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PEPTIDES—XIII*

EFFECTS OF CONFIGURATION ON DIELECTRIC INCREMENTS AND CYCLIZATION OF SOME SIMPLE PEPTIDES

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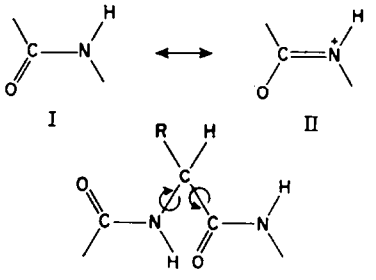
Abstract—Cyclization of the diastereoisomeric glycylleucylleucylglycylglycines, either through their *p*-nitrophenyl thiolesters or by means of dicyclohexyl carbodiimide, yields 39% of the D-L and 12% of the L-L cyclic pentapeptide. Correspondingly the dielectric increment of the open-chain D-L pentapeptide is only 64% that of the L-L, and similar differences have been found with several pairs of diastereoisomeric di- and tri-peptides. A preliminary discussion of these results in terms of preferred conformations of peptide chains is given.

CONFORMATIONS of peptide chains in proteins and synthetic polypeptides have been derived theoretically by applying two principles.[1] The first is that resonance of the amide groups between structures I and II stabilizes planar conformations of them and that *trans* conformations are preferred to *cis*. Secondly, only structures containing the maximum number of hydrogen bonds need be considered. Some attention has also been paid[2] to possible preferences for particular angles of rotation about the single bonds between the amide groups and the tetrahedral carbon atoms within the chain (termed hereafter N—C_α and C_α—CO bonds; cf. III). This paper deals with conformations of non-helical oligopeptides dissolved in water and similar solvents, which minimize influences of hydrogen-bonding on conformations. In these circumstances, any restriction of rotation about the N—C_α or C_α—CO bonds should be more apparent.



I II

III

Restriction of rotation is less easy to define for N—C_α and C_α—CO bonds in peptides than for bonds between two tetrahedral carbon atoms. Barriers to rotation between trigonal and tetrahedral carbon atoms in olefins and carbonyl compounds

* Part XII: *J. Chem. Soc.* (1962).

[1] L. Pauling, R. B. Corey and H. R. Branson, *Proc. Nat. Acad. Sci.* **37**, 205 (1951).

[2a] L. Pauling and R. B. Corey, *Proc. Nat. Acad. Sci.* **37**, 729 (1951); [b] S. Mizushima and R. Shimanouchi, *Adv. Enzymol.*, **23**, 1 (1961).

have been the subject of thermodynamic and spectroscopic investigations,[2b,3] which show that a substituent on the tetrahedral atom preferably eclipses the substituent doubly bound to the trigonal atom. This initially surprising conclusion can perhaps be qualitatively understood by considering the double bond as a pair of bent single bonds,[4] when it is seen that the preferred conformation corresponds to the familiar staggered orientation of two tetrahedral atoms.[5] The chief difficulty in applying this simple concept to C_α—CO bonds of peptides is that resonance between structures I and II imparts double-bond character to both sides of the trigonal carbon atom. In nitromethane, which has equivalent mesomeric structures, the barrier to rotation is only 0·006 kcal,[6] but acetic acid (0·48 kcal[7]) is more analogous to a peptide and barriers above 1 kcal are recorded for acetyl halides etc.[3b] Turning to N—C_α bonds, it might be thought that partial double-bond character in the link to the carbonyl group would result in this substituent being eclipsed, but the contrary assumption that the hydrogen atom is eclipsed has been made.[2b] To sum up, the situation concerning rotation around C_α—CO and N—C_α bonds is confused, and we suspect that many chemists have assumed that the barriers would be too low to have conformational consequences.

An observation, which we made several years ago,[8] indicated the actual existence of preferred conformations of the type just discussed. It was found that, whereas the *p*-nitrophenyl thiolester of glycyl-L-leucylglycyl-D-leucylglycine gave the cyclic peptide in 57% yield when it was liberated from the hydrobromide in dilute aqueous solution, the L-L-diastereoisomer afforded only 41% of cyclic peptide. We suggested that the higher yield of the L-D isomer resulted from a smaller average distance between the ends of the chain and that this difference between the diastereoisomers was a symptom of restricted rotation about some or all of the intra-chain bonds to the asymmetric carbon atoms. In order to accentuate this postulated conformational effect, we have now examined a pair of pentapeptides with neighbouring asymmetric centres, namely glycyl-D-leucyl-L-leucylglycylglycine and the L-L-diastereoisomer. Cyclization was achieved, as previously, through the *p*-nitrophenyl thiolesters in aqueous solution buffered with basic magnesium carbonate, but the cyclic peptides were isolated by counter-current distribution instead of passage through ion-exchange columns.* The yields of the pure D-L and L-L isomers were 39% and 12% respectively. Since the

* The ion-exchange method appeared to give slightly higher yields, but the cyclic pentapeptides were contaminated with other non-ionic compounds. The ion-exchange method caused considerable trouble until it was discovered that the hydroxide forms of Dowex 2x8 or 1x4 and Amberlite IRA–410 resins retained the cyclic peptides; however, the bicarbonate forms could be employed satisfactorily and it is possible that the supposed hydroxide forms employed in the earlier work[8] had been inadvertently carbonated. It is not obvious why the hydroxide forms should retain neutral amides, but, bearing in mind the large change in volume of the resin when it is converted to the hydroxide form, we suggest that a molecular sieve effect may be responsible.

