THE CONFORMATION OF POLYPEPTIDES CONTAINING ALTERNATING L- AND D-AMINO ACIDS*

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I. INTRODUCTION

Proteins and enzymes of all living systems contain L-amino acids. The natural preference for the L-configuration of the amino acid in the biological macromolecules is still a riddle. On the other hand, the occurrence of D-amino acids along with L-amino acids in most of the peptide antibiotics is quite common. For example, enniatin B, c(L-methylvaline-D-hydroxyisovaleric acid)$_3$, and valinomycin, c(L-valine-D-hydroxyisovaleric acid-D-valine-L-lactic acid)$_3$, are both cyclic peptides; gramicidin A is a linear pentadecapeptide, with L- and D-amino acids occurring alternately (of course, taking into consideration the potential equivalence of Gly to a D-amino acid) — Formyl-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-ethanolamide. Also, for example, D-Ala and D-Glu occur in peptidoglycan.

During biosynthesis, the direct incorporation of D-amino acids into the peptide antibiotics is generally not known. Instead, only their L-isomers are initially used and then converted into D-configuration by the action of enzymes, known as amino acid racemases.[1] Since the discovery of alanine racemase by Wood and Gunsalus,[2] several amino acid racemases have been found in various bacteria, as reviewed by Meister.[3]

In the course of the last two and a half decades, extensive studies, theoretical as well as experimental, on polypeptides with all L-amino acids have given rise to several types of stable secondary structures, including the well-known α-helix, 3_{10}-helix, triple-helical coiled coil of collagen and the parallel and antiparallel β-structures. Some of these molecular conformations may, however, be precluded in the incorporation of one or more D-amino acids in the otherwise all L-residue polypeptide chain. Many theoretical and experimental studies have explored the possible helical structures to identify a polypeptide chain containing both L- and D-amino acids. The conformational analysis of the structural features of polypeptides with strictly alternating L- and D-amino acids was

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first reported by Ramachandran and Chandrasekaran.[4] Since then, apart from the orthodox α-helix,[5] several novel helical structures have been proposed, and their conformational characteristics have been determined using empirical energy calculations in the authors' laboratory[6] and elsewhere.[5,7]

Recently, many of these predictions have been well supported and substantiated by experimental studies in solution and in the solid state. The details of the theoretical results, the experimental evidence of agreement, and, hence, the combined contribution towards understanding the structural and functional aspects of ionophoric antibiotics — particularly gramicidin A — will be described in the following pages.

The second section will include the details of theoretical studies on novel single and double helices of polypeptides in which L- and D-amino acids alternate. It is indeed a phenomenal observation that the synthetic polymer, poly(γ-benzyl-LD-glutamate) (PBLDG), can exhibit a variety of helical structures, depending mainly on its environment, both in solution and in the solid state. Much experimental evidence pertaining to this polymer has been accumulated in recent years from NMR, CD, and IR studies in solution and X-ray and electron diffraction analyses in the solid state. Results from the above investigations are described in the third section of this review. The next section is devoted to the structural and functional aspects, currently available from theoretical and experimental studies, of the ionophore gramicidin A.

Throughout this article, the main focus will be to describe the conformational features of polypeptides with strictly repeating LD-sequences. These results may, however, be extrapolated to structures which deviate, either minimally or substantially, from such a strict repetition.

It is customary to denote the strictly alternating polymer (of say Ala) as poly(L-Ala-D-Ala) or, in short, poly(LD-Ala) and the randomly distributed copolymer as poly(L,D-Ala). For the sake of uniformity, we will use poly(LD-Ala) for the alternating polymer.

II. THEORETICAL STUDIES

Considering the regular structure with alternating L- and D-amino acids, denoted by $(\text{LD})_n$ where n is the degree of polymerization, the repeating unit in such cases is a pair of peptide units (or residues) with alternating configurations at the α-carbon atoms. Following the usual practice, the peptide units should always be in the *trans* planar configuration, unless otherwise specified. The two pairs of dihedral angles (ϕ_L,ψ_L) and (ϕ_D,ψ_D) will suffice to describe the conformation of the polymer in its helical state. The conformational angles are defined according to the recommendations of the IUPAC-IUB Commission.[8] This review will be confined to those helices which have internal hydrogen bonds which involve the amide and carbonyl groups of the peptide units.

A. Conventional Helices

The essential nature of the resultant conformation, for polypeptides containing alternate L- and D-amino acids, has been derived by applying standard theories of peptide conformations.[9] Helical structures stabilized by regular NH···O hydrogen bonds between neighboring peptide units are the well-known 3_{10}-,α-, and ω-helices, either right- or left-handed. The existence of such structures has, however, been doubted[10] under the presumption that steric hindrance between the side chains of L- and D-residues would disrupt the helix. To resolve the question as to whether poly-LD-peptides can exist in α-helical form,[10] Hesselink and Scheraga[5] carried out conformational energy calculations using the procedure employed by Ooi et al.[11] Thus, the values of dihedral angles ϕ and ψ and the χ's have been assumed to be the same for each residue. The bond angles, bond lengths, and planar *trans* peptide groups have been maintained fixed and a poly(amino acid) chain of n LD-pairs has been treated. The conformational energy function includes contributions from internal rotations and nonbonded, electrostatic, and hydrogen bond interactions. The computations show that the energy of a regular repeating sequence of LD-alanine is −7.9 kcal/mol per residue, approximately the same as that for a right- or left-handed α-helix (denoted as α_P and α_M) which is comparable to the energy per residue in the α-helical region of poly-L-alanine — −8.0 and −7.7 kcal/mol for α_P and α_M, respectively.[5]

From similar calculations on poly(LD-valine), poly(LD-phenylalanine), and poly(LD-lysine analogue), namely poly(LD-aminoheptanoic acid), it has been shown that poly(LD-peptides) would adopt the α-helical conformation.[5] Both the α_P- and α_M-helices of poly-LD-peptides are in local minima of energy comparable to that of the right-handed α-helix of poly-L-peptides. Since these are not the global minima for poly-LD-peptides, it has been suggested[5] that poly-LD-peptides might be able to form stable structures completely different from α-helical conformation.

Of the other two possible helices, namely 3_{10}- and π-helices, which occur in the neighborhood of the α-helical region, the latter can be eliminated for the poly-LD-peptides, since the side chain-side chain interactions between the L- and D-residues are sterically unfavorable. This is due mainly to the low axial rise per residue (1.0 A) of the π-helix. On the other hand, the 3_{10}-helix seems to be a stereochemically satisfactory model. It is, however, expected that the α-helix would be energetically more stable than the 3_{10}-helix, as is always true with the poly-L-peptides.

B. Novel Helical Structures

1. Single Helices

Ramachandran and Chandrasekaran,[4,6] using semiempirical energy calculations, have predicted that single helical conformations other than the α- or 3_{10}-helix may exist, and one or more of these might readily be applicable to the structure of gramicidin A.[7] For steric reasons, such helical models can only be built with strictly repeating LD-sequences. Recently, Blout and colleagues[12] have proposed possible double helical structures as models for poly-LD-peptides based on their solution studies on gramicidin A.

a. Hydrogen Bonding Schemes in Conventional and Novel Single Helices

Before discussing the methodology and details of the novel helices common only to poly-LD-peptides, we shall first describe the types of hydrogen bonding patterns[6] in single helices of poly-L-peptides. This is essential in order to appreciate the differences between the two types of helices.

Considering only right-handed helices, hydrogen bonds can occur between a donor (NH) or an acceptor (CO) group, in peptide unit 1 and an acceptor or a donor group, in peptide unit j, in a polypeptide chain containing either all L- or all D-residues. Thus, there are two types of hydrogen bonds which are denoted[6] by

$$\begin{array}{ll} \text{Type A: } j \rightarrow 1 & \\ & j = 2, 3, 4, 5 \ldots \\ \text{Type B: } 1 \rightarrow j & \end{array}$$

The arrow indicates the direction from the donor to the acceptor unit. Whereas the type A hydrogen bond occurs in the well-known 3_{10}-, α-, and π-helices, corresponding to j = 3, 4, and 5, respectively, the type B hydrogen bond is stereochemically impossible in any polypeptide helix with all L- or all D-residues.

The situation is quite different in the case of poly-LD-peptides. Ramachandran and Chandrasekaran[6] have shown that the two pairs of conformational angles, (ϕ_L,ψ_L) and (ϕ_D,ψ_D), suitably chosen to be in their respective β-regions, could readily produce satisfactory helical structures stabilized by systematic hydrogen bonds between the peptide units. Such a helix consists of successive NH (or CO) groups pointing in opposite directions, nearly parallel to the helical axis. This arrangement leads to the formation of adjacent NH···O hydrogen bonds alternating between type A and type B. These authors[6] have designated the new helices as LD-helices and, following their nomenclature, a schematic representation of the helix and the hydrogen bonding pattern is illustrated in Figure 1.

It may be seen that the hydrogen bonds occur between donor and acceptor groups in LD-units 1 and k and, hence, the helix is denoted by the symbol LD_k. The corresponding right- and left-handed structures are designated as LD_{kP} and LD_{kM}, respectively. The various kinds of LD-helices and their hydrogen-bonding patterns, together with the number of atoms in the ring formed by the hydrogen bond, are listed in Table 1. An interesting feature is that the type A hydrogen bonds are always between D-peptide units and, similarly, the type B hydrogen bonds are only between L-peptide units for one sense of twist of the helix and vice versa for the other sense of twist. If the two L- and D-amino acids in the repeating unit are of the same kind, the right-handed helix and its inverse conformation (the corresponding left-handed helix) are isoenergetic. These two are mirror images of each other.

b. Conformational Analysis of LD-Helices

Ramachandran and Chandrasekaran[6] and Urry[7] have initially considered cyclic structures

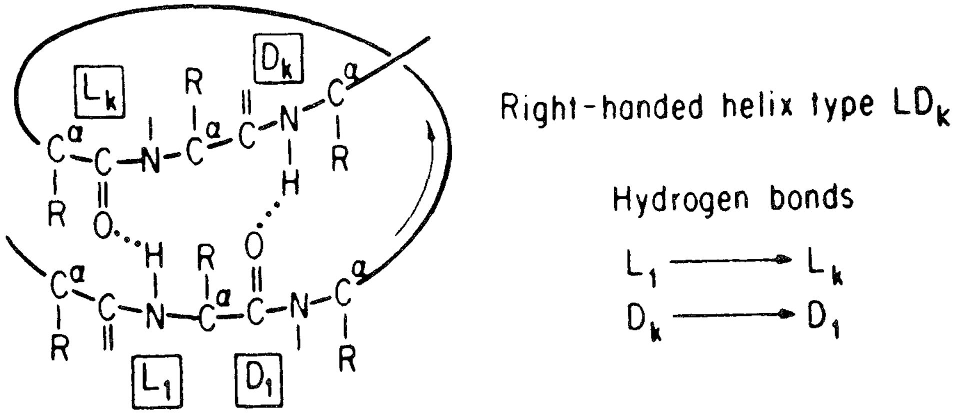

FIGURE 1. Schematic representation of hydrogen bonding pattern in right-handed single helices (LD_k) for poly-LD-peptides. The $L_1 \to L_k$ and $D_k \to D_1$ NH···O bond are shown by dotted lines. (Adapted from Ramachandran, G. N. and Chandrasekaran, R., *Ind. J. Biochem. Biophys.*, 9, 1, 1972. With permission.)

TABLE 1

Types of Hydrogen Bonds in Various Right-handed Single-helical Structures for Poly-LD-peptides

	H-bond type A		H-bond type B		Number of LD-units per turn (*n*)
Helix	Peptide units linked	*r*[a]	Peptide units linked	*r*[a]	
LD_2	$D_2 \to D_1$	10	$L_1 \to L_2$	8[b]	2.1
LD_3	$D_3 \to D_1$	16	$L_1 \to L_3$	14	2.3
LD_4	$D_4 \to D_1$	22	$L_1 \to L_4$	20	3.2
LD_5	$D_5 \to D_1$	28	$L_1 \to L_5$	26	4.1

[a] Number of atoms in ring formed by the hydrogen bond.
[b] This hydrogen bond is stereochemically not possible.

which have an integral value of *n* and for which the unit height *h* is zero. Because of symmetry considerations, cyclization is readily possible when the dihedral angles of the repeating LD-unit are (ϕ,ψ) for the L-residue and ($-\phi,-\psi$) for the D-residue. Subsequently, as the chain is wound up or down (i.e., $h \neq 0$), it gives rise to helical structures with systematic hydrogen bonds between the NH and CO groups, as depicted in Figure 1. The allowed conformations of the LD-unit characterized by (ϕ_L,ψ_L) and (ϕ_D,ψ_D) have been studied in the entire region of the four-dimensional conformational space at intervals of 10° in order to generate the desired LD_k-helices having $k = 3$, 4, and 5, values of n close to 2, 3, and 4, respectively, and also possessing good hydrogen bonds.

To build the poly(LD-Ala) chain, the standard dimensions of planar *trans* peptide unit and the Ala side group, with methyl hydrogens in the staggered positions, have been used. The bond angle $\tau(N-C^\alpha-C)$ has been generally retained at the standard value of 110° and varied only when necessary. The helical parameters have been evaluated using the matrix method described by Ramachandran and Sasisekharan.[9] For conformations with satisfactory hydrogen bonds, the total energy per repeating LD-unit has been calculated by taking into account the nonbonded and electrostatic interactions, hydrogen bonds, and torsional potentials.[9,13]

In view of the equivalence between the right- and left-handed LD-helices mentioned earlier, the conformational features of only the right-handed helices are described here. Of the three kinds of LD-helices stereochemically possible (viz., LD_3, LD_4, and LD_5 (Table 1), although LD_4 and LD_5, which have n around 3.15 and 4.10 and h about 1.55 and 1.2 Å, respectively, can be easily built with the standard value of 110° for τ), the LD_3-helix requires a considerable decrease in this bond angle to 106°. This is necessary in order to form satisfactory hydrogen bonds. This helix corresponds to values of n and h close to 2.3 and 2.4 Å, respectively.

c. Properties of LD-helices

The structural features of the low-energy conformations which are stabilized by the type A-type B NH···O hydrogen bonds are listed in Table 2. It may be noted that the two types of hydrogen bonds are not exactly equivalent but are, in general, of nearly the same energy.

An analysis of Table 2 shows that, as one goes from LD_3 to LD_5, the ranges of the dihedral angles which are generally in the β-regions of the corresponding L- and D-residues are significantly reduced. Beyond LD_5, the helices become wide and shallow ($h < 1$ Å) and are unstable due to severe steric repulsions[6] between atoms in neighboring turns.

Among these three helices, the LD_3-helix is more tightly wound about the helical axis, as the bond angle τ is only 106°, than others and has the lowest energy, −9.7 kcal/mol per residue (Table 2). This may be compared with −10.4 kcal/mol per residue for the right-handed α-helix of poly-L-alanine.[14] The LD_4-helix is slightly less stable (−9.0 kcal/mol per residue) than the LD_3-helix. However, the minimum energy of the LD_5-helix is rather high (−7.1 kcal/mol per residue), indicating substantial reduction in its relative stability.

A persistent feature in all these helices is the presence of a large central core (in contrast to its absence in the conventional all L- or all D-helices, namely the 3_{10}- and α-helices), whose size increases with n. The actual radii are approximately 0.6, 1.7, and 2.8 Å for LD_3-, LD_4-, and LD_5-helices, respectively. These and other characteristics of the LD-helices[6] are summarized in Table 3. A perspective diagram of the LD_4-helix is shown in Figure 2.

Ramachandran and Chandrasekaran[6] have discussed the ion-binding abilities of these helices in terms of the inner core sizes. The large size of the central hole as well as the inherent short contact between a carbonyl oxygen and an H^α atom in the neighboring turn have led to the elimination of the LD_5-helix as a possible ionophore. Similarly, despite its extreme stability and good stereochemistry, the LD_3-helix has also been ruled out for this purpose owing to the narrowness of its central core. This leaves only the LD_4-helix as a possible model for ionophoric LD-peptides. This aspect is discussed in a later section with respect to the structure and function of gramicidin A. Also, Urry[7] has independently proposed very similar single helices, called π_{LD}-helices, for the structure of the ion-conducting channel of gramicidin A.

