Thermodynamic, Motional, and Structural Aspects of Gramicidin-Induced Hexagonal H_{II} Phase Formation in Phosphatidylethanolamine

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ABSTRACT: The effect of gramicidin incorporation on the thermodynamic properties of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) dispersions was investigated by differential scanning calorimetry. The results show that incorporation of gramicidin in PC systems results in a decrease of the energy content of the gel to liquid-crystalline phase transition. When incorporated in PE systems, however, the peptide does not affect the properties of the gel to liquid-crystalline phase transition with the exception that at high gramicidin concentrations the onset of the melting process is shifted to a slightly lower temperature. We therefore assume that in the lamellar gel state of PE aggregation of the peptide occurs. To get more insight into the nature of the gramicidin–PE interaction, we studied the motional and structural details of H_{II} phase formation in gramicidin/PE systems with the use of ^{31}P and ^{13}C nuclear magnetic resonance (NMR) and small-angle X-ray diffraction. In agreement with earlier results [Van Echteld, C. J. A., Van Stigt, R., de Kruijff, B., Leunissen-Bijvelt, J., Verkleij, A. J., & De Gier, J. (1981) *Biochim. Biophys. Acta 648*, 287–291] it was shown that gramicidin incorporation lowers and broadens the bilayer to hexagonal H_{II} phase transition in PE systems. ^{31}P NMR chemical shift anisotropy (CSA) measurements indicated that a phase separation occurs between a gramicidin-poor lamellar phase and a gramicidin-rich H_{II} phase. From combined CSA and spin–lattice relaxation time (T_1) measurements it was suggested that in the H_{II} phase gramicidin decreases the molecular order and increases the rate of motion of the phosphate moiety of PE. In addition, ^{13}C NMR line width measurements indicated that the acyl chains are more disordered in the H_{II} phase than in the lamellar phase and that a similar disorder occurs in the H_{II} phase of the pure PE as in the gramicidin-rich H_{II} phase. This interpretation was supported by the X-ray diffraction data, which show similar first-order repeat distances in both types of H_{II} phase. From saturation-transfer NMR experiments in PE and gramicidin–PE mixtures it was shown that no exchange occurs between the lamellar and the H_{II} phases in the time scale of 1–2 s, suggesting a macroscopic phase separation. Finally, we discussed the gramicidin–lipid interaction and in particular the H_{II} phase formation by gramicidin in PE and in PC systems. It is proposed that aggregation of the peptide plays a crucial role in H_{II} phase formation.

Biological membranes are commonly described as fluid bilayers in which proteins are embedded. Such an organization explains well the main barrier function of membranes. However, there are several processes occurring in biological membranes, such as fusion, protein insertion, and translocation across membranes, during which the fluid bilayer has to be temporarily disrupted. In these and related processes nonbilayer structures have been suggested to play a role [for a review, see de Kruijff et al. (1985a,b)]. From model membrane experiments detailed insight has been obtained into the way in which bilayer to nonbilayer transitions can be induced. While dispersion of pure membrane lipids in aqueous solution under physiological conditions will in general exclusively result either in spontaneous organization of the lipids in lamellar structures or in the formation of a hexagonal H_{II} phase depending on the type of lipid, it appeared possible to affect this lipid-phase preference, e.g., by changes in pH (Farren et al., 1983; Seddon et al., 1983), temperature (Cullis & de Kruijff, 1978a), or hydration (Seddon et al., 1984), by the use of divalent cations (Rand & Sengupta, 1972; Vasilenko et al., 1982) or by lipid–protein interactions [for a review, see de Kruijff et al. (1985a,b)]. As modulators of local lipid structure in membrane functioning these latter interactions most likely are the biologically most relevant. In studies of lipid–protein interactions gramicidin has been widely used as a model for the membrane-spanning part of intrinsic membrane proteins (Chapman et al., 1977; Rice & Oldfield, 1979). This hydrophobic peptide consists of 15 alternating L- and D-amino acids and is able to form transmembrane channels through which small cations can pass the bilayer. In its channel configuration the peptide is believed to be present as an N–N terminal dimer, held together by hydrogen bonds (Urry, 1971).

Only recently it was discovered that gramicidin exerts a dramatic influence on lipid-phase behavior in model membrane systems. When incorporated in PC's,[1] a typical bilayer-forming class of lipids, gramicidin is able to induce the formation of a hexagonal H_{II} phase when the length of the acyl chain exceeds 16 carbon atoms (Van Echteld et al., 1982). Mixtures of gramicidin and lysophosphatidylcholine, which on its own prefers a micellar organization, form lamellar

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[1] Abbreviations: CSA, chemical shift anisotropy; DEPC, 1,2-dielaidoyl-*sn*-glycero-3-phosphocholine; DEPE, 1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine; DLPE, 1,2-dilauroyl-*sn*-glycero-3-phosphoethanolamine; DMPE, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DSC, differential scanning calorimetry; EDTA, ethylenediaminetetraacetic acid; H_{II} phase, hexagonal phase of type II; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; T_1, spin–lattice relaxation time; Tris, tris(hydroxymethyl)aminomethane; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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structures with an apparently fixed stoichiometry of one gramicidin molecule per four lipid molecules (Killian et al., 1983). In PE systems, which show a temperature-dependent bilayer to H_{II} phase transition, gramicidin lowers and broadens this transition (Van Echteld et al., 1981). This bilayer destabilization is so strong that even under conditions in which PE in the absence of gramicidin would be organized in a lamellar gel state gramicidin can induce the formation of the liquid-crystalline H_{II} phase.

To obtain a better molecular understanding of the way in which gramicidin is able to induce these changes in lipid-phase behavior, we studied the thermodynamic details of the interaction of gramicidin with PE and PC systems by means of DSC, and we further investigated the structural and motional aspects of the gramicidin–PE interaction by characterizing and comparing the H_{II} phase formation both in the pure lipid system and in mixed lipid/peptide dispersions, using ^{31}P and ^{13}C NMR and small-angle X-ray diffraction.

Materials and Methods

Chemicals. Gramicidin from *Bacillus brevis* was obtained from Sigma (St. Louis, MO) and used as such. DEPE, DOPE, DMPE, DLPE, DPPC, and DEPC were synthesized according to the procedure of Van Deenen & De Haas (1964). The lipids were purified by high-performance liquid chromatography as described by Dekker et al. (1983) and judged chromatographically pure by the use of high-performance thin-layer chromatography. All other chemicals were of analytical grade.

Differential Scanning Calorimetry (DSC). Samples of 10 μmol of lipid and a variable amount of gramicidin in chloroform/methanol (1/1 v/v) were dried under a stream of N_2 and stored overnight under high vacuum. The samples were then dispersed in 1 mL of a 150 mM NaCl, 10 mM Tris-HCl, and 0.2 mM EDTA buffer, pH 7.0 at temperatures of 40 and 45 °C and at room temperature for DEPE, DPPC, and DEPC, respectively. Subsequently, the samples were spun for 15 min at 4 °C at 30000*g*. The pellet was transferred to a 20-μL volatile aluminum sample pan. The samples were scanned with a heating rate of 5 °C/min in a Perkin-Elmer DSC-2 calorimeter. All samples were scanned at least 3 times, yielding identical thermograms. After the measurements the pans were opened with a forceps, and the lipids were dissolved by sonication in chloroform/methanol (1/1 v/v). After subsequent perchloric acid destruction of the lipids the amount of phospholipid originally present in the sample pans was determined by the method of Fiske & Subbarow (1925). Enthalpies were calculated by integrating the peak areas with a Hewlet-Packard digitizer (type 9864A) and relating them to the total amount of phospholipid present. The estimated maximal experimental error was 0.4 kcal/mol. The calorimeter was calibrated by using the enthalpy of the gel to liquid-crystalline phase transition of DPPC (8.0 kcal/mol; Petri et al., 1980) as a reference.

