1967, **89**, 1278.

chromatograms of the resulting polymers (yields given in Table 1) showed only weakly ninhydrin-positive spots at the origin, all faster-running contaminants having been removed by the methanol extraction. Two cyclic products were isolated from the methanol extracts, *viz.* the dioxopiperazine (VIII; $n = 1$, $x = 1$) from the dipeptide (VII; $n = 1$) and the cyclohexapeptide (VIII; $n = 2$, $x = 2$) from the tripeptide (VII; $n = 2$). All the polymers, except that from the pentapeptide (VII; $n = 4$), of which we had little, were further purified by fractional precipitation from chloroform with methanol; the purified polymers gave satisfactory elemental analyses.

The determination of the molecular weights of polydisperse macromolecular polypeptides presents many difficulties.[6] We had hoped to be able to use membrane osmometry with our polymers, but the presence of small amounts of material of relatively low molecular weight, even in the reprecipitated products, prevented routine use of this method, diffusion through the membrane causing drift and long delay in the establishment of equilibrium. We therefore turned to gel filtration on Sephadex; although not highly accurate this method gives a sufficiently good estimate of molecular weight for our purpose. Since the polybenzyl esters (I) are insoluble in water it was first necessary to convert them into the water-soluble carboxylic acids by removal of the benzyl ester groups. Small samples of the polymers were therefore debenzylated with hydrogen bromide in acetic acid and the resulting poly-acids were chromatographed on a column of Sephadex G-150. The column eluate was monitored at 220 nm and the molecular weights corresponding to the maxima of the elution profiles were calculated from a calibration curve relating the logarithms of the molecular weights of a series of standards to their elution volumes.[25] Initially we used globular proteins as standards, but when a sample of poly-(glutamic acid) of independently determined molecular weight was examined the estimated molecular weight was much too high. The gel filtrations were carried out in ammonium formate buffer, pH 9·0, and it seems likely that at this pH the repulsion between ionised side-chain carboxy-groups causes more extended conformations to be adopted by the sequential polypeptides than is the case with the less acidic globular proteins, some of which have additional conformational constraints in the form of disulphide bridges. Gel filtration separates molecules on the basis of both molecular shape and size, and our experiences emphasise that the use of this method for molecular weight determination requires standards as similar as possible in structure to the compounds to be investigated; since our work was completed, Chao and Einstein[26] have reached a similar conclusion in relation to the determination of the molecular weights of disordered proteins by gel filtration. Our calibration curve was therefore constructed by using as standards poly-(α-amino-acids) of known molecular weight (determined by membrane osmometry), most of which had been prepared by polymerisation of *N*-carboxy-anhydrides.

The molecular weights so determined are given in Table 2; the values are those for the benzyl esters (I), calculated from the directly determined molecular weights of the corresponding acids on the assumption that no degradation accompanies debenzylation. Since the molecular weights used to construct the calibration curve are number averages (M_n), and since the elution profiles of the sequential polypeptides are similar in shape to those for the standards, the molecular weights in Table 2 approximate to number averages.

TABLE 2

Molecular weights of sequential polypeptides (I)

Polymer	M_n	x	No. of amino-acid residues
$n = 1$; fraction 2	13,500	40	80
$n = 2$; fraction 2	21,600	40	120
$n = 2$; fraction 3	12,100	23	69
$n = 3$; fraction 1	11,400	15	60
$n = 3$; fraction 2	23,400	32	128
$n = 3$; fraction 3	19,500	26	78
$n = 4$	31,500	33	165

The optical purity of the sequential polypeptides was checked by an enzymic method. Samples of the polypeptides were hydrolysed with 6M-hydrochloric acid at 110 °C and the leucine in the hydrolysate was separated from the glutamic acid by preparative t.l.c. before digestion with L-amino-acid oxidase;[27] this separation step is essential since the enzyme is inhibited by D-glutamic acid. The amount of D-leucine remaining after digestion was determined with an amino-acid analyser. Control experiments were run with samples of L-leucine to which known amounts (up to 5%) of D-leucine had been added. The polypeptide hydrolysates contained 2·3—3·6% of D-leucine; in the control experiments the amount of D-leucine found was 1·2—2·5% more than that added. We conclude that the amount of racemisation of the *C*-terminal L-leucine residues in the polymerisations was certainly not more than 2% and probably as little as 1%.

The satisfactorily high molecular weights and the high degree of optical purity of the sequential polypeptides (I) renders them suitable for spectroscopic studies of conformation and these will be reported elsewhere. The succinimidyl ester method is of general utility for the preparation of sequential polypeptides by the polymerisation of oligopeptides and we regard it as a method of choice for work of this kind.

