# Use of 2-Hydroxyphenyl Esters for a Racemization-free Synthesis of Alternating Diastereoisomeric Poly-(γ-Benzyl Glutamate)s

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Poly-(γ-benzyl-D-glutamyl-γ-benzyl-L-glutamate) and its mirror image were prepared by polycondensation of the tetrapeptide 2-hydroxyphenyl esters. No racemization was detected by optical rotation measurements on the polymers dissolved in helicoclastic solvents. Fractionation of the crude polypeptides led to the pure α- and β-forms, characterized by their i.r. spectra and differing in their molecular weights. The α-form can undergo a transition to a new helical form, indicating that the optical purity of the residues is largely preserved during the synthesis. Poly-(γ-benzyl-L-glutamyl-γ-benzyl-D-glutamyl-γ-benzyl-L-glutamate) was prepared similarly.

Two aspects of the conformations of alternating poly-D-L-peptides † have aroused interest: first the influence of enantiomeric residues on the stability and geometry of the α-helical conformation, and secondly the existence of novel conformations. Experimental results for synthetic poly-D-L-peptides in solution or in the solid phase are consistent only with the α-helical structure, which appears stable, although somehow distorted as compared with the conformation of optically pure polypeptides.[1-3] The existence of another conformation has, however, been suggested.[1] These results were obtained with samples the primary structure of which was not perfectly alternating, since the method of preparation had led to some racemization.

Energy calculations have predicted[4,5] that helical conformations other than the α-helix may exist, one of which might apply to gramicidin A, an antibiotic polypeptide with an alternating D-L primary structure.[6] For steric reasons such helical models can only be built with strictly alternating copoly-D-L-peptides, and we therefore required a racemization-free synthesis of such systems in order to investigate this point.

† D-L is used to denote an alternating sequence, and D,L a random sequence in a copolypeptide.

[1] P. M. Hardy, J. C. Haylock, D. I. Marlborough, H. N. Rydon, H. T. Storey, and R. C. Thompson, *Macromolecules*, 1971, **4**, 435.
[2] F. Heitz and G. Spach, *Macromolecules*, 1971, **4**, 429.
[3] F. A. Bovey, J. J. Ryan, G. Spach, and F. Heitz, *Macromolecules*, 1971, **4**, 433.
[4] F. T. Hesselink, and H. A. Scheraga, *Macromolecules*, 1972, **5**, 455.
[5] G. N. Ramachandran, and R. Chandrasekaran, *Indian J. Biochem. Biophys.*, 1972, **9**, 1.
[6] D. W. Urry, *Proc. Nat. Acad. Sci., U.S.A.*, 1971, **68**, 672.

The polypeptide synthesis reported here had four objectives: (i) to obtain a perfect alternation of D- and L-residues by avoiding racemization at all stages; (ii) to obtain a good yield in the polymerization by avoiding dioxopiperazine formation; (iii) to reach high molecular weights; and (iv) to cross-check the results by employing two different preparations.