[3a] W. G. Dauben and K. S. Pitzer, *Steric Effects in Organic Chemistry* (Edited by M. S. Newman) pp. 58–59. John Wiley, New York (1956); [b] E. B. Wilson, *Advanc Chem. Phys.* **2**, 367 (1959); [c] R. J. Abraham and J. A. Pople, *Mol. Phys.* **3**, 609 (1960).

[4] Cf. J. A. Pople, *Quart. Rev.* **11**, 273 (1957).

[5] L. Pauling, *Proc. Nat. Acad. Sci.* **44**, 211 (1958).

[6] E. Tannenbaum, R. J. Myers and W. D. Gwinn, *J. Chem. Phys.* **25**, 42 (1956).

[7] W. J. Tabor, *J. Chem. Phys.* **27**, 974 (1957).

[8] G. W. Kenner, P. J. Thomson and J. M. Turner, *J. Chem. Soc.* 4148 (1958).

completion of our earlier work,[8] Wieland and Ohly[9] have shown that free peptides can be cyclized directly in aqueous methanol by means of dicyclohexylcarbodiimide and therefore this technique was also employed. Within experimental error, the yields were identical with those from the thiolester cyclizations. Too much significance should not be attached to this coincidence because the yield in closure of a macrocycle is influenced by so many factors of concentrations and competing reaction velocities, but it does suggest that there is a fundamental stereochemical cause for the trend to higher yields of the diastereoisomers containing D and L residues. The consistency of the thiolester method was shown when the cyclization of glycyl-L-leucylglycyl-L-leucylglycine, which had been accomplished previously by Drs. Thomson and Turner with yields between 38 and 44%,[8] was repeated for comparative purposes in yields of 36, 39, and 39%. In our opinion, such cyclizations provide reliable and theoretically simple, although experimentally laborious, qualitative demonstrations of a conformational effect. On the other hand the results are not susceptible to detailed analysis, and therefore, although further examples of the same kind would have some merit, we must look elsewhere for clues to a more profound understanding of the situation.

The dielectric increments of peptides[10] provide a striking and useful demonstration of this conformational effect. Dipolar ions generally increase the dielectric constant of water when they are dissolved in it, and in dilute solutions the increase is proportional to the concentration of solute. Their dielectric increment specifies the increase in dielectric constant calculated for a molar solution, assuming "dilute" properties; our measurements were made in the region up to 0·04M. The dielectric increment is related to the dipole moment, and thus it can provide information about the length, and hence the conformations, of flexible molecules. In part VIII,[8] we reported that the dielectric increment of glycyl-L-leucylglycyl-D-leucylglycine is considerably lower than that of the L-L isomer. Values for five more diastereoisomeric pairs are given in Table 1. The dielectric increments of di-, tri-, and penta-glycine are 70, 114, and 202 respectively, and thus the D-L or L-D isomers may perhaps be regarded as abnormal.

TABLE 1. DIELECTRIC INCREMENTS OF PEPTIDES IN AQUEOUS SOLUTION AT 30·5°

Structure	Dielectric increments of diastereoisomers	
	L–L	D–L or L–D
Leucylleucine	83	40
Leucyltyrosine	90	45
Glycylleucylleucine	112	55
Leucylleucylglycine	113	81·5
Glycylleucylleucylglycylglycine	204	130
Glycylleucylglycylleucylglycine	207*	

* This value differs from that previously recorded[8] (186) by much more than the variations in the present measurements with this compound (±1·5%), but we are unable to account for the discrepancy. The recorded value (169) for the diastereoisomer must likewise be regarded with reserve until another sample has been examined.

[9] T. Wieland and K. W. Ohly, *Liebigs Ann.* **605**, 179 (1957).

[10] E. J. Cohn and J. T. Edsall, *Proteins, Amino Acids, and Peptides* pp. 152–154. Reinhold, New York (1943).

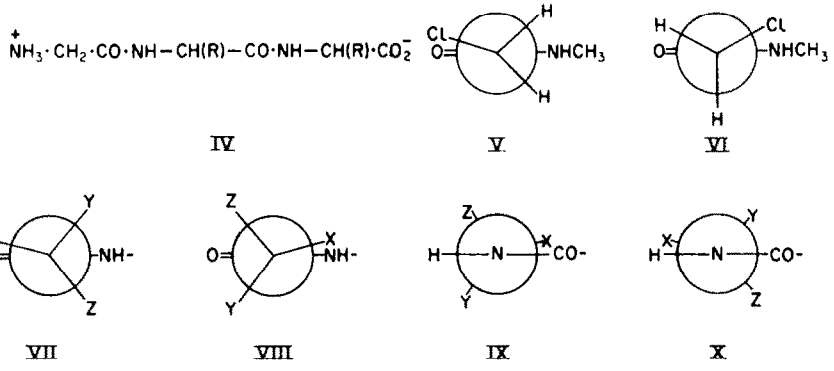
In any consideration of relations between dielectric increments and conformations, the first question is how many conformations have major roles. The large differences between the increments of diastereoisomers dispose of the supposition,[10,11] based on the proportionality of increment to number of residues in oligopeptides of glycine, that there is a random distribution of conformations. In the following discussion, going to the other extreme, we assume that, except where residues of glycine are involved, one conformation is dominant and that the conformations of two diastereoisomers are governed by the same rules. (It is conceivable that we are merely observing the effects of direct interaction of the two side-chains, but it is difficult to see why they should influence each other when so widely separated.) This is a working hypothesis to be tested by the consistency of deductions from it, but it is given some support by examination of molecular models. This reveals considerable variations between the dipole moments of conformational isomers, and consequently it would be surprising if the mean values corresponding to mixtures of a few conformational isomers showed considerable dissimilarities between pairs of diastereoisomers. Dr. A. D. Buckingham has drawn our attention to the desirability of testing the hypothesis by making measurements at different temperatures, and we intend to do this.