EXPERIMENTAL

The purity of all intermediates and end-products was confirmed by t.l.c. on Kieselgel G, whenever possible in two

[25] J. R. Whitaker, *Analyt. Chem.*, 1963, **35**, 1950; P. Andrews, *Biochem. J.*, 1964, **91**, 222; 1965, **96**, 595.

[26] L.-P. Chao and E. R. Einstein, *J. Chromatog.*, 1969, **42**, 485.

[27] *Cf.* R. Riniker and R. Schwyzer, *Helv. Chim. Acta*, 1961, **44**, 658; F. F. Richards, R. W. Sloane, and E. Haber, *Biochemistry*, 1967, **6**, 476.

solvent systems. Compounds with free α-amino-groups were revealed with 0·3% ninhydrin in butanol at 100°; these conditions caused partial development of spots from *N*-t-butoxycarbonyl compounds, but these were best detected by treatment with formic acid before spraying with ninhydrin. These and other *N*-protected peptides were also revealed by the chlorine–starch–iodide method;[28] other organic compounds were revealed either by the quenching of the fluorescence of Kieselgel GF 254 or by spraying with aqueous 5% ammonium sulphate followed by heating at 300°.[29]

Organic solutions were dried over magnesium sulphate. All evaporations and concentrations were carried out under reduced pressure in a rotary evaporator. Light petroleum was the fraction b.p. 60—80°. M.p.s are corrected. Optical rotations were measured with a Bendix-NPL Polarimeter, Model 143C.

A. Synthesis of Oligopeptides

1. *γ-Benzyl* N-*t-Butoxycarbonyl*-D-*glutamate*.—This was prepared by the following procedure, which we found greatly superior to those previously reported[16,17] for the preparation of the L-isomer by use of t-butyl azidoformate. γ-Benzyl D-glutamate[30] (95 g, 0·4 mol) and t-butyl 2,4,5-trichlorophenyl carbonate[15] (14·9 g, 0·5 mol) were stirred at room temp. for 5 days in a mixture of redistilled dimethylformamide (600 ml), water (100 ml), and triethylamine (81 g, 0·8 mol). The mixture was evaporated at 60° and the residue taken up in water (400 ml) and ethyl acetate (400 ml). The pH was brought to 3 with 2M-hydrochloric acid, with continuous shaking; the layers were then separated and the aqueous layer was further extracted with ethyl acetate (2 × 200 ml). The combined ethyl acetate solutions were extracted with saturated aqueous sodium hydrogen carbonate (1 × 500 ml and 2 × 200 ml); the extract was brought to pH 3 with 2M-hydrochloric acid and extracted with ether (3 × 200 ml). The extract was dried and evaporated and the residue dissolved in anhydrous ether (1500 ml) and treated with dicyclohexylamine (72·5 g, 0·4 mol). After 12 h at 0°, the crystals (194 g; m.p. 137—138°) were filtered off and recrystallised from ethyl acetate–light petroleum to give the pure dicyclohexylammonium salt (186 g, 90%), m.p. 139—140°, $[\alpha]_D^{25}$ −11·8° (*c* 1·0 in MeOH) (lit.,[16] m.p. 138—139°, $[\alpha]_D^{25}$ +11·9°, for the L-enantiomorph). This salt was dissolved in ethyl acetate (1000 ml) and washed with 0·5M-hydrochloric acid (3 × 500 ml) and water (3 × 500 ml). The remaining ethyl acetate solution was dried and evaporated. Gradual addition, at 0°, during several days, of light petroleum to a solution of the residue in anhydrous ether (200 ml) gave *γ-benzyl* N-*t-butoxycarbonyl*-D-*glutamate* (108 g, 80%), m.p. 65—66°, $[\alpha]_D^{25}$ −13·2° (*c* 6·0 in $CHCl_3$) (Found: C, 60·5; H, 6·7; N, 4·3. $C_{17}H_{23}NO_6$ requires C, 60·5; H, 6·8; N, 4·3%).

2. *Derivatives of Glutamyl-leucine*.—L-Leucine 2-methylthioethyl ester hydrochloride[14] (29·5 g, 122 mmol) was dissolved in chloroform and treated with a 1·5M-ammonia in the same solvent (90 ml). After 10 min at 0°, the mixture was filtered through Kieselguhr; the filtrate was evaporated to dryness and the residue freed from ammonia by dissolution in dichloromethane and evaporation. After two repetitions of this process, the residual oil was dissolved in dichloromethane (300 ml), together with *γ*-benzyl *N*-t-butoxycarbonyl-D-glutamate (41·1 g, 122 mmol), and dicyclohexylcarbodi-imide (25·4 g, 128 mmol) was added to the stirred solution at 0°. After stirring for 12 h at 4°, acetic acid (0·5 ml) was added and the mixture was kept for 30 min at room temp.; it was then filtered to remove dicyclohexylurea and the filtrate was evaporated to dryness. Two recrystallisations from di-isopropyl ether, the first at −10°, gave *γ-benzyl 2-methylthioethyl* N-*t-butoxycarbonyl*-D-*glutamyl*-L-*leucinate* (II; *n* = 1) (53 g, 83%), m.p. 48—50°, $[\alpha]_D^{25}$ +4·5° (*c* 5·0 in $CHCl_3$) (Found: C, 59·3; H, 7·7; N, 5·3. $C_{26}H_{40}N_2O_7S$ requires C, 59·6; H, 7·6; N, 5·3%).