Nuclear Magnetic Resonance (NMR). NMR samples were prepared as follows: 150 μmol of lipid and the appropriate amount of gramicidin in chloroform/methanol (1/1 v/v) were evaporated to dryness in a 10-mm NMR tube under a stream of N_2, followed by overnight storage under high vacuum. Dispersions were prepared by adding 1.0 mL of a 150 mM NaCl, 10 mM Tris/acetate, and 0.2 mM EDTA buffer, pH 7.0, containing 25% (v/v) D_2O, and incubating for several hours at 40 °C. All spectra were recorded on a Bruker WP-200 spectrometer.

^{31}P NMR. Proton noise decoupled 81.0-MHz ^{31}P NMR spectra were obtained by using a gated decoupling method with a 5-W input power during the data acquisition (0.1 s). A spectral width of 25 kHz, an 18-μs 90° pulse, a memory of 4K data points, and an interpulse delay of 1 s were employed. A total of 1000–5000 free induction decays were accumulated, and prior to the Fourier transformation an exponential multiplication was applied resulting in a 50-Hz line broadening.

The relative amounts of bilayer and H_{II} components in the ^{31}P NMR spectra were determined either by computer subtraction of the pure bilayer component and integration or, with similar results, by estimating the line shape of the pure components, followed by cutting and weighing. The maximal error was estimated to be 5%. The residual chemical shift anisotropy was measured as 3 times the distance between the chemical shift position of isotropically moving phospholipids and the high- or low-field peak in spectra of phospholipids organized in a lamellar or H_{II} phase, respectively. The estimated error in the CSA measurements is approximately 1 ppm.

T_1 relaxation times were measured by using the conventional 180°–τ–90° pulse sequence. A relaxation delay of 10 s was used.

Saturation-transfer experiments were performed as described by Van Duijn et al. (1985). Prior to the 90° radio-frequency (rf) pulse, selective saturation was obtained by irradiating the sample during 1 s with a frequency corresponding to the resonance frequency of the low-field shoulder of the bilayer component of the spectrum. An attenuation of 30 dB was used. Control spectra were obtained by using a frequency setting 14-kHz upfield. A 10-μs interval was used between saturation and the observed pulse. The interpulse time in the saturation transfer experiments was 1.1 s.

^{13}C NMR. Natural abundance ^{13}C NMR spectra were recorded at 50.3 MHz. A total of 5000–10 000 free induction decays were accumulated; 8K data points, a spectral width of 10 kHz, an 18-μs 90° rf pulse, and a 1-s interpulse time were used. To obtain maximal sensitivity without affecting dipolar ^1H–^{13}C interactions, the spectra were recorded with inverse gated decoupling and with a 2-W input power during 0.9 s prior to the rf pulse. Prior to the measurements the field homogeneity was optimized by shimming the magnet on a pure D_2O sample. Care was taken to ensure an identical positioning of the NMR tubes between the receiver coils in order to obtain a similar field homogeneity in the various samples during the measurements. The reported line widths at half-height were corrected for the 20-Hz line broadening applied to the free induction decay prior to Fourier transformation. The final error in the line width measurements was estimated to be 10%.

Small-Angle X-ray Diffraction. X-ray experiments were performed on a Kratky camera with a 10 × 0.2 mm Cu Kα beam (40 kV, 20 mA) equipped with a position-sensitive detector (LETI). Pelleted samples of hydrated lipid/peptide films were mounted in a temperature-controlled slit (16 × 1.5 × 1.5 mm) between two sheets of cellophane. X-ray diffraction profiles were obtained from 5–10-min exposure times after 15 min of temperature equilibration.

Results

Differential Scanning Calorimetry. The thermodynamic aspects of gramicidin–lipid interactions were investigated by using DSC. In Figure 1 heating curves of DEPE dispersions and mixtures of DEPE and gramicidin are shown. Pure DEPE shows a major peak at 37 °C and a minor peak at 62 °C, which correspond to the gel to liquid-crystalline ($L_\beta \rightarrow L_\alpha$) and the bilayer to hexagonal H_{II} phase transitions, respectively (Gallay & de Kruijff, 1984). When gramicidin is incorporated in DEPE in a peptide to lipid molar ratio of 1 to 50, the chain melting transition is not visibly affected. Even when present in a high molar ratio of 1 to 5, as is shown in Figure 1,

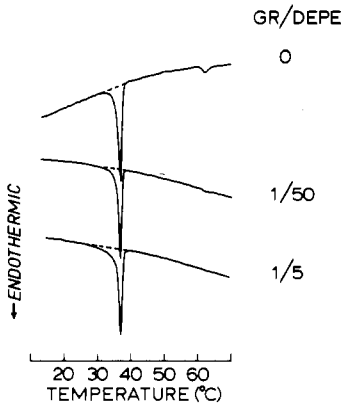

FIGURE 1: DSC heating curves of aqueous dispersions of mixtures of DEPE and gramicidin. The molar ratios of peptide to lipid are as indicated.

gramicidin does not have much effect on the main transition. The peak maximum as well as the width of this transition at half-height were measured and were found to be similar for all gramicidin concentrations used (data not shown). However, the endotherm of the sample with a molar ratio of peptide to lipid of 1 to 5 shows a slightly different line shape in that the onset of the melting process, defined as the temperature at which the heating curve starts to deviate from the base line, is shifted to a slightly lower temperature. While in this sample with a high gramicidin content the melting process begins already at 20–25 °C, in pure DEPE dispersions it starts at approximately 32 °C. Due to the characteristic, slightly asymmetric line shape of the main melting peak in PE dispersions (Chowdry et al., 1984) the exact onset of the melting process is difficult to determine.

The effect of gramicidin on the bilayer to hexagonal phase transition is more pronounced. Incorporation of already low amounts of the peptide, as is shown for a molar ratio of 1:50, results in a reduction of the peak area. At a higher gramicidin concentration of 1:5 (mol/mol) this transition completely disappears. In all samples with a molar ratio of peptide to lipid of less than 1:50 the bilayer to hexagonal H_{II} phase transition was clearly detectable, and after vertical expansion of this endotherm it appeared that the presence of gramicidin did not affect either the transition temperature or the width of the transition at half-height.

The enthalpies of both the gel to liquid-crystalline and the bilayer to hexagonal H_{II} phase transition were calculated and plotted as a function of the gramicidin concentration (Figure 2A). For pure DEPE the enthalpies of both transitions are found to be 6.7 ± 0.2 and 0.5 ± 0.1 kJ/mol, respectively, in fair agreement with previous observations of 7.4 and 0.7 kJ/mol (Gallay & de Kruijff, 1983) and of 8.8 and 0.45 kJ/mol (Epand et al., 1985). Incorporation of gramicidin shows only a minor effect on the enthalpy of the gel to liquid-crystalline transition. At low gramicidin concentrations, up to a molar ratio of 1:50, a slight increase is observed, whereas the presence of larger amounts of the peptide, even at a molar ratio of as high as 1:5, results in only a small decrease of the transition enthalpy. As could be expected from the results in Figure 1 the bilayer to hexagonal H_{II} phase transition is largely affected by the presence of gramicidin. While incorporation of small amounts of the peptide already results in a considerable decrease of the transition enthalpy, it seems that when higher gramicidin concentrations are used, the molecular efficiency slightly decreases.