The relation between dipole moment and dielectric increment has been studied several times,[10,12] and probably the most satisfactory treatment is that of Buckingham,[13] who derived dipole moments for glycine and β-alanine in good agreement with their molecular dimensions. The main difficulty in applying this theory to peptides is that the shape of the molecule has to be incorporated in the calculation. This information can be obtained from separate determinations at very high frequencies *via* calculation of relaxation times,[14] but we have relied on the simple data, employing calculation by successive approximation and feeding back the shape corresponding to the dipole moment until constancy was reached.[15] We regard these calculations with some reserve and the deduced dipole moments are probably not absolutely significant, because some of the corresponding separations of charges are too small to be simulated with Dreiding molecular models. Moreover, the separation does not increase with chain length to a superficially reasonable extent. However, the calculations are useful in confirming that the average separation of charges in a D-L-diastereoisomer is distinctly less than in the L-L. The following charge separations (Å) were calculated for the di- and tri-peptides; leucylleucine, 4·3 and 6·3; glycylleucylleucine, 4·1 and 6·2; leucylleucylglycine, 4·8 and 6·2.

Ideally the dominant conformations of peptides could be deduced from the average charge separations by equating them to chain lengths, provided that sufficient examples had been studied and that, in each case, one conformer is so dominant that contributions from the others can be neglected. In the present work, we have been less ambitious and our attention has been confined to peptides containing only two asymmetric carbon atoms. The pentapeptides display a large difference in increment, concordant with the difference in yields from cyclizations, but it is difficult to make any conformational deductions because the residues of glycine, which are not subject

[11] W. P. Conner, R. P. Clarke and C. P. Smyth, *J. Amer. Chem. Soc.* **64**, 1397 (1942).
[12a] J. G. Kirkwood, ref. 10 p. 294; [b] J. L. Oncley, ref. 10 p. 546.
[13] A. D. Buckingham, *Aust. J. Chem.* **6**, 323 (1953).
[14] W. P. Conner and C. P. Smyth, *J. Amer. Chem. Soc.* **64**, 1870 (1942).
[15] P. M. Hardy, Ph.D. Thesis, Liverpool (1961).

to so much stereochemical control, are responsible for too many uncertainties. On the other hand, in the dipeptides the amide group is not in a typical environment. The carboxylate group may be sufficiently equivalent to an amide substituent for the C_α—CO bond to be equivalent to one in a chain, but the same cannot be said of the N—C_α bond because the ammonium substituent cannot be equated for even these purposes to an amide without further justification. For these reasons we selected glycylleucylleucine as the theoretically simplest case for initial analysis; in it, the three bonds depicted in IV by lines may be regarded as typical of a polypeptide. Dreiding models of the two diastereoisomers were arranged in thirty six ways, which were derived from the following considerations.



IV V VI

VII VIII IX X

Apparently the only closely relevant investigation of rotational barriers is a study of N-methylchloroacetamide.[16] In the solid state and in polar solutions the preferred conformation is that depicted in V, while VI predominates in the vapour and in non-polar solutions. Even these results cannot be carried directly over to peptides and therefore, bearing in mind the arguments put forward at the beginning of this paper, we have considered the six arrangements of the C_α—CO bonds summarized by VII or VIII with x = R, H, or NH. "Perpendicular" forms, similar to VI, are probably sufficiently near to VII and VIII to be covered in these approximate calculations. Likewise we have considered six arrangements of the N—C_α bonds, which are summarized in IX and X with x = R, H, or CO.

Measurements were made with Dreiding models fitted with collars to restrict rotation in the chain. The carboxylate end group was treated like an amide group, the measurement being taken from the point midway between the two oxygen atoms. Except in two columns (VII and VIII, x = NH), there are two positions for the glycyl residue because R = H; this C_α—CO bond was placed so as to bisect the angle between these two positions. Table 2 records the charge separations measured on Dreiding models of these thirty six conformations of each diastereoisomer of glycylleucylleucine. Some of the thirty six conformations of a diastereoisomer must have identical charge separations but they have all been considered individually in order to check the accuracy of the measurements; the discrepancies are no more than 0·2 Å. Sixteen arrangements of this tripeptide, including all those with (IX, x = CO) or (X, x = CO), have identical or closely similar (<0·4 Å) charge separations in the diastereoisomers

[16] S. Mizushima, T. Shimanouchi, I. Ichishima, T. Miyazawa, I. Nakagawa and T. Araki, *J. Amer. Chem. Soc.* **78**, 2038 (1956).

TABLE 2. MEASURED DISTANCES (Å) BETWEEN CHARGES IN 36 CONFORMATIONS OF THE DIASTEREOISOMERIC GLYCYLLEUCYLLEUCINES

C_α—CO / N—C_α		VII						VIII					
		x = H		x = R		x = NH		x = H		x = R		x = NH	
		LL	LD	LL	LD	LL	LD	LL	LD	LL	LD	LL	LD
	x =												
	H	5·3	7·3	**7·7**	**5·6**	9·9	9·3	**9·4**	**7·6**	9·1	10·0	5·7	5·5
IX	R	**7·7**	**5·7**	5·3	7·2	9·9	9·3	9·0	10·0	**9·5**	**7·7**	5·8	5·4
	CO	7·7	7·6	7·7	7·7	6·1	6·2	6·3	6·4	6·3	6·3	7·6	7·8
	H	**8·2**	**6·4**	6·6	8·0	8·0	7·1	6·8	8·3	**8·5**	**6·0**	7·2	7·0
X	R	6·5	8·0	**8·2**	**6·5**	8·0	7·1	**8·4**	**6·0**	6·8	8·3	7·1	7·1
	CO	6·1	6·1	6·1	6·0	10·3	10·3	9·6	9·6	9·6	9·7	4·7	4·7

