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

J. Antoinette Killian*,‡ and Ben de Kruijff*,§

Department of Biochemistry and Institute of Molecular Biology, State University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands

Received May 30, 1985

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

* Correspondence should be addressed to either author.
‡ Department of Biochemistry.
§ Institute of Molecular Biology.

[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.

0006-2960/85/0424-7890$01.50/0

gramicidin molecules self-associate and reduce lipid head group order. These gramicidin-rich patches convert upon further increasing the water content into a gramicidin-rich H_{II} phase probably because the self-associated gramicidin molecules prefer a tubular organization.

Materials and Methods

Materials. Gramicidin (from *Bacillus brevis*) and deuterium-depleted water were purchased from Sigma (St. Louis, MO). ^2H_2O was obtained from Merck (Darmstadt, FRG). 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was synthesized according to Van Deenen & De Haas (1964). *N-methyl*-$^2H_3$-Labeled DOPC was prepared from 1,2-dioleoyl-*sn*-glycero-3-(*N*,*N*-dimethylphosphoethanolamine) by methylation using C^2H_3I (Merck, Sharpe & Dohme, Münich, FRG) as described before (de Kruijff et al., 1977). All lipids were finally purified by preparative high-performance liquid chromatography (Van Duijn et al., 1984) and were considered to be pure as judged from high-performance thin-layer chromatography on silica gel using $CHCl_3$/MeOH/H_2O/NH_3 (68:28:2:2 by volume) as the eluent.

Sample Preparation. DOPC/gramicidin samples containing defined amounts of ^2H_2O were prepared by using the method of Ulmius et al. (1977). A $CHCl_3$/MeOH (2:1) solution of 50–100 μmol of DOPC containing the appropriate amount of gramicidin was evaporated at 40 °C in a rotating Pyrex tube (length, 5 cm; o.d., 8 mm) under a stream of N_2. The thin DOPC/gramicidin film was further dried overnight under oil pump vacuum. The tube was sealed with a silicone stopper, and the weight of the sample was determined. The desired amount of ^2H_2O was added whereafter the sealed tube was equilibrated for 30 min at 37 °C, followed by a 2–12-h 5000*g* centrifugation at 40 °C in a swing-out rotor during which the hydrated mixed film settled as a homogeneous translucent pellet at the bottom of the tube. The samples containing high amounts of gramicidin or low amounts of ^2H_2O required the longer centrifugation times. The quoted ^2H_2O/DOPC molar ratios (*N*) were calculated from the weight of the sample after the centrifugation step. In general, less than 5% of the added ^2H_2O was lost during this centrifugation and subsequent measurements. The Pyrex tube was inserted in a conventional 10-mm NMR tube for ^2H and ^{31}P NMR studies. After the measurements an excess of ^2H_2O (200–300 μL) was added, followed by extensive vortexing to homogenize the sample. The *N* values of these excess ^2H_2O-containing samples range from 100 to 300.

In some experiments the dry DOPC/gramicidin film was hydrated with 20% ^2H_2O in H_2O instead of pure ^2H_2O. The ^{31}P and ^2H NMR characteristics of both types of samples were found to be similar, suggesting that ^2H_2O and H_2O interact in a comparable way with the DOPC and gramicidin molecules. In case of *N*-C^2H_3-labeled DOPC the samples were hydrated with deuterium-depleted water.