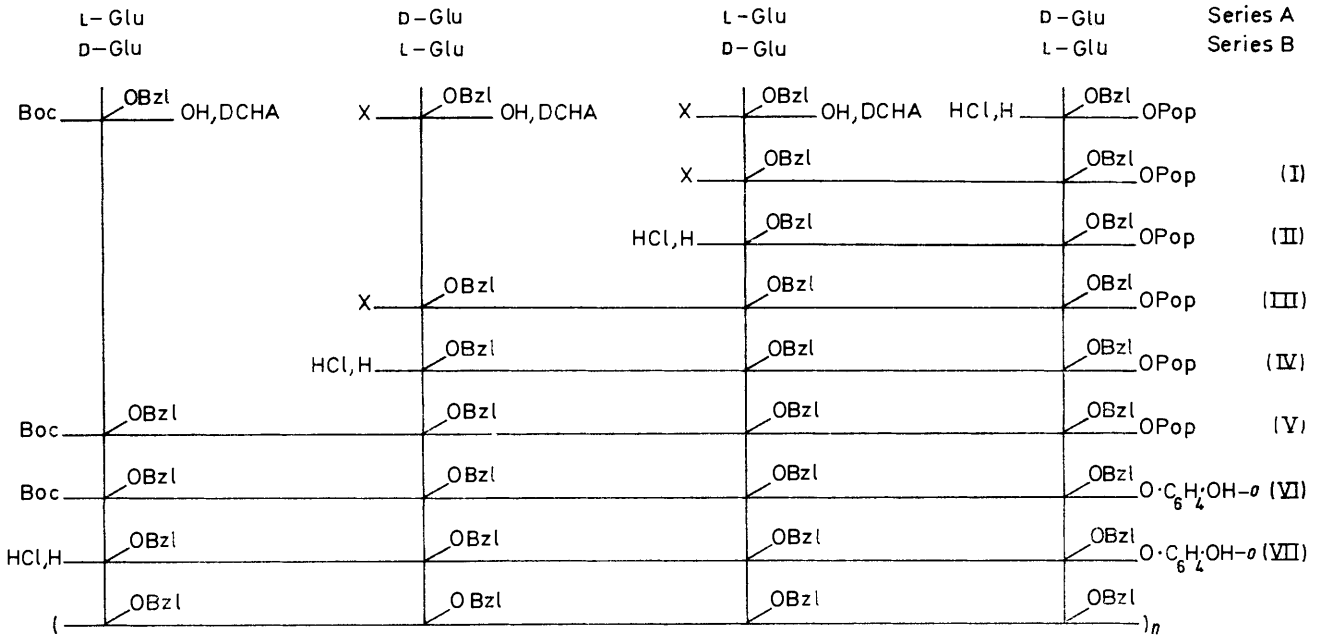
Earlier results [7,8] have shown that a 2-hydroxyphenyl ester is a useful peptide derivative for meeting the first requirement. When its hydroxy-group is protected by phenacylation,[8] it furnishes a convenient intermediate for the stepwise synthesis of the monomeric peptide through elongation at the *N*-terminal residue: as this protected ester is not reactive no self-condensation can occur, at least at room temperature. The use of t-butoxycarbonyl as the *N*-terminal protecting group during removal of the phenacyl group has been recommended.[8] Use of the 2-hydroxyphenyl ester is known to lead to polymers with molecular weights as high as those obtained from polymerization of the usual peptide active esters.[9] However, when applied to the polymerization of a dipeptide it is reported to lead only to a substituted dioxopiperazine even under conditions of high concentration.[8] This also occurred in our case and we therefore synthesized a tetrapeptide ester as polymerization substrate, by a stepwise rather than a fragment condensation method in order to avoid racemization completely.

The results were cross-checked by synthesizing the mirror image of the polymer, using different protecting groups in the intermediate stages.

† Particular samples obtained, differing in their viscosities, are designated $DL_{cat}$I, $LD_{cat}$II, and $LD_{cat}$III (Table 2).

One of the intermediates was an *N*-t-butoxycarbonyl-tripeptide phenacyloxyphenyl ester, which was used for synthesis of the corresponding polytripeptide.

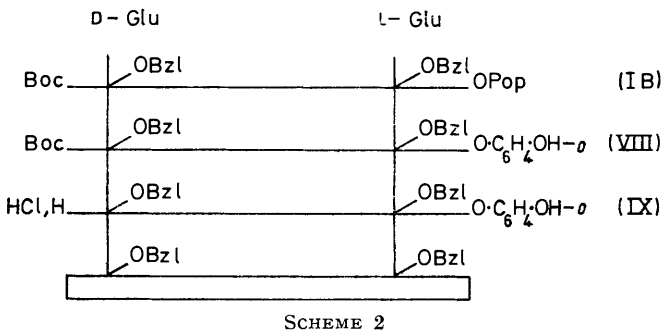
All the synthesis steps are summarized in the Schemes 1 [for poly-(γ-benzyl-D-glutamyl-γ-benzyl-L-glutamate) ($DL_{cat}$) †] and its mirror image ($LD_{cat}$) †], 2 (for the substituted dioxopiperazine), and 3 {for poly-(γ-benzyl-L-glutamyl-γ-benzyl-D-glutamyl-γ-benzyl-L-glutamate) [$(LDL)_n$]}.




