Post, R. L., Hegyvary, C., & Kume, S. (1972) *J. Biol. Chem. 247*, 6530.
Robinson, J. (1981) *Biochim. Biophys. Acta 642*, 405.
Saenger, W. (1984) *Principles of Nucleic Acid Structure*, Springer-Verlag, New York.
Scheiner-Bobis, G., Buxbaum, E., & Schoner, W. (1987) Proceedings of the Fifth International Conference on Na,K-ATPase, Aarhus, Denmark, Abstract 66a.
Shull, G. E., Schwartz, A., & Lingrel, J. B. (1985) *Nature (London) 316*, 691.
Skou, J. C. (1960) *Biochim. Biophys. Acta 42*, 6.
Skou, J., & Hilberg, C. (1965) *Biochim. Biophys. Acta 110*, 359.
Solomon, I. (1955) *Phys. Rev. 99*, 599.
Solomon, I., & Bloembergen, N. (1956) *J. Chem. Phys. 25*, 261.
Stewart, J. M. MacD. (1987) Ph.D. Dissertation, University of Virginia.
Vilsen, B., Andersen, J. P., Petersen, J., & Jørgensen, P. L. (1987) *J. Biol. Chem.* (in press).
Viswamitra, M. A., Hosur, M. V., Shakked, Z., & Kennard, O. (1976) *Cryst. Struct. Commun. 5*, 819.

The Membrane as an Environment of Minimal Interconversion. A Circular Dichroism Study on the Solvent Dependence of the Conformational Behavior of Gramicidin in Diacylphosphatidylcholine Model Membranes†

J. Antoinette Killian,‡ Kari U. Prasad, Dorothy Hains, and Dan W. Urry*
Laboratory of Molecular Biophysics, School of Medicine, University of Alabama at Birmingham, P.O. Box 311, Birmingham, Alabama 35294

Received October 21, 1987; Revised Manuscript Received February 8, 1988

ABSTRACT: The conformation of gramicidin in diacylphosphatidylcholine model membranes was investigated as a function of the solvent in which peptide and lipid are initially codissolved. By use of circular dichroism it is demonstrated that, upon removal of the solvent and hydration of the mixed gramicidin/lipid film, it is the conformational behavior of the peptide in the organic solvent that determines its final conformation in dimyristoylphosphatidylcholine model membranes. As a consequence, parameters that influence the conformation of the peptide in the solvent also play an essential role, such as the gramicidin concentration and the rate of interconversion between different conformations. Of the various solvents investigated, only with trifluoroethanol is it possible directly to incorporate gramicidin entirely in the $\beta^{6.3}$-helical (channel) configuration. It is also shown that the conformation of gramicidin in the membrane varies with the peptide/lipid ratio, most likely as a result of intermolecular gramicidin–gramicidin interactions at higher peptide/lipid ratios, and that heat incubation leads to a conformational change in the direction of the $\beta^{6.3}$-helical conformation. Using lipids with an acyl chain length varying from 12 carbon atoms in dilauroylphosphatidylcholine to 22 carbon atoms in dierucoylphosphatidylcholine, it was possible to investigate the acyl chain length dependence of the gramicidin conformation in model membranes prepared from these lipids with the use of different solvent systems. It is demonstrated for each solvent system that the distribution between different conformations is relatively independent of the acyl chain length but that the rate at which the conformation converts toward the $\beta^{6.3}$-helical configuration upon heating of the samples is affected by the length of the acyl chain. The conversion to the $\beta^{6.3}$-helical configuration is fastest in the short-chained dilauroylphosphatidylcholine. Finally, the effect of chemical modification of gramicidin on its conformational behavior was investigated by using the N-terminal-modified derivatives desformylgramicidin and *N*-acetylprolyldesformylgramicidin, the tryptophan-substituted analogue 9-phenylalanylgramicidin, and tryptophan-formylated gramicidin. It is shown that, by codissolving peptide and lipid in trifluoroethanol, all these analogues can be incorporated in the $\beta^{6.3}$-helical conformation but that they differ in their interaction with Na ions, as determined by ^{23}Na NMR measurements.

The peptide antibiotic gramicidin is a hydrophobic linear pentadecapeptide produced by *Bacillus brevis*, strain ATCC 8185 (Katz & Demain, 1977). Although it is a relatively simple peptide, gramicidin exhibits a complex conformational behavior. Among the various conformations that have been proposed are single-stranded helices, which can be left- or right-handed and may vary in pitch (Urry et al., 1971; Ramachandran & Chandrasekaran, 1972), and double-stranded helices, which can also be left- or right-handed and vary in pitch and which in addition can run either parallel or antiparallel (Veatch et al., 1974; Sychev et al., 1980). Hybrid structures, consisting of partially intertwined helices, may also occur (Heitz et al., 1986), and interconversion via hybrid structures has been suggested (Urry et al., 1975). In organic solvents the distribution of conformations, as well as the kinetics of interconversion from one conformation to another,

† This work was supported in part by NIH Grant GM26898 to D. W.U. J.A.K. is a recipient of a stipend from the Netherlands Organization of Pure Research (ZWO).
‡ Present address: Department of Biochemistry, State University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands.

0006-2960/88/0427-4848$01.50/0

has been shown to be dependent upon the type of solvent, the gramicidin concentration, and the temperature (Veatch & Blout, 1974; Urry et al., 1975; Sychev et al., 1980).

In the membrane gramicidin can form ion-selective transmembrane channels (Hladky & Haydon, 1970, 1972; Myers & Haydon, 1972; Bamberg et al., 1976; Eisenman et al., 1978). The channel conformation of the peptide is believed to be an N-terminal to N-terminal hydrogen-bonded, single-stranded $\beta^{6.3}$-helical dimer (Urry et al., 1971, 1983; Weinstein et al., 1979; Wallace, 1986). The $\beta^{6.3}$-helical conformation in lipid has well-defined spectral features when measured with circular dichroism (CD)[1] (Masotti et al., 1980; Urry et al., 1983) which differ from spectral features found in organic solvents (Wallace, 1986). The conformational behavior of gramicidin in the membrane is complex as well. When gramicidin is added to lysophosphatidylcholine micelles or diacylphosphatidylcholine model membranes from a solution in TFE or dimethyl sulfoxide (DMSO), it directly incorporates in the $\beta^{6.3}$-helical configuration (Masotti et al., 1980; Tournois et al., 1987), whereas upon addition of the peptide as a dry powder or an ethanolic solution (Masotti et al., 1980) or a methanolic solution in the presence of Cs ions (Wallace, 1984), heating appears to be necessary to incorporate gramicidin in its channel configuration. When gramicidin-containing model membranes are prepared via hydration of a mixed lipid/peptide film, subsequent heating results in a change in the CD characteristics toward those indicative of the $\beta^{6.3}$-helix while the channel properties become more pronounced (Shungu et al., 1986).