Nuclear Magnetic Resonance (NMR). Proton noise decoupled ^{31}P NMR spectra were recorded at 81.0 MHz on a Bruker WP-200 spectrometer. The decoupler was gated, and the 5-W input power was on during 10% of the pulse cycle. Typically, 1000–10000 transients were recorded by using 18-μs 90° radio-frequency (rf) pulses, employing a 25-kHz sweep width, 4K data points, and a 1-s interpulse time. An exponential multiplication corresponding to a 50-Hz line broadening was applied to the accumulated free induction decays prior to Fourier transformation. For phospholipids organized in lamellar phases the residual chemical shift anisotropy ($\Delta\sigma$) of the ^{31}P NMR line shape was determined with identical results (estimated error = 0.2 ppm) as either the distance from the low-field shoulder to the high-field peak or as 3 times the distance from the high-field peak to the chemical shift position of isotropically moving DOPC molecules as present in a solution of sonicated vesicles. In composite spectra the amount of DOPC giving rise to a "hexagonal" line shape was determined by computer subtraction of the closest corresponding ^{31}P NMR "bilayer" spectrum of a pure DOPC dispersion (estimated error = 10%). ^2H NMR spectra were recorded at 30.7 MHz in a conventional high-resolution probe from 1000–5000 transients by using 46-μs 90° rf pulses, a 10-kHz sweep width, 4K data points, and a 0.2-s interpulse time. The accumulated free induction decays were exponentially filtered, resulting in a 20-Hz line broadening. T_1's were recorded by using the inversion recovery method (estimated error in T_1 = 10%).

Unless otherwise indicated, all measurements were carried out at 25 °C. ^2H NMR spectra were computer-simulated according to Siminovitch et al. (1984).

X-ray Diffraction. The samples used for the NMR experiments were transferred with a spatula to a slit in a sample holder of a Kratky camera. The sample was immediately mounted between two sheets of cellophane, whereafter subsequently the X-ray diffraction pattern was recorded at 25 °C by using a 10 × 0.2 mm Cu Kα beam (λ = 1.54 Å) and an exposure time of 30–60 s. After 1 min each sample was measured a second time with identical results, demonstrating the stability of the sample under the measuring conditions. The camera was equipped with a LETI position-sensitive detector interfaced to a microcomputer.

Results

X-ray Diffraction. The macroscopic structure of DOPC/gramicidin mixtures was studied by small-angle X-ray diffraction as a function of the state of hydration. For pure DOPC up to a molar ratio of ^2H_2O/DOPC (*N*) of 10, one sharp reflection is observed (Figure 1). Above $N = 10$ a second reflection emerges for which the repeat distance (*d*) relates as $^1/_2$ to the major reflection, which is typical for a lamellar organization. In agreement with previous studies (Södermann et al., 1983) and in conjunction with the forthcoming ^{31}P NMR and ^2H NMR data, it can be concluded that the DOPC molecules at 25 °C are organized in a lamellar phase at all stages of hydration. The repeat distance of this lamellar phase is nearly constant for *N* up to 10, then increases some 15 Å for *N* up to 25, and remains constant at higher water concentrations (Figure 2A). This classical picture of PC hydration suggests that the first approximately 10 ^2H_2O molecules bind to the head group and the next 15 molecules cause swelling of the lamellae, whereafter at higher ^2H_2O concentrations phase separation occurs between a maximally swollen DOPC lamellar phase and excess ^2H_2O.

Incorporation of gramicidin drastically affects the X-ray diffraction patterns, which is exemplified in Figure 1 for a DOPC/gramicidin (10:1 mol/mol) sample. In agreement with previous data (Killian et al., 1985) in excess water two major and some minor reflections are present whose *d* values relate as $1:(1/\sqrt{3}):^1/_2:(1/\sqrt{7})$, which is characteristic for hexagonally organized tubes. Lowering the water content down to $N = 11.1$ results in a loss of the $1/\sqrt{3}$ reflection, suggesting that no hexagonal phase exists below this amount of water. As the water content is further lowered, the remaining dominant reflection decreases in intensity with the growth of a new strong reflection at shorter *d* values. For $N = 7.4$ clearly two reflections are visible; their *d* values do not show a simple relationship and thus indicate two different structures. For $N < 7.4$ again one major first-order reflection is observed. The