2. LD-Ribbon Structure

This structure is one of the various types of the LD-helices (Table 1), designated as LD_2-helix. It has a peculiar feature in that the hydrogen bond with a ring of of eight atoms is stereochemically impossible in the peptide chain. Consequently the LD_2-structure with alternating L- and D-residues has only one systematic hydrogen bond for every two residues. The hydrogen bonding scheme produces a folded chain structure, called the LD-ribbon,[6] due to its ribbon-like appearance (Figure 3), and its inverse structure is called the DL-ribbon. The ribbon structure has approximately twofold symmetry. However, the best hydrogen bonds occur when the twist angle for an LD-unit is around 160° ($n \sim 2.2$). The hydrogen bonds are all between NH and CO groups of D-peptide units in the LD-ribbon structure, while all the hydrogen bonding groups belong to the L-peptide units in the DL-ribbon structure. Moreover, these hydrogen bonds are approximately in the same direction as the length of the ribbon. The peptide units not taking part in the hydrogen bonding scheme have their planes nearly at right angles to the plane of the ribbon, thereby making the free NH or CO groups alternately point up and down.

The LD-ribbon structure[6] is, in fact, formed essentially by the repetition of a favorable LD-bend, also known as the β-turn (or DL-bend for the DL-ribbon).[15] The energy values for the LD-ribbon of poly(LD-Ala) have been computed[6] following the method of Ramachandran and Sasisekharan,[9] as described earlier for LD-helices. Both right- and left-handed models for all the combinations of (ϕ_L,ψ_L) and (ϕ_D,ψ_D) in the

TABLE 2

Characteristics of the Low-Energy Conformations of LD_k-helices[a]

Helix	Conformational angles (°) ϕ_L	ψ_L	ϕ_D	ψ_D	Helical parameters n	h (Å)	Hydrogen bonds Type A R_1 (Å)	θ_1 (°)	Type B R_2 (Å)	θ_2 (°)	Energy (kcal/mol per LD + unit)
LD_3	−80	100	125	−85	2.28	2.41	2.87	19	3.04	15	−19.4
	−80	105	120	−85	2.30	2.43	2.92	20	3.03	12	−19.3
	−85	105	120	−80	2.29	2.41	2.86	16	2.97	13	−19.3
	−85	100	125	−80	2.29	2.39	2.81	15	2.99	16	−19.3
	−80	110	115	−85	2.31	2.44	2.95	20	3.03	15	−19.3
LD_4[b]	−106	122	140	−130	3.14	1.56	2.96	26	2.87	14	−17.9
	−106	120	142	−130	3.13	1.56	2.96	26	2.85	16	−17.8
	−104	122	140	−132	3.15	1.58	3.04	28	2.94	14	−17.8
	−104	120	142	−132	3.14	1.58	3.04	28	2.92	15	−17.7
	−104	118	144	−132	3.13	1.57	3.04	29	2.91	18	−17.6
LD_5[b]	−126	146	140	−144	4.11	1.19	3.12	26	3.07	23	−14.2
	−126	148	138	−144	4.12	1.18	3.05	25	3.09	26	−14.2
	−126	144	142	−144	4.09	1.20	3.19	27	3.05	19	−13.9
	−120	152	132	−148	4.10	1.18	3.13	31	3.04	28	−13.7
	−126	150	136	−144	4.14	1.17	2.98	23	3.11	29	−13.5

[a] R is the distance N···O (Å) and θ is the angle between NH and N···O.

[b] Conformational angles are varied at 2° intervals.

TABLE 3

Important Conformational Features of Novel LD$_k$-helices and Conventional α- and 3_{10}-Helices of Poly(LD-Ala)

Characteristics		LD$_3$	LD$_4$	LD$_5$	α_{DL}	3_{10}-$_{DL}$
Value of τ(N—C$^\alpha$—C) (°)		106	110	110	110	110
Ranges of dihedral angles (°) covering up to 1.2 kcal/mol above the minimum energy	ϕ_L	−100 to −80	−112 to −102	−128 to −120	−65 to −40	−70 to −50
	ψ_L	100 to 115	112 to 124	144 to 152	−65 to −40	−35 to −10
	ϕ_D	110 to 130	140 to 152	132 to 142	−65 to −40	−70 to −50
	ψ_D	−65 to −85	−124 to −134	−142 to −148	−65 to −40	−35 to −10
n		2.30	3.15	4.10	3.6	3.3
h (Å)		2.40	1.55	1.20	1.5	1.7
Lowest energy (kcal/mol per LD-unit)		−19.4	−17.9	−14.2	−17.0	−13.5
Maximum length for 15 residues (Å)		18.0	12.6	9.0	22.5	28.5
Radius of the inner core (Å)		0.6	1.7	2.8	0.0	0.0

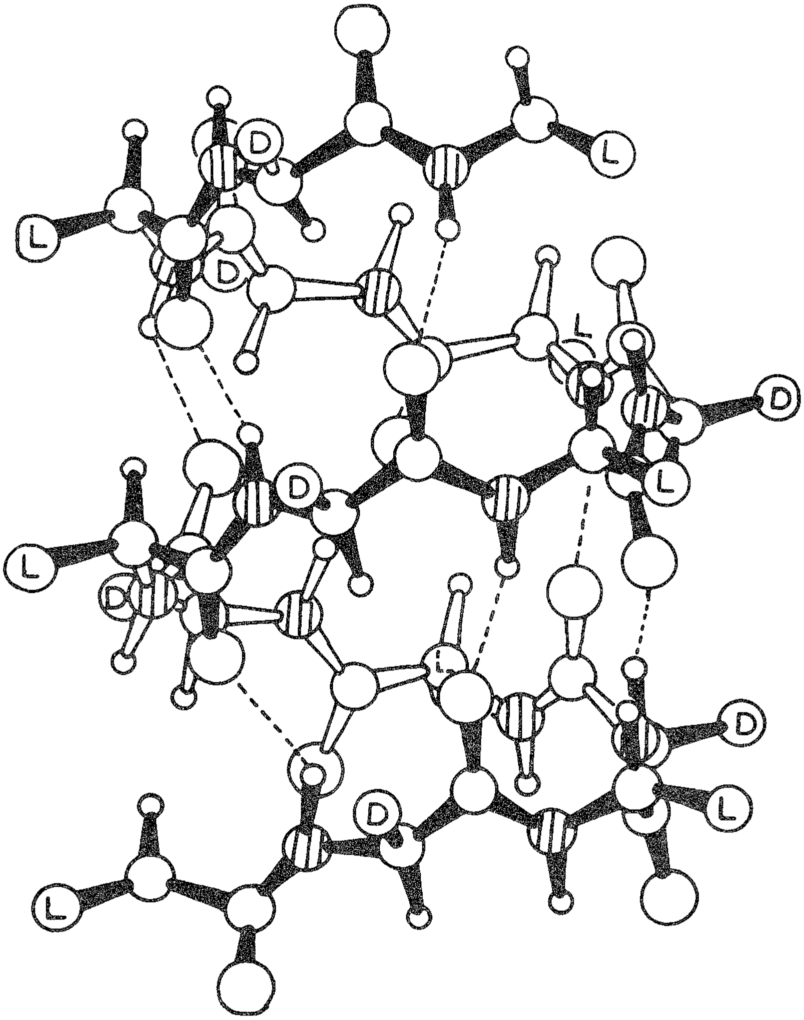

FIGURE 2. A perspective diagram of the LD$_4$-helix. Small circles indicate hydrogen, medium circles carbon, and large circles oxygen atoms; striated circles indicate nitrogen atoms. NH···O hydrogen bonds are shown by dashed lines. The symbols L and D denote the configurations at the α-carbon atoms.

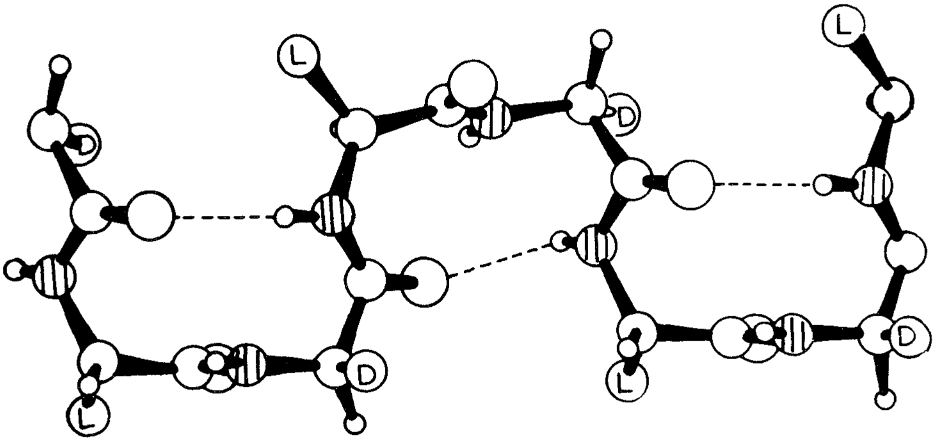

FIGURE 3. A perspective diagram of the LD-ribbon structure. Atoms are represented as in Figure 2.

allowed conformational space have been considered in these calculations.

a. Properties of the Ribbon Structure

The structural features of the low-energy conformations of the LD-ribbon[6] are summarized in Table 4. The number of LD-units per turn is always greater than two, indicating that the structure is not completely extended, but is slightly twisted. The axial rise per LD-unit is approximately 4.3 Å. As shown in Table 4, the major conformational difference between the right- and the left-handed structures is associated with $\Delta\phi = 70°$ and $\Delta\psi = -75°$ in the orientation of the D-peptide unit, which runs across the length of the ribbon, whereas the orientation of the L-peptide unit remains practically the same. Of the two conformers, the right-handed structure has a lower minimum energy of −11.0 kcal/mol per LD-unit (or −5.5 kcal/mol per residue), almost 0.8 kcal/mol per LD-unit below the other. For an isolated single chain of poly-LD-peptide, the ribbon structure may not be a very favorable conformation, as its minimum energy is significantly higher than either the α-helix or any one of the LD-helices mentioned before. On the other hand, Ramachandran and Chandrasekaran[6] have argued that since one set of NH and CO groups for every two residues is freely exposed and not involved in intramolecular hydrogen bonds, this loss in energy may be readily compensated by complementary association between individual chains, particularly in the solid state. The resulting structure would then be analogous to the molecular packing of the classical β-structure with all L-residues.[9] The regularly stacked LD-ribbons in polar solvents would, however, be disrupted by the solvent-solute interactions unless large hydrophobic L- and D-side groups shield the intermolecular NH···O hydrogen bonds.

b. Relative Stabilities of the Classical Helices, LD-helices, and the Ribbon Structure

The detailed conformational analyses[5,6] of the α-helix, LD-helices, and the ribbon structure for poly(LD-Ala), modeled for poly-LD-peptides, converge to almost similar inferences on their relative stabilities. This agreement is remarkable in view of the differences in the potential functions and in the methods employed. Hesselink and Scheraga[5] have suggested that the α-helix is by far the most stable structure for the isolated molecules of poly(LD-Ala). Also, both the analyses have yielded the minimum energy for the LD- or DL-ribbon to be approximately −5.5 kcal/mol per residue, thereby showing that, in isolation, this structure is definitely less favorable. This does not imply that the ribbon structure is excluded for the polymer. Instead, as mentioned earlier, this model may derive additional stability by the stacking of the ribbons connected via intermolecular NH···O hydrogen bonds.

Hesselink and Scheraga[5] have computed the energy of the LD_4-helix alone, and not of the other two members (LD_3 and LD_5) in the LD-he-

TABLE 4

Characteristics of the Low-Energy Conformations of the LD-ribbon Structure of Poly (LD-Ala)

	Connormational angles(°)				Helical parameters		Hydrogen bond $D_2 \rightarrow D_1$		
Ribbon	ϕ_L	ψ_L	ϕ_D	ψ_D	n	h (Å)	R (Å)	θ (°)	Energy (kcal/mol per LD-unit)
Right-handed	—60	100	65	45	2.17	4.24	3.00	27	-11.0
	—60	95	70	45	2.17	4.23	2.97	27	-11.0
	—55	100	60	45	2.22	4.20	2.89	25	-11.0
	—55	105	55	45	2.22	4.22	2.92	25	-11.0
	—60	105	60	45	2.17	4.26	3.03	27	-10.8
Left-handed	—65	100	135	—35	-2.13	4.28	2.94	26	-10.2
	—60	110	125	—40	-2.10	4.32	2.94	28	-10.2
	—60	110	125	—35	-2.14	4.37	2.90	23	-10.1
	—60	100	140	—40	-2.16	4.37	2.91	24	-10.0
	—70	100	135	—30	-2.13	4.29	3.04	23	-9.9

lix family. Ramachandran and Chandrasekaran,[6] who have analyzed the features of all of the helices, have shown that the LD-helix is, in general, energetically more stable than the LD-ribbon but is definitely less stable than the α-helix. This deduction is in complete agreement with the results of Hesselink and Scheraga.[5] The stabilities decrease from LD$_3$ through LD$_5$ in that order, according to the results of Ramachandran and Chandrasekaran[6] which were derived from the energies of the molecules in their isolated states (Table 2); however, it is not wise to interpret this observation further to determine the most favorable model for the polymers. It is quite possible that once a particular helix is nucleated, due to environmental conditions (such as solvent, temperature, etc.) the helix thus nucleated would thereafter continue to grow. In this way, helices other than those corresponding to minimum energy can be expected to be observed. Such experimental observations have been reported by Spach and associates[16,17] in recent years on the synthetic polymer PBLDG. These will be described in Section III.

3. Double Helices

Recently, from solution studies on gramicidin A, Blout and colleagues[12] first proposed novel double helices as possible structures for this ionophore. Subsequently, the results of X-ray and electron diffraction analyses[17] of PBLDG have indicated that this polymer can also exist in one or more double helical forms. Previously, double helices were a unique feature only for polynucleotides[18] and polysaccharides.[19] Venkataram Prasad and Chandrasekaran[20] have discussed in great detail the conformational characteristics of different possible types of double helices for poly-LD-peptides. A brief outline of the conformational analysis of the double helix as given by these authors[20] follows.