The observed absence of any substantial effect of gramicidin on the gel to liquid-crystalline transition of DEPE is in sharp

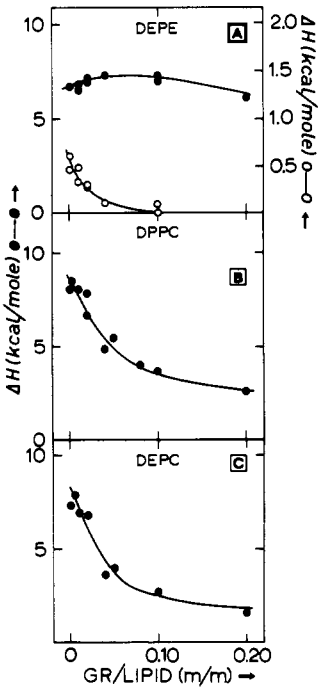

FIGURE 2: Effect of gramicidin incorporation on the enthalpy of the gel to liquid-crystalline transition (●) in aqueous dispersions of DEPE (A), DPPC (B), and DEPC (C) and on the bilayer to hexagonal H_{II} phase transition (○) in aqueous dispersions of DEPE (A).

contrast with the results of Chapman et al. (1977) concerning the gramicidin–DPPC interaction. These authors showed that incorporation of the peptide broadens the main transition of DPPC and reduces the enthalpy of this transition. We found a similar interaction of gramicidin with DPPC. As indicated in Figure 2B, the enthalpy of the main transition decreases linearly with the gramicidin concentration until a molar ratio of peptide to lipid of approximately 1 to 15 is reached. Incorporation of higher amounts of gramicidin results in only a relatively minor further decrease of the transition enthalpy. Chapman et al. (1977) suggested that this deflection corresponds to the onset of an aggregation process in which localized gramicidin/lipid clusters are formed.

To discriminate between contributions of the head group and of the acyl chain moiety to the difference in interaction of gramicidin with DEPE and DPPC, we studied the effect of the peptide on the transition enthalpy of DEPC. As is shown in Figure 2C, gramicidin induces a similar reduction of the enthalpy of the gel to liquid-crystalline transition in DEPC as in DPPC, which indicates that the phospholipid head group of the lipid plays a determinant role in its interaction with gramicidin. These results furthermore suggest that while in PC systems in the gel state aggregation of the peptide occurs only at concentrations of peptide to lipid of 1:15 (mol/mol) or higher, in PE systems gramicidin forms aggregates at all concentrations below the gel to liquid-crystalline phase transition.

Nuclear Magnetic Resonance. The interaction of gramicidin with DEPE was further investigated by NMR techniques. To obtain detailed insight into the structural and dynamic aspects of H_{II} phase formation in pure DEPE and in DEPE/gramicidin systems, a variety of NMR techniques was employed. In order to gain a sufficient sensitivity in the various experiments, we used concentrated lipid samples. As an additional advantage samples with a relatively low water content in general give rise to better line shapes. Also, the samples were hydrated carefully to avoid the formation of smaller structures which, due to faster tumbling, could lead to a partial averaging

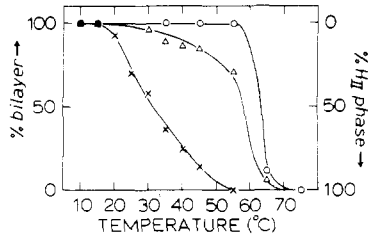

FIGURE 3: Percentages of bilayer and H_{II} component in the ^{31}P NMR spectra of dispersions of DEPE (○) and gramicidin/DEPE mixtures at molar ratios of 1:100 (△) and 1:10 (×) as a function of temperature.

of the orientation of the phospholipid molecules.

We first characterized the gramicidin/DEPE systems by conventional ^{31}P NMR. This is a convenient technique to obtain quantitative information about the amount of phospholipids present in a bilayer or in a hexagonal H_{II} phase, since both types of organization show a characteristic ^{31}P NMR spectrum. While a bilayer organization of phospholipids gives rise to an asymmetric line shape with a low-field shoulder and a high-field peak, the hexagonal H_{II} type of spectrum has a reversed asymmetry and a reduced chemical shift anisotropy due to rapid diffusion of the phospholipid molecules around the aqueous cylinders (Cullis & de Kruijff, 1978b; Seelig, 1978).

Figure 3 shows the extent of H_{II} phase formation at the various gramicidin concentrations as a function of temperature. These data are in good agreement with the results obtained by Van Echteld et al. (1981) in the temperature range of 25–60 °C in which a gramicidin to DEPE molar ratio of 1:25 was used as the highest ratio. Pure DEPE dispersions show a sharp transition from a bilayer organization to a hexagonal H_{II} phase at 55–65 °C. When gramicidin is incorporated in a molar ratio of 1 to 100, this transition begins at a lower temperature and is extended over a larger temperature range, most of the transition still taking place between 55 and 65 °C. At a higher gramicidin to lipid ratio of 1:10 (mol/mol) H_{II} phase formation starts already at 20 °C and increases rapidly with temperature. Also, with freeze–fracture electron microscopy, carried out as described elsewhere (Killian et al., 1985), it was shown that upon quenching of this sample from 25 °C tubes are present, characteristic of an organization of the lipids in an H_{II} phase (data not shown).

Combined with the DSC results these data suggest that upon gramicidin incorporation part of the DEPE molecules, most likely those interacting with the peptide, give rise to a broad bilayer to hexagonal H_{II} transition, which is shifted to lower temperatures and which, due to its width and energy content, cannot be detected in the thermogram. The remainder of the molecules still show an unperturbed bilayer to hexagonal H_{II} phase transition. As the gramicidin concentration is increased, the fraction of these molecules decreases such that eventually only a broad bilayer to hexagonal H_{II} transition is present, which already starts below the gel to liquid-crystalline phase transition.

At 40 °C the amount of H_{II} phase induced by low concentrations of gramicidin (gramicidin:DEPE ≤ 1:25 mol/mol) is proportional to the peptide content as could be calculated from our data and from those obtained by Van Echteld et al. (1981). At this temperature per gramicidin molecule approximately 15 phospholipid molecules are organized in the hexagonal H_{II} phase. At higher concentrations the molecular efficiency of the gramicidin-induced H_{II} phase formation decreases.

Further information on the interaction of gramicidin with DEPE and in particular on the effect of the peptide on local

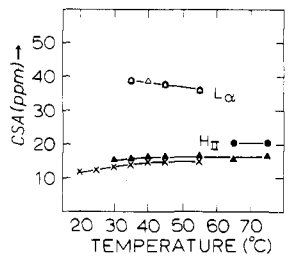

FIGURE 4: Temperature dependence of the chemical shift anisotropy of the bilayer components of dispersions of DEPE (○) and a mixture of gramicidin and DEPE in a molar ratio of 1:100 (△) and of the hexagonal components of dispersions of DEPE (●) and gramicidin/DEPE mixtures in molar ratios of 1:100 (▲) and 1:10 (×).

head group order and motion can be obtained by measuring the chemical shift anisotropy of the bilayer and H_{II} components in the ^{31}P NMR spectra. The results are given in Figure 4. The chemical shift anisotropy of the bilayer components was measured in pure DEPE and in a sample with a molar ratio of peptide to lipid of 1 to 100. Because of the large amount of H_{II} phase present, the position of the high-field peak of the bilayer component could not be accurately determined in samples with a higher molar ratio of peptide to lipid. The pure DEPE bilayer at 40 °C has a CSA of approximately 40 ppm, as was also found by Van Echteld et al. (1981), and which is typical for organization of phospholipids in a liquid-crystalline lamellar phase (Cullis & de Kruijff, 1979; Seelig, 1978). When the temperature is raised, a slight decrease in CSA is observed, as might be expected since at higher temperature the motion of the acyl chains will increase and hence, the surface area per phospholipid and consequently the motional freedom of the phosphate moiety of the lipid molecule will increase. Incorporation of gramicidin in DEPE in a molar ratio of 1 to 100 does not influence the CSA of the bilayer component. In both the pure DEPE and the gramicidin/DEPE sample an identical temperature-dependent decrease of the CSA is observed.

In the absence of changes in local order of the phosphate region a transition from a bilayer organization to a hexagonal H_{II} phase results in a decrease of the CSA by a factor of 2 (Cullis & de Kruijff, 1978b). Pure DEPE, when organized in the hexagonal H_{II} phase, shows a CSA of approximately 20 ppm, as was also found by Van Echteld et al. (1981). In the gramicidin/DEPE (1:100 mol/mol) sample the position of the low-field peak of the H_{II} phase component can be measured over a large temperature range. The presence of such a low amount of the peptide already results in a significant decrease of the CSA, indicating an increased disorder of the phospholipid head groups. Incorporation of higher amounts of gramicidin leads to a further reduction of the CSA. At temperatures above the gel to liquid-crystalline phase transition of DEPE no temperature dependency of the CSA of the hexagonal H_{II} component of the spectrum is observed. When the H_{II} phase is induced at temperatures below the gel to fluid transition, the CSA further decreases, indicating that the phospholipid head groups are disordered to a larger extent. Summarizing these CSA measurements, it can be concluded that the presence of gramicidin is not felt in the bilayer component of the ^{31}P NMR spectra but that the phospholipid head group order of the H_{II} component is significantly affected when gramicidin is incorporated. These data suggest that a phase separation occurs between a gramicidin-poor lamellar phase and a gramicidin-rich H_{II} phase.