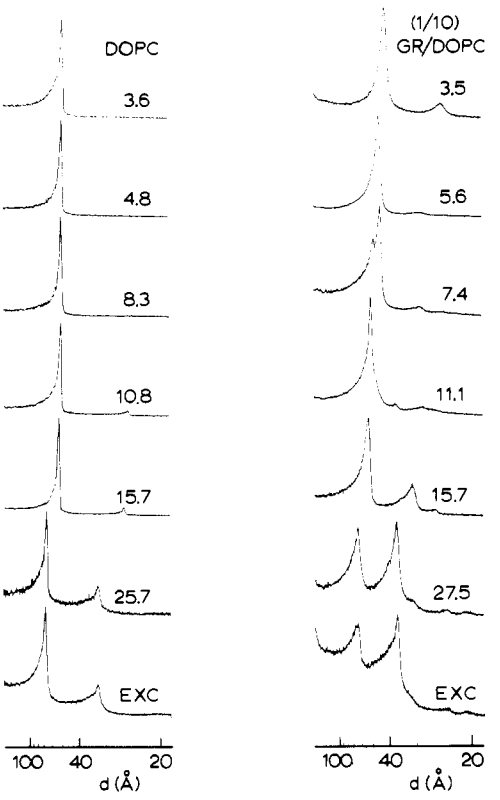

FIGURE 1: Small-angle X-ray diffraction patterns of DOPC and DOPC/gramicidin (10:1 mol/mol) model membranes with a varying ^2H_2O content (N = ^2H_2O/DOPC, mol/mol). EXC refers to the condition in which excess ^2H_2O is present.

origin of the weak second reflection in the N = 3.5 sample is unknown. At lower concentrations of gramicidin qualitatively similar behavior is observed. A $1/\sqrt{3}$ relfection is present for N in excess of approximately 10, and for N between 11 and 5 two reflections are present. The intensity of the $1/\sqrt{3}$ reflection and the second reflection with the smaller d value in the N = 5–10 range is approximately proportional to the gramicidin content. The d values of the first-order reflections are shown in Figure 2A as a function of the gramicidin and ^2H_2O content. Two main observations can be made: (1) for $N > 10$ the d values are independent of gramicidin content and are very similar for the lamellar and hexagonal phase in agreement with previous observations (Killian et al., 1985); (2) below N = 10 and in the presence of gramicidin two phases appear to be present, one with a repeat distance of the pure DOPC bilayer and another one with a 5–7-Å smaller d value. The diameter of the tubes of which the gramicidin-induced H_{II} phase is composed increases with increasing hydration up till a limiting value of 72 Å (Figure 2B) in agreement with the behavior of the hexagonal H_{II} phase formed by PE's (Seddon et al., 1984).

^{31}P NMR. ^{31}P NMR is a very convenient tool for distinguishing between bilayer and H_{II} types of organizations of phospholipids since due to the additional motional averaging of the chemical shift anisotropy by lateral diffusion of the phospholipids around the H_{II} tubes the residual chemical shift anisotropy changes sign and is reduced by a factor of 2 in the absence of changes in local conformation, resulting in characteristically different line shapes (Seelig, 1977; Cullis & de Kruijff, 1979). In the case of DOPC at all stages of hydration the characteristic bilayer type of line shape is observed with a low-field shoulder and a high-field peak (Figure 3). The residual chemical shift anisotropy ($\Delta\sigma$), which is a measure of the local order in the phosphate region, slightly but significantly increases with increasing ^2H_2O content (Figure 4B), suggesting a more ordered phosphate segment of the head group at full hydration.