The double helix is formed essentially by the association of two conformationally identical poly-LD-peptide strands wound about a common helical axis. The two chains interact through NH···O hydrogen bonds between the NH and CO groups, similar to the situation in conventional polypeptide structures.[9] As in the single-chain LD-helix, succesive NH (or CO) groups are pointed in opposite directions relative to the helical axis. There are two possible ways of intertwining the two strands coaxially to form the double helix. In one case, the strands have opposite polarities and are related by a twofold symmetry perpendicular to the common helical axis, as observed in the famous Watson-Crick DNA double helix.[18] In the other, the polarities of the strands are the same. The two resulting models are generally referred to as the antiparallel and parallel double helices. In the former model, the hydrogen bonds are systematically formed between L-residues of

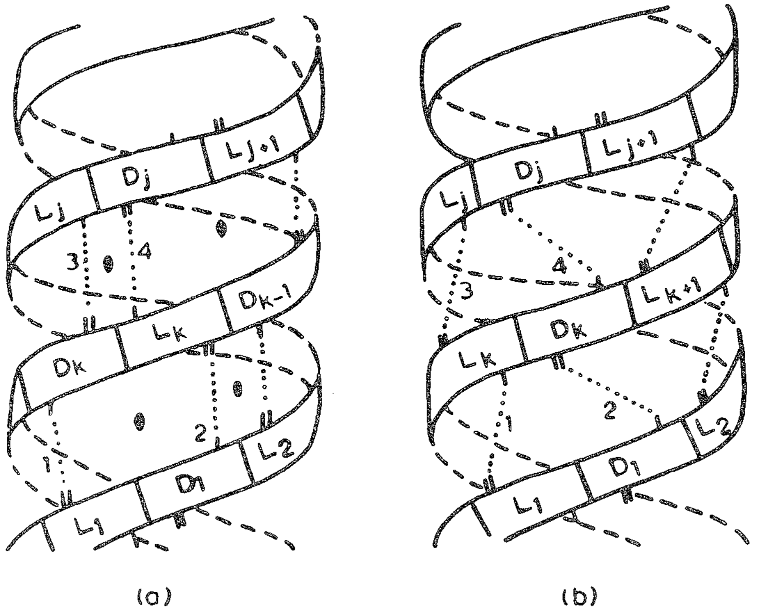

FIGURE 4. Hydrogen-bonding pattern in double helices of poly-LD-peptides. The interchain NH···O bonds are shown by dotted lines. (a) The antiparallel double helices A_3, A_4, A_5, and A_6 correspond to j = 3, 4, 5, and 6, respectively, and the hydrogen bonds are formed between L and D peptide units. Because of dyad symmetry, the hydrogen bonds marked 1 and 2, as well as 3 and 4, are equal. (b) In the parallel double helices P_3 and P_4, having j = 3 and 4, respectively, the interchain hydrogen bonds link peptide units L to L and D to D. (From Venkataram Prasad, B. V. and Chandrasekaran, R., *Int. J. Peptide Protein Res.*, 10, 129, 1977. With permission.)

one strand and D-residues of the other and vice versa. In the case of the parallel model, these bonds are only between like residues (L to L and D to D). The very nature of this pattern is reminiscent of the β-sheet structures of poly-L-peptides. In fact, the new double helices can be alternatively conceived of as being generated by wrapping two strands of a β-structure around a cylinder so that its surface is lined with NH···O=C groups. This scheme imposes a constraint on the pitch of the helix to vary within a narrow range of about 10 to 12 Å.

In Figure 4 are shown the hydrogen bonding patterns that occur in the antiparallel and parallel double helices. Hydrogen-bonded duplexes are possible in each kind only for four different values of j (namely 3, 4, 5, and 6) for the repeating unit L_jD_j in this diagram. Accordingly, the antiparallel models are designated as A_3, A_4, A_5, and A_6 and the corresponding parallel models as P_3, P_4, P_5, and P_6. This is done in conformity with the notation employed for LD-helices.[6] While the repeating unit L_kD_k in the second strand is related to L_1D_1 in the first strand by a local dyad for the antiparallel model (Figure 4a), such a symmetry is absent for the parallel model (Figure 4b). In both models, L_kD_k of the second strand is hydrogen bonded to L_1D_1, as well as L_jD_j, of the first strand. As the pitch remains almost the same in all of them, the diameter of the double helix increases proportionately with the value of j. Consequently, a large empty core is an essential and common aspect in all these double helices, as was also reported for LD-helices.[6]

a. Conformational Analysis of the Double Helix

The procedure employed for building the double helix is, due to the presence of two strands, slightly more complicated than for the single-stranded LD-helices. Therefore, the basic principles underlying the generation of the double helix will be briefly mentioned.[20]

The input parameters, such as the dimensions of the peptide unit, are essentially the same as those in the LD-helix calculations previously described. The coordinates (r,θ,z) of the repeating unit L_1D_1 of the first chain for given (ϕ_L,ψ_L) and (ϕ_D,ψ_D), which are nearly in the β-regions of the respective L- and D-residues, are computed in the helical system in which the x-axis passes through the first α-carbon atom, using the general matrix method,[9] and the helical parameters n and h are evaluated. If these are within the range amenable for the formation of all the four interchain hydrogen bonds, marked 1, 2, 3, and 4 in Figure 4, the coordinates of additional peptide units spanning more than a turn of the helix are computed. The double helix is then generated by appropriately interspersing the second-strand coaxial with the first, using a rotation $(\Delta\theta)$ about, and a translation (Δz) along, the helical axis. Consequently, if (r,θ,z) are the coordinates of L_1D_1 in the helical system, the corresponding coordinates of L_kD_k will be $(r, -\theta+\Delta\theta, -z+\Delta z)$ for the antiparallel chain (Figure 4a) and $(r, \theta+\Delta\theta, z+\Delta z)$ for the parallel chain (Figure 4b).

For the double helix having satisfactory hydrogen bonds, 12 peptide units in each strand with Ala side group (methyl hydrogens in staggered positions) at successive α-carbon atoms, alternately in L- and D-configurations, have been considered[20] to compute the total energy. The method is very similar to that employed for the LD-helices previously described. Contributions from all those interactions arising from the repeating unit L_1D_1 within itself and with the rest of the double helix have been summed up to determine the total energy per LD-unit of the double helix.

For the four different types of double helices, either parallel or antiparallel, which correspond to j = 3, 4, 5, and 6 as already described, the number of repeating LD-units (n) per turn is approximately 2.5, 3.5, 4.5, and 5.5, respectively. In the computational analysis,[20] the entire allowed conformational space has been searched by varying the four conformational angles (ϕ_L,ψ_L) and (ϕ_D,ψ_D) generally at intervals of 5° and, in some cases, at intervals of 2°. Moreover, the ranges of n have been centered around the above values with suitable deviations on either side so that none of the stereochemically acceptable models is left unnoticed. The combinations of (ϕ_L,ψ_L) and (ϕ_D,ψ_D) which produce right-handed helices alone have been analyzed in detail, as their properties are implicitly related to the corresponding left-handed helices obtained by mirror symmetry.

b. Properties of Double Helices

Venkataram Prasad and Chandrasekaran[20] have demonstrated that stereochemically satisfactory models of all of the four types of antiparallel double helices (A_3, A_4, A_5, and A_6) can be generated. On the other hand, while they find that P_3 and P_4 are possible, P_5 and P_6 of the parallel double helix family are impossible. The difficulty is mainly due to severe short contacts between the two strands in the latter.

The relevant conformational features giving the ranges of dihedral angles, helical parameters, etc. for the six allowed models of double helices are summarized in Table 5; the minimum energy values as well as the inner core radii are also listed.

An important characteristic, inferred from the values of the inner core radii, is that all double helices, like single helices (LD-helices), possess large central empty cores. Their sizes, by virtue of the hydrogen-bonding pattern, increase from A_3 to A_6 (or from P_3 to P_6) in that order from 0.9 to 4.4 A. The core sizes of A_3 and A_4 (or P_3 and P_4) and A_5 are comparable to those of the single helices LD_3, LD_4, and LD_5, respectively. Moreover, an examination of the positions of the C^α and C^β atoms indicate that the $C^\alpha-C^\beta$ bonds stick out almost perpendicular to the helical axis, as in the LD-helices. Perspective views of the double helices A_4 and P_4 are shown in Figure 5.

The detailed structural characteristics of the low-energy conformations of the antiparallel and parallel double helices of poly(LD-Ala) are listed in Tables 6 and 7, respectively. In the antiparallel models, the minimum energies are −29 kcal/mol per LD-unit for A_3 and approximately −24 kcal/mol per LD-unit for A_4, A_5, and A_6, which is almost 5 kcal/mol per LD-unit higher than for the former model. Therefore, in isolation, A_3 appears to be energetically more stable than others. However, within this family, the low energies are distributed from −21 to −29 kcal/mol per LD-unit. In all the allowed conformations, the hydrogen bonds are satisfactory, but are always nonlinear.

Another important observation is a strong correlation between ϕ_L and ψ_D and the relative

TABLE 5

Summary of the Relevant Features of the Double Helices of Poly(LD-Ala)

Characteristics		A_3	A_4	A_5	A_6	P_3	P_4
Allowed regions of conformational angles (°)	ϕ_L	−145 to −115	−160 to −125	−145 to −130	−165 to −155	−130 to −115	−150 to −140
	ψ_L	100 to 180	100 to 180	130 to 180	140 to 180	120 to 140	140 to 150
	ϕ_D	100 to 180	100 to 180	130 to 180	150 to 180	160 to 180	145 to 160
	ψ_D	−75 to −110	−95 to −125	−130 to −145	−130 to −180	−85 to −105	−110 to −100
Lowest energy (kcal/mol per LD-unit)		−28.8	−23.7	−24.0	−24.4	−23.3	−20.9
Range of n (pitch: 10.0 to 11.5 Å)		2.6 to 2.9	3.35 to 3.65	4.35 to 4.65	5.40 to 5.60	2.60 to 2.90	3.35 to 3.65
Maximum length (Å) for 15 residues (in each chain)		31.5	24.0	18.0	13.5	31.5	21.8
Radius of the inner core (Å)		0.9	2.0	3.3	4.4	0.9	2.0

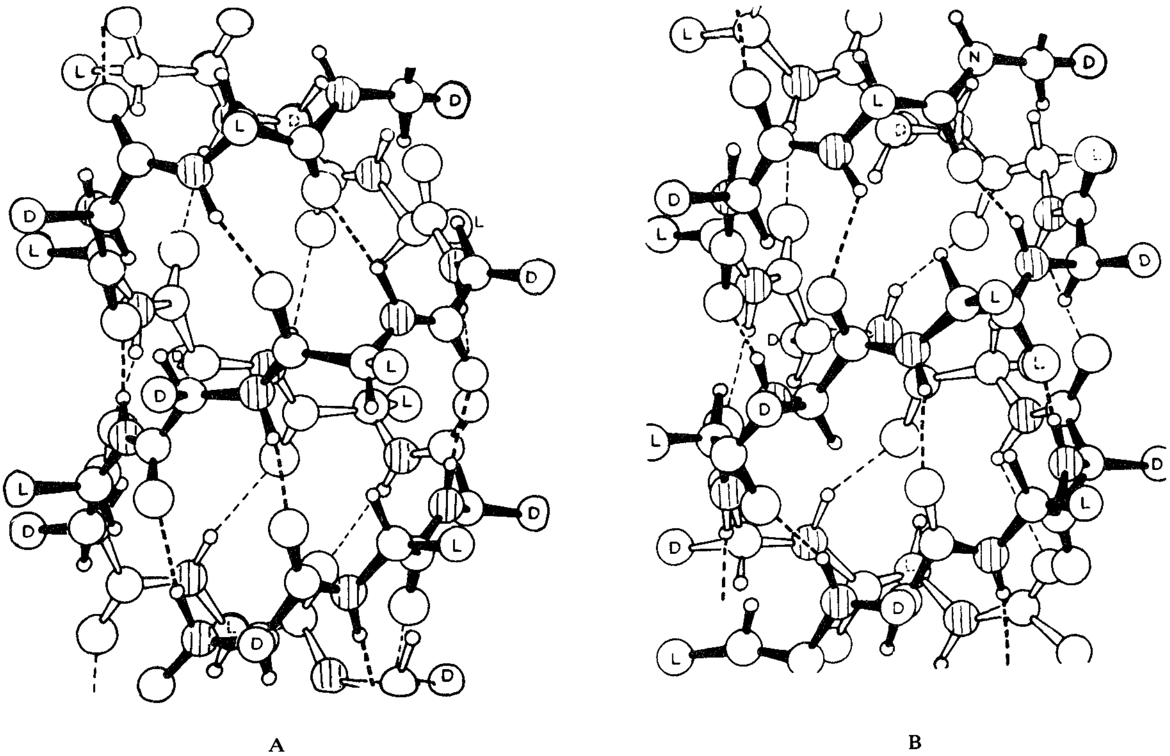

FIGURE 5. Perspective diagram of the double helix of poly-LD-peptides. (A) Antiparallel model A_4; (B) parallel model P_4. The interchain hydrogen bonds are marked by dashed lines. The symbols L and D denote the configurations at the α-carbon atoms. The atoms are represented as in Figure 2. (From Venkataram Prasad, B. V. and Chandrasekaran, R. *Int. J. Peptide Protein Res.*, 10, 129, 1977. With permission.)

population of conformers in each type of antiparallel double helix.[20] The general trend is such that as ϕ_L decreases, the corresponding ψ_D increases in magnitude. Consequently, the greater the difference in magnitude between the two, the larger the number of sterically allowed conformations. A_3 and A_4 follow this relationship and record more of conformations in the allowed conformational space (Table 6) than the other two types (A_5 and A_6), which show a smaller difference in the ϕ_L- and ψ_D-values, thereby resulting in less conformations.

In the case of the parallel double helices, the minimum energies of the two permissible models, P_3 and P_4 (analogous to A_3 and A_4), are seen from Table 7 to be −23 and −20 kcal/mol per LD-unit, respectively. The actual low energies vary from −19 to −23 kcal/mol per LD-unit. The number of allowed conformations is much smaller than for the corresponding antiparallel analogues. This is due mainly to the difficulty encountered in making all the hydrogen bonds equally satisfactory. The distribution of conformational angles is again very similar to the behavior of the antiparallel models.

Two important aspects of the hydrogen bonding pattern, present only in the parallel double helices are worth reporting. The first pertains to the existence of short contacts in the region 2.0 to 2.2 Å between an H^α atom in one strand and a carbonyl oxygen atom just above it in the other strand. The orientations of the relevant C^α—H^α bonds and the carbonyl groups are suggestive[20] of weak CH···O hydrogen bonds similar to observations in the structures of polyglycine II[21] and collagen.[22] The second aspect refers to the fact that the backbone NH···O hydrogen bonds are at an angle to the axis of the helix, which is considerably more pronounced than in the antiparallel family. This can be easily visualized from Figure 5.

The implication of these double helical structures to the polymorphism reported for PBLDG[16,17] and the studies on gramicidin A[12] will be discussed in the following sections.

TABLE 6

Characteristics of the Low-Energy Conformations of the Antiparallel (A_k) Double Helices of Poly(LD-Ala)

Double helix	Conformational angles(°)				Helical parameters		Parameters for[a] positioning the second strand		Hydrogen bond parameters		Energy (kcal/mol per LD-unit)
	ϕ_L	ψ_L	ϕ_D	ψ_D	n	h (Å)	$\Delta\theta$ (°)	Δz (Å)	N···O (Å)	NH∧NO (°)	
A_3	−145	180	115	−75	2.93	3.53	101.6	8.36	2.94	28.6	−28.9
	−120	165	135	−85	2.63	4.25	94.7	8.86	2.84	21.5	−26.6
	−135	165	135	−85	2.88	3.89	91.8	8.69	2.93	20.5	−26.4
	−120	170	130	−90	2.73	4.13	89.6	8.74	2.81	18.3	−25.8
	−130	155	145	−90	2.86	3.93	87.8	8.68	2.89	20.3	−25.2
A_4	−145	165	135	−105	3.59	2.92	76.0	8.12	3.00	9.1	−23.3
	−145	170	130	−105	3.62	2.89	72.7	8.04	3.02	8.7	−23.1
	−160	145	155	−95	3.58	2.75	85.5	7.99	3.07	29.5	−23.0
	−140	160	140	−110	3.58	2.97	73.5	8.16	2.99	7.4	−22.8
	−135	140	160	−120	3.64	2.85	67.7	7.92	2.90	16.2	−22.7
A_5	−140	150	155	−135	4.43	2.28	54.2	7.40	2.90	12.0	−24.1
	−140	160	145	−135	4.51	2.23	54.5	7.42	2.91	11.2	−23.8
	−140	145	160	−135	4.38	2.28	58.5	7.50	2.86	10.6	−23.6
	−135	140	165	−140	4.37	2.33	58.4	7.68	2.96	12.5	−22.3
	−135	165	140	−140	4.58	2.25	50.9	7.52	3.07	18.4	−21.5
A_6[b]	−160	155	160	−135	5.52	1.79	47.1	7.23	3.04	15.4	−24.4
	−159	157	157	−134	5.41	1.82	53.1	7.36	2.96	8.8	−24.3
	−163	149	167	−134	5.57	1.75	45.9	7.19	3.08	22.4	−23.6
	−157	155	159	−136	5.40	1.85	54.5	7.49	3.03	9.8	−22.3
	−161	149	167	−136	5.59	1.76	45.2	7.27	3.14	21.5	−22.1

[a] The coordinates of the repeating unit L_kD_k in Figure 4a of the second strand are given by $(r, -\theta+\Delta\theta, -z+\Delta z)$, where (r,θ,z) are the cylindrical polar coordinates of the repeating unit L_1D_1 of the first strand.