Because the motional freedom of the phosphate moiety of the DEPE molecules in the H_{II} phase apparently increases when gramicidin is present, we decided to investigate whether

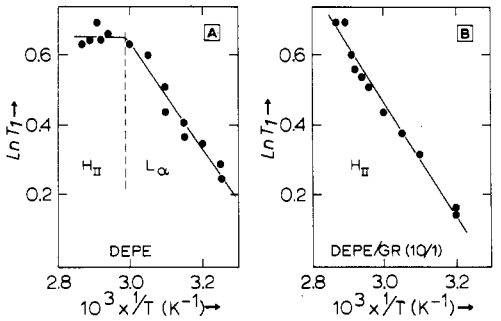

FIGURE 5: Arrhenius plots of the temperature dependence of the T_1 relaxation time (in seconds) for a pure DEPE dispersion (A) and for a mixture of gramicidin and DEPE in a molar ratio of 1 to 10 (B).

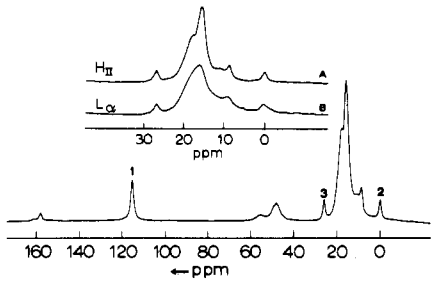

FIGURE 6: 50.3-MHz natural abundance ^{13}C NMR spectrum of a pure DEPE dispersion at 65 °C. Peaks 1, 2 and 3 represent the resonances of the $\Delta^{9,11}$ trans double bond of the lipid, the terminal methyl group, and the α-carbon atom of the ethanolamine, respectively. The insert shows the expanded region of the acyl chain methylene resonances at 65 °C (A) and at 55 °C (B).

the peptide also affects the rate of motion in this part of the lipid molecule. Therefore, we measured the spin–lattice relaxation times of the phosphorus nuclei in a pure DEPE and a gramicidin/DEPE (1:10 mol/mol) mixture at different temperatures. Arrhenius plots are shown in Figure 5. In pure DEPE for the L_α phase a straight line is observed from which an activation energy of 12.2 kJ/mol can be calculated. The sign of the activation energy indicates that relaxation occurs in the fast-correlation time region. In the hexagonal H_{II} phase no such temperature dependency is observed. This difference might very well reflect a decrease of motion of the phospholipid head groups upon transition from a bilayer organization to a hexagonal H_{II} phase, which might be due to the tighter packing of the lipid head groups in the latter case. In the gramicidin-containing sample almost all the lipids are organized in the hexagonal H_{II} phase at the temperatures measured. In contrast to the Arrhenius plot of the H_{II} phase of pure DEPE in this sample a negative slope was found (activation energy, 13.4 kJ/mol). Together with the decrease in CSA as was observed in the H_{II} phase of DEPE, these results suggest that upon incorporation of gramicidin the area per phospholipid head group increases, leading to an increase in motional freedom and rate of motion of the phosphate groups.

To get more insight into the gramicidin–DEPE interaction, we also used ^{13}C NMR to characterize the various samples. Measurement of the line widths of the natural abundance ^{13}C resonances of the lipid in unsonicated dispersions can provide useful qualitative information on its motional characteristics (Brainard & Cordes, 1981; Lancee-Hermkens & de Kruijff, 1977). Since ^{13}C atoms from different places in the lipid molecule give rise to distinct resonances, the local interaction of gramicidin with the surrounding phospholipids can be monitored. Figure 6 shows the natural abundance ^{13}C NMR spectrum of a pure DEPE dispersion at 65 °C. The resonances could be assigned according to previously published natural

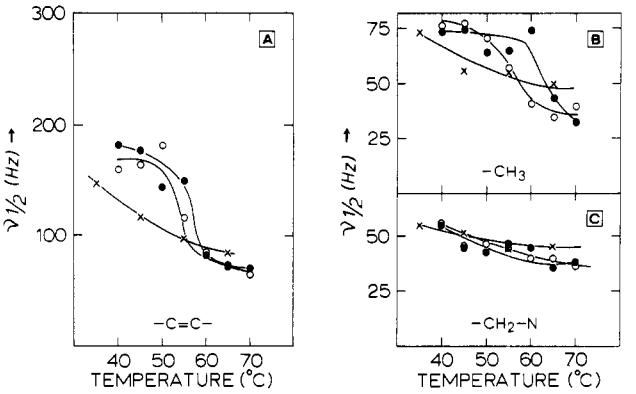

FIGURE 7: Temperature dependence of the line widths of the natural abundance ^{13}C resonances at 50.3 MHz of the $\Delta^{9,11}$ trans double bond (A), the terminal methyl group (B), and the α-carbon atom of the ethanolamine (C) in a pure DEPE dispersion (●) and in mixed gramicidin/DEPE dispersions in molar ratios of 1:100 (○) and 1:10 (×).

abundance ^{13}C NMR spectra of egg PE (Van Duijn et al., 1984) and of egg PC (Brainard & Cordes, 1981). In the low-field region resonances are visible from the carbonyl atoms of the lipid (160 ppm). In the upfield direction there occur successively a sharp resonance of the acyl chain $\Delta^{9,11}$ trans double-bond carbons (117 ppm; peak 1), broad resonances of the glycerol backbone and the β-carbon atom of the ethanolamine (46–58 ppm), and resonances of the α-carbon atom of the ethanolamine (27 ppm; peak 3), of the methylene carbon atoms (5–25 ppm), and finally of the terminal methyl group (0 ppm; peak 2). In the gramicidin-containing samples the presence of the peptide did not lead to the appearance of additional NMR signals. This must be due to the highly restricted motion of the peptide carbon atoms. Below the gel to liquid-crystalline phase transition the entire spectrum was lost, which also must be due to the slow rate of motion of the lipid molecules. Above the transition the line widths of peaks 1–3, which originate from different parts of the PE molecule, could be measured as a function of temperature (Figure 7). In the pure DEPE dispersion a sharp decrease in line width of the resonance of the trans double-bond carbon atoms (peak 1) is observed at the bilayer to hexagonal H_{II} phase transition temperature, indicating that the motion in this part of the acyl chain is increased when the lipids are organized in the hexagonal H_{II} phase (Figure 7A). In the sample containing gramicidin in a molar ratio of 1 to 100 the line widths in both phases are similar, and the H_{II} phase promoting ability of the peptide is expressed in that a decrease of line width occurs at a slightly lower temperature. When a high amount of gramicidin is present (1:10 mol/mol), most of the lipids are organized in the hexagonal H_{II} phase in the whole temperature range of 35–65 °C. In this sample a gradual decrease of the line width is observed with increasing temperature, until at 60 °C the line widths of the trans double-bond carbon atoms of the lipids in the H_{II} phase in the various samples are all similar.

At the terminal part of the acyl chain similar temperature-dependent behavior is observed (Figure 7B). The smaller line widths as compared to Figure 7A reflect the increased intrinsic motion at the end of the acyl chain. In the terminal part of the polar head group no change in line width is observed upon transition from the lamellar to the hexagonal H_{II} phase (Figure 7C), and neither does gramicidin incorporation significantly affect the line width.