The conformation of gramicidin is not only important for its channel properties; it also plays a decisive role in the effect of the peptide on the macroscopic organization of membrane lipids [for review, see Killian and De Kruijff (1986)]. In phosphatidylcholines with a chain length larger than 16 carbon atoms gramicidin induces a transition from a bilayer organization of the lipids to a hexagonal H_{II} phase (Van Echteld et al., 1982). Studies in which CD measurements were combined with the use of lipid-structure probing techniques strongly suggest that it is the $\beta^{6.3}$-helical conformation of gramicidin that is responsible for its H_{II} phase inducing activity (Tournois et al., 1987).

In view of the importance of the gramicidin structure for its various functional properties [see De Kruijff and Killian (1987)] and the number of efforts under way to carry out involved physical characterizations of the channel structure in lipids, it is necessary to investigate in greater detail the factors that determine the conformation of gramicidin in a lipid environment. Gramicidin-containing model membranes are usually prepared by codissolving peptide and lipid in an organic solvent followed by the removal of the solvent and hydration of the mixed lipid/peptide film. Circular dichroism evidence is presented here which demonstrates that the conformation of gramicidin in the organic solvent determines the conformation of the peptide in the membrane. However, heating of the samples as routinely done in this laboratory (Urry et al., 1979a; Masotti et al., 1980) leads to a slow conversion of the conformation of gramicidin toward the $\beta^{6.3}$-helical configuration, regardless of the solvent used. This conversion even occurs, though more slowly, at room temperature, and therefore in the lipid membrane, the $\beta^{6.3}$-helical conformation is clearly the thermodynamically preferred conformation.

[1] Abbreviations: CD, circular dichroism; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; T_1, longitudinal relaxation time; TFE, trifluoroethanol.

Since peptide aggregation and lipid acyl chain length may be of importance for both the channel properties of gramicidin and its H_{II} phase inducing activity, the effect of these parameters on the conformation of the peptide in the membrane was also investigated. It is demonstrated that the CD patterns of gramicidin are affected by the gramicidin/lipid ratio in that there is a shift in equilibrium between channel and nonchannel states but that the dependence on acyl chain length is more accurately described as changes in the intensity of the ellipticities of the channel state.

Finally, it was investigated whether N-terminal-modified analogues, which interfere with head to head hydrogen-bonded dimer formation, and tryptophan-modified analogues, which have a different conformational behavior (Prasad et al., 1983; Killian et al., 1987), can be incorporated in the $\beta^{6.3}$-helical conformation. The results are discussed in light of the implications of the conformational behavior of gramicidin for its functional properties and in terms of developing a standard procedure of sample preparation for the study of gramicidin structure, transport mechanisms, and lipid interactions.

Materials and Methods

Materials. Gramicidin from *B. brevis* was obtained from ICN Nutritional Biochemicals Corp. (Cleveland, OH) as a mixture of gramicidins A, B, and C (Sarges & Witkop, 1965a–c) and lyophilized from methanol/water before use. 1,2-Dilauroyl-*sn*-glycero-3-phosphocholine (di-C12:0-PC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (di-C14:0-PC), and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (di-C16:0-PC) were purchased from Avanti Biochemicals (Birmingham, AL). 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (di-C18:1_c-PC) and 1,2-dierucoyl-*sn*-glycero-3-phosphocholine (di-C22:1_c-PC) were a kind gift of W. S. M. Geurts van Kessel and were synthesized as described elsewhere (Van Deenen & De Haas, 1964).

All chemicals were of analytical grade. Trifluoroethanol was distilled before use.

Synthesis of Gramicidin Analogues. Gramicidin in which the indoles of the tryptophans were N-formylated was prepared as described previously (Killian et al., 1985), and the synthesis was verified with ^{1}H NMR. [1-^{13}C]Phe^9-gramicidin A was obtained via solid-phase synthesis and purified as described elsewhere (Prasad et al., 1983). For preparation of desformylgramicidin, the *N*-formyl group was removed from gramicidin A by reacting with HCl/dioxane for 4 h essentially following the earlier procedure (Sarges & Witkop, 1964) except that the acidolysis step was carried out in N_2 atmosphere. After removal of the solvent, the peptide was dissolved in methanol and passed through a column of AG 50W-X2 ion-exchange resin column (H^+ form) at 4 °C. Following initial elution with methanol to remove unreacted gramicidin, desformylgramicidin was obtained by eluting the column with methanolic ammonia solution. The solvent was evaporated, and the peptide was purified by precipitating from methanol/water. Desformylgramicidin A (0.29) in dimethylformamide (DMF) (2 mL) was reacted with *N*-acetyl-L-proline *p*-nitrophenyl ester (44.3 mg, 1.5 equiv). After the reaction was stirred overnight at room temperature, triethylamine (0.01 mL) was added and stirring continued until thin-layer chromatography (TLC) indicated the completeness of the reaction. After removal of DMF, the peptide was dissolved in methanol and precipitated by the addition of water. The precipitate was washed with saturated $NaHCO_3$ solution and water. *N*-Acetyl-L-prolyldesformylgramicidin in methanol was then passed through an AG 50W-X2 (H^+ form) ion-exchange resin column to remove any unreacted desformylgramicidin.

The syntheses of [1-^{13}C]Phe^9-gramicidin A, *N*-acetyl-L-prolyldesformylgramicidin, and desformylgramicidin were confirmed with ^{13}C NMR.

Preparation of Lipid Vesicles. Stock solutions of gramicidin and lipid were prepared in chloroform/methanol (1/1 v/v). The appropriate amounts of gramicidin and lipid were mixed and the solvent was removed on a rotary evaporator. The dry mixture was then dissolved in the organic solvent of choice. Details of gramicidin concentration and amounts of solvent used are given in the figure legends for each experiment. After incubation for 1 h at room temperature the solvent was removed by rotary evaporation (water bath at 45 °C) and the samples were dried overnight under high vacuum. The mixtures were hydrated in 10 mM NaCl in H_2O (pH 6.5) to a concentration of 0.25 mM gramicidin. The lipids were allowed to swell for 30 min at room temperature or, in case of di-C16:0-PC, at 45 °C. Next, the samples were sonicated at 25 °C (or at 45 °C in case of di-C16:0-PC) by using a Heat Systems Ultrasonics, Inc., sonicator cell disrupter (W-225R). After sonication for 2–3 min at power level 8 with a macrotip and a 30% duty cycle, the samples were centrifuged for 15 min at 20000*g* at 25 °C. The gramicidin concentration in the supernatant was determined by measurement of the absorbance after 30-fold dilution of the sample in methanol, using a molar extinction coefficient of 20 700 cm^{-1} M^{-1} at 280 nm. For *N*-acetyl-L-prolyldesformylgramicidin and desformylgramicidin, the same extinction coefficient was used. For Phe^9-gramicidin and for tryptophan-formylated gramicidin, 14 100 and 13 700 cm^{-1} M^{-1} were used, respectively (Killian et al., 1987). The lipid concentration was determined by a phosphorus assay (Dittmer & Wells, 1969). Prior to CD measurements and Na NMR experiments the sample was diluted with 10 mM NaCl to obtain a 0.2 mM gramicidin concentration. Significant pH changes were found neither upon hydration of the samples nor after sonication.