and eight, including that of the α-helix viz. VII (x = H) with X (x = R), lead to a smaller charge separation in the L-L isomer. Some of these twenty four arrangements may contribute to the entire assembly of conformations but only to a minor extent. We are left with twelve arrangements as possible major contributors to the actual mixture. Of these twelve the four based on VII (x = NH) are eliminated by the results with leucylleucylglycine, leucylleucine and leucyltyrosine because in these instances diastereoisomers would be indistinguishable. The remaining arrangements, which are picked out in heavy type in Table 2, are (1) VII (x = H) with X (x = H); (2) VII (x = R) with X (x = R); (3) VII (x = H) with IX (x = R); (4) VII (x = R) with IX (x = H); (5) VIII (x = H) with IX (x = H); (6) VIII (x = R) with IX (x = R); (7) VIII (x = H) with X (x = R); (8) VIII (x = R) with X (x = H). Examination of peptides containing several asymmetric carbon atoms would help to distinguish between these possibilities but it should be noted that there are pairs, viz. (1) and (2), (3) and (4), (5) and (6), (7) and (8), which have identical dipole moments not only in this instance but in all peptides, and they cannot in principle be differentiated by this method. Molecular models of these eight arrangements are instructive. There is severe repulsion between the alkyl substituent and the N—H hydrogen atom next along the chain in VIII (x = R) i.e. (6) and (8). In VIII (x = H) i.e. (5) and (7) the hydrogen atom on the asymmetric carbon atom and the N—H hydrogen atom just mentioned are separated by 2·1 Å; this repulsion would not be large enough to prevent the arrangement if other circumstances were favourable. In IX (x = R) i.e. (3) and (6) the alkyl group and the carbonyl preceding it in the chain are separated by 2·2 Å, perhaps a permissible amount. Arrangements (1) and (2) incorporate a hydrogen bond in a seven-membered ring and they correspond to the 2_7 helix.[17] Although intramolecular hydrogen bonds are unlikely to play an important role in favouring conformations in aqueous media there is no obvious reason why they should be excluded. Arrangement (4) is apparently without appreciable repulsions but it cannot be selected on that score alone because some of the effects mentioned are probably small.

[17] C. H. Bamford, A. Elliott and W. E. Hanby, *Synthetic Polypeptides* pp. 119–121. Academic Press, New York (1956).

Independent evidence of conformational selection in peptides has been published recently. The p$Ka_{1,2}$ of D-leucyl-L-tyrosine are 3·12, 8·38 compared with 3·46, 7·84 for the L-L isomer, indicating a smaller charge separation in the D-L isomer, and some other examples have been recorded.[18] Moreover, the proton magnetic resonance spectrum of the D-L isomer, unlike that of the L-L isomer, shows resonances of the leucine side-chain shifted to higher field by proximity of the aromatic ring in the tyrosyl residue,[19] again indicating a more compact molecule in the D-L instance. It seems likely that more detailed information concerning preferred conformations, at least about the N—C_α bonds, may be obtained from proton magnetic resonance spectroscopy by consideration of the NH—C_αH coupling constants. It is to be expected that these coupling constants will be related to the angles between the NH and CH bonds, just as is the case for protons attached to tetrahedral[20] and trigonal carbon atoms.[3c]

We believe that the present work gives good ground for the theory of conformational selection at N—C_α and C_α—CO bonds in peptides, and it goes some way towards defining the effect. It should be possible to go considerably further by examining tetra- and penta-peptides composed entirely of optically active residues, unless their solubilities are too low. The importance of these studies lies in understanding the behaviour of peptides. For example in enzymic reactions the free energy of activation will be influenced by the average conformation of a peptide in free solution, because it must adopt a particular conformation on the surface of the enzyme.

EXPERIMENTAL

General directions are given in Part XI.[21]

Syntheses of Cyclic Pentapeptides

Benzyloxycarbonylleucylglycylglycine ethyl ester

(a) L-*form.* The optical rotation of this compound, with m.p. 103–104·5°, has been erroneously recorded as $[\alpha]_D^{25}$ −10·98° (*c* 5, 95% ethanol.[22] Our crude product (83%) from condensation of benzyloxycarbonyl-L-leucine and glycylglycine ethyl ester hydrochloride via the sulphuric anhydride (method c[23]) had m.p. 102–103° and $[\alpha]_D^{24}$ −8·0°, altered by recrystallization from ethylacetate–ether to m.p. 104–105°, $[\alpha]_D^{21}$ −5·5° ± 0·2° (*c* 5, 95% ethanol) (Found: C, 58·9; H, 7·3; N, 9·7. Calc. for $C_{20}H_{28}N_3O_6$: C, 58·95; H, 7·2; N, 10·3%). Similar material was obtained from benzyloxycarbonyl-L-leucylglycine and glycine ethyl ester hydrochloride by means of ethyl chloroformate and triethylamine. The compound has also been prepared independently by Heaton[24] in several ways, and he has also noted the mistake.

(b) D-*form.* This ester was prepared from benzyloxycarbonyl-D-leucine in the same way as the L-isomer, and it had m.p. 102–103°, $[\alpha]_D^{21}$ +5·7° ± 0·2° (*c* 5, 95% ethanol) (Found: C, 59·0; H, 7·2; N, 10·0%). Hydrolysis by sodium hydroxide in aqueous methanol at room temp afforded the

[18a] E. Ellenbogen, *J. Amer. Chem. Soc.* **78,** 369 (1956); [b] N. C. Li, G. W. Miller, N. Solony and B. T. Gillis, *Ibid.* **82,** 3737 (1960).