[b] Conformational angles varied at 2° interval.

TABLE 7

Characteristics of the Low-Energy Conformations of the Parallel (P_k) Double Helices of Poly(LD-Ala)

	Conformational angles (°)				Helical parameters		Parameters for[a] positioning the second strand		Hydrogen bond parameters				Energy (kcal/mol per LD-unit)
									1		2		
Double helix	ϕ_L	ψ_L	ϕ_D	ψ_D	n	h (Å)	$\Delta\theta$ (°)	Δz (Å)	N···O (Å)	NH NO (°)	N···O (Å)	NH∧NO (°)	
P_3	−120	140	160	−100	2.82	3.97	−53.0	3.94	2.95	14.5	2.83	17.0	−23.3
	−120	135	170	−100	2.78	4.14	−50.6	4.13	3.16	22.2	2.98	19.4	−21.6
	−115	125	175	−105	2.78	3.91	−50.5	3.89	2.81	17.9	3.08	29.1	−21.2
	−130	130	170	−85	2.68	3.99	−46.0	3.97	3.00	30.6	2.81	27.0	−21.0
	−125	135	170	−100	2.88	4.01	−54.7	4.01	3.10	15.2	3.15	22.5	−20.7
P_4[b]	−145	144	155	−106	3.51	2.89	−26.5	4.32	3.10	31.5	2.84	19.5	−20.9
	−147	148	151	−104	3.52	2.88	−27.2	4.30	3.10	32.7	2.83	20.5	−20.4
	−145	152	147	−106	3.55	2.88	−27.5	4.33	3.10	30.3	2.87	23.0	−20.1
	−147	144	157	−106	3.56	2.92	−28.3	4.38	3.18	30.5	3.00	21.7	−19.8
	−145	146	155	−108	3.58	2.93	−28.9	4.40	3.18	28.0	3.02	21.5	−19.7

[a] The coordinates of the repeating unit L_kD_k (Figure 4b) in the second strand are given by $(r, \theta+\Delta\theta, z+\Delta z)$, where (r,θ,z) are the cylindrical polar coordinates of the repeating unit L_1D_1 of the first strand.

[b] Calculations are done at 2° interval.

III. EXPERIMENTAL STUDIES ON POLY(γ-BENZYL-LD- GLUTAMATE)

Several properties of polypeptides with alternating L- and D-amino acids have been determined in detail from experiments both in solution and in the solid state, mostly on the synthetic polymer, poly(γ-benzyl-LD-glutamate). Among the experimental methods, ORD, CD, IR, and NMR spectroscopy have liberally contributed, in conjunction with theoretical studies, to the elucidation of the variety of structures exhibited by this polymer in different solvents. In addition, X-ray and electron diffraction analyses of films or fibers in the solid state have confirmed many of the above results. These studies will be described in this section.

As our concern will be mainly with the structural features of PBDLG, we will restrict ourselves to the relevant information provided by the solution and solid state studies. Therefore, certain aspects, such as the chemical synthesis of any of the polymer systems, will be omitted. For such details, the reader may refer to the original papers.

Although many of the structural characteristics worked out from conformational analysis are based on a strictly alternating L- and D-primary sequence, solution studies clearly indicate the occurrence of helical structures even for copolymers in which the repetition of the LD-sequence is violated. Therefore, these examples will be discussed as the need arises.

Before considering the actual details, a word of caution about the nomenclature of the alternating polymer of γ-benzyl glutamate is in order. The LD- and DL-polymers are quite different in that the N-terminal position in these molecules corresponds to an L- and a D-residue, respectively. Hence, their helical senses, in anticipation of the results to be described shortly, are not the same in many instances. Since, for an infinite chain length, both right-handed and the corresponding mirror image, left-handed helical forms are isoenergetic (from conformational energy calculations) we will primarily concentrate on the characteristics of the helical structures and will somewhat disregard the sense of the helix arising from the difference between the LD- and DL-polymers which are synthesized.

A. Preliminary Studies on Copolypeptides with Randomly Distributed L- and D-Residues

One of the crucial problems in all these studies is, of course, the chemical synthesis[23] of the polymer in which the L- and D-amino acids are incorporated in a strictly alternating fashion. It was not until 1974 that a racemization-free synthesis[24] of the alternating LD-polymer of γ-benzyl glutamate could be achieved with the use of 2-hydroxyphenyl esters. Prior to this, considerable interest also existed in understanding the properties of polypeptides containing both L- and D-amino acids in different proportions. For example, as recently as 20 years ago, Downie et al.[25] studied the variation of the molar rotation $[m']_D$ with the ratio L/(L + D) in different solvents and concluded that PB(D,L)G may exist in a helical conformation. They also showed the helical sense to be right-handed for the polymer molecules with less than 30% of D-residues. Doty and Lundberg[26] reached the same conclusion from their calculations on the rotatory dispersion of a right-handed α-helix of PB(D,L)G.

Hydrodynamic studies[27,28] of PB(D,L)G in *N,N*-dimethylformamide (DMF) revealed that the polymer could exhibit two forms, A and B (referred to as DL-A and DL-B), of identical molecular weight which are characterized by different intrinsic viscosities, as well as primary structures. The two forms have been obtained by the polymerization of the N-carboxyanhydride using aprotic initiators — form A in benzene and form B in dioxane or DMF. Form A has been found to be flexible in DMF and, in fact, has some helical structure in chloroform as shown by a transition in viscosity in mixed solvents.[28] On the other hand, form B is like a rigid rod in DMF with axial ratio proportional to mass and its behavior is very similar to the hydrodynamic behavior of PBLG.[27,28] From stability measurements, it has been established that form B is not a mixture of PBLG and PBDG, which would otherwise form a trival explanation for these observations. These results have been confirmed by dielectric absorption studies and Kerr-effect measurements in ethylene dichloride solution.[29]

Detailed X-ray studies on the molecular conformations of PB(D,L)G have been conducted by Tsuboi et al.[30] They demonstrated that a fiber diagram of 50-50% PB(D,L)G with an average degree of polymerization (DP) of 192 contains most of the strong reflections, but not some of the weak off-meridional reflections, characteristic of PBDG fibers. They also observed better crystallinity with higher molecular weight samples. Hence, they argue that in the PB(D,L)G fiber there are many fairly large crystallites, in addition to a less crystalline portion. Incidentally, this observation is similar to that reported later for the α-helical form of PBLG.[31]

By monitoring the polarized infrared spectra of a wide variety of PB(D,L)G samples, Tsuboi et al.[30] have confirmed that the samples are not just mixtures of PBLG and PBDG. Variation of the L/D ratio from 1:0 (i.e., PBLG) to 0.5:0.5 (i.e., PB(D,L)G with equal proportion of L- and D-residues) in the copolymer has resulted mainly in two distinct types of spectral changes, A and B. Those of type A, which are concerned with relatively weak bands of unknown origin, occur soon after the fraction of D-amino acids is increased from zero; the other type, dealing with the amide I, II, III, and V bands characteristic of α-helix, occur when the fraction of D-monomers approaches 50%. Thus, based on the correlations between amide band frequency and conformation, the type B spectral changes have been associated with an increase in the disordered form or, conversely, a decrease in the α-helical form. Similarly, the spectral changes of type A have been interpreted as a possible conversion of a "regular" α-helix corresponding to that of pure PBLG to a "perturbed" or "distorted" helix of right-handedness as the D-residues are incorporated.

It has, therefore, been suggested,[30] by combining the X-ray and infrared results, that PB(D,L)G film consists of three parts, namely, a regular α-helical region, a perturbed helical region, and a disordered region. From an estimation of the fraction of amino acids in each fragment by measuring the infrared absorption intensities of certain bands undergoing spectral changes of type A (say 563 cm^{-1}) or type B (say 613 cm^{-1}), these authors[30] have reported that the regular α-helical content decreases rapidly with the increase of D-residues.

In order to rationalize these observations, Tsuboi et al.[30] have proposed a model for the mechanism of polymerization, which is a modification of that of Wada[32] from dielectric dispersion studies of PB(D,L)G in ethylene dichloride. At the beginning of polymerization, the composition of the growing chain in D- and L-residues is random and, hence, there is no helical conformation. However, as soon as a certain number of residues (not less than four) of the same kind are incorporated in a sequence, a seed helix is formed whose handedness will be right for L and left for D. Once the seed helix is initiated, during the final stages of copolymerization, it continues to grow by the predominant incorporation of one type of monomer units over the other.

With this background, we shall now return to the structural investigations on PBDLG for which steric interactions were originally considered to be less favorable for any conventional helical structure.[23] We shall describe, in what follows, the various experimental results available on regular structures proposed for this polymer.

B. α_{DL}-Helix

1. ORD and CD Studies

Heitz and Spach[23] have conducted ORD and CD studies on PBDLG prepared using the active ester method. However, racemisation during polycondensation could not be prevented, which resulted in polymers with about 53% D-residues and 47% L-residues. Hence, it has been interpreted that the primary structure is essentially the repetition of a DL-sequence, but with some randomly distributed D-D defects. The optical rotation $[\alpha]_D$ obtained for PBDLG samples is high and negative in DMF and in helicogenic solvents such as chloroform, dioxane, and *m*-cresol. On the other hand, the A and B forms of random PB(D,L)G give values of $[\alpha]_D$ near zero.

From a comparison of the calculated value of $[m']_D$ with that obtained by linear extrapolation by Downie et al.[25] in DMF and *m*-cresol, Heitz and Spach[23] have suggested the presence of a high proportion of the left-handed helical conformation in PB(D,L)G. This has been further substantiated by a fairly good agreement between the observed rotatory dispersion and the calculated curve[26] for a left-handed helix of PB(D,L)G. The Moffitt parameters a_0 and b_0 are −445 and 427 for the observed curve and −680 and 500 for the calculated.

Taking a value of b_o equal to zero as indicative of the absence of any helical structure and a value of +600 for a fully left-handed α-heli-

TABLE 8

Optical Rotatory Properties of PBDLG and PBLDG Samples in Different Solvents

	Dioxane		CHCl$_3$		DMF		Pyridine		Benzene	
Sample	$[\alpha]_{546}^{25}$	b$_0$	$[\alpha]_{546}^{25}$	b$_0$	$[\alpha]_{546}^{25}$	b$_0$	$[\alpha]_{546}^{25}$	b$_0$	$[\alpha]_{546}^{25}$	b$_0$
DL$_{cat}$ I	+19.0	−160	+24.2	−125	+10.0	−90				
LD$_{cat}$ II	−15.8	+80	−25.0	+135	−13.1	+105	−31.2	+140	−27.4	+175
LD$_{cat}$ III	−15.4	+130	−24.8	+180	−9.4	+100			−24.1	+130
DL$_{cat}$ IV			+31.0							
DL-7	−39.0	+400								

Adapted with permission from Heitz, F. and Spach, G., *Macromolecules*, 8, 740, 1975. Copyright by the American Chemical Society.

cal structure, and assuming the absence of any other ordered structure, a value of +300 would indicate the presence of a mixture of 50% left-handed helices and 50% random coil, or, in the case of a mixture of fully helical molecules, 75% left-handed and the rest right-handed. Therefore, in the specimen studied by Heitz and Spach,[23] the PB(D,L)G polymer is at least partially helical and mostly left-handed.

Such a suggestion is also supported by the CD spectrum of a solution in chloroform.[23] The presence of left-handed helices is indicated by a positive maximum at 225 nm. Also, in hexafluoro-2-propanol, between 195 and 250 nm, the CD spectrum is in agreement with a left-handed helical structure ($\Delta\varepsilon = +4$ at 222 and 209 nm).

It should, however, be noted that during the synthesis of the alternating polymers cited above, some racemisation could occur and produce a sequence of two or more successive residues in the same configuration. This in turn would leave some uncertainty as to whether the above results are a true reflection of perfectly alternating DL-sequences in the polymer.

To resolve this ambiguity, Caille et al.[24] have carried out ORD and CD experiments on pure samples of PBDLG prepared through a racemisation-free technique. Three of the specimens, denoted by DL$_{cat}$ I, LD$_{cat}$ II, and LD$_{cat}$ III, which differ mainly in their viscosities, have been studied in detail. It is to be noted that whereas the first sample contains D-residue at the N-terminus, the other two have L-monomers at this end.

The optical rotatory properties[33] of the above polymers in different solvents are listed in Table 8. CD measurements[33] in chloroform and trimethyl phosphate reveal an extremum at 222 nm, in agreement with an α-helical conformation.[34] Also, the optical rotation $[\alpha]_D$ and the b$_0$ values for these samples (Table 8) suggest that the optical activity is a genuine property of the polymer. This is more so as the optical rotation of DL$_{cat}$ I, and its mirror images, LD$_{cat}$ II and LD$_{cat}$ III, are opposite in sign and of nearly the same order. The actual magnitudes would in turn indicate that DL$_{cat}$ I is at least partially right-handed and LD$_{cat}$ II or LD$_{cat}$ III is left-handed.

Although these results unequivocally confirm the existence of helical structures for the various samples, the deduction of the helical sense is not straightforward. The original argument of Heitz and Spach[23] that the helical sense and hence the ORD curves could arise from an excess of one helical sense due to an excess of one of the enantiomers would no longer be valid,[33] since a racemisation-free technique[24] has been employed in the synthesis of these alternating polymers containing exactly equal amounts of D- and L-monomers. Instead, Heitz and Spach[33] have proposed that the favored helical sense could be explained as resulting from side chain-side chain interactions occurring in an α-helical poly(DL-peptide). For example, Hesselink and Scheraga[5] have shown from conformational analysis that the interactions between the side chains of poly(LD-Val) or poly(LD-Ala) are always attractive and stronger than the same interactions in the corresponding α-helical structures of poly(L-peptides). Therefore, for poly(DL-peptide) with 2n residues, the N-terminal residue of which has a D-configuration, the right-handed helical sense should be favored because there are 2n-2 interacting side chains as compared with 2n-4 in the other screw sense. In these interactions, the nearest neighbors involved are the C^{β} atoms of the nth L-residue and the (n-3)th D-residue in the right-handed helix or the (n + 3)th D-residue in the other helical sense.

a. Infrared Spectra

Heitz et al.[16] have obtained infrared spectra for films of PBDLG synthesized by the racemisation-free technique. The spectra show characteristic bands at 3290 cm^{-1} (amide A), 1664 cm^{-1} (amide I), and 1550 cm^{-1} (amide II). The infrared dichroism is also the same as that expected for the α-helical conformation.

Heitz and Spach[33] also have recorded the spectra of this polymer in helicogenic solvents such as dioxane. The characteristic bands (Table 9) in dioxane are very similar to those observed in the solid state. The bands observed for PBLG and for the random forms DL-A and DL-B are also listed in Table 9 for comparison. The amide I band for PBDLG occurs at a slightly higher wave number than that found in PBLG and is almost the same as that found for random PB(D,L)G. The amide II band position corroborates the existence of an α-helix as no absorption at 1535 cm^{-1}, characteristic of a random coil,[35] is found.

b. X-ray and Electron Diffraction

From moderately oriented films of pure samples of PBDLG of molecular weight 30,000 or higher, Heitz et al.[16] have recorded X-ray and electron diffractograms. The patterns are suggestive of minor deviations from regular α-helical structure. The meridional reflection at 1.47 Å and a streak on a layer line at 5.6 Å are the prominent features. Thus, the α_{DL}-helix[16] consists of 3.8 residues per turn (*n*) and an axial rise of 1.47 Å per residue (*h*) instead of 3.6 and 1.5 Å observed for the conventional α-helix. The interchain spacing corresponds to about 15.2 Å as measured from the X-ray or electron diffraction pattern.