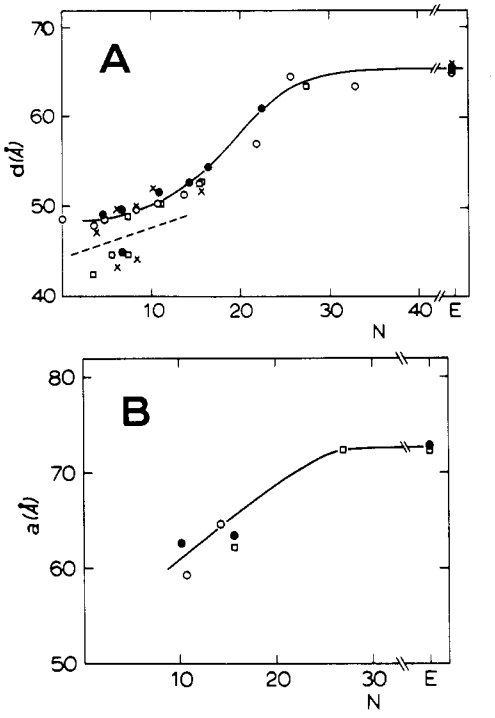

FIGURE 2: (A) Repeat distances (d) of first-order reflections in X-ray diffraction patterns of DOPC/gramicidin mixtures at various ^2H_2O contents. Below N = 10 in some cases two strong reflections are present, which are both indicated in this figure. E indicates the excess ^2H_2O situation. Molar ratio gramicidin/DOPC was 0 (○), 1:50 (●), 1:25 (×), or 1:10 (□). (B) Hydration dependency of the tube diameter (a) of the gramicidin-induced H_{II} phase in DOPC systems. The tube diameter is calculated as 2 times the $1/\sqrt{3}$ reflection observed in the X-ray diffraction pattern. Molar ratio gramicidin/DOPC was 1:50 (○), 1:25 (●), or 1:10 (□).

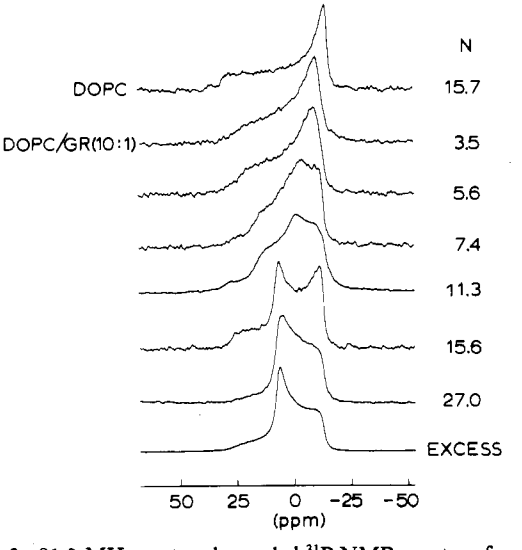

FIGURE 3: 81.0-MHz proton-decoupled ^{31}P NMR spectra of aqueous dispersions of DOPC and DOPC/gramicidin (10:1 mol/mol) samples containing different amounts of ^2H_2O.

Gramicidin incorporation changes the ^{31}P NMR spectra in three ways. Figure 3 illustrates this for 10:1 DOPC/gramicidin (mol/mol) mixtures. First, from excess water down to N = 11.3 a hexagonal type of line shape is observed, superimposed on a bilayer component. The fraction of DOPC in the hexagonal phase decreases with decreasing gramicidin and