[19] F. A. Bovey and G. V. D. Tiers, *J. Amer. Chem. Soc.* **81,** 2870 (1959).

[20] H. Conroy, *Advances in Organic Chemistry* (Edited by R. A. Raphael, E. C. Taylor and H. Wynberg) Vol. II, p. 311. Interscience, New York (1960).

[21] M. T. Leplawy, D. S. Jones, G. W. Kenner and R. C. Sheppard, *Tetrahedron* **11,** 39 (1960).

[22] H. F. Schott, J. B. Larkin, L. B. Rockland and M. S. Dunn, *J. Org. Chem.* **12,** 493 (1947).

[23] D. W. Clayton, J. A. Farrington, G. W. Kenner and J. M. Turner, *J. Chem. Soc.* 1403 (1957).

[24] G. S. Heaton, Ph.D. Thesis, Manchester (1960).

corresponding *acid*, m.p. 145–147° $[\alpha]_D^{23}$ +10·9° ± 0·4° (*c* 5, 95% ethanol). (Found: N, 11·4. $C_{18}H_{25}N_3O_6$ requires N, 11·1%) after one crystallization from aqueous methanol. The rotation of the enantiomer has been reported as −10·7° [24], −12.8° [25], and −14·28° [22] with m.p. 144–145° in each case. The purity of our material was checked by hydrogenolysis with palladized carbon to the tripeptide, $[\alpha]_D^{25}$ −51·7° ± 0·4° (*c* 5, water) (reported[22] for the enantiomer +57·6°), which was hydrolyzed with 6N hydrochloric acid at 100° during 21 hr to a solution having optical rotation corresponding to a specific rotation of −14·6° ± 0·6° for the D-leucine, assumed to be quantitatively liberated.

Benzyloxycarbonylglycylleucylleucylglycylglycine ethyl ester

(a) L-L-*form*. Benzyloxycarbonyl-L-leucylglycylglycine ethyl ester was hydrogenated with 10% palladized carbon at atm press and room temp in ethanol containing 15% of acetic acid. After removal of the catalyst and addition of ethanolic hydrogen chloride, the solution was evaporated to small bulk and diluted with ether. The hygroscopic crystalline hydrochloride (9·15 g) of L-leucylglycylglycine ethyl ester obtained in this manner was condensed with benzyloxycarbonylglycyl-L-leucine (9·52 g) via the sulphuric anhydride (method c[23]). The *pentapeptide ester* (12·77 g, 75%) crystallized from aqueous ethanol in needles, m.p. 123·5–124·5° $[\alpha]_D^{20}$ −34·8° ± 0·2° (*c* 4·5, ethanol). (Found: C, 58·1; H, 7·4; N, 12·3. $C_{29}H_{43}N_5O_8$ requires C, 58·2; H, 7·5; N, 12·1%).

(b) L-D-*form*. This preparation was like the preceding one except that the hydrobromide of D-leucylglycylglycine ethyl ester was prepared from the benzyloxycarbonyl derivative by treatment with hydrogen bromide in acetic acid at room temp. The *pentapeptide ester* was a colourless amorphous powder with m.p. 120–123° $[\alpha]_D^{20}$ −19·8° ± 0·3° (*c* 4, ethanol). (Found: C, 58·1; H, 7·4; N, 12·1%).

Benzyloxycarbonylglycylleucylleucylglycylglycine

(a) L-L-*form*. N NaOH (11·4 cc) was added to a solution of the pentapeptide ester (6 g) in ethanol (60 cc). After 2½ hr the solution was acidified (pH 2) and evaporated. A portion of the acidic material, obtained by ethyl acetate extractions, was distributed (30 transfers) between ethyl acetate and 0·5 M potassium phosphate (pH 6·0). The purified glass (K 0·26) crystallized from ethyl acetate–light petroleum, and the main bulk could then be crystallized. The *pentapeptide* (95% yield) had m.p. 124–126° $[\alpha]_D^{20}$ −36·5° ± 0·2° (*c* 4, ethanol). (Found: C, 56·4; H, 7·1; N, 12·5. $C_{26}H_{39}N_5O_8$ requires: C, 56·8; H, 7·15; N, 12·7%). If the time of hydrolysis was increased to 12 hr, the yield fell to 74%.

(b) L-D-*form*. This *pentapeptide* was obtained likewise as an amorphous powder, m.p. 106–108° (after drying for 3 hr at 60°/0·05 mm) $[\alpha]_D^{18}$ −24·9° ± 0·2° (*c* 4, ethanol). (Found: C, 56·4; H, 7·1; N, 12·6%).

Glycylleucylleucylglycylglycine

The free peptides were prepared from the benzyloxycarbonyl derivatives by the technique described below for di- and tri-peptides.

(a) L-L-*form*. The pentapeptide crystallized in needles, $[\alpha]_D^{22}$ −53·7° ± 0·5° (*c* 2, N HCl). (Found: C, 49·9; H, 8·2; N, 16·1. $C_{18}H_{33}N_5O_6 \cdot H_2O$ requires: C, 49·9; H, 8·1; N, 16·2%).

(b) L-D-*form*. The *pentapeptide* crystallized in needles, $[\alpha]_D^{22}$ −17·7° ± 1·3° (*c* 1·5, N HCl). (Found: C, 48·3; H, 8·0; N, 15·8. $C_{18}H_{33}N_5O_6 \cdot 1^1/_2H_2O$ requires: C, 48·0; H, 8·0; N, 15·6%).