TABLE 9

Infrared Characteristics (cm^{-1}) of Various Poly(γ-benzyl glutamate) Samples in Dioxane and in the Solid State

Sample	Amide I	Amide II
PBLG	1652	1545
DL A and B	1660—1662	1550
DL-7	1660	1554
PBDLG α-helical	1665	1552
PBDLG α-helical (solid state)	1664	1550
PBDLG LD_3-helical	1648	1548
PBDLG LD_3-helical (solid state)	1645	1540

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c. NMR Spectra

The existence of α-helical structures in both the statistical and alternating copolymers has been demonstrated from 100-MHz NMR spectra[36] and confirmed in great detail using high-resolution NMR studies at 270 MHz.[37]

In recent years, it has been well established that the α-CH and NH protons of polypeptides undergo significant and characteristic downfield and upfield shifts, respectively, when transition occurs from α-helix to a random coil[38-41] and that in organic solvents the helix and coil exhibit separate resonances for these protons in the transition region.[36] Bovey et al.[36] have employed this technique to examine the conformations of three samples — DL-7, which is PB(D,L)G containing D- and L-residues in the ratio 53:47, DL-A, and DL-B(*vide supra*). The 100-MHz spectra recorded in $CDCl_3$ are all found to be indistinguishable, for these samples within the probable experimental error. These authors have also observed that the positions of the two α-CH and the two NH peaks in $CDCl_3$ and subsequent changes in their positions and intensities due to the addition of TFA (a strong helix-breaker) are very similar to the behavior of helical poly(γ-benzyl-L-glutamate) in similar solvents. Thus, it has been concluded[36] that PB(D,L)G is capable of forming α-helical structures in $CDCl_3$.

Paolillo et al.[42] reached the same conclusion, but they observed in the α-CH region an unusual secondary peak at 3.65 ppm. This has not been clearly assigned but seems to be related to the increase of D-residue amount in a right-handed L-polymer helix. These authors suspected that this peak is due to some distortion of the regular α-helices.

Heitz et al.[37] could resolve the ambiguities with the use of copolymers prepared by the racemisation-free technique and of the high-resolution 270-MHz NMR spectrometer. They obtained the spectra of several samples of PBDLG and PB(D,L)G in chloroform, dioxane, and dimethyl formamide. The chemical shifts of the NH and α-CH protons at room temperature in $CDCl_3$/0.5% TFA which they reported are listed in Table 10 and the spectra are compared in Figure 6. The spectrum of DL-A with its α-CH main peak at 3.94 ppm is very similar to that of PBLG but with considerable differences in the side-chain spectrum. This is attributed to some conformational heterogeneity in DL-A implying that the polymer is composed of rigid helical blocks linked by more flexible parts, as in the block model proposed earlier.[27] The secondary peak at 3.65 ppm, originally reported for PB(D,L)G,[42] is also observed for LD 80/20 with a spectrum resembling that of PB(D,L)G.

TABLE 10

Chemical Shifts of the NH and α-CH Protons of Various Poly(γ-benzyl glutamate) Samples in $CDCl_3$/0.5% TFA

Samples	Chemical shifts δ (ppm)	
	NH	α-CH
PBLG	8.23	3.92
DL-A	8.1	3.94
LD 80/20	8.25	3.91 + 3.66
LDL	8.71 + 8.31	3.91 + 3.83 + 3.70
DL-7	8.50	3.92 + 3.83 + 3.655
LD_{cat} II	8.56	3.82 + 3.655
LD_{cat} III	8.56	3.82 + 3.655

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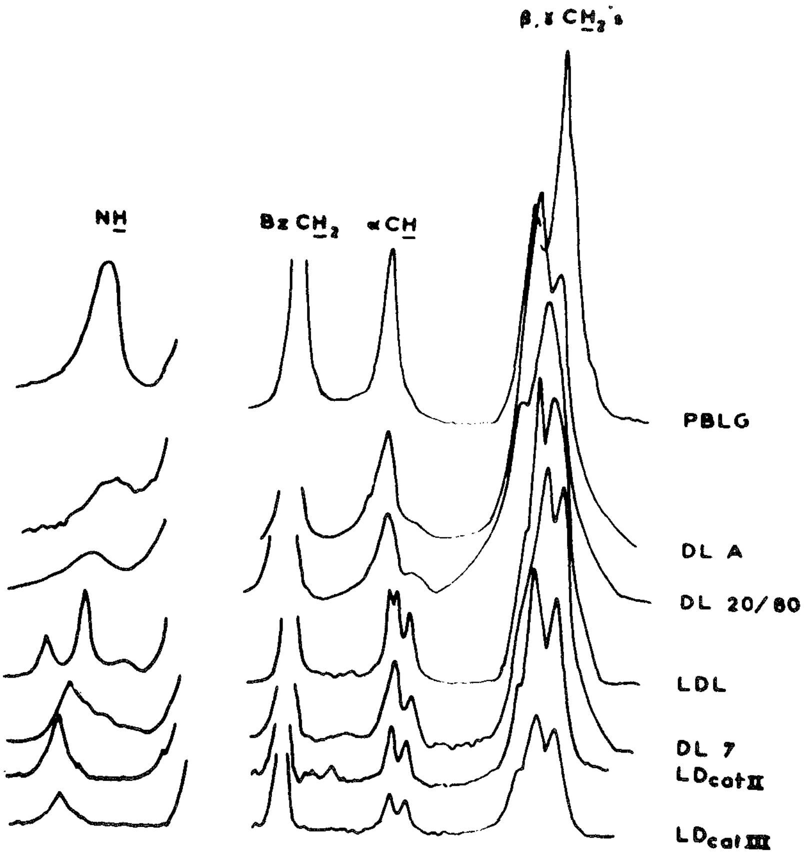

FIGURE 6. 270-MHz NMR spectra of several samples of PB(D,L)G at 18°C in $CDCl_3$/0.5% TFA. (Reprinted with permission from Heitz, F., Cary, P. D., and Crane-Robinson, C., *Macromolecules*, 8, 745, 1975. Copyright by the American Chemical Society.)

As the primary sequence in LD 80/20 is (L$_n$-D$_m$-L$_p$) so that (n + p):m is 80:20, the secondary peak at 3.65 ppm is said to have its origin in the introduction of D-residues into an L-polymer (or vice versa).

An interpretation of the peak at 3.65 ppm does not follow immediately from a measurement of the relative intensities of the two α-CH peaks at 3.95 and 3.65 ppm, as this ratio is the same in LD 80/20 and the 52/48 sample of Paolillo et al.[42] The analysis of the distribution of peaks and their intensities in the other four periodic copolymers in Table 10 provides a more exact explanation to the origin of the 3.65-ppm peak.[37] The intensity of this peak (Figure 6) gradually increases for LDL (the repeating sequence is L-D-L), DL-7, and LD$_{cat}$ in that order as D- (or L-) residues are introduced up to 50% into a chain initially formed by L- (or D-) residues. This behavior suggests that this peak arises due to the introduction of D- (or L-) residues into an L- (or D-) chain while the conformation remains helical.

It is thus obvious that although the 3.65 ppm is absent in PBLG, its intensity gradually increases as the amount of regularly alternating sequences increases until, in LD$_{cat}$ III, it constitutes half the total α-CH area. As both right- and left-handed helices are present for this polymer,[33] Heitz et al.[37] have proposed that the 3.65-ppm peak must be due to the combined effect of D-residues on right-handed helices and L-residues on left-handed helices (i.e., on the "wrong sense"). Similarly, the 3.83 ppm, which corresponds to 3.95 ppm in PBLG, must be due to L-residues on right-handed helices and D-residues on left-handed helices (i.e., on the "correct sense"). On the basis of these proposals, these authors have explained all the observed features in the spectra of the various samples. They have also confirmed the earlier suggestions[36] that PB(D,L)G essentially takes up α-helical structure in CDCl$_3$/0.5% TFA.

Heitz et al.[37] have also suggested that multiple α-CH peaks would be present in the NMR spectra of D,L-copolymers since the environments of α-CH groups of L- and D-residues are not the same in right- or left-handed α-helical conformation. For example, in the right-handed model, the D-residue has its α-CH close to two NH groups, while it is close to two CO groups in the L-residue. As an alternative suggestion, different side chain, rather than main chain, conformations are implicated to explain the observed chemical shifts. Since the basis of the α-CH chemical shifts has not yet been determined unequivocally, Heitz et al.[37] have concluded that the probable distortions in the α-helical conformation cannot be established from NMR spectra.

C. π_{DL}-Helix and α_{DL}-π_{DL} Transition

The existence of an LD$_3$-helix for PBDLG has been shown experimentally both in the solid state[16] and in solution.[33,37] The helix is also denoted as the π^4_{LD}-helix, where the superscript defines approximately the number of peptide units per turn, or, in short, is denoted as the π_{DL}-helix. Although three different types of LD$_k$-helices have been predicted to be possible from conformational analysis,[6] PBDLG is found to exist only as an LD$_3$-helix.

1. X-ray and Electron Diffraction

X-ray patterns from fibers of α-helical PBDLG, heated to 120°C under vacuum and cooled to room temperature, indicate an expansion of the hexagonal lattice from the α-helical value of a = 15.2 to 17.0 Å and a strong meridional reflection of spacing 2.33 Å.[16] The electron diffractograms are also quite similar and exhibit several other meridional and near-meridional reflections. Analysis of the diffractograms yields the helical parameters, n = 2.2 dipeptide units and h = 2.33 Å. These are consistent with the LD$_3$-helix (Table 3) and not with any of the well-known single helices composed of only L- (or D-) residues.

2. Infrared Spectra

Infrared spectra[16] of oriented samples, the same as those used in X-ray analysis, are shown in Figure 7. The corresponding spectrum of the α-helical form is also shown for comparison. It may be seen that the dichroic effects for the amide A and amide I bonds are similar to those of the α-helix, whereas the amide II band shows almost no dichroism.

As is evident from the LD$_3$ (or π_{DL}) model, the two peptide units of the LD-repeat do not have the same orientation relative to the helical axis. Consequently, from the atomic coordinates of this model and the directions of the transition moments of the peptide units reported,[43] the re-

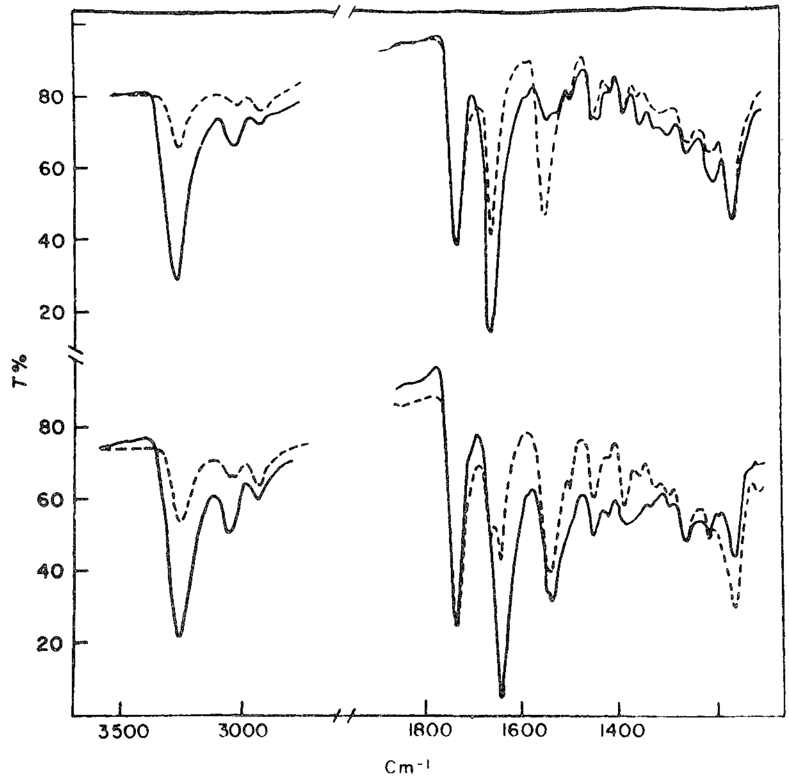

FIGURE 7. Polarized infrared spectra of PBDLG. Upper spectrum corresponds to α_{DL}-helical structure and lower spectrum to LD_3-helix. Continuous line represents the spectrum for electric vector parallel to the direction of stroking, and the dashed line represents the electric vector perpendicular to the direction of stroking. (From Heitz, F., Lotz, B., and Spach, G., *J. Mol. Biol.*, 92, 1, 1975. With permission.)

sultant dichroic ratio for the amide II band is expected to be very small ($D\|/D\perp = 1.6$) for a perfectly oriented sample and smaller still when considering disorder in the fiber axis directions.[16] The observed value of unity (Figure 7) is in good agreement with that expected for the π_{DL}-helix. On the other hand, the dichroic ratios $D\|/D\perp$ for amide A and amide I bands are of the order of 14, but the experimental values are much lower due to the distribution of orientations in the sample.[16]

The existence of π_{DL}-helix for PBDLG in solvents such as dioxane has been demonstrated.[33] As the solution is heated from 25 to 95°C, the positions of the amide I and II bands in the infrared spectrum at 95°C resemble those observed in the solid state (Figure 7) for PBDLG in the π_{DL}-helical conformation. Since the successive carbonyl (or NH) groups in the LD_k-helix are pointing in opposite directions (Figure 2), the dipole moment of the polymer in this conformation should be quite low, and from dipole moment measurements in solution this is shown to be true.[33]

3. *ORD and CD Studies*

Heitz and Spach[33] have reported that both the ORD and the CD are modified (Figures 8 and 9) as the α-helical sample LD_{cat} II undergoes transconformation to the π_{DL} form due to heat treatment. The transition not only causes a change of sign of the CD, but also shifts the extremum corresponding to the peptide n-π^* transition from 222 to 227 nm. These two features indicate the presence of dissymmetry in the π_{DL}-helix, as in the α-helix.

The origin of optical activity in the π_{DL}-helix has been attributed, probably, to a favored helical sense which should arise mainly from the number of hydrogen bonds formed in one helical sense or the other.[33] In a sample of PBDLG, in which the N-terminal residue has an L-con-

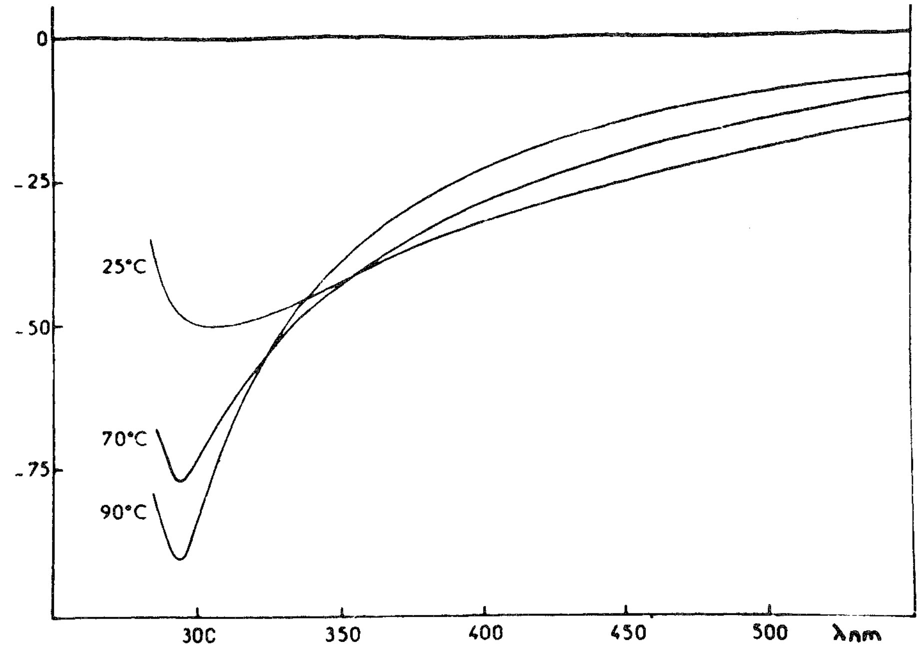

FIGURE 8. Optical rotatory dispersion curves of PBLDG (sample LD_{cat} II) in dioxane (concentration 0.5%) at 25, 70, and 90°C. (Reprinted with permission from Heitz, F. and Spach, G. *Macromolecules*, 8, 740, 1975. Copyright by the American Chemical Society.)