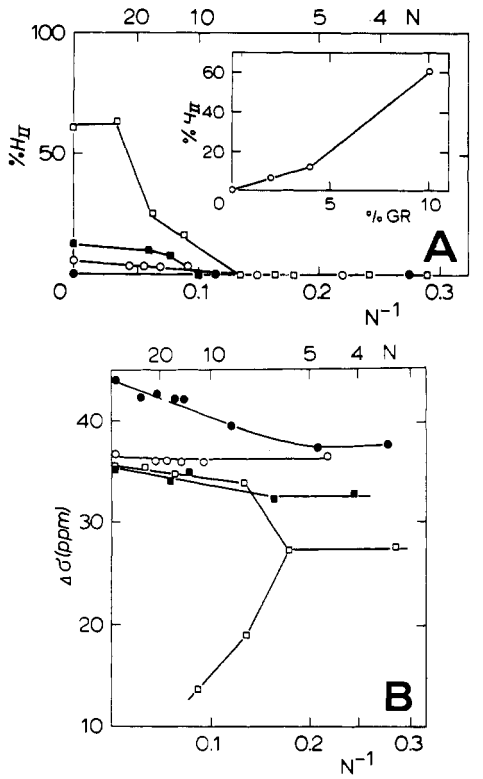

FIGURE 4: (A) Quantification by ^{31}P NMR of the hexagonal H_{II} phase formation in DOPC/gramicidin systems of varying ^2H_2O content. The insert shows the relation between the amount of H_{II} phase vs. gramicidin concentration (mol %) in excess ^2H_2O. Molar ratio gramicidin/DOPC was 0 (●), 1:50 (○), 1:25 (■), or 1:10 (□). (B) Residual chemical shift anisotropy ($\Delta\sigma$) of the bilayer component present in the ^{31}P NMR spectrum of DOPC/gramicidin mixtures at various ^2H_2O contents. For 10:1 DOPC/gramicidin (mol/mol) $\Delta\sigma$ is given for both axially symmetrical line shapes observed in the ^{31}P NMR spectrum at intermediate ^2H_2O contents. Molar ratio gramicidin/DOPC was 0 (●), 1:50 (○), 1:25 (□), or 1:10 (■).

^2H_2O content (Figure 4A). The molecular efficiency of the H_{II} phase formation by gramicidin is rather low: 2.5 mol of DOPC/gramicidin in excess water for DOPC/gramicidin ratios up to 25:1 (insert, Figure 4A). Second, below $N = 15.6$ only bilayer types of ^{31}P NMR spectra are observed, demonstrating that hydration is a prerequisite for H_{II} phase formation in this system. Interestingly, for $6 < N < 12$, where X-ray data indicated two phases, two superimposed axially symmetrical ^{31}P NMR line shapes are present, one of which has a greatly reduced $\Delta\sigma$ (Figure 4B). The amount of this bilayer component decreases with decreasing gramicidin concentration. Exact quantification of this component by spectral subtraction of the broad bilayer component is very difficult in view of the decrease in $\Delta\sigma$ of that component induced by gramicidin (Figure 4B). It is estimated to be 50% of the total intensity for the $N = 11.3$ sample. The decrease in $\Delta\sigma$ of both bilayer spectral components must be the result of decreased lipid phosphate order induced by gramicidin in these lamellar DOPC/gramicidin phases. Third, below $N = 6$ the bilayer component with the very small $\Delta\sigma$ vanishes, and now single-component bilayer types of ^{31}P NMR spectra are observed with an increased line width as compared to the peptide-free bilayer. In these liquid-crystalline systems this increase in line width indicates together with the decrease in $\Delta\sigma$ a decrease in head group order (Burnell et al., 1980).

^2H NMR Head Group Labeled DOPC. To further characterize the head group conformation of DOPC upon gramicidin incorporation, we studied by ^2H NMR the characteristics of [*N-methyl*-$^2H_3$]DOPC in mixtures with gramicidin at

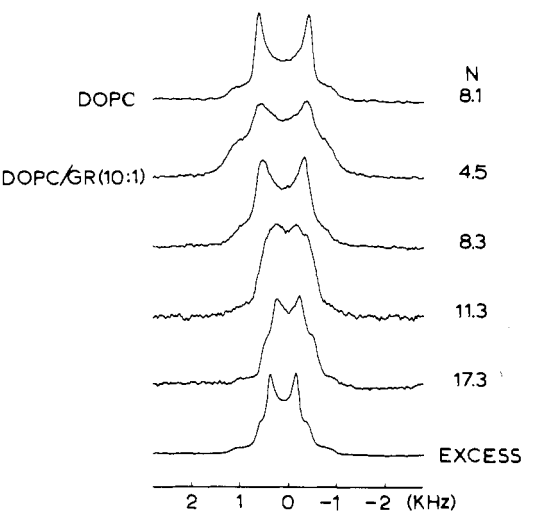

FIGURE 5: 30.7-MHz ^2H NMR spectra of [*N-methyl*-$^2H_3$]DOPC and [*N-methyl*-$^2H_3$]DOPC/gramicidin (10:1 mol/mol) samples at various H_2O contents.