Benzyloxycarbonylglycylleucylleucylglycylglycine p-*nitrophenyl thiolester*

Procedure (c, through the lithium salts of the foregoing acids) was used as described in Part VII.[26]

(a) L-L-*form*. This amorphous *thiolester* (90% yield) had m.p. 115–120°. (Found: C, 55·7; H, 6·2; N, 12·5; S, 5·0; $C_{32}H_{42}N_6O_9S$ requires: C, 56·0; H, 6·2; N, 12·2; S, 4·75%).

(b) L-D-*form*. Bis-*p*-nitrophenyl disulphide was removed by repeated precipitation from aqueous acetone, but even so the amorphous *thiolester* (87% yield), m.p. 116–119°, was probably not quite uncontaminated. (Found: C, 55·6; H, 6·1; N, 12·6; S, 5·4%).

[25] M. Bergmann, L. Zervas and J. S. Fruton, *J. Biol. Chem.* **111**, 225 (1935).
[26] J. A. Farrington, P. J. Hextall, G. W. Kenner and J. M. Turner, *J. Chem. Soc.* 1410 (1957).

Cycloglycylleucylleucylglycylglycyl

(1) *Thiolester method*

Cyclization of the thiolesters was accomplished by the technique described in Part VIII,[8] but after disulphide and magnesium carbonate had been removed, the aqueous filtrate was brought to pH 3 (dil HCl) and extracted with ethyl acetate–butan-l-ol (6 × 100 cc of 1:1, v:v). The combined extracts were washed with sodium hydrogen carbonate solution, dried ($MgSO_4$) and evaporated.

(a) L-L-*form*. The crude product (0·305 g, after drying at 40°/0·05 mm, from 1·078 g of benzyloxycarbonylpentapeptide thiolester) was distributed (50 transfers) in ethyl acetate–water–methanol (10v–9v–1v) in a Craig machine with 20 cc phases. All the material could be dissolved in the first tube, but a settling period of 15 min was necessary. Tubes 8–23 yielded the crystalline *cyclic peptide* (0·068 g, 11·1%), d. 300–320°, K 0·45 (r_{max} 15 containing 8·8 mg, good agreement between experimental and theoretical distribution of material) $[\alpha]_D^{16}$ −81·4° ± 2·8° (*c* 0·73, methanol). (Found: C, 54·6; H, 8·0; N, 17·7. $C_{13}H_{21}N_5O_5$ requires: C, 54·4; H, 7·9; N, 17·6%). Its IR absorption spectrum had maxima at 3279, 3049, 2933, 1639, 1534, 1468, 1445, 1385, 1368, 1326, 1272, 1222, 1166, 1139, 1117, 1096, 1046, 1029, 977, 919, 856, 823 cm^{-1} (KBr disc). Repetition of the cyclization gave a 12% yield.

(b) L-D-*form*. The crude product (0·405 g from 1·008 g of benzyloxycarbonylpentapeptide thiolester) was placed in the first three tubes of the Craig machine and distributed (70 transfers) as in the preceding experiment. Tubes 26–45 yielded the *cyclic peptide* (0·222 g, 38·3%) K 1·03 (r_{max} 35 containing 20·7 mg, good agreement between experimental and theoretical curves), and it was recrystallized from methanol in clumps of needles, d. 310–330°, $[\alpha]_D^{22}$ −15·5° ± 2·6° (*c* 0·81, trifluoracetic acid; the compound was insufficiently soluble in methanol for this measurement). (Found: C, 54·3; H, 8·0; N, 17·3%; M.W. (isothermal distillation in trifluoroacetic acid) 380 (±40). $C_{18}H_{31}N_5O_5$ requires: M.W. 397). Its IR absorption spectrum had maxima at 3236, 3058, 2924, 1634, 1548, 1447, 1433, 1412, 1381, 1366, 1326, 1276, 1236, 1208, 1168, 1142, 1122, 1104, 1044, 1017, 986, 932, 912, 888, 864 852 cm^{-1} (KBr disc). Repetition of the cyclization gave a 40% yield.

(2) *Carbodi-imide method*[9]

(a) L-L-*form*. A solution of glycyl-L-leucyl-L-leucylglycylglycine (0·857 g) in water (224 cc) was diluted with methanol (860 cc) and cooled to 0°. A solution of dicyclohexylcarbodi-imide (2·5 g) in methanol (4 cc) was added in one portion. After 4 days the mixture was allowed to reach 20° and then it was kept for 3 more days. The solution was evaporated and the residue was dissolved in methanol (50 cc), to which glacial acetic acid (5 cc) was added. When the solution had been concentrated to 20 cc, the colourless crystalline solid A (1·0 g) was collected. Evaporation of the filtrate yielded an oil which was boiled with ethyl acetate (30 cc). The pale brown solid B (0·84 g) was collected when the mixture had been cooled to 0°. The pale brown oil (1·87 g) remaining in the filtrate was partitioned between ether and water. The ether layer contained an oil (0·69 g) which appeared from its IR spectrum to be an N-acylurea; the aqueous layer yielded 0·45 g (53%) of the original pentapeptide. The combined solids A and B, consisting mainly of dicyclohexylurea, were extracted with water (150 cc) in a Soxhlet apparatus during 6 hr. A little material which separated from the cooled extract was rejected and the remainder was evaporated (0·145 g) and distributed (18 transfers) between ethyl acetate–water–methanol as before. Tubes 3–11 furnished 0·104 g (12·7%) of cyclic pentapeptide, identical in IR spectrum with that already described.

(b) L-D-*form*. Cyclization of the pentapeptide (0·232 g) was carried out just as in the L-L-series, and 0·079 g (34%) was recovered. Tubes 6–14 of the 18 transfer distribution yielded 87 mg (39%) of crystalline cyclic peptide, identified by IR spectroscopy with that already described.