The ^{13}C NMR spectra of the various samples showed another interesting feature. In the region of the acyl chain

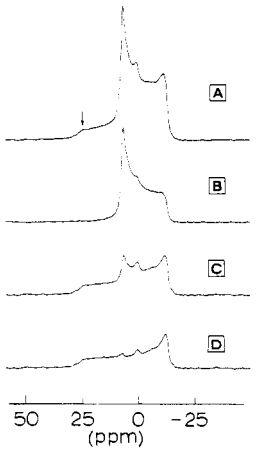

FIGURE 8: 81.0-MHz ^{31}P saturation-transfer NMR spectra at 60 °C of a pure DEPE dispersion (A), of the same dispersion after saturation at a resonance position as indicated by the arrow (B), of the saturated component, obtained after subtraction of spectrum B from spectrum A (C), and of the selectively saturated component, obtained from spectrum C after correction for aselective saturation as described in the text (D).

methylene resonances, i.e., between 5 and 25 ppm, a difference in resolution is observed between organization of lipids in a lamellar and in a hexagonal H_{II} phase (insert, Figure 6). Transition to this latter phase apparently results in a decrease in line width of at least part of the methylene resonances. In the sample with a molar ratio of gramicidin to lipid of 1 to 100 this spectral change was observed at a slightly lower temperature, reflecting the H_{II} phase promoting ability of gramicidin. When the peptide was present in a 1 to 10 molar ratio, the characteristic line shape in the region of the methylene carbon resonances, associated with an H_{II} organization of the phospholipids, was observed at all temperatures above the gel to liquid-crystalline phase transition.

From these ^{13}C NMR measurements it can be concluded that the effect of gramicidin on the line widths mainly reflects the H_{II} phase promoting ability of the peptide and, furthermore, that the peptide does not seem to have a significant effect on the internal motion of the various parts of the lipid molecule in the model membrane.

So far we used ^{31}P NMR and ^{13}C NMR to characterize the interaction of gramicidin with DEPE, thereby focusing on the effect of the peptide on the molecular order and motion of the lipid molecules. To get further insight into the nature of both the temperature-dependent and the gramicidin-induced H_{II} phase formation, we also studied the dynamic characteristics of the gramicidin/PE systems at a macroscopic level.

With ^{31}P saturation-transfer NMR, information can be obtained on the rate of exchange between lipids organized in a bilayer and lipids in a hexagonal H_{II} phase. Only when a fast exchange occurs, i.e., faster than the spin–lattice relaxation time, which is 1–2 s for the various gramicidin/DEPE systems, will selective saturation in one organization result in a transfer of saturation to the other lipid organization. Figure 8A shows the ^{31}P NMR spectrum of a pure DEPE dispersion at 63 °C. At this temperature it can be seen from their characteristic lineshapes that both a bilayer and a hexagonal H_{II} component are present. When this sample is irradiated with a frequency corresponding to the resonance frequency of the low-field shoulder of the bilayer component, spectrum B results, which is an almost pure H_{II} type of spectrum. Subtraction of both spectra yields Figure 8C, representing the part of spectrum A that is saturated. While the percentage of H_{II} phase present in the original spectrum amounts to 63, the saturated spectrum

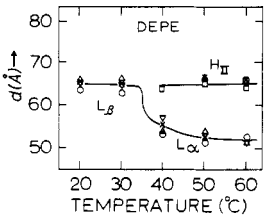

FIGURE 9: Temperature dependence of the first-order repeat distance in a pure DEPE dispersion (○) and in dispersions of mixtures of gramicidin and DEPE in molar ratios of 1:100 (▽), 1:50 (△), 1:10 (×), and 1:5 (□).

only shows a small H_{II} component. The presence of this component could be the result of either a partial fast exchange between lipids in the bilayer and the H_{II} phase, or it could originate from aselective saturation. We investigated this latter possibility by repeating the experiment after a 6 °C rise in temperature in order to obtain a pure H_{II} type of spectrum. From this experment it appeared that 24% of the H_{II} component was aselectively saturated. From the percentage of H_{II} phase in the original spectrum and of aselective saturation in a pure H_{II} type of spectrum we calculated the absolute intensity of the aselectively saturated H_{II} component and subtracted this from the saturated spectrum, as shown in Figure 8C. Figure 8D shows the resulting selectively saturated component. Since this is an almost pure bilayer type of spectrum, it can be concluded that the exchange of lipids between the bilayer and H_{II} phase in a pure DEPE dispersion occurs at a slower rate then 1–2 s.

A similar experiment was performed on a gramicidin/DEPE sample (1:100 mol/mol) at 58 °C to see if there is any fast exchange between lipids in the gramicidin-induced H_{II} phase and the lamellar phase. As a control sample to measure aselective saturation we used the gramicidin/DEPE (1:10 mol/mol) sample at the same temperature. Similar results were obtained, indicating also that no fast exchange of lipids occurs between the gramicidin-induced H_{II} phase and a lamellar phase of DEPE.

Small-Angle X-ray Diffraction. Further information on structural characteristics of the gramicidin/PE systems was obtained with the use of small-angle X-ray diffraction. With this technique not only can the macroscopic structure itself be defined but also the interbilayer repeat distance in the lamellar phase and the tube diameter in the H_{II} phase can be determined. Although in DEPE systems in the lamellar phase no higher order reflections are found, as was previously observed in pure DEPE systems by Valtersson et al. (1985), organization in the H_{II} phase yielded its characteristic diffraction pattern with reflections at distances relating as 1 to $1/\sqrt{3}$ to $^1/_2$. We recorded the diffraction profiles of gramicidin/DEPE samples at various peptide concentrations in the temperature range of 30–60 °C (data not shown). From the patterns obtained in the various samples the H_{II} phase promoting effect of gramicidin was again obvious and in good agreement with the present (Figure 3) and previous (Van Echteld et al., 1981) ^{31}P NMR results. To see if the repeat distances in the lamellar or in the H_{II} phase would be affected by the presence of the peptide, we measured the first-order spacings of both the H_{II} component and the lamellar component in the gel and in the fluid state. These values are plotted in Figure 9 as a function of temperature. Below the gel to fluid transition the interbilayer distance is approximately 64 Å and appears to be independent of the gramicidin concentration. In all samples the transition to a liquid-crystalline phase between 30 and 40 °C is accompanied by an approximately 10-Å decrease in first-order repeat distance, due to the

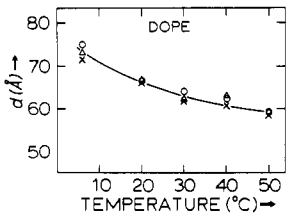

FIGURE 10: Temperature dependence of the first-order repeat distance of the H_{II} component in pure DOPE dispersions (○) and in dispersions of mixtures of gramicidin and DOPE in molar ratios of 1:50 (△) and 1:10 (×).

decrease in effective acyl chain length. In the hexagonal H_{II} phase the first-order spacing is 64 Å and is independent of the gramicidin concentration. When pure lipids are organized in this latter phase, it was found by Seddon et al. (1984) that, due to an increased motion of the acyl chains, the first-order spacing and therewith the tube to tube distance decrease with temperature. The gramicidin-induced H_{II} phase does not show such a temperature dependence, at least not below the bilayer to hexagonal H_{II} phase transition temperature of the pure DEPE dispersion. This could be due to the fact that the H_{II} phase present in these samples is induced under the combined influence of both gramicidin and temperature. Above 60 °C it is difficult to perform reliable measurements in our X-ray set-up, because at such high temperatures dehydration of the sample easily occurs. Therefore, the temperature dependence of the first-order spacings at temperatures above the bilayer to hexagonal H_{II} transition temperature was measured by using DOPE systems with a variable gramicidin content. The bilayer to hexagonal H_{II} transition of the lipid occurred in the temperature range of 4–10 °C. In agreement with the ^{31}P NMR data reported by Van Echteld et al. (1981), gramicidin also promotes H_{II} phase formation for this PE species in that at 6 °C the mixed bilayer–H_{II} diffraction pattern of the pure lipid was converted to an H_{II} pattern upon incorporation of gramicidin in a molar ratio of peptide to lipid of 1 to 100 or higher. The first-order repeat distances in the hexagonal phase of various DOPE/gramicidin mixtures are shown in Figure 10 as a function of temperature. From this figure it is clear that in agreement with the data of Seddon et al. (1984) and Kirk & Gruner (1985) the first-order spacings decrease with temperature but are not affected by the incorporation of gramicidin.