To this ester (5·2 g, 10 mmol), dissolved in acetone (50 ml) and water (10 ml), aqueous 0·3M-ammonium molybdate (3·0 ml) and hydrogen peroxide (30% w/v; 23 ml, 200 mmol) were added. After 2 h at room temp., the acetone was removed (water-bath, <40°), and the residue was diluted with water (50 ml) and extracted with chloroform (2 × 50 ml). The extract was washed with saturated brine (3 × 50 ml), dried, and evaporated. Crystallisation, by adding a solution in anhydrous ether (30 ml) dropwise with vigorous stirring to di-isopropyl ether (200 ml) and keeping the mixture at −10° for 12 h, gave *γ-benzyl 2-methylsulphonylethyl* N-*t-butoxycarbonyl*-D-*glutamyl*-L-*leucinate* (IV; *n* = 1) (4·7 g, 84%), m.p. 58—60°, $[\alpha]_D^{25}$ +7·5° (*c* 4·5 in $CHCl_3$) (Found: C, 56·3; H, 7·4; N, 5·0. $C_{26}H_{40}N_2O_9S$ requires C, 56·1; H, 7·2; N, 5·0%). The ester (4·7 g, 8·4 mmol) was dissolved in acetone (AnalaR; 36 ml) and water (18 ml). The apparent pH (glass and calomel electrodes) was brought to 10·0 and kept between 10·0 and 10·5 by addition of 0·25M-sodium hydroxide, controlled by an autotitrator. The reaction was stopped after 24 h, by which time 1 equiv. of alkali had been added and the rate of addition had become slow. The apparent pH was brought to 7·0 with M-hydrochloric acid and the acetone was removed. The aqueous solution was basified with saturated aqueous sodium hydrogen carbonate, extracted with ether (3 × 20 ml), acidified to pH 3 with M-hydrochloric acid, and again extracted with ether (3 × 50 ml). The combined second ether extracts were dried and evaporated. Crystallisation of the residue from aqueous ethanol gave *γ-benzyl* N-*t-butoxycarbonyl*-D-*glutamyl*-L-*leucinate* (V; *n* = 1) (3·6 g, 95%), m.p. 53—55°, $[\alpha]_D^{25}$ +6·1° (*c* 5·0 in $CHCl_3$) (Found: C, 61·5; H, 7·7; N, 6·1. $C_{23}H_{34}N_2O_3$ requires C, 61·3; H, 7·6; N, 6·2%).

This ester (4·5 g, 10·1 mmol), dissolved in dichloromethane (50 ml), was stirred at 0° for 10 min with dicyclohexylcarbodi-imide (2·2 g, 10·6 mmol). *N*-Hydroxysuccinimide[31] (2·3 g, 20 mmol) was added and stirring was continued for 1 h at 0° and then for 12 h at room temp. Glacial acetic acid (0·1 ml) was then added and after a further 30 min the precipitated dicyclohexylurea was filtered off. The filtrate was washed with saturated brine (3 × 50 ml), dried, and evaporated. The residue was then taken up in acetone (50 ml) and kept for 12 h at −10°. A little more urea was filtered off and the filtrate was evaporated to dryness. Two recrystallisations from anhydrous ether (200 ml) gave *γ-benzyl* N-*succinimidyl* N-*t-butoxycarbonyl*-D-*glutamyl*-L-*leucinate* (VI; *n* = 1) (3·6 g, 65%), m.p. 110°, $[\alpha]_D^{25}$ −8·8° (*c* 5·0 in $CHCl_3$) (Found:

[28] H. N. Rydon and P. W. G. Smith, *Nature*, 1952, **169**, 922.
[29] T. Ziminski and E. Borowski, *J. Chromatog.*, 1966, **23**, 480.
[30] S. Guttmann and R. A. Boissonas, *Helv. Chim. Acta*, 1958, **41**, 1852.
[31] E. Wünsch and E. Jaeger, *Z. physiol. Chem.*, 1966, **346**, 301.