Circular Dichroism (CD). CD spectra were recorded at 30 °C on a Cary 60 spectropolarimeter as described earlier (Masotti et al., 1980), using a 0.2-mm optical path length cell. Unless otherwise indicated, spectra were recorded within 4 h after hydration of the samples.

^{23}Na Nuclear Magnetic Resonance. ^{23}Na NMR spectra were obtained on a JEOL FX-100 spectrometer operating at 26.3 MHz. Typical conditions were as follows: a memory of 2K, a 5000-Hz spectral width, a 300-ms interval time, and a 90° pulse of 35 μs. Spin–lattice relaxation times were measured by using the inversion–recovery method. All measurements were performed immediately after sonication and centrifugation at concentrations of 10 mM NaCl and 0.2 mM gramicidin.

Results

Solvent Dependence of the Conformation of Gramicidin in Di-C14:0-PC Dispersions. A common method for incorporating peptides in model membranes is to codissolve the peptide and the appropriate lipid in an organic solvent, followed by removal of the solvent by evaporation and drying under high vacuum. Hydration of the dry film then results in the formation of peptide-containing model membranes. Application of this method of incorporation to gramicidin/lipid systems indicates however that whether or not in the membrane gramicidin adopts the $\beta^{6.3}$-helical configuration, which has been concluded to be the functionally active channel conformation of gramicidin (Urry et al., 1983), is clearly solvent dependent (see Figure 1). The most extreme differences in conformation in the membrane were observed when TFE and ethanol were used to cosolubilize peptide and lipid. When gramicidin and

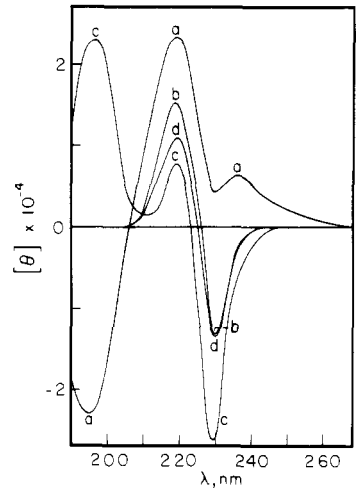

FIGURE 1: CD spectra of sonicated dispersions of gramicidin/di-C14:0-PC (1/10 molar ratio) mixtures in 10 mM NaCl, prepared from solutions containing 0.25 mM gramicidin in 1 mL of TFE (a), methanol (b), ethanol (c), or chloroform/methanol (1/1 v/v) (d).

di-C14:0-PC are originally codissolved in TFE, dichloromethane, or dichloroethane, the CD spectra of the peptide in the membrane are characteristic of the $\beta^{6.3}$-helix (Urry, 1985), displaying positive extrema at 218 and 235 nm with a weak negative inflection in between (at 229 nm) and negative ellipticity below 208 nm (Figure 1). When ethanol is used, the spectra are characterized by a large negative peak at 229 nm, a weaker positive peak at 218 nm, and most distinctively, a positive ellipticity below 208 nm. For methanol, and for a methanol/chloroform (1/1 v/v) mixture (as shown in Figure 1) as well as for all other solvents used (benzene, dichloromethane, dichloroethane, chloroform, and dioxane), intermediate situations are encountered, ranging from close to the CD pattern obtained with ethanol (dioxane) to close to that obtained from TFE (dichloromethane, dichloroethane). These spectra could reflect either a noninterconverting mixture of conformations or a dynamic equilibrium between different conformations.

The solvent dependence of the conformational behavior of gramicidin in phosphatidylcholine model membranes was further investigated by using TFE, ethanol, and chloroform/methanol (1/1 v/v) because (1) the solvents cover well the range of differences in conformational behavior, as judged from the CD spectra, and (2) they do not result in solubility problems over a wide range of gramicidin/lipid ratios. Of particular interest is chloroform/methanol, because it is a widely used solvent system in studies on gramicidin–lipid interactions.

If the conformation of gramicidin is determined by its conformational behavior in the solvent, in which peptide and lipid are originally codissolved, then one should expect that factors which affect the conformation of gramicidin in these particular solvents also affect the conformation in the membrane. One obvious parameter is the peptide concentration in the solvent. Previous CD studies (Urry et al., 1975) have shown that, upon increasing the concentration of hydrogenated gramicidin in various solvents, the ellipticity near 230 nm becomes increasingly negative. Indeed, when the gramicidin concentration in ethanol or chloroform/methanol is increased from 0.01 to 1 mM, drastic changes occur in the CD characteristics of the peptide in the membrane (Figure 2B,C) and the ellipticity at 230 nm becomes more negative. For TFE, the changes are less apparent. However, even in TFE, on further increasing the gramicidin concentration to 10 mM, differences are observed for this solvent as well (Figure 2A).

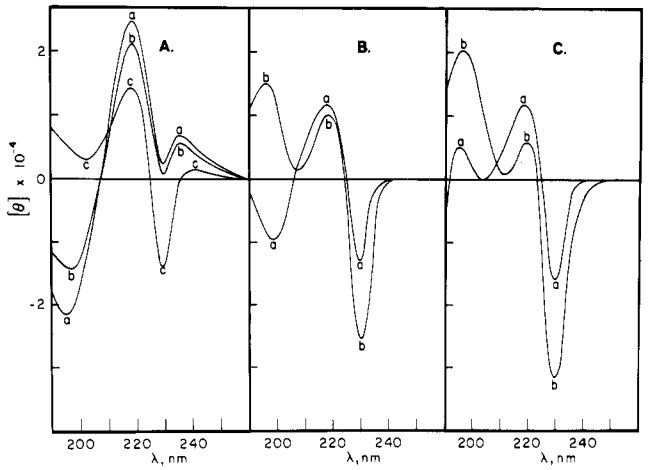

FIGURE 2: CD spectra of sonicated dispersions of gramicidin/di-C14:0-PC (1/10 molar ratio) mixtures in 10 mM NaCl, prepared from TFE (A), chloroform/methanol (1/1 v/v) (B), and ethanol (C) as a function of the gramicidin concentration in the organic solvent: (a) 0.01 mM, (b) 1.0 mM, and (c) 10 mM. The total amount of gramicidin was 0.5 μmol. For additional details, see text.