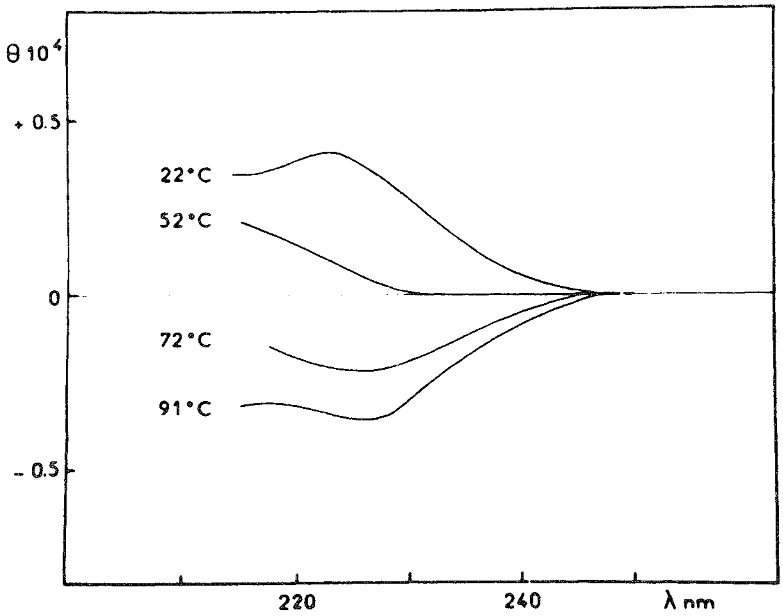

FIGURE 9. Temperature effect on the CD spectra of PBLDG (sample LD_{cat} II) in dioxane (concentration 0.03%). θ in deg cm^2 $dmol^{-1}$. (Reprinted with permission from Heitz, F. and Spach, G., *Macromolecules*, 8, 740, 1975. Copyright by the American Chemical Society.)

figuration, the left-handed π_{DL}-helix can have 2n-3 hydrogen bonds including the terminal NH_2 and COOH groups, whereas it can have only 2n-5 for the right-handed helix. Hence, there is preference for the former.

4. NMR Spectra

Heitz et al.[37] have monitored the α_{DL}-π_{DL} transition by heating LD_{cat} II and LD_{cat} III samples from 18 to 86°C and recording the proton spectra in dioxane at 270 MHz. Both samples exhibit a new peak at 4.45 ppm, the former sample in the entire temperature range, while the latter only at the highest temperature. For the LD_{cat} II sample, the area of this peak is found to increase with temperature, thereby indicating a corresponding increase in the proportion of the π_{DL}-helix. No such peak has, however, been observed for the PBLG sample either at 18 or at 86°C as it does not take up the π_{DL}-form.

The addition of TFA to dioxane results in a decrease in the area of the peak at 4.45 ppm and also a simultaneous increase in the α-CH peak at 3.8 ppm, which is characteristic only of a helical structure. Hence, it follows that the 4.45-ppm peak is not due to the formation of a random coil but to a novel helix, which is possible only with a polymer with strictly alternating L- and D-residues, such as the π_{DL}-helix. Whereas the α-CH protons lie alternately between two CO bonds and two NH bonds in the α_{DL}-helix, they are always positioned between an adjacent NH and CO bonds in the π_{DL}-helix. This difference in environment is probably responsible for the occurrence of the 4.45-ppm peak.[37]

It is remarkable that the α_{DL} to π_{DL} transconformation takes place even in the solid state, which involves a transition between two basically different patterns of hydrogen bonding. In contrast to the 13-member hydrogen-bonded ring system in the α_{DL}-helix, there are alternately 14- and 16-member hydrogen-bonded ring systems in the π_{DL}-helix and, hence, all the hydrogen bonds are ruptured during the transition effected by heat treatment. In this process, there is almost 180° rotation around the axis defined by the α-carbon atoms of every other peptide unit in the α_{DL}-helix. In turn, the overall conformation at every α-carbon atom is altered from the original α-helical to alternating β^+ and β^- conformations (the + and − corresponding to L- and D-residues in their respective β-regions) as required by the chemical sequence. During this conversion, the helical sense remains unaltered. The transformation may begin at one end of the helix and then gradually move on along the chain.[16] The conversion will, however, be inhibited due to steric hindrance arising, for example, from a succession of two or more residues of the same configuration.

D. ω_{DL}-Helix

When casting solutions of PBDLG, for example, in dioxane, or dimethyl formamide, that contains a small amount of water, Heitz et al.[16] have always obtained only unoriented films. The infrared spectrum of these films differs from those of α_{DL}- or π_{DL}-helices. It is characterized by an amide A band at 3290 cm^{-1} with a satellite at 3350 cm^{-1}; the amide I and II bands are at 1676 and 1532 cm^{-1}, respectively. This spectrum closely resembles that of the ω-form of poly(β-benzyl-L-aspartate).[44,45]

Also, the electron diffraction pattern[16] exhibits a hexagonal symmetry with a spacing of 13.6 Å, which is different from, and actually intermediate between, those observed with samples in the α_{DL}-(13.2 Å) and π_{DL}-(14.75 Å) forms, as may be expected for an ω_{DL}-helix. The pattern is consistent with an axial rise per residue of 1.32 to 1.37 Å, which is again in agreement with an ω-helical structure.

E. Double Helices

On the basis of very recent X-ray, electron diffraction, and infrared studies, Heitz et al.[46] and Lotz et al.[17] have obtained evidence to propose that, in addition to α_{DL}- and π_{DL}-structures, several double-helical structures (originally postulated for gramicidin A)[12] are equally possible for PBDLG. Analogous to Urry's nomenclature,[7] Lotz et al.[17] have designated the double helices as $\pi\pi^{n}_{DL}$, where n is the number of amino acid residues per turn in each helical chain.

1. A_3 or P_3 Double Helix

After heating an oriented sample of PBDLG, initially present either in α_{DL}- or $LD_3(\pi^{4.4}_{DL})$-modification, to 220°C, Lotz et al.[17] could produce a new diffraction pattern. The equatorial reflections indicate a hexagonal lattice with *a* = 18.2 Å as compared to 15.2 and 17.0 Å for the α_{DL}- and LD_3-structures, respectively. Also,

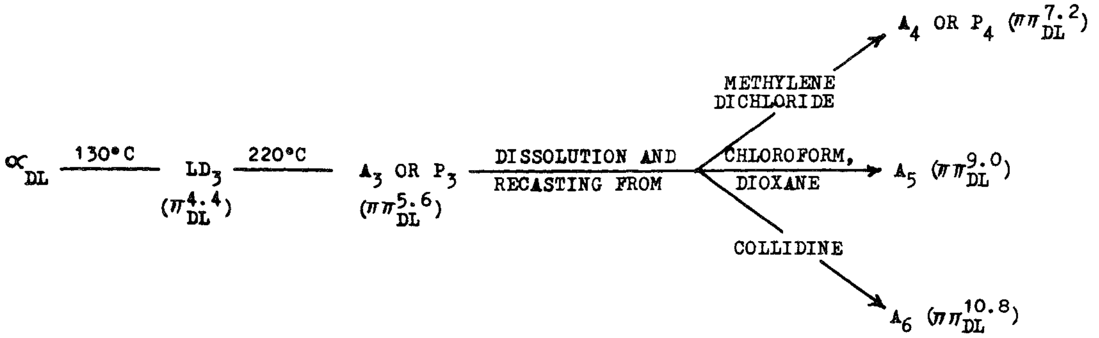

FIGURE 10. Experimental conditions used to obtain the various crystalline modifications of PBDLG. (From Lotz, B., Colonna-Cesari, F., Heitz, F., and Spach, G., *J. Mol. Biol.* 106, 915, 1976. With permission.)

the axial rise per repeating dipeptide unit is 4.02 Å in the new form, as compared to 2.97 and 2.33 Å in α_{DL}- and LD_3-helices. The corresponding first-layer line spacings, linked to the pitch of the helix, are 5.63, 5.6, and 5.12 Å.

The infrared spectrum of this new form closely resembles those of conventional β-structures.[47] The band observed at 1630 cm^{-1} and a shoulder at 1690 cm^{-1} are typical of the two components of the amide I band of intramolecularly hydrogen-bonded pleated sheet structures. Based on these details, the pattern has been identified with a double helical structure,[17] in which each strand has 5.6 residues per turn, designated as $\pi\pi^{5.6}_{DL}$. This double helix is the same as either A_3 or P_3,[20] the features of which were described in Section II.

Lotz et al.[17] have suggested that both antiparallel and parallel double helices are possible structures for $\pi\pi^{5.6}_{DL}$. The Fourier transforms calculated by them for both models are in good agreement with the observed intensity data. However, these authors prefer the antiparallel model, since it is in accordance with the presence of a shoulder at 1690 cm^{-1} in the infrared spectrum, which is usually associated with antiparallel arrangement of chains.[47]

2. *A_4 (or P_4), A_5, and A_6 Double Helices*

These three double helices are derived mainly from the A_3- (or P_3-) form, always by implying an organic solvent, either by exposure to it or its vapors or by dissolution and recasting. A schematic representation of the experimental conditions used[17] to obtain the various crystalline modifications of PBDLG is shown in Figure 10. The different double helical forms in solution cannot be solely distinguished from their respective infrared spectra since they are almost identical. On the other hand, by casting these solutions into films in the solid state, they can be individually characterized by X-ray and electron diffraction analyses.[17] The details are given below.

When PBDLG in the A_3 or P_3 helical form is dissolved and recast in dichloromethylene, the new structure produces an X-ray diffractogram suggestive of a hexagonal cell with $a = 22.6$ Å and an axial rise of 2.94 Å per dipeptide repeating unit, corresponding to the double helix A_4 or P_4, designated as $\pi\pi^{7.2}_{DL}$-helix.

Starting with the same A_3 or P_3 form dissolved in chloroform or dioxane leads to an enlarged hexagonal unit cell with $a = 24.7$ Å. Another modification also exists with an improved and oriented sample yielding a monoclinic cell with $a = 25.3$ Å, $b = 24.4$ Å, and $\gamma = 117°$. The spacings provide a lower axial rise of 2.25 Å, which is consistent with the double helix A_5 or the so-called $\pi\pi^{9.0}_{DL}$-helix.

However, diffraction patterns from single crystals obtained by controlled evaporation of PBDLG in collidine (Figure 10) provide a still enlarged monoclinic cell with $a = 26.4$ Å, $b = 25.6$ Å, and $\gamma = 118°$. The axial rise per dipeptide is the lowest, approximately 1.87 Å, the same as that for A_6 or the $\pi\pi^{10.8}_{DL}$-helix.

Lotz et al.[17] have proposed both antiparallel and parallel models, the same as A_4 and P_4,[20] for $\pi\pi^{7.2}_{DL}$, but preferred A_4 due to steric reasons. Their choice is in complete agreement with the results of Venkataram Prasad and Chandrasekaran,[20] which were summarized in the previous section. The remaining two double-helical models, according to Lotz et al.,[17] are again consistent only with antiparallel structures since parallel models are impossible[20] to construct for these cases.

The conformational features and atomic coordinates of the various double helical structures[17] are very similar to those described[20] in the previous section. The progressive increase in the helical diameter and the related decrease in the axial rise per repeat in the family of double helices seem to be quite compatible with the size of the solvent molecules appropriate for each kind.

To a first approximation, Lotz et al.[17] have used the system poly(DL-Ala) in the Fourier transform calculations and found reasonable agreement with the observed intensity distribution. The rather poor quality of the patterns has enabled them to learn very little about the regularity of the side-chain conformations in PBDLG. Similarly, although the solvent molecules appear to complex with these double helices (inferred from the correlation between the solvent size and modification of the helical form), a paucity of data has led to no details regarding the location of solvent molecules and their interactions with the polymer itself. On the basis of the solvent-polymer composite structure, Lotz et al.[17] have also predicted that one of the structures observed by Ascoli et al.[48] for poly(L-Ala-D-Val) might be a $\pi\pi^{9.0}{}_{DL}$- (or A_5-) helical structure.

3. Transconformations of PBDLG

Lotz et al.[17] have described the various possible transconformations which they have hitherto observed using appropriate experimental conditions. They also caution that failure to observe others might be due to the fact that the right experimental conditions have not yet been found.

The transition from the single helix α_{DL} to the double helix A_3 or P_3 is generally achieved via the intermediate LD_3. The α_{DL}-LD_3 transition is accomplished by heating the sample in the solid state or in solution from room temperature to about 130°C. Further heating to 240°C (Figure 10) leads to the double helix A_3 (or P_3). In order to obtain the other three double helices (A_4, A_5, and A_6) from A_3, the appropriate solvent molecules indicated in Figure 10 must be used. The conversion within the double helix family is also possible in solution. On the other hand, the reverse conversion from double to single helix is generally not observed in the solid state; such a change in solution is, however, possible with the use of random-coil-promoting solvents such as TFA.[17]

IV. STRUCTURAL STUDIES ON GRAMICIDIN A

The primary structure of the polypeptide antibiotic gramicidin A, given in the first section, was originally elucidated by Sarges and Witkop in 1965.[49,50] Gramicidins B and C are structural analogues of gramicidin A. The forms A, B, and C are distinguished by the presence of L-Trp, L-Phe, and L-Tyr in the 11th position. Gramicidin A constitutes the major component of the antibiotic synthesized by the bacteria.

The influence of gramicidins on biological membranes has been thoroughly studied.[51-56] Among the membranes investigated are those of mitochondria, erythrocytes, and electroplax, and in each case it is concluded that gramicidin produces a passive permeability of the membranes to alkali metal ions. Also, it is found that gramicidins produce permeability to alkali metal ions in lipid bilayers as well.[57-59] Thus, in both natural and artificial membranes gramicidin has effects somewhat similar to those of smaller cyclic antibiotics such as nonactin and valinomycin.

A. Transport Mechanisms

Two mechanisms have been proposed for the selective transport of ions across lipid layer membranes of antibiotics. In the carrier mechanism, the ion is sequestered by the antibiotic, and the resulting complex diffuses through the hydrophobic interior of the membrane. The second mechanism involves the formation of channels through the membrane which in turn provide pathways for the ion transport. It is generally agreed that nonactin and valinomycin[60,61] are carriers, while gramicidin, nyastin, and alamethicin form transmembrane channels.[62]

The general agreement seems reasonable in view of the fact that gramicidin A is a linear molecule, capable of forming many internal hydrogen bonds, and also in terms of its solubility properties in aqueous and hydrocarbon phases.

Whereas nonactin and valinomycin are soluble in both these media, gramicidin is not. Therefore, the gramicidin molecules can easily get localized and aggregated in a suitable way to form transmembrane channels. Further evidence is obtained from conductance measurements on gramicidin A-mediated ion flux across lipid bilayer membranes which show steps of uniform height; this suggests that channels are formed and that each channel constitutes a specific conductance which tends towards space charge saturation with increasing electrolyte concentration.[62]

By studying the dependence of single-channel parameters on membrane thickness and composition, Hladky and Haydon[63] have discovered that single-channel conductance remains almost the same regardless of the type of membrane used over a wide range of thicknesses and compositions. This evidence, supported by the measured value of diffusion coefficient for K^+ ions through the channel, has enabled these authors to conclude that gramicidin forms a pore through the membrane. This pore must possess a fixed structure independent of the membrane in which it is situated; for example, if the membrane is thicker than the length of the pore, it must presumably thin down locally or "dimple" when the conductance channel is formed.[64] From the hydrocarbon thickness of the membranes employed in these studies, Hladky and Haydon[63] have estimated the length of the pore to be less than 35 Å.