C, 59·0; H, 6·7; N, 7·8. $C_{27}H_{37}N_3O_9$ requires C, 59·2; H, 6·8; N, 7·7%); this compound, like all the other succinimidyl esters, was too unstable on silica gel for satisfactory t.l.c. even in anhydrous solvents. To the ester (2·0 g, 3·6 mmol) in ethyl acetate (20 ml), 3·5M-hydrogen chloride in the same solvent (10 ml) was added. After 1 h at room temp., the solution was evaporated to dryness and kept in a vacuum desiccator (NaOH) for 12 h. Crystallisation from hexafluoroacetone sesquihydrate–ether gave the *hydrochloride* of *γ-benzyl* N-*succinimidyl* D-*glutamyl*-L-*leucinate* (VII; $n = 1$) (1·33 g, 76%), m.p. 105—106°, $[\alpha]_D^{24}$ −38·6° (*c* 3·2 in $CHCl_3$) (Found: C, 52·9; H, 6·4; N, 8·5. $C_{22}H_{30}ClN_3O_7,H_2O$ requires C, 52·6; H, 6·4; N, 8·4%).

3. *Derivatives of Diglutamyl-leucine.*—γ-Benzyl 2-methylthioethyl *N*-t-butoxycarbonyl-D-glutamyl-L-leucinate (20 g, 38·2 mmol) in ethyl acetate (100 ml) was kept at room temp. for 1 h with 3·5M-hydrogen chloride in ethyl acetate (110 ml). The solvent was removed and the oily residue kept in a vacuum desiccator (NaOH) for 12 h. It was then dissolved in dichloromethane (100 ml) and γ-benzyl *N*-t-butoxycarbonyl-D-glutamate (12·2 g, 36·2 mmol), triethylamine (3·66 g, 36·2 mmol), and dicyclohexylcarbodi-imide (7·8 g, 37·8 mmol) were added successively to the solution cooled to 0°. After stirring at room temp. for 12 h, acetic acid (0·1 ml) was added to the solution, which was then stirred for a further 30 min and filtered. The filtrate was evaporated and the residue taken up in ether (200 ml) and washed with M-hydrochloric acid (3 × 50 ml), saturated aqueous sodium hydrogen carbonate (3 × 50 ml), and saturated brine (2 × 50 ml). The solution was dried and evaporated and the residue kept at −10° for 12 h in acetone (200 ml). Filtration, evaporation, and recrystallisation, first from ether at −10° and then from acetone–ether, gave *di-γ-benzyl 2-methylthioethyl* N-*t-butoxycarbonyldi*-D-*glutamyl*-L-*leucinate* (II; $n = 2$) (19·8 g, 74%), m.p. 84—86°, $[\alpha]_D^{25}$ +16·4° (*c* 5·0 in $CHCl_3$) (Found: C, 61·0; H, 7·0; N, 5·6. $C_{38}H_{53}N_3O_{10}S$ requires C, 61·4; H, 7·1; N, 5·7%).

Oxidation of this ester (7·4 g) as described in A2 for the corresponding dipeptide gave *di-γ-benzyl 2-methylsulphonylethyl* N-*t-butoxycarbonyl-di*-D-*glutamyl*-L-*leucinate* (IV; $n = 2$) (6·2 g, 80%), m.p. 83—84°, $[\alpha]_D^{25}$ +22·9° (*c* 3·7 in $CHCl_3$) (Found: C, 59·0; H, 6·9; N, 5·6. $C_{38}H_{53}N_3O_{12}S$ requires C, 58·8; H, 6·8; N, 5·4%); an oxidation in which the ammonium molybdate was omitted gave a mixture from which was isolated, by preparative t.l.c. ($CHCl_3$–MeOH, 98 : 2), the corresponding *2-methylsulphinylethyl ester*, m.p. 89°, $\nu_{max.}$ 1025s cm^{-1} (S=O stretch) (Found: C, 60·1; H, 7·2; N, 5·5. $C_{38}H_{53}N_3O_{11}S$ requires C, 60·1; H, 7·0; N, 5·5%). The methylsulphonylethyl ester (6·2 g, 8·0 mmol), in acetone (60 ml) and water (30 ml), was split with sodium hydroxide at pH 10—10·5, as described in A2. The acidified reaction solution was evaporated to dryness and the oily residue was taken up in ether (100 ml) and washed with saturated aqueous sodium hydrogen carbonate (2 × 50 ml). The lowest of the resulting three layers was discarded and the remaining two were thoroughly extracted with water (5 × 50 ml). The combined aqueous extract was brought to pH 3 with M-hydrochloric acid and extracted with ether (2 × 50 ml). Evaporation of the dried extract gave *di-γ-benzyl* N-*t-butoxycarbonyldi*-D-*glutamyl*-L-*leucinate* (V; $n = 2$) as an oil (4·4 g, 82%), which could not be crystallised but was characterised as its *dicyclohexylamine salt*, m.p. 101—102° (from ethyl acetate–light petroleum) (Found: C, 65·9; H, 8·6; N, 6·5. $C_{47}H_{70}N_4O_{10}$ requires C, 66·4; H, 8·2; N, 6·6%). The partially protected tripeptide was condensed with *N*-hydroxysuccinimide, as described in A2 for the corresponding dipeptide, to afford *di-γ-benzyl* N-*succinimidyl* N-*t-butoxycarbonyldi*-D-*glutamyl*-L-*leucinate* (VI; $n = 2$) which was twice recrystallised from acetone–ether; yield 68%, m.p. 94—95° and 113—114° (dimorphous), $[\alpha]_D^{25}$ +4·2° (*c* 6·0 in $CHCl_3$) (Found, for low-melting form: C, 61·4; H, 6·6; N, 7·2. For high-melting form: C, 61·1; H, 6·5; N, 7·2. $C_{39}H_{50}N_4O_{12}$ requires C, 61·1; H, 6·5; N, 7·3%). Treatment of this with hydrogen chloride, as described for the corresponding dipeptide, gave the *hydrochloride* of *di-γ-benzyl* N-*succinimidyl di*-D-*glutamyl*-L-*leucinate* (VII; $n = 2$) (98% yield) which could not be recrystallised; m.p. 70—80°, $[\alpha]_D^{25}$ −24·6° (*c* 3·1 in $CHCl_3$) (Found: C, 58·3; H, 6·3; N, 8·1. $C_{34}H_{43}ClN_5O_{10}$ requires C, 58·1; H, 6·1; N, 8·0%).