SCHEME 1

X = Boc in series B
X = Nps in series A

During these investigations, we found that the 2-hydroxyphenyl ester method suffers from certain weaknesses in addition to dioxopiperazine formation from dipeptides. For instance the yields of phenacyloxyphenyl esters are poor, despite several modifications of the method.[8] Also the products obtained after removal of the phenacyl protecting group are often discoloured and purification results in loss of material.

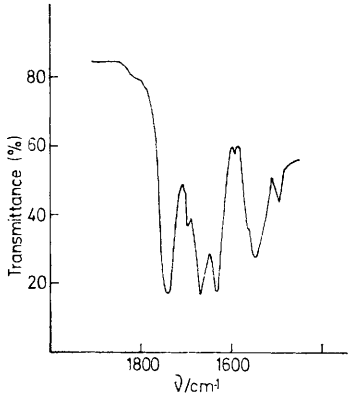



SCHEME 2

[7] R. D. Cowell and J. H. Jones, *J. Chem. Soc.* (*C*), 1971, 1082.
[8] Y. Trudelle, *J.C.S. Perkin I*, 1973, 1001.
[9] Y. Trudelle, *Chem. Comm.*, 1971, 639.

The 2-nitrophenylsulphenyl group was found more suitable for *N*-protection in some intermediary steps than the t-butoxycarbonyl group, because the hydrochlorides resulting from its removal are less hygroscopic, perhaps as no acetic acid is used for the cleavage. No other conditions were tried for removal of the latter group.

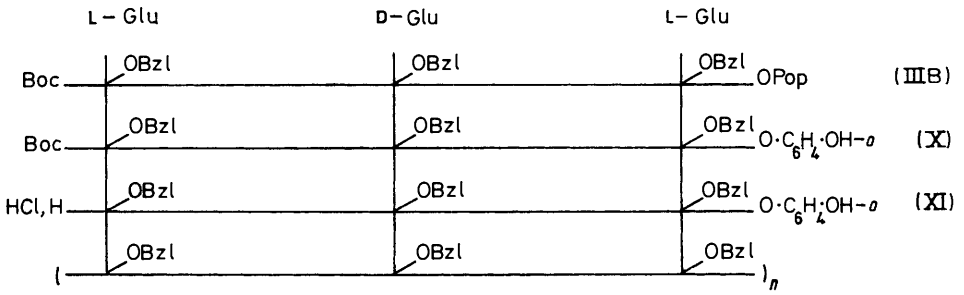
After removal of the by-products of the polycondensation, the i.r. spectrum of the polymers revealed the existence of two fractions, α and β (see Figure). Separation to obtain pure α- and β-fractions was achieved with difficulty, due to the partial interconvertibility of these two species.




I.r. spectrum of crude poly-(γ-benzyl-D-glutamyl-γ-benzyl-L-glutamate) (film)

The weight average molecular weight of sample $LD_{cat}II$ only was determined by light scattering measurements in dimethylformamide ($\bar{M}_w$ 31,000). The various samples were characterized by their viscosity in dichloroacetic acid. As the values obtained are all in the same range (Table 2) we conclude that all samples have similar molecular weights.