In the case of chloroform/methanol (1/1 v/v), it was found that the total volume of solvent for a given gramicidin concentration is also an important parameter. Distinct differences were observed in CD patterns of gramicidin in di-C14:0-PC systems, dried from a 0.25 mM gramicidin containing solution in either 1 or 10 mL total volume. When the volume was reduced, the CD characteristics changed toward those of the channel configuration.

A possible explanation for this is that, during evaporation of the solvent, the samples become concentrated. In this case, whether the total volume of solvent at a given concentration would affect the final configuration of gramicidin in the membrane would depend upon the rate of interconversion between the different conformations in the particular solvent systems. When ethanol or TFE was used, only minor differences in CD pattern were observed upon varying the total amount of solvent; by the above reasoning then the rate of interconversion in these solvents would have to be slow when compared to the rate of solvent evaporation. That the 1-h incubation time which was used in our method of sample preparation was sufficient to reach conformational equilibrium in these solvent systems was demonstrated by the observation that very similar CD patterns were obtained upon prolonging the incubation time to 12 h. Another explanation would involve the differential rate of solvent evaporation in the mixed solvent chloroform/methanol. As chloroform has the greater vapor pressure, rotary evaporation of larger volumes would result in conversion of the 1:1 mixed solvent to a greater proportion of methanol, with the resulting CD pattern reflecting more the mixture of conformations in methanol.

Since gramicidin–gramicidin interactions are thought to be important for H_{II} phase formation (Killian et al., 1987; Chupin et al., 1987) and since they also might play a role in channel functioning (Stark et al., 1986; Spisni et al., 1983), it was investigated whether the conformation of gramicidin in the lipid was dependent on the gramicidin/lipid ratio. From Figure 3 it can be seen that lowering the gramicidin/di-14:0-PC molar ratio from 1/10 to 1/50 while keeping the gramicidin concentration in the organic solvent constant leads to a change in the CD spectra toward the $\beta^{6.3}$-helical conformation, when chloroform/methanol or ethanol are used. In case of TFE only a minor difference is observed. In none of these samples did the gramicidin/lipid ratio change upon sonication and subsequent centrifugation. It should be noted

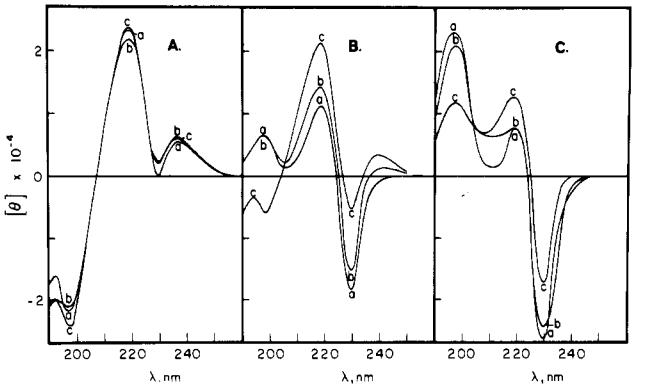

FIGURE 3: CD spectra of sonicated dispersions of mixtures of gramicidin and DMPC in molar ratios of 1/10 (a), 1/25 (b), and 1/50 (c), prepared from TFE (A), chloroform/methanol (1/1 v/v) (B), and ethanol (C). For each sample, 0.5 μmol of gramicidin was used in a total amount of 2 mL of solvent. For details, see text.

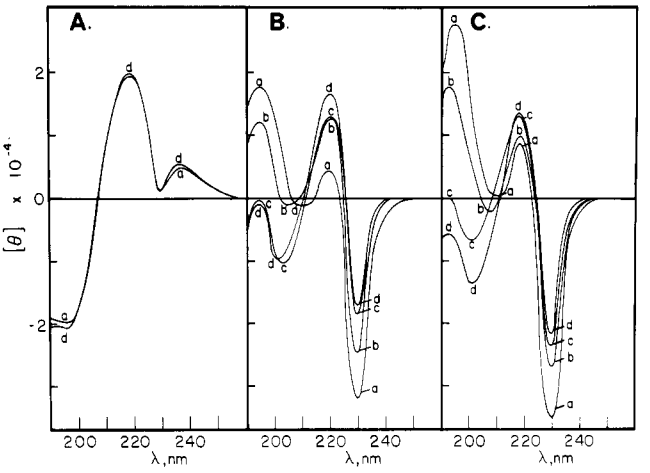

FIGURE 4: CD spectra of sonicated dispersions of gramicidin/di-C14:0-PC (1/10 molar ratio) mixtures in 10 mM NaCl, prepared from TFE (A), chloroform/methanol (1/1 v/v) (B), and ethanol (C) with different periods for heat incubation at 68 °C: (a) 0 h, (b) 1 h, (c) 3 h, and (d) 8 h. Heat incubation was carried out with unsonicated samples, prepared from a solution containing 2 μmol of gramicidin in 8 mL of solvent. Similar results were obtained when sonicated samples were used.

that, since the lipids themselves do not give rise to significant ellipticities in this concentration range, the observed differences in CD characteristics cannot be due to varying the absolute amount of lipids, and therefore it is proposed that they reflect an increase in gramicidin–gramicidin interactions within the membrane at higher peptide/lipid ratios. This is particularly likely since in the solvents themselves the CD characteristics did not show any change due to the presence of lipids (data not shown).

A factor that affects the conformational behavior of gramicidin in the membrane itself is heat incubation. This treatment has been reported to result in changes in the conformation of gramicidin toward the $\beta^{6.3}$-helical conformation (Masotti et al., 1980; Urry et al., 1979a,b) and is now even becoming a common step in sample preparation in studies on the channel conductance and channel structure (Short et al., 1987; Shungu et al., 1987). Figure 4 shows that, when gramicidin/di-C14:0-PC (1/10 molar ratio) samples are prepared from TFE, the $\beta^{6.3}$-helical conformation is obtained directly upon hydration and no spectral changes occur when the sample is incubated at 68 °C. In contrast, when ethanol or chloroform/methanol are used, a considerable proportion of the peptide is present in another conformation upon hydration of the peptide/lipid film. For samples prepared from

chloroform/methanol, a nearly identical spectrum is observed to those prepared from ethanol. This is in contrast to the results presented in Figure 1, in which quite different CD patterns were obtained upon using chloroform/methanol or ethanol, and is ascribed to the larger amount of solvent used. Upon heat incubation the spectra change and the spectral characteristics slowly convert toward those indicative of the $\beta^{6.3}$-helical conformation. These spectral changes are found to occur over prolonged incubation times and even occur at room temperature, though much more slowly. For a sample prepared with ethanol as the original solvent after 20 h of incubation at 68 °C, changes in CD pattern were still occurring, and even after 50 h the channel state had not been completely achieved. After 50-h incubation time of an unsonicated sample, ^{31}P NMR measurements (carried out as will be described elsewhere) showed that the characteristic "bilayer" type of line shape was unchanged, demonstrating that the bilayer organization of the lipids was still fully intact. Thin-layer chromatography on these samples did not show any lipid breakdown.