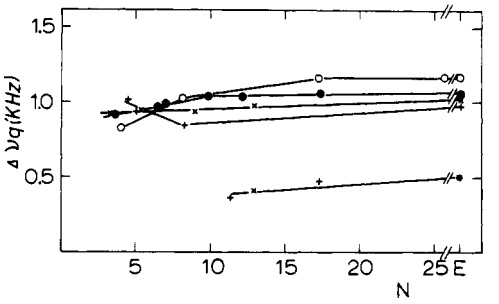

FIGURE 6: Quadrupolar splittings ($\Delta\nu_q$) at 30.7 MHz of [*N-methyl*-$^2H_3$]DOPC/gramicidin mixtures as a function of the H_2O content. Molar ratio gramicidin/DOPC was 0 (○), 1:50 (●), 1:25 (×), or 1:10 (+).

various H_2O contents. For pure DOPC bilayers at all stages of hydration ^2H NMR spectra are observed that are characterized by two peaks and two shoulders (Figure 5). These spectra arise because of the incomplete averaging of the quadrupolar interactions due to rapid anisotropic motion of the C–D bonds (Seelig, 1978). The distance between the peaks is the quadrupolar splitting ($\Delta\nu_q$), which is a measure of the order of the C–D bond. For DOPC $\Delta\nu_q$ slightly increases with increasing ^2H_2O content (Figure 6), which demonstrates an ordering of the head group with increasing hydration in agreement with the ^{31}P NMR data.

Some typical ^2H NMR spectra of DOPC/gramicidin (10:1 mol/mol) mixtures are represented in Figure 5. Like in ^{31}P NMR, gramicidin affects the ^2H NMR characteristics of [*N-methyl*-$^2H_3$]DOPC differently at three stages of hydration. In excess water down to $N = 12$–13, two quadrupolar splittings are observed with values that differ by approximately a factor of 2 (Figure 6). The smaller splitting most likely originates from hexagonally organized DOPC molecules for which the quadrupolar interaction in the choline methyl group is further averaged by the fast diffusion of the lipid molecules around the tubes of which this phase is composed (Seelig, 1978). The theoretically expected and experimentally observed 2-fold reduction of $\Delta\nu_q$ demonstrates that the choline methyls have a similar local conformation and motion in the lamellar phase of DOPC and hexagonal H_{II} phase of DOPC/gramicidin mixtures. The outer splitting slightly decreases with increasing gramicidin concentration (Figure 6). In the case of $N = 11.3$ where there is virtually no H_{II} phase present, the spectrum is broadened, which is probably due to structural heterogeneity,

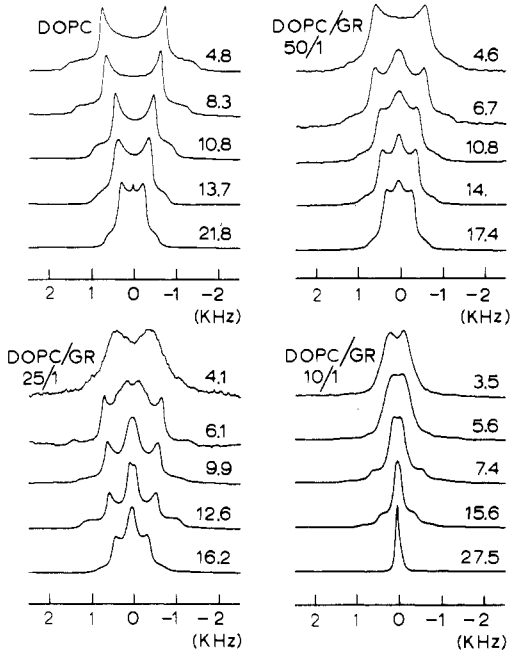

FIGURE 7: 30.7-MHz ^2H NMR spectra of DOPC/gramicidin/^2H_2O samples.