Use of Doty's relationship [10,11] between the viscosity in dichloroacetic acid and molecular weight established for poly-(γ-benzyl-L-glutamate) leads for the sample $LD_{cat}II$ to a molecular weight of 7000, much lower than the measured value. This result confirms qualitatively the theory of Miller *et al.*,[12] who predicted that in a poly-D,L-peptide the chain dimensions would show a marked dependence on the distribution of D- and L-residues for a polymer in the random coil conformation, and would go through a minimum for an alternating sequence. We note that the discrepancy between molecular weights measured and estimated from viscosity is higher for the sample $LD_{cat}II$ than for a sample prepared by the pentachlorophenyl ester method [2] ($\bar{M}_w$ 16,000 from viscosity and 21,000 by light scattering measurements). This implies that the chain dimensions are different and thus suggests that the distribution of the residues in the two polymers is not the same, possibly owing to a greater extent of racemization in the samples prepared by the pentachlorophenyl ester method.




SCHEME 3

DCHA = dicyclohexylamine
Pop = 2-phenacyloxyphenyl

Other abbreviations as recommended by the I.U.P.A.C.–I.U.B. Commission on Biochemical Nomenclature (*Biochem. J.*, 1972, **126**, 773).

All the polymers [except of course $(LDL)_n$] have no measurable optical activity at wavelengths above 300 nm when dissolved in trifluoroacetic acid. Thus the amount of racemization, if any, is less than 1%; *i.e.* less than *ca.* 1 in 200 residues is racemized.

The small extent (or absence) of racemization is confirmed by the conformational behaviour of the samples prepared by the 2-hydroxyphenyl ester method. As already indicated above, only optically pure poly-D-L-peptides may exist in the $\pi_{DL}$ helical form. This helical conformation has indeed been detected for the samples mentioned here.[13] No $\pi_{DL}$ helix was detected for the samples prepared by the pentachlorophenyl ester method. Thus, the 2-hydroxyphenyl ester method appears the best from the point of view of racemization. Other anchimeric esters, or the coupling reagent of Kemp,[14] may also be useful, but no information is available on their ability to give periodic polymers of high molecular weight.

For the sample $(LDL)_n$ the value of $[\alpha]_{546}^{25}$ measured in trifluoroacetic acid is not in agreement with that obtained from a linear interpolation of the optical rotation from zero to −54·7° [the value for poly-(γ-benzyl-L-glutamate)]

[10] P. Doty, J. H. Bradbury, and A. M. Holtzer, *J. Amer. Chem. Soc.*, 1956, **78**, 947.
[11] J. C. Mitchell, A. E. Woodward, and P. Doty, *J. Amer. Chem. Soc.*, 1957, **79**, 3955.
[12] W. G. Miller, D. A. Brant, and P. J. Flory, *J. Mol. Biol.*, 1967, **23**, 67.
[13] G. Spach and F. Heitz, *Compt. rend.*, 1973, **276C**, 1373.
[14] D. S. Kemp and S. W. Chien, *J. Amer. Chem. Soc.*, 1967, **89**, 2743.

and is about half than expected from its composition. As we have no reason to suspect that racemization occurred during the synthesis of this polymer, this means that the interpolation procedure cannot be employed straightforwardly for estimation of the amount of racemization. It follows that for samples prepared by the *p*-nitrophenyl and pentachlorophenyl ester methods, the amounts of racemization quoted are probably underestimates.

When dissolved in chloroform the DL$_{cat}$ polypeptides show optical rotation at all wavelengths between 290 and 589 nm. This result is not an artefact as shown by the fact that the pairs of symmetrical derivatives (Table 1) and the two polymers (Table 2) of series A and B have identical scalar physical properties. The optical activity indicates rather that conformational effects occur within the polymers; they are due to intramolecular interactions which generate a dissymmetry in the handedness of a helical polymer chain of finite length. Experimental evidence of this will be given elsewhere.

It is noteworthy that the sign of $[\alpha]_{546}^{25}$ of the sample DL$_{cat}$I in chloroform is opposite to that measured for samples prepared by the conventional active ester methods [2,15] (+24 as opposed to −39°). This again shows the influence of racemization on conformation.