Preparation of Benzyloxycarbonyl Dipeptides

Benzyloxycarbonylleucylleucine

(a) L-L-*form*[27]. The methyl ester, m.p. 95–96° $[\alpha]_D^{18}$ −39·0° ± 0·3° (*c* 5·3, methanol), prepared by carbodi-imide condensation in methylene chloride, was hydrolysed with potassium hydroxide in aqueous methanol at room temp. The resultant acid crystallized from ethyl acetate–light petroleum in leaves, m.p. 89·5° $[\alpha]_D^{21}$ −28·2° ± 0·3° (*c* 3, methanol).

[27a] M. Brenner and C. H. Burckhardt, *Helv. Chim. Acta* **34**, 1070 (1951); [b] E. L. Smith, D. H. Spackman and W. J. Polglase, *J. Biol. Chem.* **199**, 801 (1952).

(b) D-L-*form.* The methyl ester, m.p. 78·5–79·5° $[\alpha]_D^{25}$ −2·3° ± 0·6° (*c* 1·8, methanol). (Found: C, 64·3; H, 8·4; N, 7·2. $C_{21}H_{32}N_2O_5$ requires: C, 64·3; H, 8·2; N, 7·1%) (ref. 27*b* gives m.p. 81·5–82·5° $[\alpha]_D^{24}$ +2·30° (*c* 1, ethanol) for the L-D-form was prepared in 74% yield from benzyloxycarbonyl-D-leucine, L-leucine methyl ester hydrochloride, triethylamine, and dicyclohexylcarbodiimide in methylene chloride. Hydrolysis gave an oily acid, characterized as its *dicyclohexylammonium salt*, m.p. 163·5–164·5°. (Found: C, 68·7; H, 9·4; N, 7·5. $C_{32}H_{53}N_3O_5$ requires: C, 68·7; H, 9·5; N, 7·5%).

Preparation of Benzyloxycarbonyl Tripeptides

Benzyloxycarbonylleucylglycylglycine

See the beginning of the Experimental section.

Benzyloxycarbonylleucylleucylglycine

(a) L-L-*form.* Benzyloxycarbonyl-L-leucylglycine methyl ester,[28] m.p. 91–92° $[\alpha]_D^{19}$ −27·4° ± 0·3° (*c* 3·6, ethanol) was prepared in 82% yield from benzyloxycarbonyl-L-leucine and glycine methyl ester hydrochloride by procedure c of the sulphuric anhydride method.[23] Hydrogenolysis in methanol containing 5% acetic acid over 5% palladized charcoal, followed by addition of HCl, gave an oily hydrochloride, which was condensed with benzyloxycarbonyl-L-leucine in the same way (yield 70%). The *tripeptide ester* crystallized from ether in prisms, m.p. 111–112° $[\alpha]_D^{18}$ −50·4° ± 0·3° (*c* 2·9, methanol). (Found: C, 61·3; H, 7·8; N, 9·2. $C_{23}H_{35}N_3O_6$ requires: C, 61·45; H, 7·85; N, 9·35%). Hydrolysis with sodium hydroxide in aqueous methanol (70 min at 20°) afforded an oil, which could not be crystallized even after counter-current distribution (21 transfers; K 0·30) between ethyl acetate and M phosphate buffer (pH 6·7). The *dicyclohexylammonium salt* crystallized from methanol–ether in needles, m.p. 180–181° $[\alpha]_D^{18}$ −38·0° ± 0·8° (*c* 1·3, 40% aqueous ethanol). (Found: C, 66·1; H, 9·0; N, 9·2. $C_{44}H_{66}N_4O_6$ requires: C, 66·2; H, 9·15; N, 9·1%).

(b) D-L-*form.* The *methyl ester* crystallized from aqueous ethanol in fibrous needles, m.p. 123–124° $[\alpha]_D^{16}$ −24·3° ± 0·3° (*c* 3·5, ethanol). (Found: C, 61·0; H, 7·7; N, 9·5%). The *benzyloxycarbonyltripeptide* crystallized from ethyl acetate–light petroleum in prisms, m.p. 148–150°. (Found: C, 60·4; H, 7·7; N, 9·7. $C_{32}H_{53}N_3O_6$ requires: C, 60·7; H, 7·6; N, 9·65%).

Benzyloxycarbonylglycylleucylleucine

(a) L-L-*form.* The *ethyl ester* was obtained in 80% yield from benzyloxycarbonylglycyl-L-leucine ethyl ester hydrochloride by procedure c of the sulphuric anhydride method,[23] and it crystallized from ethyl acetate–light petroleum in prisms, m.p. 122°. (Found: C, 62·2; H, 8·1; N, 9·1. $C_{24}H_{37}N_3O_6$ requires: C, 62·2; H, 8·05; N, 9·1%). The benzyloxycarbonyltripeptide crystallized from ethyl acetate–light petroleum and had m.p. 131–132° (lit.[29] m.p. 137°) $[\alpha]_D^{18}$ −38·3° ± 0·8° (*c* 1·3, ethanol). (Found: C, 60·8; H, 7·7; N, 9·6%).

(b) L-D-*form.* Obtained likewise (yield 71%), the methyl ester[29] crystallized from ethyl acetate–light petroleum in fibrous needles, m.p. 118° $[\alpha]_D^{18}$ −16·8° ± 0·4° (*c* 2·6, ethanol). (Found: C, 61·5; H, 7·9; N, 9·3%). The benzyloxycarbonyltripeptide[29] crystallized from ethyl acetate–light petroleum in needles, m.p. 145–147°. (Found: C, 60·3; H, 7·7; N, 9·8%).