4. *Derivatives of Triglutamyl-leucine and Tetraglutamyl-leucine.*—The following were prepared by the procedures used for the tripeptide derivatives (A3) except that the initial couplings were carried out in chloroform instead of dichloromethane.

Tri-γ-benzyl 2-methylthioethyl N-*t-butoxycarbonyltri*-D *glutamyl*-L-*leucinate* (II; $n = 3$) (83% yield), m.p. 114—115° (from ethanol–ether), $[\alpha]_D^{25}$ +9·5° (*c* 5·0 in $CHCl_3$) (Found: C, 62·7; H, 7·2; N, 6·4. $C_{50}H_{66}N_4O_{13}S$ requires C, 62·4; H, 6·9; N, 5·8%), and the corresponding *2-methylsulphonylethyl ester* (IV; $n = 3$) (86% yield), m.p. 120—121° (from ethanol–ether), $[\alpha]_D^{25}$ +12·6° (*c* 5·0 in $CHCl_3$) (Found: C, 60·3; H, 6·7; N, 5·7. $C_{50}H_{66}N_4O_{15}S$ requires C, 60·4; H, 6·6; N, 5·6%); *tri-γ-benzyl* N-*t-butoxycarbonyltri*-D-*glutamyl*-L-*leucinate* (V; $n = 3$) (86% yield), m.p. 77—78° (from aqueous ethanol), $[\alpha]_D^{23}$ +12·2° (*c* 5·6 in $CHCl_3$) (Found: C, 62·3; H, 6·6; N, 6·2. $C_{47}H_{60}N_4O_{13},H_2O$ requires C, 62·3; H, 6·8; N, 6·2%), and its N-*succinimidyl ester* (VI; $n = 3$) (77% yield), m.p. 127—128° (from ethanol), $[\alpha]_D^{25}$ +1·7° (*c* 4·6 in $CHCl_3$) (Found: C, 62·0; H, 6·4; N, 7·1. $C_{51}H_{63}N_5O_{15}$ requires C, 62·1; H, 6·4; N, 7·1%); *tri-γ-benzyl* N-*succinimidyl tri*-D-*glutamyl*-L-*leucinate hydrochloride* (VII; $n = 3$) (75% yield), m.p. 74—76° (from acetone–ether), $[\alpha]_D^{25}$ −28·6° (*c* 4·8 in $CHCl_3$) (Found: C, 59·9; H, 6·1; N, 7·7. $C_{46}H_{56}ClN_5O_{10}$ requires C, 59·9; H, 6·1; N, 7·6%).