In order to obtain more information about the conformation of gramicidin in the various samples, ^{23}Na NMR longitudinal (T_1) relaxation experiments were performed. In its channel conformation gramicidin has a strong interaction with Na ions. When there is a fast exchange between Na ions interacting with the gramicidin channel and Na ions in the bulk solution, a marked decrease in T_1 occurs (Urry et al., 1979a). Indeed, when gramicidin/di-C14:0-PC (1/10 molar ratio) samples were prepared from TFE, the value of T_1 for 10 mM NaCl decreased from about 60 ms for pure di-C14:0-PC to about 40 ms in the presence of 0.2 mM gramicidin and did not change upon subsequent heating of the sample (data not shown). This is in agreement with the observation on the basis of the CD data that the conformation of the peptide remains unaltered (Figure 4). In contrast, and again in agreement with the CD data, in a gramicidin/lipid sample prepared from ethanol no significant decrease of T_1 was observed after hydration, whereas heat incubation led to a similar decrease in T_1 as found for samples prepared from TFE (data not shown).

Conformational Behavior of Gramicidin in Phosphatidylcholines with Different Acyl Chain Lengths. The data presented so far have examined the solvent dependence of the conformational behavior of gramicidin in di-C14:0-PC model membranes; in this lipid system gramicidin has no effect on the macroscopic lipid organization. The ability of gramicidin to induce H_{II} phase formation depends on the acyl chain length as well as on the gramicidin/lipid ratio (Van Echteld et al., 1982; Killian & DeKruijff, 1986). In di-C18:1$_c$-PC gramicidin induces H_{II} phase formation only at ratios of gramicidin to lipid of about 1/25 and higher. However, efforts to prepare sonicated vesicles at these or higher gramicidin/di-C18:1$_c$-PC ratios resulted in a loss of gramicidin during the centrifugation step. The amount of peptide loss increased with the gramicidin/lipid ratio and was almost quantitative at ratios higher than 1/15, a value that corresponds to the bilayer solubility of gramicidin in di-C18:1$_c$-PC model membranes (Killian et al., 1987). At a molar ratio of gramicidin to lipid of 1/30 no significant loss of peptide or lipid was observed, and therefore this ratio was used to investigate the chain-length dependence of the conformation of gramicidin in the saturated lipids di-C12:0-PC, di-C14:0-PC, and di-C16:0-PC and in the unsaturated lipids di-C18:1$_c$-PC and di-C22:1$_c$-PC. The unsaturated lipids were chosen not only because of the current interest in the gramicidin–di-C18:1$_c$-PC interaction but also because of the low gel to liquid-crystalline phase transition temperature

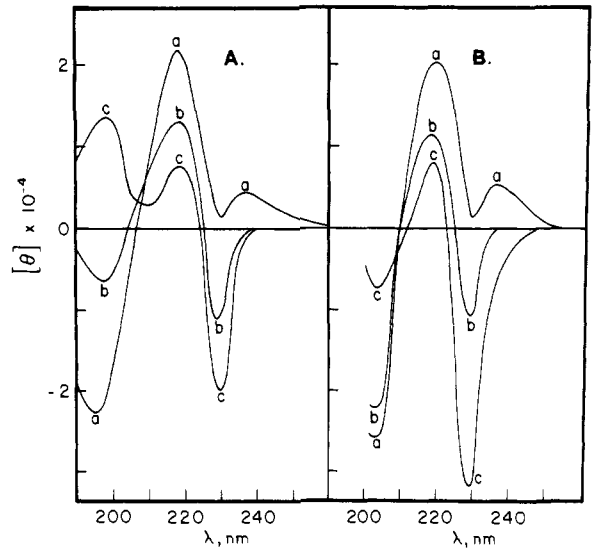

FIGURE 5: CD spectra of sonicated dispersions of mixtures of gramicidin with di-C12:0-PC (A) and di-C22:1$_c$-PC (B) in a 1/30 molar ratio of peptide to lipid prepared from TFE (a), chloroform/methanol (1/1 v/v) (b), and ethanol (c). For each experiment, 0.5 μmol of gramicidin was used in a total amount of 2 mL of solvent.

of this lipids as compared to their saturated homologues, which made it possible to avoid possible artifacts by sonication at very high temperature and by measurements during which the lipids are in the gel state. As is shown in panels A and B of Figure 5 for each solvent system used, CD spectra are obtained that are quite analogous for gramicidin incorporated into di-C12:0-PC and into di-C22:1$_c$-PC membranes. In these, as in the other lipid systems investigated (data not shown), the use of TFE leads to direct incoporation in the $\beta^{6.3}$-helical conformation, whereas for ethanol or chloroform/methanol, different CD spectra are obtained. For the latter solvent systems, the CD patterns differed slightly depending on the lipid system used. In the unsaturated lipids, the ellipticity could not be measured below 202 nm, and at that wavelength, a negative ellipticity was found even when ethanol was used as the solvent, as is shown in Figure 5B for gramicidin incorporated in di-C22:1$_c$-PC. For the saturated lipids, rather similar spectra were found, except that for ethanol the low-wavelength ellipticity was positive (Figure 5A). In samples prepared from ethanol and chloroform/methanol, heat incubation resulted in a change in the spectral characteristics toward those indicative of the $\beta^{6.3}$-helical conformation. After 4 h of incubation at 68 °C, the conformation of the peptide in the shorter chain lipid di-C12:0-PC was essentially fully converted to the $\beta^{6.3}$-helical conformation, whereas for the longer chain lipids this conversion was still incomplete (data not shown). No distinct differences in kinetics were observed among the longer chain lipids used. Incubation at room temperature for 24 h led to a relatively small change in spectral characteristics toward the $\beta^{6.3}$-helical configuration for samples prepared from both ethanol and chloroform/methanol, whereas in the gramicidin/di-C12:0-PC sample again the most effective conversion was observed, as deduced from the CD spectra (data not shown). For samples prepared from TFE no significant changes occurred upon heating or incubation at room temperature.