Preparation of Free Peptides

Generally a solution of the benzyloxycarbonyl derivative (0·5–1·0 g) in 50% aqueous methanol (50 cc) was added to a suspension of 10% palladized charcoal (0·20 g) in acetic acid (5 cc). Hydrogen was bubbled through the solution for at least 4 hr, and it was warmed to 60° before filtration. The catalyst was washed with 50% aqueous methanol. When the filtrates had been concentrated, they were partitioned between ethyl acetate and water (25–50 cc). Evaporation of the aqueous layer yielded the peptide.

Glycyl-L-leucine

This peptide decomposed at 240–245° $[\alpha]_D^{20}$ −34·7° ± 0·5° (*c* 2, water). (Found: C, 51·1; H, 8·9; N, 14·9. Calc. for $C_8H_{16}N_2O_3$: C, 51·05; H, 8·6; N, 14·9%).

[28] R. W. Young, K. H. Wood, R. J. Joyce and G. W. Anderson, *J. Amer. Chem. Soc.* **78**, 2128 (1956).
[29] S. Simmonds, J. I. Harris and J. S. Fruton, *J. Biol. Chem.* **188**, 260 (1951).

Leucylleucine

(a) L-L-*form.*[27b] The dipeptide was recrystallized from water as the monohydrate, $[\alpha]_D^{22}$ −13·9° ± 1·2° (*c* 1·6, N NaOH) −20·0° ± 2·0° (*c* 1·1, N HCl). (Found: C, 54·9; H, 10·0; N, 10·9. Calc. for $C_{12}H_{24}N_2O_3.H_2O$: C, 54·9; H, 10·0; N, 10·7%).

(b) D-L-*form.* This *dipeptide*, $[\alpha]_D^{22}$ −63·2° ± 1·8° (*c* 1·1, N HCl), was only sparingly soluble (<1%) in hot water. (Found: C, 51·0; H, 10·1; N, 10·0. $C_{12}H_{24}N_2O_3.2H_2O$ requires: C, 50·9; H, 10·0; N, 10·0%). Ref. 27b records $[\alpha]_D^{27}$ +74·4° (*c* 1, N HCl) for the L-D-form.

Leucylleucylglycine

(a) L-L-*form.* The *tripeptide* had $[\alpha]_D^{22}$ −13·8° ± 3·5° (*c* 0·6, N HCl). (Found: C, 55·4; H, 8·6; N, 13·5. $C_{14}H_{27}N_3O_4$ requires: C, 55·8; H, 9·0; N, 13·9%).

(b) D-L-*form.* The *tripeptide* had $[\alpha]_D^{23}$ −64·6° ± 2·0° (*c* 0·8, N HCl). (Found: C, 52·5; H, 9·0; N, 13·1. $C_{14}H_{27}N_3O_4.H_2O$ requires: C, 52·5; H, 9·1; N, 13·2%).

Glycylleucylleucine

(a) L-L-*form.*[29] The tripeptide crystallized from water in needles, $[\alpha]_D^{24}$ −55·5° ± 2° (*c* 1, N HCl). (Found: C, 54·1; H, 9·1; N, 13·6. Calc. for $C_{14}H_{27}N_3O_4.\frac{1}{2}H_2O$: C, 54·2; H, 9·0; N, 13·6%).

(b) L-D-*form.*[29] The tripeptide had no detectable optical rotation (*c* 0·7, N HCl). (Found: C, 52·7; H, 8·9; N, 13·1. Calc. for $C_{14}H_{27}N_3O_4.H_2O$: C, 52·5; H, 9·1; N, 13·2%).

Paper Chromatography of Peptides

In butan-1-ol 100:acetic acid 17:water 38 descending chromatography on Whatman no. 1 paper gave the following R_F values: glycine 0·11, leucine 0·56, glycylglycine 0·15, glycylleucine 0·54, leucylglycine 0·49, leucylleucine 0·83 (L-L) and 0·81 (D-L), glycylleucylleucine 0·80 (L-L) and 0·76 (L-D), leucylleucylglycine 0·80 (L-L) and 0·76 (D-L), leucylglycylglycine (0·39), tetraglycine (0·06), glycylleucyl-leucylglycylglycine 0·62 (L-L) and 0·59 (L-D), glycylleucylglycylleucylglycine 0·65 (L-L).

Measurements of Dielectric Increments[8]

The peptides were recrystallized if a balance point could not be reached, and in the cases of glycylleucylleucine (L-L and L-D forms) and leucylleucylglycine (L-L form) electrodialysis through cellophane was necessary for removal of salts. The leucyltyrosines were commercial samples (Nutritional Biochemicals Corp., Cleveland). In addition to the values recorded in Table 1, the following dielectric increments were determined: alanine 22·0, leucine 20·7, glycylleucine 68·4, leucylglycylglycine 115, tetraglycine 162·5. The measurements with glycine (calibration) were made at several concentrations up to 0·5 M and those with glycyl-L-leucyl-D-leucine up to 0·011 M. In all the remaining instances low solubility of the peptides was a limiting factor and the maximum molar concentrations were as follows: leucylleucine, 0·043 (L-L) and 0·023 (D-L); leucyltyrosine, 0·009 (L-L) and 0·015 (D-L); glycylleucylleucine, 0·020 (L-L) and 0·011 (L-D); leucylleucylglycine, 0·021 (L-L) and 0·020 (D-L); glycylleucylleucylglycylglycine, 0·015 (L-L) and 0·016 (L-D); glycylleucyl leucylglycylglycine, 0·015 (L-L) and 0·016 (L-D); glycylleucylglycylleucylglycine, 0·011 (L-L); tetraglycine, 0·031.

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