Tetra-γ-benzyl 2-methylthioethyl N-*t-butoxycarbonyltetra*-D-*glutamyl*-L-*leucinate* (II; $n = 4$) (88% yield), m.p. 175—176° (from ethanol), $[\alpha]_D^{23}$ +5·35° (*c* 5·0 in $CHCl_3$) (Found: C, 62·9; H, 5·9; N, 6·7. $C_{62}H_{79}N_5O_{16}S$ requires C, 63·0; H, 5·9; N, 6·7%), and the corresponding *2-methylsulphonylethyl ester* (IV; $n = 4$) (92% yield), m.p. 144—146° (from ethanol), $[\alpha]_D^{25}$ +8·8° (*c* 5·0 in $CHCl_3$) (Found: C, 61·6; H, 6·7; N, 5·8. $C_{62}H_{79}N_5O_{18}S$ requires C, 61·3; H, 6·5; N, 5·8%); *tetra-γ-benzyl* N-*t-butoxycarbonyltetra*-D-*glutamyl*-L-*leucinate* (V; $n = 4$) (77% yield), m.p. 132—133° (from aqueous ethanol), $[\alpha]_D^{25}$ +3·9° (*c* 4·5 in $CHCl_3$) (Found: C, 63·9; H, 6·6; N, 6·2. $C_{59}H_{73}N_5O_{16}$ requires C, 64·0; H, 6·6; N, 6·3%), and its N-*succinimidyl ester* (VI; $n = 4$) (83% yield), m.p. 148—150° (from ethyl acetate–ether), $[\alpha]_D^{25}$ +1·90° (*c* 5·4 in $CHCl_3$) (Found: C, 62·9; H, 6·3; N, 6·8. $C_{63}H_{76}N_6O_{15}$ requires C, 62·8; H, 6·3; N, 7·0%); *tetra-γ-benzyl* N-*succinimidyl tetra*-D-*glutamyl*-L-*leucinate hydrochloride* (VII; $n = 5$) (77% yield), decomp. 180° (from ethyl acetate–ether), $[\alpha]_D^{25}$ −33·7° (*c* 1·5 in $CHCl_3$) (Found: N, 7·2. $C_{58}H_{69}ClN_6O_{16}$ requires N, 7·4%).

B. Preparation and Properties of Polymers

1. *Small-scale Polymerisations.*—The hydrochloride of the succinimidyl ester (VII) (50—100 mg) was dissolved or suspended in solvent (usually 0·02—0·2 ml); 1 equiv. of triethylamine was added, and the mixture was stirred by hand for several min. It was kept in a stoppered tube at room temp. for 4 days; then chloroform (0·5 ml) was added, followed by a further 0·5 ml after 2 more days. After a further 2 days the solution was washed with water (3 × 5 ml), the chloroform layer was evaporated, and the residue was dried in a vacuum desiccator (P_2O_5). The product was dissolved in chloroform–ethanol (80 : 20 v/v) to give a 0·5% solution, 1 ml of which was applied to a column (100 × 2·5 cm) of Sephadex LH 20. The column was developed with the same solvent mixture, the effluent being monitored in a flow-through cell at 254 nm (LKB Uvicord). The chart-trace was converted into a plot of optical density against volume; the areas of the various peaks were measured and the corresponding weights calculated on the basis of a calibration with solutions of poly-(β-benzyl-L-aspartate). The results are summarised in Tables 3—6. The structures suggested for the products $V_e/V_o > 1·0$ are based on the values of V_e/V_o and the fact that they were all ninhydrin-negative.

TABLE 3

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Polymerisation of H–D-Glu–Leu–OSu

Solvent	Concn. (mol kg^{-1})	Products V_e/V_o and % yield 1·00 [a]	1·05—1·25 [b]	1·64 [c]
Light petroleum	0·3	51	33	16
Benzene	1·2	47	15	26
Tetrahydrofuran	2·0	50	25	20
Dimethylformamide	1·2	41	45	14

[a] Polymer [b] Cyclohexapeptide, $V_e/V_o = 1·15$, and cyclodecapeptide, $V_e/V_o = 1·07$. [c] Dioxopiperazine.

TABLE 4

OBzl
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Polymerisation of H–D-Glu$_2$–Leu–OSu

Solvent	Concn. (mol kg^{-1})	Products V_e/V_o and % yield 1·00 [a]	1·09 [b]	1·15 [c]	>1·25 [d]
Light petroleum	0·05	29	10	54	7
	0·15	36	8	50	6
	0·4	40	11	44	4
	1·5	29	11	44	16
Benzene	0·3	29	8	53	10
	1·0	28	15	54	3
	2·4	38	11	51	0
	3·0	32	15	47	6
Tetrahydrofuran	2·7	21	17	58	4
Dimethylformamide	0·12	0	24	68	5
	0·3	0	28	64	0
	1·0	16	28	49	4
	3·0	27	19	46	4
Dimethyl sulphoxide	0·3	17	19	56	4
	1·0	28	26	46	0
	3·0	21	25	54	0
None		25	23	38	14

[a] Polymer [b] Cyclotetrapeptide. [c] Cyclohexapeptide. [d] Cyclotripeptide.