Conformational Behavior of Gramicidin Analogues in Phosphatidylcholine Model Membranes. It is aparent that in the long-chain di-C22:1$_c$-PC gramicidin can also adopt the $\beta^{6.3}$-helical configuration (see Figure 5B); this is in spite of the now obvious mismatch in length between the channel structure [25–30 Å (Urry et al., 1971); mean end to end length

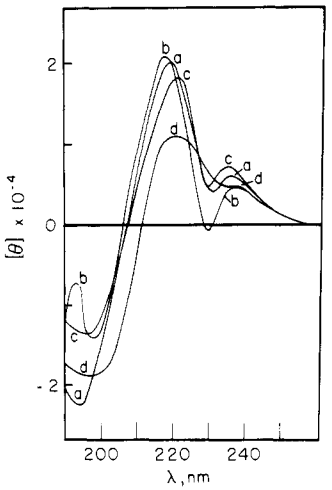

FIGURE 6: CD spectra of sonicated dispersions of *N*-acetyl-L-prolyldesformylgramicidin (a), desformylgramicidin (b), [1-^{13}C]Phe^9-gramicidin (c), and tryptophan-formylated gramicidin (d) in di-C14:0-PC in a 1/10 molar ratio of peptide to lipid. For each experiment, 0.5 μmol of gramicidin was used, dissolved in 2 mL of TFE.

of 26 Å (Urry et al., 1979b)] and lipid layer (thickness of hydrophobic layer = 34 Å; distance between phosphate groups = 45 Å) (Lewis & Engelman, 1983). Either the N–N terminal hydrogen bonds stay intact, preserving the dimeric nature of the channel possibly by lipid dimpling, or the dimer dissociates into two monomers with each in the $\beta^{6.3}$-helical conformation. In order to get more insight into this matter and especially into the possibility of the formation of gramicidin monomers in the $\beta^{6.3}$-helical conformation, the effects of N-terminal modification of the peptide on its CD characteristics were investigated. For this purpose desformylgramicidin was used; this does not as readily form conducting channels (Urry et al., 1971; Bamberg et al., 1976) presumably because the formation of a head to head dimer at neutral pH would be severely hampered by the presence of a positive charge at the N-terminus. Also *N*-acetyl-L-prolyldesformylgramicidin was used where the *N*-acetylL-prolyl grouping is expected to perturb the structure of the helix at the N-terminus, making the head to head association to form dimers less favorable.

Figure 6 (curves a and b) shows that incorporation of the peptide in di-C14:0-PC model membranes from TFE results in CD characteristics indicative of the $\beta^{6.3}$-helical conformation for *N*-acetyl-L-prolyldesformylgramicidin as well as for desformylgramicidin. This raises the possibility that indeed gramicidin monomers can be present in the $\beta^{6.3}$-helical conformation. However, large differences were found in the interaction of both analogues with Na ions (see Table I). For *N*-acetyl-L-prolyldesformylgramicidin a reduction in T_1 of Na ions was found as compared to the T_1 in a sonicated dispersion of pure di-C14:0-PC, whereas no effect on T_1 was observed when desformylgramicidin was incorporated. Thus, the binding sites of desformylgramicidin seem to be inaccessible to Na ions although the peptide appears to be in the channel conformation and with the tryptophan side chains apparently in similar orientation as in gramicidin itself, as deduced from the CD pattern. One possible explanation for this behavior (Jakobsson & Chiu, 1987) could be that the positive charge at the N-terminus induces an alignment of the dipoles of the water molecules within the channel which causes a repulsion of cations at the mouth of the channel. Alternatively, it is possible that, due to the positively charged N-terminus, desformylgramicidin prefers an orientation with the N-terminus at the lipid/water interface. The resulting repulsive electrostatic forces would block cation interactions with the channel.

Table I: ^{23}Na NMR Longitudinal (T_1) Relaxation Times of Sonicated Dispersions of Di-C14:0-PC and Mixtures of Di-C14:0-PC and Gramicidin Analogues in a 1/10 Molar Ratio of Peptide to Lipid[a]

sample	T_1 (ms)
di-C14:0-PC	60
di-C14:0-PC	
+gramicidin	40
+desformylgramicidin	60
+*N*-acetyl-L-prolyldesformylgramicidin	50
+Phe^9-gramicidin A	48
+tryptophan-N-formylated gramicidin	59

[a] Samples contained 0.25 mM peptide and 10 mM NaCl. Measurements were performed at 30 °C. The experimental error is estimated to be ±3 ms.

Whether, by using TFE, it is possible to incorporate the tryptophan-substituted analogue Phe^9-gramicidin in the $\beta^{6.3}$-helical conformation was also investigated. For this analogue it was shown that the conventional method of incorporation by addition of the peptide as a dry powder to lysophosphatidylcholine (LPC) micelles, followed by heating, does not result in an adquate incorporation in the $\beta^{6.3}$-helical conformation for ion NMR studies (Prasad et al., 1983; Hinton et al., 1987). Figure 6, curve c, shows that, by using TFE, a CD spectrum of the Phe^9 analogue can be obtained, in di-C14:0-PC model membranes, which is characteristic of channel formation. A decrease in T_1 of Na ions was observed upon incorporation of this analogue which was somewhat less than for gramicidin itself (Table I). When the same method of sample preparation is applied with tryptophan-N-formylated gramicidin, the CD data suggest that incorporation of the peptide in the $\beta^{6.3}$-helical conformation has occurred, with differences in CD pattern as would be expected due to modification of the chromophore of the tryptophan indole rings (Figure 6, curve d). However, in contrast to Phe^9-gramicidin, this analogue exhibited no Na interaction at all (Table I). This illustrates the importance of the tryptophan residues for proper functioning of gramicidin as a channel.

DISCUSSION

An important observation in this paper is that the conformation of gramicidin in model membranes is determined by the solvent from which the dispersions are prepared. Although the conformation of the peptide in the membrane is not demonstrably identical with the one in the solvent, there is a unique relationship between them. The CD characteristics of hydrogenated gramicidin in TFE [positive ellipticity at 220 nm with no band at 230 nm (Urry et al., 1975)], of gramicidin methanol [a slightly negative ellipticity at 230 nm (Prasad et al., 1983)], and of gramicidin in ethanol [a very large negative ellipticity at 230 nm (Veatch & Blout, 1974; Veatch et al., 1974)] are reflected in the CD spectra of gramicidin in a lipid dispersion prepared from these solvents. Even more convincing is the observation that the concentration-dependent changes in the conformational equilibrium of gramicidin conformers in these solvents are expressed in the CD characteristics of gramicidin in the membrane (see Figure 2).