It has been argued by some authors,[65,66] on the basis of the relationship between the membrane conductance and gramicidin concentration, that two molecules are required to form the conducting channel. The molecular weight data[49] for gramicidin A in several organic solvents suggest that the nature of aggregation is entirely solvent dependent and yet clearly indicate that the dimer is indeed the effective species in gramicidin-mediated ion transport across a lipid bilayer. For example, in less polar solvents such as dioxane and ethyl acetate, gramicidin attains a molecular weight which approaches the dimer value even at low concentrations. However, in anhydrous aliphatic alcohols, the proportion of monomers is higher.[67] Still, the dimer species is favored for increasing the size of solvent alkyl groups. Over a wide concentration range in the highly polar TFE, the molecular weight is found to be consistently near the monomer value. It may, therefore, be expected that in less polar solvents such as lipids, the proportion of dimer will be appreciable.

B. Conformational Aspects

Knowledge of the molecular conformation of gramicidin will be immensely helpful in understanding its pharmacological, transport, and physical properties. Considerable work has been done in this direction, and a number of models have been reported in the literature. The very first proposal was by Sarges and Witcop[49] as early as in 1965; they suggested a β-structure for gramicidin, purely on the basis of IR evidence. In this model, two molecules are cyclically joined head to tail (formyl end to hydroxyl end). Features such as steric hindrances between bulky side chains and presence of three *cis* peptide bonds per molecule led to a rejection of this model.

In order to explain the functional aspects of this antibiotic, on the basis of helix radius and total length for fifteen residues, Urry[7] eliminated many of the conventional structures such as the α- and 3_{10}-helix as plausible models for gramicidin. Instead, he has proposed structures such as the LD_k- or π_{LD}-helices, also called the β-helices,[69] originally formulated in detail by Ramachandran and Chandrasekaran[6] and described in Section II. These LD-helices fulfill the requirements of channel formation when two of them are coaxially joined head-to-head or tail-to-tail or head-to-tail by means of NH···O hydrogen bonds. Urry et al.[68] have suggested that the gramicidin channel consists of two molecules, each having a lipophilic, left-handed, LD_k-helix, coupled at the formyl ends, i.e., joined head to head. Of the two models considered,[68] namely $\pi^4{}_{LD}$- and $\pi^6{}_{LD}$-helices (LD_3 and LD_4), the former has a radius much smaller than the radii of some of the ions which are readily conducted by gramicidin, as well as a total length for the head to head dimer too long (about 40 Å) to span the lipid bilayer. However, the $\pi^6{}_{LD}$-helix (LD_4) possesses the correct dimensions (see Table 3) so that the resulting channel can account for the passage of various cations with the aid of local ion-induced conformational fluctuations. These fluctuations result in the relaxation of the helical coordinates in which the peptide C=O moieties librate such that the oxygen atoms move towards the helical axis. The channel then becomes lined with negative dipoles as the peptide oxygens satisfy coordination requirements of the cation while it is in the channel. Thus, ion selectivity is provided by the size of the channel and by the amount of deformation energy involved in the peptide libration. If this is true, greater selectivity and activation energy for the diffusion of ions might be expected.

C. Experimental Studies

In order to formulate suitable models for the structure of gramicidin A, several groups[12,67,70,71] have examined its conformationally related properties through several experiments involving techniques such as UV, ORD, IR, and NMR. Urry et al.[70] and Glickson et al.[71] sought evidence for π_{LD}-helices for gramicidin A in solution. Although some of these observations have been interpreted[70,71] to be indicative of the existence of π_{LD}-helices, alternate interpretations have been advanced.[12,67] Scrutinizing the facts obtained from systematic experimental studies, Blout and his group[12] have proposed novel double helices for gramicidin A. An account of these studies follows.

1. Single Helical Models

UV absorption studies[70] have been carried out on hydrogenated gramicidin in which the interference due to the tryptophan chromophores is eliminated. The spectra show marked hypochromism in TFE and to a lesser extent in dioxane. The observed peptide band at 190 nm is an expected feature either for the intramolecularly hydrogen-bonded helical structures or for more extended and intra- or intermolecularly hydrogen-bonded β-structures. The absorption data on the hydrogenated sample are, however, interpreted by Urry et al.[70] as an indication of helix formation. However, this enhanced helix formation in polar solvents such as TFE on hydrogenation of aromatic residues cannot be ruled out[67] when it pertains to the conformation of natural gramicidin. In other words, the helix formation may be a feature of hydrogenated gramicidin in TFE, but it may not be the case for unhydrogenated gramicidin.

The solvent effects on the CD spectra of gramicidin A[67,70] are shown in Figure 11. The spectrum in anhydrous *n*-propanol (Figure 11A, Curve 1), which is concentration independent, shows intense bands centered at about 230 and 195 nm with $[\theta]_{res}$ values of about -1.6×10^4 and $+3.9 \times 10^4$, respectively. This pattern bears considerable similarity both in sign and magnitude to the spectra of model homopolymers in the β-conformation.[72,73] Moreover, the positive bands in the aromatic region (255 to 310 nm) in the spectrum indicate a largely structured form. The complexity of the negative CD bands may be due to the tryptophan residues in this ordered conformation.[67]

In a more polar solvent as TFE, the spectrum of hydrogenated gramicidin shows two positive extrema at 224 and 212 nm and a negative extremum at 194 nm, which is somewhat redshifted from the α-helix positions of 222, 208, and 190 to 192 nm.[70] Consequently, these features are interpreted as characteristic of left-handed helices.[70] However, Isbell et al.[67] have reported that the CD spectrum recorded in propanol reduces progressively by the addition of water (Curves 2 and 3) and the final spectrum resembles that in TFE (Curve 4) and is very similar to that obtained for *N*-acetyl-Trp-amide in band location, sign, and magnitude. Hence, they argue that in anhydrous *n*-propanol, the spectrum is indicative of an ordered structure and the residual dichroism observed at limiting water content arises completely from the contributions by intrinsic tryptophan residues rather than from the conformation of the backbone as interpreted by Urry et al.[70] On this basis, the CD spectrum in TFE should reflect the unstructured form of gramicidin A.

On the other hand, the behavior of gramicidin A in anhydrous dioxane (Figure 11B) is extremely concentration dependent. At low concentration, the CD spectrum (Curve 3) is remarkably similar to that observed in propanol (Figure 11A). At very high concentrations, the spectrum is inverted (Curve 1). This is very similar to the spectrum recorded for poly-L-Trp chromophores.[74] At intermediate concentration, the spectrum (Curve 2) appears to be an average of those at low and high concentrations. According to Isbell et al.,[67] Curves 1 and 3 of Figure 11B are probably indications of two extremes of conformational equilibrium. If that were the case, Curve 2 could easily be matched by adding approximately equal

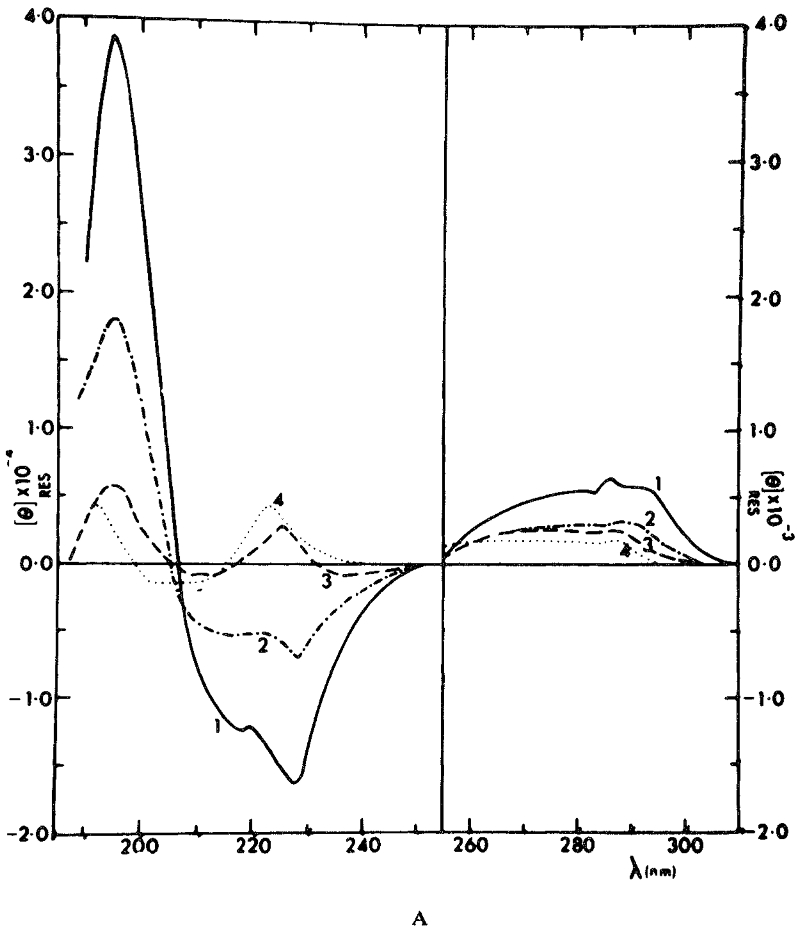

FIGURE 11. Circular dichroism of gramicidin A at 22°C. (A) in different solvents: (1) *n*-propanol, 1.0 mg/ml; (2) 95% v/v *n*-propanol:water, 0.94 mg/ml; (3) 75% v/v *n*-propanol:water, 0.75 mg/ml; (4) TFE, 1.12 mg/ml. (B) in anhydrous dioxane at different concentrations. (1) 4.86 mg/ml; (2) 1.16 mg/ml; (3) 0.8 mg/ml. (From Isbell, B. E., Rice-Evans, C., and Beaven, G. H. *FEBS Lett.*, 25, 192, 1972. With permission.)

contributions of the two extreme forms. Even at lower concentrations, as already mentioned, from molecular weight data,[67] gramicidin A exists in dimer form. Hence, the concentration dependence of the CD spectrum in dioxane appears to be related to the dimerization.

The formation of dimer species of gramicidin A at higher concentrations in dioxane is also supported by concentration-dependent IR spectrum.[67] The decrease in the relative intensity of the 1650 cm^{-1} band and the simultaneous increase in that of the 1630 cm^{-1} band with increasing concentration are again suggestive of the formation of dimer species. Since the spectra characterized by a strong 1630 cm^{-1} band and a resolved 1680 cm^{-1} band with a shoulder at 1650 cm^{-1} are generally accepted to be indicative of an antiparallel β-structure,[75] Isbell et al.[67] have proposed a β-structure to the dimer species. Although the broad amide II transition at 1545 cm^{-1} could be used in favor of an α-helical structure, the evident complexity and possible solvent effects make the assignment of the 1545 cm^{-1} band rather vague.[67]

Glickson et al.[71] have carried out NMR studies (220 MHz) to obtain evidence for the π_{LD}-helical conformation of gramicidin A. Any conclusion from these studies depends upon the accuracy with which the assignment of the peaks can be made. Due to the complex nature of the gramicidin A spectra in DMSO, only one fourth of the possible NH peaks have been uniquely identified and the corresponding NH−CH coupling constants determined.[71]

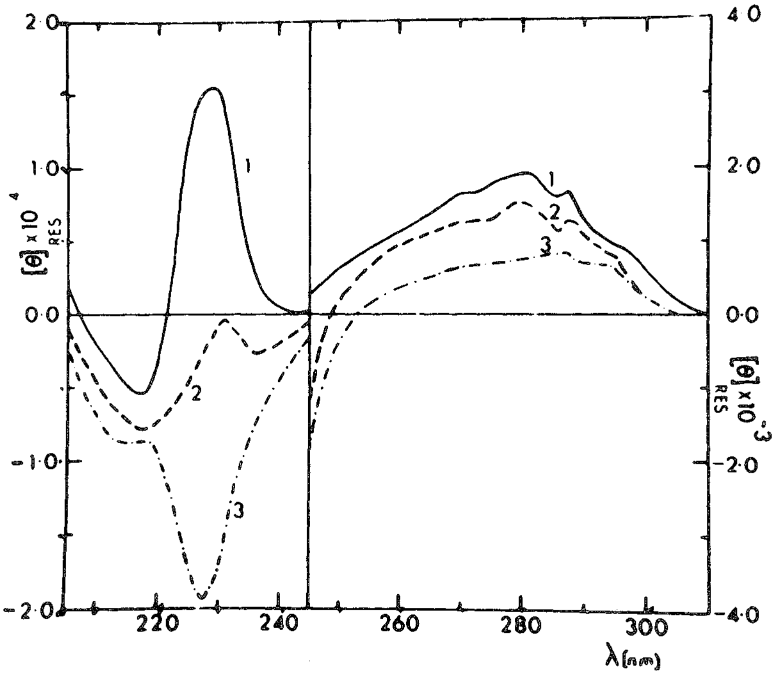

FIGURE 11B

From these values, the conformational angles (ϕ) about N−C$^\alpha$ bonds have been estimated by using the empirical relationships[76] between coupling constants and dihedral angles. Certainly, the large coupling constants experimentally measured are well outside the range associated with the α-helical form. Thus, according to Glickson et al.,[71] the dihedral angles, ϕ, are around 160°, compatible with the values necessary for the construction of π_{LD}- (or β-) helices.

All the conclusions drawn by Urry and coworkers[70,71] are based on the observations in relatively more polar solvents such as TFE and DMSO in which gramicidin A is not likely to form dimer species. Thus, the relevance of their conclusions may be questioned. However, it must be kept in mind that the biological environment for gramicidin is a heterogeneous mixture of polar and nonpolar regions.

2. Double Helical Models

Blout and colleagues[12,77,78] have conducted extensive solution studies on four different conformational species of gramicidin which have been physically isolated from a single nonpolar solvent system such as methanol, ethanol, or dioxane. They have reported that π_{LD}-helices as models for gramicidin are not fully consistent with some of the experimental data and, in order to explain the observations, have postulated double helices. The details of these double helices[20] were described in Section II.

The four species isolated are designated 1, 2, 3, and 4 in increasing order of mobility in thin-layer chromatography. Veatch et al.[12] have observed multiple spots in the two-dimensional thin-layer chromatography which clearly represent interconversion among the various conformational states of these species. A similar observation was also reported[49] as early as 1965. The characterization of these species has been done using CD, IR, and NMR.[12]

The CD spectra[12] for these four species in dioxane and 2-propanol (solvents in which gramicidin is known to form dimers)[67] show interesting characteristics, as shown in Figure 12. The spectra for species 1 and 2, in either solvent, are almost similar. The spectrum of species 4 is approximately the mirror image of that of species 1 and 2. In contrast, species 3 has a spectrum which differs significantly from the rest.