2. *Preparative Polymerisations.*—(a) *Poly-(γ-benzyl-D-glutamyl-L-leucine)* (I; $n = 1$). The hydrochloride of the succinimidyl ester (VII; $n = 1$) (4·6 g, 9·5 mmol) was mixed with redistilled tetrahydrofuran (4·5 ml). Triethylamine (0·96 g, 9·5 mmol) was added and the mixture was stirred for 5 min and then kept for 4 days at room temp. More chloroform (2 ml) was added at the end of this period and again after a further 2 days. After 8 days in all the mixture was diluted with chloroform (25 ml). The resulting suspension was washed with brine (3 × 50 ml) and the chloroform layer was dried ($MgSO_4$) after the addition of a few drops of trifluoroacetic acid, filtered, and evaporated. The residue was triturated with methanol and the solid product extracted with this solvent in a Soxhlet apparatus for 48 h. The residual *polymer* (1·6 g, 51%) was split into the following fractions with chloroform–methanol: fraction 1, insoluble in chloroform (60 ml), 200 mg; fraction 2, insoluble in 1 : 1 chloroform–methanol (120 ml), 1300 mg, m.p. 320—340° (decomp.), $[\alpha]_D^{27}$ +27·4° (*c* 3·5 in $CHCl_3$) [Found: C, 64·3; H, 7·0; N, 8·3. $(C_{18}H_{24}N_2O_4)_x$ requires C, 65·1; H, 7·2; N, 8·4%]; fraction 3, soluble in 1 : 1 chloroform–methanol, 80 mg.

TABLE 5

OBzl
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Polymerisation of H–D-Glu$_3$–Leu–OSu

Solvent	Concn. (mol kg^{-1})	Product V_e/V_o and % yield 1·00 [a]	1·09 [b]	>1·2
Light petroleum	0·1	50	36	10
	0·3	49	39	4
Benzene	0·3	51	31	5
	1·0	52	29	6
	3·3	60	22	10
Tetrahydrofuran	0·3	66	28	
	1·0	76	17	
	2·0	70	22	
Dioxan	0·3	53	37	
	1·0	56	20	
	2·0	65	25	
Dimethylformamide	0·3	8	78	
Dimethyl sulphoxide	2·1	50	40	6
None		53	30	4

[a] Polymer. [b] Cyclo-octapeptide. [c] Cyclotetrapeptide.

TABLE 6

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Polymerisation of H–D-Glu$_4$–Leu–OSu

Solvent	Concn. (mol kg^{-1})	Product V_e/V_o and % yield 1·0 [a]	1·06 [b]	1·19 [c]
Light petroleum	1	40	>10	25
Benzene	1	58	0	20
Tetrahydrofuran	1	48	10	31
Dioxan	1	51	5	27

[a] Polymer. [b] Cyclodecapeptide. [c] Cyclopentapeptide.

Evaporation of the Soxhlet methanol extract gave a solid; preparative t.l.c. with chloroform-methanol (99 : 1 v/v) gave the dioxopiperazine, *cyclo-γ-benzyl-D-glutamyl L-leucine* (VIII; $n = 1$, $x = 1$), m.p. 175—177° [Found: M_r (mass spectrometry), 332. $C_{18}H_{24}N_2O_4$ requires M_r, 332].

(b) *Poly[di-(γ-benzyl-D-glutamyl)-L-leucine]* (I; $n = 2$), was prepared similarly from the hydrochloride of (VII; $n = 2$) (1·86 g) in light petroleum (12 ml). Fractionation of the methanol-extracted *polymer* (460 mg) (M_r, by osmotic pressure in *m*-cresol, 25,000) gave: fraction 1, insoluble

in chloroform (20 ml), 50 mg; fraction 2, insoluble in 1 : 2 chloroform–methanol (90 ml), 300 mg, m.p. 250—260° (decomp.), $[\alpha]_D^{27}$ −45·0° (*c* 4·1 in $CDCl_3$) [Found: C, 65·5; H, 6·6; N, 7·7. $(C_{30}H_{37}N_3O_7)_x$ requires C, 65·3; H, 6·7; N, 7·6%]; fraction 3, soluble in 1 : 2 chloroform–methanol, 70 mg, $[\alpha]_D^{27}$ −26·8° (*c* 4·0 in $CDCl_3$).

Evaporation of the methanol extract and fractionation of the resulting solid by preparative t.l.c. (chloroform–methanol 97 : 3) gave *cyclodi-(γ-benzyl-D-glutamyl)-L-leucyl-di-(γ-benzyl-D-glutamyl)-L-leucine* (VIII; $n = 2$, $x = 2$), m.p. 240° (decomp.) [Found: C, 64·9; H, 6·8; N, 7·7%; M_r (vapour pressure osmometry), 1052. $C_{60}H_{74}N_6O_{14}$ requires C, 65·3; H, 6·7; N, 7·6%; M_r, 1102].