Dependence on Acyl Chain Length. It is clearly shown that gramicidin strongly promotes H_{II} phase formation in DEPE as well as in DOPE systems. We thought it of interest to see whether gramicidin would also be able to induce the hexagonal H_{II} phase in PE's with a shorter chain length, such as DMPE and DLPE, in which no H_{II} phase formation has been observed at temperatures up to 150 °C (Seddon et al., 1983). Therefore, we used ^{31}P NMR to study the effect of gramicidin incorporation on lipid structure in these systems at 50 °C, which is above the gel to liquid-crystalline phase transition of both lipids (data not shown). We found that incorporation of the peptide in a molar ratio of 1 to 10 indeed results in the appearance of an additional spectral component with an increased intensity at the resonance position of the low-field peak of lipids organized in the H_{II} phase.

Discussion

In this study the thermodynamic, motional, and structural parameters of gramicidin–lipid interactions have been investigated. The results of this study allow us to gain a better insight into the mechanism of gramicidin-induced H_{II} phase formation in model membranes. To get an overall picture of the molecular details of the modulation of lipid polymorphism by gramicidin, it is useful to first consider the gramicidin–PE interaction. Subsequent extrapolation to PC systems, combined with literature data on gramicidin–PC interactions, might then also lead to a better molecular understanding of the gramicidin-induced H_{II} phase formation in PC systems.

In our view the most striking new information in the gramicidin–PE system was that gramicidin incorporation has virtually no effect on the energy content of the gel to liquid-crystalline phase transition. Since gramicidin lowers the enthalpy of this transition in PC systems (Chapman et al., 1977) and incorporation of a similar rigid molecule like cholesterol lowers the enthalpy in PE and PC systems similarly (Van Dijck et al., 1976), we suggest that gramicidin is highly aggregated in the gel state of PE. Although the presence of the peptide apparently does not affect the main transition temperature, it is suggested from the shift of the onset of the melting process to a lower temperature that part of the lipids, probably at the phase boundaries of the aggregated gramicidin, are fluidized already below the transition temperature. With the observation that at high peptide concentrations H_{II} phase formation also starts to occur below the temperature of the gel to liquid-crystalline transition of the pure PE system this suggests a direct transition from a gramicidin-rich lamellar gel state to a liquid-crystalline H_{II} phase. This is furthermore suggested by the ^{31}P NMR line shapes of the gramicidin-containing samples at temperatures just below the gel to liquid-crystalline phase transition temperature (data not shown), which seem a superposition of line shapes of a lamellar gel state and a liquid-crystalline H_{II} phase. Such a direct transition could in principle give rise to a slight increase in ΔH, since the enthalpy of the bilayer to H_{II} phase transition would then be included in the main transition. Within the experimental errors we did not observe such an increase. As the temperature is increased, more PE molecules, possibly from boundary regions between the lamellar and the H_{II} phases, are affected by the peptide to adopt the H_{II} phase. Part of the PE molecules are unaffected by the presence of gramicidin and undergo a normal bilayer to hexagonal H_{II} phase transition. This implies that also in the liquid-crystalline state there is a phase separation between a gramicidin-poor lamellar phase and a gramicidin-rich H_{II} phase.

That indeed phase separation is induced between a gramicidin-poor lamellar phase and a gramicidin-rich H_{II} phase is furthermore suggested by CSA measurements in the ^{31}P NMR spectra. While with this technique the presence of gramicidin could not be demonstrated in the bilayer component, in the H_{II} phase clearly an effect of the peptide was shown on the local order of the phospholipid head group. That gramicidin prefers an organization in the H_{II} phase is furthermore suggested by the decrease in molecular efficiency of H_{II} phase formation at high gramicidin concentrations, indicating an increase of the gramicidin/lipid ratio in this phase.

From the combined ^{31}P NMR CSA and T_1 measurements we suggest that gramicidin decreases the molecular order and increases the rate of motion of the phosphate moiety of DEPE in the H_{II} phase. This result is compatible with earlier suggestions (Van Echteld et al., 1981) on the parallel allignment of the gramicidin molecules along the acyl chains, whereby the peptide spaces the lipid head groups, resulting in an increased head group motion and disorder.

The bilayer to hexagonal H_{II} phase transition in pure DEPE is accompanied by a large reduction in ^{13}C NMR line width of the acyl chain carbons, demonstrating an increased disorder or motion of that part of the molecule. This is most likely not

due to an averaging of dipolar ^1H–^{13}C interactions caused by the diffusion of the lipid molecules around the pipes of the H_{II} phase, since the line width of the α-carbon resonance is not affected by the transition. Instead, it appears in agreement with electron spin resonance (ESR) (Hardman, 1982), Fourier transform infrared (FT-IR) (Mantsch et al., 1981), and ^2H NMR (Tilcock et al., 1982; Gally et al., 1980) data that the acyl chains in the H_{II} phase are more disordered in agreement with the inverted nature of that phase. The gramicidin-induced decrease in line width of the acyl chain carbons is paralleled by the H_{II} phase formation induced by the peptide. Within the limits of the interpretation of ^{13}C NMR line width data in terms of changes in order or motion and the accuracy of its determination we propose that the line width changes induced by gramicidin are predominantly due to H_{II} phase formation. Furthermore, it appears that within the H_{II} phase the local order of the acyl chain carbons is not very much affected by gramicidin.

This is also suggested by the results of small-angle X-ray studies on DEPE/gramicidin systems. The first-order repeat distance in the H_{II} phase is sensitive to changes in effective chain length of the lipid and therefore to changes in ordering of the acyl chains. Since the first-order repeat distances of the gramicidin-induced H_{II} phase in the 40–60 °C temperature range are found to be equal to the first-order spacings of the pure DEPE H_{II} phase at 65 °C, also from the X-ray data, a similar disorder of the acyl chains in both H_{II} phases is suggested, assuming that the diameter of the aqueous channel in both phases is identical. When present in the H_{II} phase at temperatures above the bilayer to hexagonal H_{II} phase transition temperature of the pure PE, gramicidin does not seem to affect the effective lipid chain length, as is suggested by the X-ray measurements with DOPE. At these temperatures the first-order repeat distance is independent of the concentration of the peptide but decreases with increasing temperature, due to the resulting increase in chain motion, which leads to a reduction of the effective chain length. In all cases where ^{31}P NMR revealed an H_{II} phase, X-ray diffraction showed one sharp set of reflections with a $1:(1/\sqrt{3}):{}^1/_2$ relationship, suggesting the presence of only one type of H_{II} phase.

The saturation-transfer ^{31}P NMR experiments show that in the case of a coexisting lamellar and H_{II} phases in pure DEPE and in DEPE/gramicidin mixtures the majority of the PE molecules do not exchange between the phases in the 1–2-s time range, suggesting either that there is a profound diffusion barrier between the two phases or, more likely, that the phase separation is macroscopic, thereby reducing the exchange efficiency.

In summary, this study shows that aggregation of the peptide occurs in PE/gramicidin systems in the gel state followed by a phase separation between a gramicidin-poor lamellar phase and a gramicidin-rich H_{II} phase. It also shows that gramicidin apparently has a strong tendency to organize in the H_{II} phase. The gramicidin-induced H_{II} phase shows all the characteristics of a normal temperature-induced H_{II} phase and only differs from it by an increased disorder and rate of motion of the phosphate moiety of the lipid.