Before discussing what the various CD patterns in the membrane represent, a short review of the literature on the conformation of gramicidin in organic solution is warranted. In TFE gramicidin most likely has a conformation similar to that in DMSO (Urry et al., 1972), in which solvent the structure was considered consistent with a left-handed $\beta^{6.3}$-helix. This has been demonstrated by the recent two-dimensional NMR data of Hawkes et al. (1987). It would explain

well the ease with which gramicidin is incorporated as the $\beta^{6.3}$-helical configuration when dispersions are prepared from TFE, and it is also in agreement with the observation that, upon external addition of the peptide to preformed vesicles, the $\beta^{6.3}$-helical conformation is obtained immediately when the peptide is introduced dissolved in either DMSO or TFE (Masotti et al., 1980; Tournois et al., 1987). In methanol, for which similar results were obtained as for the (1/1) mixture of chloroform and methanol (see Figure 1), a double-helical structure was proposed, on the basis of ^{13}C NMR data (Fossel et al., 1974), whereas HPLC measurements demonstrated a monomeric conformation in methanol (Braco & Abad, 1986). This discrepancy can be easily attributed to the differences in peptide conformation in the solvent in both studies and is in agreement with the concentration-dependent differences observed in the CD spectra (Urry et al., 1975). The HPLC measurements were performed at a low peptide concentration, under which conditions for ethanol an equilibrium between monomers and dimers was shown (Braco & Abad, 1986). Earlier studies (Veatch & Blout, 1974; Veatch et al., 1974) indicated the presence of four different conformational species in ethanol, the dominant one being an antiparallel dimer. This was recently confirmed with the use of two-dimensional NMR techniques (Arseniev et al., 1984) using dioxane, a solvent in which the dominant conformation in ethanol could be isolated.

Extrapolating these findings to the situation in the membrane suggests the following. When gramicidin/lipid films are prepared from TFE, the peptide is present in the single-stranded $\beta^{6.3}$-helical conformation and as such is readily incorporated into the membrane. There are differences in the CD patterns between when gramicidin is in a given organic solvent and when it is in the membrane after having been incorporated into the membrane by using that organic solvent. This may be due to the lipid environment altering the orientations of the tryptophan side chains and possibly due to the backbone becoming less flexible and having differences in helical pitch. When gramicidin/lipid dispersions are prepared from ethanol, it is suggested that the peptide initially incorporates primarily as an antiparallel dimer, whereas upon drying from methanol or chloroform/methanol the peptide is incorporated as a mixture of conformers. However, prolonged heat treatment of these samples leads to a conversion to the $\beta^{6.3}$-helical conformation, which appears to be the thermodynamically preferred configuration of gramicidin in the membrane. Since the kinetics of this conformational change are very slow, especially at room temperature, it is argued that the membrane can be regarded as an environment of minimal interconversion for gramicidin conformers.

A second important implication of this study is that the N–N hydrogen-bonded dimeric structure (the dimeric channel configuration) may not be a prerequisite to obtain a CD pattern characteristic of the $\beta^{6.3}$-helical conformation. Furthermore, even though the $\beta^{6.3}$-helical conformation is present, ion interaction can be blocked as in the case of desformylgramicidin. Two especially intriguing observations in view of the effect of gramicidin on lipid structure are (1) that, in lipids with a bilayer thickness larger than the gramicidin channel length, the peptide appears to be present in the $\beta^{6.3}$-helical conformation and (2) that gramicidin may be present as a monomer in membranes in the $\beta^{6.3}$-helical configuration, on the basis of the CD patterns of N-terminal-modified analogues (Figure 6). These observations are in agreement with the notions (1) that the chain-length dependence of H_{II} phase formation (Van Echteld et al., 1982) may be attributed to dissociation of N–N dimers, induced by the mismatch between the length of the gramicidin dimer and the lipid bilayer thickness (Killian & De Kruijff, 1987), (2) that the $\beta^{6.3}$-helical conformation, but not the N–N hydrogen-bonded dimer, is required for H_{II} phase formation (Brasseur et al., 1987; Tournois et al., 1987), and (3) that the choice of solvent is not only decisive for the relative amount of gramicidin present in the $\beta^{6.3}$-helical conformation but it also determines the extent of H_{II} phase formation (Tournois et al., 1987).

The observations, in particular, of the nature of the acyl chain length dependence of the CD pattern (Figure 5), and of the effect of N-terminal modification (Figure 6), are in apparent contrast to earlier published data (Wallace et al., 1981). However, this discrepancy can most likely be accounted for by method of incorporation, that is, by the multiplicity of parameters that have been shown to influence the gramicidin conformation, such as the nature of the organic solvent, the gramicidin concentration, the amount of solvent, the temperature, the incubation time in the solvent, the rate of evaporation of the solvent, and the history of the sample after hydration. All these parameters provide a likely source of reproducible, but possibly misleading, differences in the CD characteristics after incorporation in the membrane.

One might wonder whether, in black lipid membrane studies, the solvent from which gramicidin is added could affect the properties of the channel. This could be considered less likely for two reasons: (1) in general, gramicidin is added to the membrane as an extremely dilute solution ($\sim 10^{-8}$ M) where it is most likely present as a monomer in any solvent, and (2) the gramicidin conformation is dependent on the gramicidin/lipid ratio (see Figure 4), which probably can be attributed to the occurrence of intermolecular gramicidin–gramicidin interactions at higher molar ratios. Thus, the extremely low gramicidin/lipid ratio, used in black lipid membrane studies, would favor occurrence of gramicidin in the $\beta^{6.3}$-helical conformation. It should also be appreciated however for the black lipid membrane studies that gramicidin in an organic solvent is introduced into a large aqueous bath such that gramicidin molecules insoluble in water have become dispersed in a distribution which results from the state that occurred in the organic solvent. Accordingly, the gramicidin could readily enter the membrane in the $\beta^{6.3}$-helical conformation but with different distributions of side chain rotameric states. Thus, if changing side chain distributions alter the conductance state of the gramicidin channel, then the state of gramicidin in the different solvents could result in different dispersities of conductance states.

Finally, it is important to emphasize that, in order to allow comparison of structural data on the gramicidin channel in lipid model membranes, it is necessary to use a uniform incorporation procedure. As a convenient method it is suggested, on the basis of the results reported in this paper, to first codissolve gramicidin with the appropriate lipid in TFE. Then, in particular when high absolute amounts of peptide are required, an additional heat incubation might be applied. In Figure 2, it is shown that even in TFE when a 10 mM solution of gramicidin is used, the characteristics of complete incorporation of the channel state are not obtained. Upon heating this sample at 68 °C, the conformation changed in a direction toward the $\beta^{6.3}$-helical configuration, and after 4 h no further changes were observed (data not shown). Further proof of the value of the preparation of samples from TFE is demonstrated by the CD spectra of the tryptophan-modified gramicidin analogues. Prior to this study, incorporation of such analogues in the $\beta^{6.3}$-helical conformation has not been possible (Prasad et al., 1983). Although other parameters might be

involved that are yet unknown, application of this procedure will decrease major differences in the structural distributions of gramicidin in membranes and therefore will allow for a better comparison of experimental data using different physical methods and from different laboratories.

Registry No. Di-C12:0-PC, 18194-25-7; di-C14:0-PC, 18194-24-6; di-C16:0-PC, 63-89-8; di-C18:1_c-PC, 4235-95-4; di-C22:1_c-PC, 51779-95-4; Na, 7440-23-5; trifluoroethanol, 75-89-8; gramicidin, 1405-97-6; Phe^9-gramicidin A, 109050-08-0; desformylgramicidin A, 63808-16-2; *N*-acetyl-L-prolyldesformylgramicidin, 114378-80-2; *N*-acetyl-L-proline *p*-nitrophenyl ester, 96153-30-9.