(c) *Poly-[tri-(γ-benzyl-D-glutamyl)-L-leucine]* (I; $n = 3$) was prepared similarly from the hydrochloride of (VII; $n = 3$) (3·8 g) in tetrahydrofuran (3·8 ml). Fractionation of the *polymer* (1·8 g, 56%) gave: fraction 1, soluble in chloroform but insoluble in 1 : 1 chloroform–methanol (40 ml), 1·28 g, m.p. 258—290° (decomp.), $[\alpha]_D^{27}$ −54·0° (*c* 4·2 in $CDCl_3$) [Found: C, 65·6; H, 6·8; N, 7·3. $(C_{42}H_{50}N_4O_{10})_x$ requires C, 65·5; H, 6·5; N, 7·3%]; fraction 2, immediately precipitated in 2 : 3 chloroform–methanol (50 ml), 220 mg, $[\alpha]_D^{27}$ −34·7° (*c* 4·5 in $CDCl_3$): fraction 3, slowly precipitated from this solvent mixture, 100 mg, $[\alpha]_D^{27}$ −31·0° (*c* 4·5 in $CDCl_3$).

(d) *Poly[tetra-(γ-benzyl-D-glutamyl)-L-leucine]* (I; $n = 4$) was prepared similarly from the hydrochloride of the succinimidyl ester (VII; $n = 4$) in benzene (7 ml). The *polymer* (2·2 g, 57%) was precipitated from chloroform (40 ml) with methanol (60 ml); yield 1·3 g, m.p. 260—280° (decomp.), $[\alpha]_D^{27}$ −28° (*c* 3·7 in $CDCl_3$) [Found: C, 65·5; H, 6·7; N, 7·2. $(C_{54}H_{63}N_5O_{13})_x$ requires C, 65·5; H, 6·4; N, 7·1%].

3. *Molecular Weight Determinations.*—The polymer was kept for 12 h at room temp. with 50% w/v hydrogen bromide in acetic acid; the product was evaporated and the residue triturated with ether. This debenzylated polymer (3 mg) was dissolved in 0·1M-ammonium formate buffer (pH 9·2; 0·5 ml) and applied to a column of Sephadex G 150 (300 × 25 mm). Elution was carried out with the same buffer; the optical density of the eluate was continuously monitored at 220 nm with a Hilger and Watts Uvispek recording spectrophotometer fitted with a Gilford absorbance converter. The calibration curve was constructed from measurements on polypeptides of known molecular weight (by osmotic pressure) (Table 7).

4. *Racemisation Tests.*—Sufficient polymer to give 0·3 mmol of leucine was heated in a sealed tube at 110° for 24 h with the HCl–H_2O azeotrope (5 ml). The cooled solution was evaporated and the residue applied as a band to a glass plate coated with Kieselgel HF 254 (200 × 200 × 2 mm). The chromatogram was developed (ascending) with chloroform–methanol–17% ammonia (1 : 2 : 1) and dried. The positions of the leucine and glutamic acid bands were determined by spraying the two edges of the plate with ninhydrin in butanol, followed by heating at 100°. The leucine band was scraped from the plate and packed into a small column, and the leucine was eluted with acetic acid (200 ml); amino-acid analysis showed the recovery of leucine to be 70—90%. A solution of the eluted leucine (10 μmol) in 0·2M-Tris buffer, pH 7·3, was incubated at 37° for 24 h under oxygen with dried *Crotalus adamanteus* venom (20 mg). The enzyme was precipitated with trichloroacetic acid (0·1 g) and the precipitate washed with aqueous 5% trichloroacetic acid (3 × 1 ml). The filtrate and washings were extracted with ether, to remove trichloroacetic acid, and evaporated to dryness. The residue was dissolved in 0·05M-hydrochloric acid (1 ml) and the D-leucine estimated on an amino-acid analyser. Control experiments were carried out (whole procedure) with mixtures of D-glutamic acid, L-leucine, and D-leucine in the proportions 3 : 1 : 0·00, 3 : 1 : 0·03, 3 : 1 : 0·05. The D-leucine contents found were as shown in Table 8.

TABLE 7

Polypeptide	M_n	V_e/V_o
H-(L-Met-L-Glu)$_x$-OH	37,900 ± 1500 *	1·40 ± 0·02
H-(L-Glu)$_x$-OH	37,000 ± 1500 *	1·47 ± 0·02
H-(D-Glu-D-Glu-L-Leu)$_x$-OH	16,800 ± 500 †	2·14 ± 0·02
H-(L-Asp)$_x$-OH	12,500 ± 1500 †	2·02 ± 0·02

* Calculated from M_n measured for the polymethyl ester.
† Calculated from M_n measured for the polybenzyl ester.

TABLE 8

Sample	D-Leucine (%)
Polymer (I; $n = 1$); fraction 2	2·7
Polymer (I; $n = 2$); unfractionated	2·4
Polymer (I; $n = 2$); fractions 2 + 3	2·3
Polymer (I; $n = 3$); unfractionated	3·6
Polymer (I; $n = 4$); reprecipitated	3·1
Control; 0·0% leucine	2·8, 2·1
Control; 3·0% leucine	4·5
Control; 5·0% leucine	6·2

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