The IR spectrum[12] also differentiates species 3 from 1, 2, and 4. It shows a distinct peak at

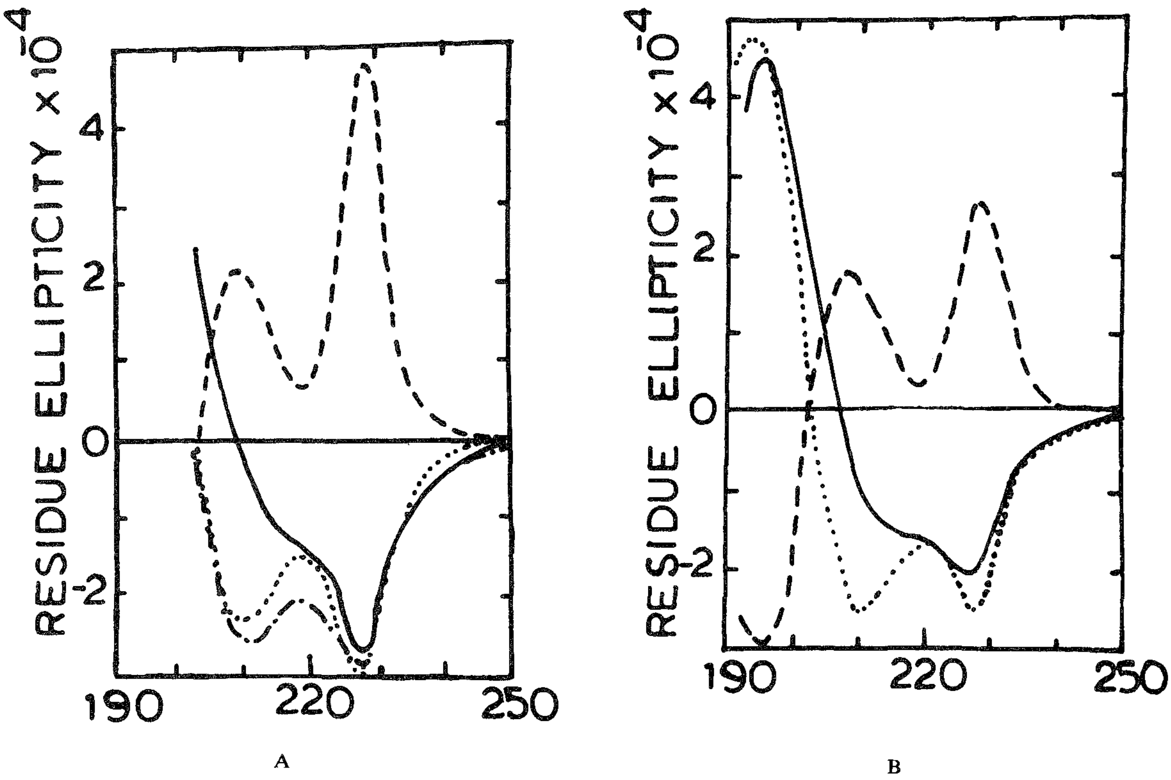

FIGURE 12. Circular dichroism spectra of gramicidin isolated species (A) from 200 to 250 nm in dioxane (. . .)1; (· -)2; (—)3; (--)4 and (B) from 190 to 250 nm in 2-propanol (· · ·)1 + 2; (—)3; (--)4. (Reprinted with permission from Veatch, W. R., Fossel, E. T., and Blout, E. R., *Biochemistry*, 13, 5249, 1974. Copyright by the American Chemical Society.)

1680 cm^{-1} for species 3, whereas for others it is absent. Peaks common for all the species are those at 1545 cm^{-1} (amide II) and 1633 cm^{-1} (amide I). Moreover, the spectra of species 1 and 2 are again very similar.

The proton nuclear magnetic resonance (pmr) spectra[12] (100 MHz) of these four isolated species are shown in Figure 13. Species 1, 2, and 4 have very similar peaks at 9.4 and 9.5 ppm which are assigned to the resonances of indole amide protons, whereas species 3 has a major peak at 9.4 ppm and a smaller peak at 9.0 ppm. There are also significant differences among the isolated species in the region above 1 ppm, which are assigned to the resonances of methyl protons of the valine and leucines adjacent to tryptophan residues. Species 4 lacks the resolved peak near 0.3 ppm observed for species 1 and 2, and species 3 has three well-resolved peaks at 0.7, 0.3, and 0.1 ppm.

Specific conformational details of each of these species have been inferred from the above observations. The 1680-cm^{-1} peak in the IR spectrum and the nature of the CD spectrum indicate that species 3 has a hydrogen bonding pattern as in antiparallel β-structures. Therefore, Veatch et al.[12] have proposed antiparallel double helices (for example, A_3 or A_4) for this species. This proposal is well supported by X-ray diffraction data. Gramicidin crystallized from methanol has $P2_1$ symmetry with two asymmetric units per unit cell, each containing a gramicidin dimer. As for the other three species, they have proposed parallel double helices, species 1 and 2 having opposite handedness to that of species 4. The IR spectra of these three species are very similar and indeed are indicative of parallel β-hydrogen bonding. The π^{LD}-helix, which is, after all, essentially similar to a rolled-up parallel β-structure, can account for all the observed facts as well as the latest double helices proposed by Veatch et al.[12] However, the formyl proton resonance attributes asymmetry for species 2, which should suggest possible head-to-tail aggregation of the π_{LD}-helices. For such an aggregation, it is difficult to con-

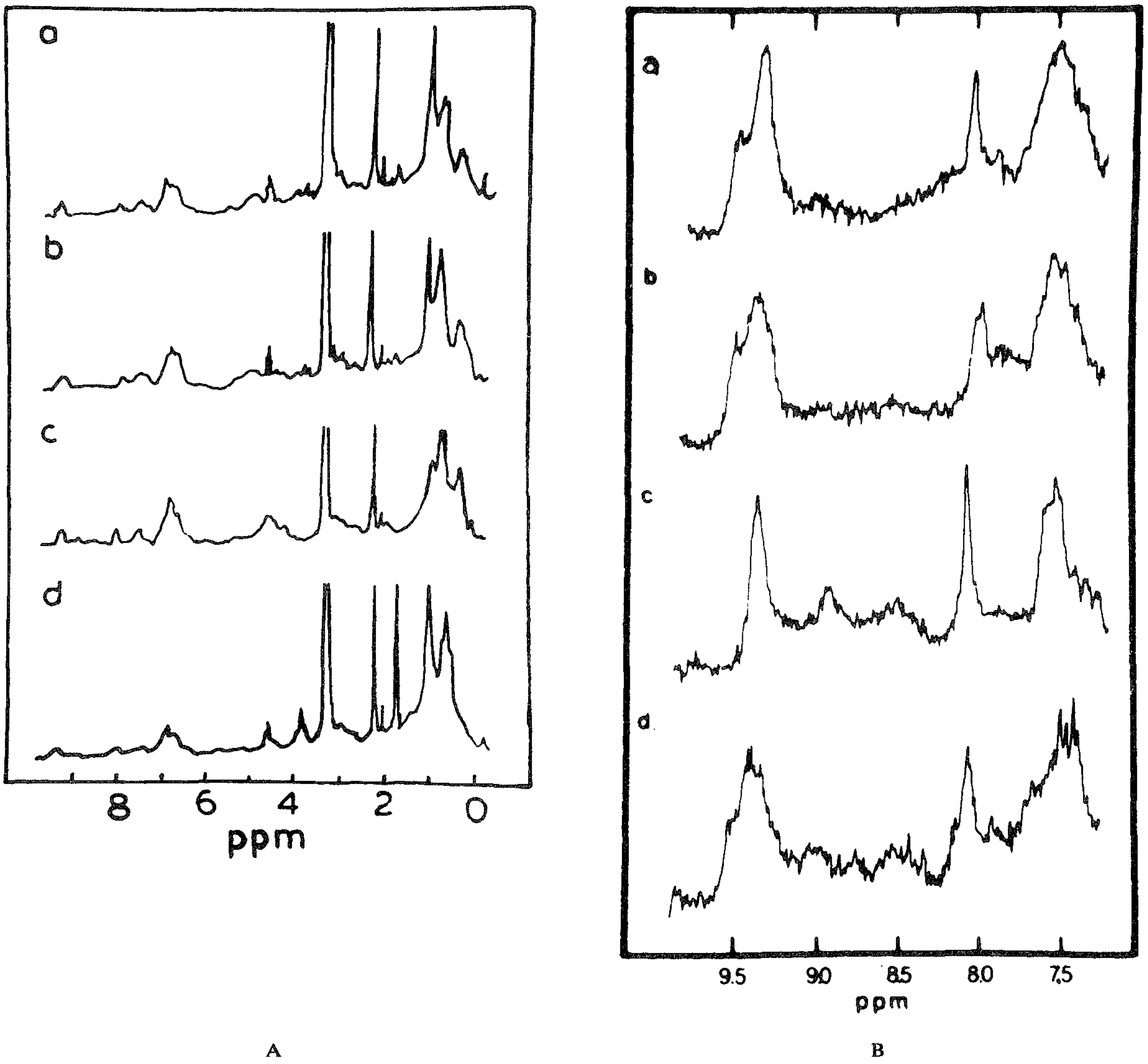

FIGURE 13. The ^{1}H NMR spectra of gramicidin isolated species in dioxane-d_8 (100 MHz). The curves a, b, c, and d correspond to species 1, 2, 3, and 4. (A) 0- to 9-ppm region and (B) expanded downfield region of 7.5 to 9.5 ppm. (Reprinted with permission from Veatch, W. R., Fossel, E. T., and Blout, E. R., *Biochemistry*, 13, 5249, 1974. Copyright by the American Chemical Society.)

ceive a mechanism of dimerization in which trimers and tetramers are ruled out.[77]

In order to examine the nature of aggregation of gramicidin molecules in different solvents, Veatch and Blout[77] have measured kinetic and equilibrium constants from the solution data obtained by circular dichroism, fluorescence intensity, fluorescence polarization, and quantitative thin-layer chromatography. It has already been mentioned that gramicidin aggregates as dimers in relatively nonpolar solvents, as evidenced from molecular weight measurements.[67] It has been noted that decreasing solvent polarity strongly favors the aggregated species, and the decay time of these species is sufficiently long for them to be physically isolated.[77] The large values of dimerization constant and, hence, the greater stabilization in nonpolar solvents are suggestive of dimers with a substantial number of intermolecular hydrogen bonds. It is quite interesting to note that all the species are found to be dimers on the measured dimerization constants in ethanol at a concentration of 0.05 *M*, which implies the presence of only about 2% monomer. The aggregation rates for the isolated species in nonpolar solvents are generally found to be very slow, indicating the formation of more complex dimer structure than perhaps the head-to-head aggregation of single helices.

No significant changes, either in the dimerization constant or in the species mole fraction, have been observed when the formyl proton in gramicidin is replaced by a methyl group in *N*-acetyldesformyl gramicidin. This invariance shows that none of the isolated species is energetically destabilized relative to the average monomer conformation in ethanol. Also, it contradicts the observation of Urry[7] that such a substitution would destabilize head-to-head helical dimers due to steric hindrance of the two methyl groups which, in turn, would disrupt the interhelix hydrogen bond pattern. Also, the measured concentration dependence of the rate of forward aggregation proves that the dimerization is a rate-limiting process. This precludes the possibility of higher order aggregation. These results are thus quite inconsistent with head-to-head, or head-to-tail, dimerization of π_{LD}-helices.

The NMR spectral studies and spin lattice relaxation measurements by Fossel et al.[78] shed some light on the structure of the aggregated species of gramicidin and the structure of the monomer which is favored only in more polar solvents. These studies have been carried out in two solvents — DMSO-d_6, in which the molecules are mostly in monomer state, having little or no regular structure, and methanol-d_4, in which the molecules are mostly in the dimer state. The ^{13}C NMR spectra of gramicidin[78] at 67.88 MHz in DMSO-d_6 show several resonances resolved in the upfield region which were originally unresolved at 20.0 and 25.16 MHz. The various chemical shifts have been shown to agree very well with those of the corresponding resonances in simple model molecules such as *N*-acetyl amino acid amides in the same solvent. This close correspondence indicates an averaging of environments through relatively rapid rotation around most bonds. In methanol-d_4, in contrast to the behavior in DMSO-d_6, there is no such close correspondence of chemical shifts to those of the model molecules. In this solvent, the largely homogeneous chemical shifts in DMSO-d_6 have been replaced by marked chemical shift nonequivalence. This heterogeneity is attributed partly to the existence of a mixture of conformationally different dimers in the solvent. In addition, some of the heterogeneity may be due to carbon atoms residing in chemically equivalent, but magnetically nonequivalent, environments. This magnetic nonequivalence would result from possible nonaveraging among different conformational states in the relatively rigid dimer states. On the contrary, the spectrum of species 3 in 2-propanol is sharper and more simple, indicating that a single conformational dimer, suggested to be an antiparallel double helix,[12] is predominant.

The spin lattice relaxation (T_1) measurements[78] supplement the conclusion drawn from other studies and provide a good account of the motion of various carbon atoms in the molecule. Using the theory of Doddrell et al.[79] for the relation between T_1 and isotropic molecular rotation, which is indicated by correlation time τ_R, Fossel et al.[78] have determined τ_R values for the α-carbon atoms in gramicidin A in DMSO-d_6 and methanol-d_4 solvents. The normalized τ_R values (taking into consideration the viscosity of the solvent) in DMSO-d_6 range from 0.4 to 2.5 nsec, whereas in methanol-d_4 they are about 25 nsec. The observation that these normalized τ_R-values in gramicidin A change about an order of magnitude going from DMSO to methanol has a bearing on the structure of the molecule in both solvents. The values of τ_R in DMSO, when compared with τ_R values of random coils of peptides, establish that their flexibilities are essentially very similar. In contrast, the τ_R values in methanol compare very well with those found for polypeptides in the helical states, thereby suggesting a more rigid structure for gramicidin A in methanol.[78]

V. CONCLUSION

On the basis of theoretical conformational analysis, the poly-LD-peptides have been shown to adopt novel structures such as LD_k single helices and antiparallel (A_k) and parallel (P_k) coaxial double helices, apart from the classical single helices. This vast conformational freedom is especially due to the presence of L- and D-residues in alternation. The common feature of all these novel helices is the presence of a large central core; in that respect, they can be good models for the antibiotic ionophore gramicidin. The $C^\alpha-C^\beta$ bond in these structures is nearly perpendicular to the helix axis, suggesting that these novel helices are feasible for poly-LD-peptides with bulkier side chains as well. However, the conformational flexibility may not be as much as is reported for the poly-LD-peptides with an alanine side chain. The region that is allowed for poly(L-Ala-D-Ala) must be very general, and perhaps not all this region is permissible for the peptides with bulkier side chains.

In the double helices, particularly in A_3 and A_4, the available conformational space is quite large. The pattern of hydrogen bonding permits the formation of each of these double helices over a significant range of pitch (9.8 to 11 Å). In this respect, double helices have more conformationally allowed freedom and space than single helices. The type of double helical structure adopted by a poly-LD-peptide is strongly dependent on the size of the solvent molecules. The actual conformation of the polymer is further influenced by the nature of the amino acid side chain.

Experimental studies on PBDLG have shown that, under suitable conditions,this polymer can take up most of these structures. It has also been demonstrated that transconformations among these helices are easily inducible. This enormous conformational versatility that is exhibited is unprecedented in the field of polypeptides, both synthetic and natural. The conformational flexibility and transconformations among these structures may be attributed to the fact that these helices (LD_k, A_k, and P_k) have similar ϕ and ψ angles, all in the same stable β-region. Energy-wise, transition from one conformation to another does not seem to be unconvincing. The stability of single helices must not be compared with that of double helices as the two models are completely different. However, the conformational energy in a single strand of the double helix is similar to that calculated for the single helix (about −18.0 kcal/mol per LD-unit). The interconversion among the different types of double helices can be easily speculated from the distribution of conformational energy values. There are conformations in each of these types with similar energy values.

For gramicidin A, LD_3 and LD_4 from the single helix family and A_3 and A_4 from the double helix family can be good models. The ion-transducting activity of the gramicidin is believed to involve a dimeric structure with dimension required to span the lipid bilayer membrane. The proposed head-to-head aggregation of LD_4 helices satisfies the requirement of length and pore size for the formation of channels through lipid bilayer membranes. However, the evidence put forward for the LD_k-helix from solution studies (UV, NMR, CD) by Urry and his group is also interpreted convincingly by others to be contrary. Thus, it is possible to determine the CD and IR results for gramicidin as to be interpreted both for and against LD_k helices. The coupling constant measurements from NMR studies also cannot provide a decisive preference for one model over the other. This is solely because the ϕ-angles correlated with coupling constants are similar both for single and double helices.

The NMR studies along with correlation time (τ_R) measurements clearly indicate that in polar solvents gramicidin A in its monomeric state has no regular structure. The NMR spectra and kinetic and equilibrium constant measurements on the isolated gramicidin species in nonpolar solvents strongly suggest the formation of a complex dimer structure such as the coaxial double helices rather than head-to-head aggregation of single helices. The structures A_3 and A_4 (or P_3 and P_4) satisfy the criteria regarding the dimensions required to be a transmembrane channel.

From what has been stated above, it may be concluded that the structure of gramicidin A is not unique, mostly because it is highly solvent dependent. In the biological environment, which is a heterogeneous mixture of both polar and nonpolar regions, gramicidin A in its dimeric form as a transmembrane channel can exist either as a head-to-head aggregation of two single helices or as a coaxial double helix. A decisive preference for one of the models is a difficult proposition at present. Detailed crystal structure investigation may provide a solution to this problem.

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