TABLE 1

Properties of peptide intermediates

| Peptide | Procedure for preparation [yield (%)] | M.p. (°C) {$[\alpha]_{546}^{25}$ (°)} | Argentometric titration (%) [$R_F$ value] | $\nu_{max.}$/cm$^{-1}$ | Analyses (%) * C | H | Cl | N | S |
|---|---|---|---|---|---|---|---|---|---|
| (IA) | (1) | 68—74 [c] | | 1770, 1735, | 65·05 | 5·1 | | 5·2 | 3·55 |
| $C_{44}H_{41}N_3O_{11}S$ | [94] | {+54·5} | | 1680 [a] | 64·45 | 5·05 | | 5·1 | 3·9 |
| (IB) | (1) | 101—103 [d] | | 1770, 1735 | 67·4 | 6·05 | | 3·65 | |
| $C_{43}H_{46}N_2O_{11}$ | [92] | {−30·8} | | 1710, 1685br [a] | 67·35 | 6·05 | | 3·65 | |
| (IIB) | (2a) | Hyg. † [e] | 97·0 | 1770, 1735 | 64·05 | 5·75 | 5·35 | 3·9 | |
| $C_{38}H_{39}ClN_2O_9$ | [97] | {−31·3} | | 1695 [a] | 64·9 | 5·6 | 5·05 | 4·0 | |
| (IIA) | (3a) | Hyg.;† 56—58 [f] | 98·0 | | | for (IIB) | | | |
| $C_{38}H_{39}ClN_2O_9$ | [75] | {+30·4} | | | | | | | |
| (IIIB) | (1) | 94—96 [g] | | 1770, 1730 | 67·25 | 6·2 | | 4·35 | |
| $C_{55}H_{59}N_3O_{14}$ | [88] | {−21·3} | | 1695, 1650 [b] | 67·0 | 6·05 | | 4·25 | |
| (IIIA) | (1) | 130—131 [h] | | 1770, 1735 | 65·15 | 5·35 | | 5·65 | 2·8 |
| $C_{56}H_{54}N_4O_{14}S$ | [90] | {+38·0} | | 1680br [a] | 64·75 | 5·25 | | 5·4 | 3·1 |
| (IVB) | (2a) | 108—110 [i] | 99·0 | 1775, 1735, | 65·2 | 5·7 | 3·8 | 4·45 | |
| $C_{50}H_{52}ClN_3O_{12}$ | [81] | {+4·15} | | 1705, 1680 | 65·1 | 5·7 | 3·85 | 4·55 | |
| (IVA) | (3b) | 108—112 [i] | 100·0 | 1645 [b] | | for (IVB) | | | |
| $C_{50}H_{52}ClN_3O_{12}$ | [84] | {−4·25} | | | | | | | |
| (VB) | (1) | 73—75 [j] | | | | | | | |
| $C_{67}H_{72}N_4O_{17}$ | [87] | {−1·0} | | 1730, 1695, | 66·65 | 5·95 | | 4·65 | |
| (VA) | (1) | 69—73 [j] | | 1670, 1640 [b] | 66·75 | 6·05 | | 4·65 | |
| $C_{67}H_{72}N_4O_{17}$ | [77] | {+1·1} | | | | for (VB) | | | |
| (VIB) | (4) | 88—90 [k] | | 1735, 1690, | 65·2 | 6·2 | | 5·05 | |
| $C_{59}H_{66}N_4O_{16}$ | [81] | {+20·5} | [0·80] | 1665 [b] | 65·15 | 6·15 | | 5·15 | |
| (VIA) | (4) | 89—90 [k] | | | | for (VIB) | | | |
| $C_{59}H_{66}N_4O_{16}$ | [73] | {−21·0} | [0·84] | | | | | | |
| (VIIB) | (2b) | 124—127 [l] | 99·0 | | | | | | |
| $C_{54}H_{59}ClN_4O_{14}$ | [89] | {−0·6} | | 1730, 1665br [a] | 62·85 | 5·9 | 3·65 | 5·3 | |
| (VIIA) | (2b) | 127—130 [l] | 100·0 | | 63·35 | 5·8 | 3·45 | 5·45 | |
| $C_{54}H_{59}ClN_4O_{14}$ | [80] | {+0·7} | | | | for (VIIB) | | | |
| (VIII) | (4) | 96—97 [j] | | 1770, 1730br, | | | | | |
| $C_{35}H_{40}N_2O_{10}$ | [65] | {+4·85} | [0·785] | 1680sh [a] | | | | | |
| (IX) | (2c) | Hyg.† [e] | 100·0 | 1770, 1735, | | | | | |
| $C_{30}H_{33}ClN_2O_8$ | | | | 1695 [a] | | | | | |
| (X) | (4) | 108—110 [j] | | 1730, 1680, | 65·0 | 6·15 | | 4·85 | |
| $C_{47}H_{53}N_3O_{13}$ | [73] | {+5·8} | [0·73] | 1660 [b] | 65·3 | 6·4 | | 4·9 | |
| (XI) | (2b) | 93—95 | 98·0 | 1740, | 62·35 | 5·1 | | 5·2 | |
| $C_{42}H_{46}ClN_3O_{11}$ | [50] | {+2·8} [m] | | 1670 [a] | 62·7 | 5·75 | | 5·2 | |