Let us now consider the possible mechanism of H_{II} phase formation induced by gramicidin in PC systems. In view of the observation that H_{II} phase formation only was observed when the acyl chain length exceeded 16 carbon atoms (Van Echteld et al., 1982) it was suggested that a mismatch in the length of the gramicidin dimer and the thickness of the lipid bilayer would result in dimple formation, which could trigger the induction of the H_{II} phase. Since we observed that

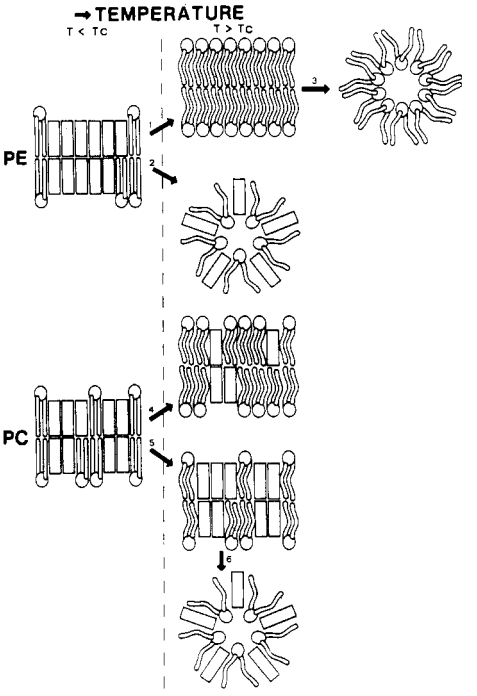

FIGURE 11: Schematic representation of the possible mechanism of gramicidin-induced H_{II} phase formation in PE and PC systems. In PE dispersions in the lamellar gel state gramicidin forms aggregates at all concentrations of the peptide. As the temperature is raised, a phase separation occurs between a fluid bilayer of pure DEPE (1) and an H_{II} phase that is very rich in gramicidin (2). Upon a further increase in the temperature more lipids will organize in this gramicidin-containing H_{II} phase. The remaining part of the lipids, the extent of which is dependent on the gramicidin concentration, will undergo a normal bilayer to hexagonal H_{II} phase transition (3). In PC systems aggregation of gramicidin in the lamellar gel state occurs only at high gramicidin concentrations, whereby probably relatively small aggregates are formed as compared to those in PE systems. As the temperature is raised, the peptide will be present in the fluid bilayer as monomers, dimers, or small aggregates at low gramicidin concentration (4). At higher concentrations of the peptide larger aggregates are formed (5). These large aggregates are able to induce H_{II} phase formation (6) when the chain length exceeds 16 carbon atoms.

gramicidin-induced H_{II} phase formation in the typical bilayer-forming DLPE and DMPE, we consider this possibility unlikely and propose that the chain length dependence of the H_{II} phase induction is related to the ability of longer chains to fit in the H_{II} phase (Seddon et al., 1983).

DSC experiments show that in contrast to PE systems in the lamellar gel state of DPPC and DEPC only aggregation occurs when the peptide is present in excess of a 1:15 molar ratio with respect to the lipid. This difference between interaction of gramicidin with PE and PC systems might be a consequence of the stronger intermolecular electrostatic interactions and hydrogen bonding of the polar head group of PE's as compared to PC's (Hauser et al., 1981), which might facilitate aggregation of proteins in the former class of lipids. Such behavior has been reported for the intrinsic red cell membrane protein glycophorin, for which it was observed by freeze–fracturing (Taraschi et al., 1982) and phosphorescence depolarization (Van Hoogevest et al., 1985) measurements that the protein was more aggregated in PE systems than in PC systems.

In the liquid-crystalline PC systems in which gramicidin induces an H_{II} phase, the peptide has a significant but limited solubility in the coexisting lamellar phase as is evident from the observed decrease in CSA of the bilayer component in the ^{31}P NMR spectra of the PC (Van Echteld et al., 1981; Killian

et al., 1985; Killian & de Kruijff, 1985). As will be shown in the following paper (Killian & de Kruijff, 1985) (and in agreement with the DSC data presented in this paper), a concentration-dependent aggregation of the polypeptide in the liquid-crystalline lamellar phase of PC occurs that is proposed to lead to H_{II} phase formation. Apparently, in the aggregated state the gramicidin molecules prefer to organize in tubular structures such as those found in the H_{II} phase. In this light it is intringuing to note that in model membranes under conditions in which part of the molecules would form the H_{II} phase and part the lamellar phase, in general intermediate lipid structures such as inverted micellar particles or cubic phases are formed (Verkleij, 1984). Up till now such structures have not been found in any of the gramicidin-containing samples that might be explained by the strong tendency of the polypeptide-containing system to adopt an H_{II} phase. The shape of the gramicidin molecule, in particular its bulky C-terminal end where the four tryptophan molecules are located, could very well be of importance for such an organization. Furthermore, it is of interest to note that high concentrations of gramicidin can cause a disordering of the acyl chains in PC systems (Chapman et al., 1977; Lee et al., 1984), which would facilitate the formation of the H_{II} phase.

In Figure 11 a schematic representation of the gramicidin-induced H_{II} phase formation in PE and PC systems is given that summarizes most of our present information on these mixtures. Another factor of importance in the formation of the H_{II} phase is the hydration of the lipid head groups. In the following paper in this issue, a detailed analysis of the phase properties of DOPC/gramicidin systems with a varying water content is given.

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Registry No. DEPC, 56782-46-8; DEPE, 19805-18-6; DLPE, 59752-57-7; DMPE, 998-07-2; DOPE, 4004-05-1; DPPC, 63-89-8.

REFERENCES

Brainard, J. R., & Cordes, E. H. (1981) *Biochemistry 20*, 4607–4617.

Chapman, D., Cornell, B. A., Eliasz, A. W., & Perry, A. (1977) *J. Mol. Biol. 113*, 517–538.

Chowdry, B. Z., Lipka, G., Dalziel, A. W., & Sturtevant, J. M. (1984) *Biophys. J. 45*, 901–904.

Cullis, P. R., & de Kruijff, B. (1978a) *Biochim. Biophys. Acta 513*, 31–42.

Cullis, P. R., & de Kruijff, B. (1978b) *Biochim. Biophys. Acta 507*, 207–218.

Cullis, P. R., & de Kruijff, B. (1979) *Biochim. Biophys. Acta 559*, 399–420.

Dekker, C. J., Geurts van Kessel, W. S. M., Klomp, J. P. G., Pieters, J., & de Kruijff, B. (1983) *Chem. Phys. Lipids 33*, 93–106.

de Kruijff, B., Cullis, P. R., Verkleij, A. J., Hope, M. J., Van Echteld, C. J. A., & Taraschi, T. F. (1985a) in *The Enzymes of Biological Membranes* (Martonosi, A., Ed.) pp 131–204, Plenum Press, New York.

de Kruijff, B., Cullis, P. R., Verkleij, A. J., Hope, M. J., Van Echteld, C. J. A., Taraschi, T. F., Van Hoogevest, P., Killian, J. A., Rietveld, A., & Van der Steen, A. T. M. (1985b) in *Progress in Protein–Lipid Interactions* (Watts, A., & de Pont, J. J. H. H. M., Eds.) pp 89–142, Elsevier, Amsterdam.

Epand, R. M. (1985) *Chem. Phys. Lipids 36*, 387–393.

Farren, S. B., Hope, M. J., & Cullis, P. R. (1983) *Biochem. Biophys. Res. Commun. 111*, 675–683.

Fiske, C. H., & Subbarow, Y. (1925) *J. Biol. Chem. 66*, 375–389.

Gallay, J., & de Kruijff, B. (1984) *Eur. J. Biochem. 142*, 105–112.

Gally, H. U., Pluschke, G., Overath, P., & Seelig, J. (1980) *Biochemistry 19*, 1638–1643.

Hardman, P. D. (1982) *Eur. J. Biochem. 124*, 95–101.

Hauser, H., Pascher, L., Pearson, R. H., & Sunbell, S. (1981) *Biochim. Biophys. Acta 650*, 21–51.

Killian, J. A., & de Kruijff, B. (1985) *Biochemistry* (following paper in this issue).

Killian, J. A., de Kruijff, B., Van Echteld, C. J. A., Verkleij, A. J., Leunissen-Bijvelt, J., & De Gier, J. (1983) *Biochim. Biophys. Acta 728*, 141–144.