REFERENCES

Arseniev, A. S., Bystrov, V. F., Ivanov, V. T., & Ovchinnikov, Y. A. (1984) *FEBS Lett. 165*, 51–56.

Bamberg, E. H., Noda, K., Gross, E., & Lauger, P. (1976) *Biochim. Biophys.* Acta *419*, 223–228.

Braco, L., & Abad, C. (1986) *J. Chromatogr. 353*, 181–192.

Brasseur, R., Killian, J. A., De Kruijff, B., & Ruysschaert, J. M. (1987) *Biochim. Biophys. Acta 903*, 11–17.

De Kruijff, B., & Killian, J. A. (1987) *Trends Biochem. Sci. (Pers. Ed.) 12*, 256–257.

Dittmer, J. D., & Wells, M. A. (1969) *Methods Enzymol. 14*, 482–530.

Eisenman, G., Sandblom, J., & Neher, E. (1978) *Biophys. J. 22*, 307–340.

Fossel, E. T., Veatch, W. R., Ovchinnikov, Y. A., & Blout, E. R. (1974) *Biochemistry 13*, 5264–5275.

Hawkes, G. E., Lian, L.-Y., Randall, E. W., Sales, K. D., & Curzon, E. H. (1987) *Eur. J. Biochem. 166*, 437–445.

Heitz, F., Heitz, A., & Trudelle, Y. (1986) *Biophys. Chem. 24*, 149–160.

Hinton, J. F., Koeppe, R. E., Shungu, D., Whaley, W. L., Paczowski, J. A., & Millett, F. S. (1986) *Biophys. J. 49*, 571–577.

Hladky, S. B., & Haydon, D. A. (1970) *Nature (London) 225*, 451–453.

Hladky, S. B., & Haydon, D. A. (1972) *Biochim. Biophys. Acta 274*, 294–312.

Jakobsson, E., & Chiu, S.-W. (1987) *Biophys. J. 52*, 33–45.

Katz, E., & Demain, A. L. (1977) *Bacteriol. Rev. 41*, 449–474.

Killian, J. A., & De Kruijff, B. (1986) *Chem. Phys. Lipids 40*, 259–284.

Killian, J. A., & De Kruijff, B. (1988) *Biophys. J. 53*, 111–117.

Killian, J. A., Timmermans, J. W., Keur, S., & De Kruijff, B. (1985) *Biochim. Biophys. Acta 820*, 154–156.

Killian, J. A., Burger, K. N. J., & De Kruijff, B. (1987) *Biochim. Biophys. Acta 897*, 269–284.

Kolb, H.-A., & Bamberg, E. (1977) *Biochim. Biophys. Acta 464*, 127–141.

Lewis, B. A., & Engelman, D. M. (1983) *J. Mol. Biol. 166*, 211–217.

Masotti, L., Spisni, A., & Urry, D. W. (1980) *Cell Biophys. 2*, 241–251.

Prasad, K. U., Trapane, T. L., Busath, D., Szabo, G., & Urry, D. W. (1983) *Int. J. Pept. Protein Res. 22*, 341–347.

Ramachandran, G. N., & Chandrasekaran, R. (1972) *Indian J. Biochem. Biophys. 9*, 1–11.

Sarges, R., & Witkop, B. (1965a) *J. Am. Chem. Soc. 87*, 2011–2027.

Sarges, R., & Witkop, B. (1965b) *J. Am. Chem. Soc. 87*, 2027–2030.

Sarges, R., & Witkop, B. (1965c) *Biochemistry 4*, 2491–2494.

Short, K. W., Wallace, B. A., Myers, R. A., Fodor, S. P. A., & Dunker, A. K. (1987) *Biochemistry 26*, 557–562.

Shungu, D. C., Hinton, J. F., Koeppe, R. E., & Millett, F. S. (1986) *Biochemistry 25*, 6103–6108.

Spisni, A., Pasquali-Ronchetti, I., Casali, E., Lindner, E., Cavatorta, P., Masotti, L., & Urry, D. W. (1983) *Biochim. Biophys. Acta 732*, 58–68.

Stark, G., Strassle, M., & Tackaz, Z. (1986) *J. Membr. Biol. 89*, 23–27.

Sychev, S. V., Nevskaya, N. A., Jordanov, St., Shepel, E. N., Miroshnikov, A. I., & Ivanov, V. T. (1980) *Bioorg. Chem. 9*, 121–151.

Tournois, H., Killian, J. A., Urry, D. W., Bokking, O. R., De Gier, J., & De Kruijff, B. (1987) *Biochim. Biophys.* Acta *905*, 222–226.

Urry, D. W. (1985) in *The Enzymes of Biological Membranes* (Martonosi, A. N., Ed.) pp 229–257, Plenum, New York.

Urry, D. W., Goodall, M. C., Glickson, J. D., & Mayers, D. F. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1907–1911.

Urry, D. W., Glickson, J. D., Mayers, D. F., & Haider, J. (1972) *Biochemistry 11*, 487–493.

Urry, D. W., Long, M. M., Jacobs, M., & Harris, R. D. (1975) *Ann. N.Y. Acad. Sci. 264*, 203–220.

Urry, D. W., Spisni, A., & Khaled, M. A. (1979a) *Biochem. Biophys. Res. Commun. 88*, 940–949.

Urry, D. W., Spisni, A., Khaled, M. A., & Long, M. M. (1979b) *Int. J. Quantum Chem., Quantum Biol. Symp. No. 6*, 289–303.

Urry, d. W., Trapane, T. L., & Prasad, K. U. (1983) *Science (Washington, D.C.) 221*, 1064–1067.

Van Deenen, L. L. M., & De Haas, G. H. (1964) *Adv. Lipid Res. 2*, 168–363.

Van Echteld, C. J. A., De Kruijff, B., Verkleij, A. J., Leunissen-Bijvelt, J., & De Gier, J. (1982) *Biochim. Biophys. Acta 692*, 126–138.

Veatch, W. R., & Blout, E. R. (1974) *Biochemistry 13*, 5257–5263.

Veatch, W. R., Fossel, E. T., & Blout, E. R. (1974) *Biochemistry 13*, 5249–5256.

Wallace, B. A. (1984) *Biophys. J. 45*, 114–116.

Wallace, B. A. (1986) *Biophys. J. 49*, 295–306.

Wallace, B. A., Veatch, W. R., & Blout, E. R. (1981) *Biochemistry 20*, 5754–5760.

Weinstein, S., Wallace, B. A., Blout, E. R., Morrow, J. S., & Veatch, W. R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 7230–7234.