* Found: upper figures; required: lower figures. † Hygroscopic.

[a] In chloroform. [b] KBr discs. [c] From propan-2-ol. [d] From di-isopropyl ether–absolute ethanol. [e] Dissolved in acetone and precipitated with di-isopropyl ether. [f] Dissolved in boiling propan-2-ol and precipitated with ether after cooling. [g] From ethanol. [h] From propan-2-ol–methanol. [i] Dissolved in acetone and precipitated with propan-2-ol–light petroleum. [j] From di-isopropyl ether–acetone. [k] From ethyl acetate–cyclohexane. [l] Dissolved in acetone and precipitated with dry ether. [m] Dissolved in chloroform and precipitated with dry ether.

TABLE 2

Properties of polymer samples

| Sample | $[\alpha]_{546}^{25}$ (°) in $CF_3{\cdot}CO_2H$ | $[\alpha]_{546}^{25}$ (°) in $CHCl_3$ | $[\eta]$ [c] | $\nu_{max}$ (film)/cm$^{-1}$ |
|---|---|---|---|---|
| β-Fraction | 0 [a] | — | 7·2 | 3290, 1690sh, 1629, 1525 |
| DL$_{cat}$I | 0 [b] | +24·2 | 12·0 | 3290, 1665, 1550 |
| LD$_{cat}$II | 0 [b] | −25·0 | 10·0 | 3290, 1665, 1550 |
| LD$_{cat}$III | 0 [b] | −24·8 | 14·4 | 3290, 1665, 1550 |
| (LDL)$_n$ | −8·5 [a] | — | 13·8 | 3290, 1662, 1555 |

[a] *c* 1·0. [b] *c* 1·5. [c] Measured in dichloroacetic acid at 25°; in ml g$^{-1}$.