Killian, J. A., Leunissen-Bijvelt, J., Verkleij, A. J., & de Kruijff, B. (1985) *Biochim. Biophys. Acta 812*, 21–26.

Kirk, G. L., & Gruner, S. M. (1985) *J. Phys. (Les Ulis, Fr.)* (in press).

Lancee-Hermkens, A. M. W., & de Kruijff, B. (1977) *Biochim. Biophys. Acta 470*, 141–151.

Lee, D. C., Durrani, A. A., & Chapman, D. (1984) *Biochim. Biophys. Acta 769*, 49–56.

Mantsch, H. H., Martin, A., & Cameron, D. G. (1981) *Biochemistry 20*, 3138–3145.

Petri, W. A., Jr., Estep, T. N., Pal, R., Thompson, T. E., Biltonen, R. L., & Wagner, R. R. (1980) *Biochemistry 19*, 3088–3091.

Rand, R. P., & Sengupta, S. (1972) *Biochim. Biophys. Acta 255*, 484–492.

Rice, D., & Oldfield, E. (1979) *Biochemistry 18*, 3272–3278.

Seddon, J. M., Cevc, G., & Marsh, D. (1983) *Biochemistry 22*, 1280–1289.

Seddon, J. M., Cevc, G., Kaye, R. D., & Marsh, D. (1984) *Biochemistry 23*, 2634–2644.

Seelig, J. (1978) *Biochim. Biophys. Acta 515*, 105–140.

Taraschi, T. F., de Kruijff, B., Verkleij, A. J., & Van Echteld, C. J. A. (1982) *Biochim. Biophys. Acta 685*, 153–161.

Tilcock, C. P. S., & Cullis, P. R. (1982) *Biochim. Biophys. Acta 684*, 212–218.

Urry, D. W. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 672–676.

Valtersson, C., Van Duijn, G., Verkleij, A. J., Chojnacki, T., de Kruijff, B., & Dallner, G. (1985) *J. Biol. Chem. 260*, 2742–2751.

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

Van Dijck, P. W. M., de Kruijff, B., Van Deenen, L. L. M., De Gier, J., & Demel, R. A. (1976) *Biochim. Biophys. Acta 455*, 576–587.

Van Duijn, G., Verkleij, A. J., & de Kruijff, B. (1984) *Biochemistry 23*, 4969–4977.

Van Duijn, G., Dekker, J., Leunissen-Bijvelt, J., Verkleij, A. J., & de Kruijff, B. (1985) *Biochemistry 24*, 7640–7650.

Van Echteld, C. J. A., Van Stigt, R., de Kruijff, B., Leunissen-Bijvelt, J., Verkleij, A. J., & De Gier, J. (1981) *Biochim. Biophys. Acta 648*, 287–291.

Van Echteld, C. J. A., de Kruijff, B., Verkleij, A. J., Leunissen-Bijvelt, J., & De Gier, J. (1982) *Biochim. Biophys. Acta 692*, 126–138.
Van Hoogevest, P., de Kruijff, B., & Garland, P. B. (1985) *Biochim. Biophys. Acta 813*, 1–9.
Vasilenko, I., de Kruijff, B., & Verkleij, A. J. (1982) *Biochim. Biophys. Acta 684*, 282–286.
Verkleij, A. J. (1984) *Biochim. Biophys. Acta 779*, 43–63.

Importance of Hydration for Gramicidin-Induced Hexagonal H_{II} Phase Formation in Dioleoylphosphatidylcholine Model Membranes

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ABSTRACT: The macroscopic organization, lipid head group conformation, and structural and dynamic properties of ^2H_2O were investigated in dioleoylphosphatidylcholine (DOPC) model systems of varying gramicidin and ^2H_2O (or H_2O) content by means of small-angle X-ray diffraction and ^{31}P and ^2H NMR. At low stages of hydration, $N < 6$ ($N = {}^2H_2O/DOPC$ molar ratio), a single lamellar phase is observed in which the gramicidin molecules become preferentially hydrated upon increasing N. For $6 < N < 12$ phase separation occurs between a gramicidin-poor and a gramicidin-rich lamellar phase. This latter phase is characterized by a smaller repeat distance and decreased DOPC head group order. For $N > 12$, the gramicidin-rich lamellar phase converts to a hexagonal H_{II} phase. Thus, hydration of gramicidin is a prerequisite for H_{II} phase formation in the DOPC/gramicidin system. The H_{II} phase is very rich in gramicidin and ^2H_2O (gramicidin:DOPC:H_2O = 1:1.1:0.9 w/w/w). A model is proposed in which self-assembly of hydrated gramicidin molecules into domains of a specific structure plays a determinant role in the formation of the H_{II} phase by gramicidin.

An intriguing aspect of the channel-forming pentadecapeptide gramicidin is that it can dramatically affect lipid polymorphism [for a review, see de Kruijff et al. (1985)]. In aqueous mixtures with lysophosphatidylcholine a lamellar phase is present (Killian et al., 1983; Pasquali-Ronchetti et al., 1983) with a defined stoichiometry (Killian et al., 1983), in contrast to the behavior of the pure lyso compound, which forms micelles under these conditions. The temperature-dependent bilayer → hexagonal H_{II} transition occurring in unsaturated phosphatidylethanolamine (PE's)[1] is shifted toward lower temperatures upon incorporation of the polypeptide (Van Echteld et al., 1981). This hexagonal H_{II} promoting ability of gramicidin is even expressed in aqueous dispersions of unsaturated phosphatidylcholines (PC's)[1] with a chain length in excess of 16 carbon atoms (Van Echteld et al., 1982).

The shape of the gramicidin molecule, the length of the acyl chains, and increased acyl chain disorder induced by the peptide have been suggested to be of importance for the lipid structure modulating activity of this polypeptide (de Kruijff et al., 1985). In the preceding paper (Killian & de Kruijff, 1985), evidence is presented that aggregation of gramicidin plays an important role in hexagonal H_{II} phase formation in PE systems.

In general, H_{II} phase formation appears to be strongly dependent on lipid head group hydration: H_{II} phase preferring lipids typically have a low head group hydration (Luzzati, 1968), decreasing water content promotes H_{II} phase formation (Seddon, 1984), and dehydration of the PE head group by trinitrophenylation results in H_{II} phase formation (Van Duijn et al., 1985). That the very hydrophobic gramicidin molecule affects the hydration properties of lipids is suggested by the visual observation that gramicidin-containing lipid systems usually disperse and swell less readily in aqueous solutions than the pure lipid systems.

To get insight into the importance of hydration for gramicidin-induced H_{II} phase formation, we studied in this paper by X-ray diffraction and ^{31}P and ^2H NMR (^2H-labeled head group) DOPC/gramicidin mixtures with varying ^2H_2O or H_2O content. The combination of these techniques can give detailed molecular information on the structural and motional properties of the molecules in such systems (Luzzati, 1968; Shipley, 1973; Seelig, 1977, 1978; Cullis & de Kruijff, 1979; Södermann et al., 1983). The choice of DOPC is based on the observation that gramicidin incorporated into (Van Echteld et al., 1981, 1982) or added through the aqueous phase (Killian et al., 1985) to model membranes of this lipid induces H_{II} phase formation. Besides, the hydration properties of this lipid have been well studied (Södermann et al., 1983; Borle & Seelig, 1983).

It will be shown that water plays a crucial role in gramicidin-induced H_{II} phase formation in that it hydrates gramicidin in preference to DOPC, resulting in a change in gramicidin conformation. In this hydrated conformation the

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[1] Abbreviations: $\Delta\sigma$, residual chemical shift anisotropy; $\Delta\nu_q$, quadrupolar splitting; DOPC, dioleoylphosphatidylcholine; H_{II}, hexagonal phase of type II; NMR, nuclear magnetic resonance; PE, phosphatidylethanolamine; PC, phosphatidylcholine; T_1, spin–lattice relaxation time.

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