## EXPERIMENTAL

M.p.s were determined with a hot-plate Leitz microscope. I.r. spectra were recorded with a Perkin-Elmer 257 spectro-

[15] P. M. Hardy, H. N. Rydon, and H. T. Storey, *J.C.S. Perkin I*, 1972, 1523.

photometer. Optical rotations of intermediates were determined with a Perkin-Elmer 141 M polarimeter (1 dm cell; *c* 1·0 in $CHCl_3$). T.l.c. was carried out on Eastman Chromatogram Sheet 6060 (silica gel) with acetic acid–butanol–water (15 : 100 : 35) as eluant. Viscosities were measured with an Ubbelohde viscosimeter (Cannon CUSMU size 75). Dialysis was carried out in thin-walled Visking dialysis tubing. Light scattering measurements were performed with a SOFICA photogoniodiffusiometer. Amino-acid analyses were run on a Beckman 120C amino-acid analyser (columns filled with Beckman spherical ion exchange resin type M72). The benzene used as solvent for polycondensations was distilled over sodium and stored over molecular sieves (4 Å). Triethylamine was distilled once from benzyloxycarbonylglycine *p*-nitrophenyl ester and then redistilled.

*Preparation of the Peptides* (*see Table* 1).—*Procedure* (1). Standard coupling procedure described by Trudelle.[8]

*Procedure* (2). Removal of the *N*-t-butoxycarbonyl group on a 24 mmol scale. The *N*-protected peptide was dissolved in 1·1N-hydrogen chloride in acetic acid (250 ml). After 15 min stirring the solvent was removed under vacuum. The resulting oil was extracted twice with benzene. The extract was evaporated and the resulting oily product was taken up in acetone (procedures 2a and 2b) or taken up in benzene, then decolourized with charcoal (procedure 2c). The crude product was precipitated with light petroleum (2a) or with anhydrous ether (2b and 2c).

*Procedure* (3). Removal of the *N*-*o*-nitrophenylsulphenyl group on a 22·4 mmol scale. To a solution of the protected peptide ester in ethyl acetate (250 ml) (procedure 3a) or in ethyl acetate–acetone (procedure 3b) was added a solution of hydrogen chloride in ether [56 mmol in 300 ml (3a) or in 9·2 ml (3b)]. Evaporation under vacuum (3a) or precipitation with light petroleum (3b) gave an oily residue which crystallized under ether.

*Procedure* (4). Standard procedure for the removal of the phenacyl group as described by Trudelle.[8]

*Polycondensations.*—The polycondensations were carried out as described by Trudelle [8] but with benzene instead of dimethylformamide. After 4 days at room temperature the crude polymer was obtained by evaporation, then taken up in dimethylformamide and precipitated with water. The three samples obtained in this way are referred to as $DL_{cat}I$, $LD_{cat}$, and $(LDL)_n$.

*Purification and Fractionation of the Polymers.*—The crude samples showed i.r. bands characteristic of both α- and β-structures. After removal of by-products, they were fractionated to yield samples having spectra characteristic of pure α and β [16] fractions. The sample $LD_{cat}$ contained a fraction poorly soluble in dimethylformamide; thus two distinguishable LD samples could be obtained, in equal amounts; the less soluble was later shown to have the higher molecular weight. In order to eliminate low molecular weight material in the soluble part and in $DL_{cat}I$ and $(LDL)_n$, further purification was carried out by dialysis in dimethylformamide for one week. All samples were then exhaustively extracted (Soxhlet) for 10 days with hot absolute ethanol. The ethanol-soluble part was identified as having the β-structure. An *X*-ray and electron diffraction study of this fraction has been published.[16] Since the i.r. spectrum of the insoluble part still showed absorption corresponding to a mixture of α- and β-structures, a final purification was carried out by dissolution in dichloroacetic acid and precipitation with methanol. This led to a fraction showing only the α-spectrum.

The characteristics of the various fractions are given in Table 2.

*Amino-acid Analyses.*—Foreign amino-acids in the samples $DL_{cat}I$ and $LD_{cat}II$ were present to the extent of $<0{\cdot}2$ mole %, and were mostly threonine, valine, and glycine. In the starting amino-acids the content of foreign amino-acids was 0·3 mole %; aspartic acid was found to the extent of 0·1 mole %.

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[16] B. Lotz, F. Heitz, and G. Spach, *Compt. rend.*, 1973, **276C**, 1715.