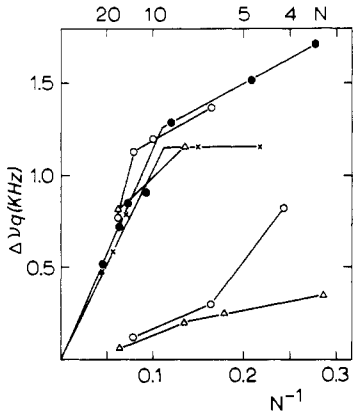

FIGURE 8: Quadrupolar splittings ($\Delta\nu_q$) at 30.7 MHz of DOPC/gramicidin/^2H_2O samples. Only data on spectral components giving rise to quadrupolar splittings are included. Molar ratio gramicidin/DOPC was 0 (●), 1:50 (×), 1:25 (○), or 1:10 (△).

for instance, the coexistence of the two lamellar phases. The $\Delta\nu_q$ measured from the peak maximum is very similar to that observed in the H_{II} phase (Figure 6), which is in agreement with the ^{31}P NMR data that show a greatly increased disorder of the lipid head group in part of the bilayer-organized DOPC molecules. At still lower hydration more simple one-component ^2H NMR spectra are observed that display a larger line width again in agreement with the ^{31}P NMR data. At the lowest water contents tested (N = 3–5) $\Delta\nu_q$ increases with increasing gramicidin concentration, opposite to the situation in excess H_2O (Figure 6).

^2H NMR of ^2H_2O. ^2H_2O quadrupolar splittings and spin–lattice relaxation times (T_1) of ^2H_2O can give valuable information on hydration properties of phospholipids such as DOPC (Södermann et al., 1983; Borle & Seelig, 1983). Figure 7 shows typical ^2H NMR spectra of DOPC/gramicidin/^2H_2O samples at various gramicidin and ^2H_2O concentrations. For pure DOPC from the lowest amount of ^2H_2O tested up to N = 21 a single quadrupolar splitting is observed whose value decreases in a biphasic way with increasing ^2H_2O content (Figure 8). Above N = 21 an isotropic component becomes visible with an intensity that increased with further increase

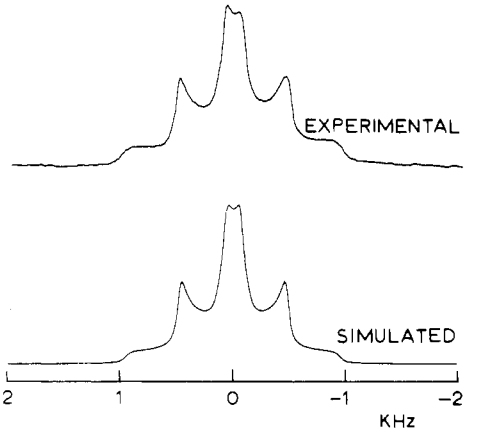

FIGURE 9: Experimental and simulated ^2H NMR spectrum of a DOPC/gramicidin/^2H_2O (25:1:315 mol/mol/mol) sample. The simulated spectrum is obtained by assuming the presence of two quadrupolar splittings of 0.95 and 0.14 kHz, respectively, with relative intensity ratios of 7:3.

of N. These results are in perfect agreement with the data of Södermann et al. (1983) but differ from the results reported by Borle & Seelig (1983) in that these authors reported an increase in $\Delta\nu_q$ upon raising N from 4 to 8. Furthermore, these authors observed an isotropic component at much lower N values. The reason for this discrepancy is not understood. Most likely it is not caused by the use of $H_2O/^2H_2O$ mixtures in that study, since control experiments with 4:1 $H_2O/^2H_2O$ mixtures gave very similar results to those obtained for pure ^2H_2O samples (data not shown). In following Södermann et al. (1983) the observed ^2H NMR spectra of DOPC/^2H_2O mixtures can be understood in terms of a three-pool model. One pool consists of approximately 10 ^2H_2O molecules that bind firmly to various sites at the head group (primary hydration shell). Their motion is restricted due to the binding, but there is rapid exchange of ^2H_2O between these sites, the last occupied site having the smallest intrinsic $\Delta\nu_q$. The second pool consists of the next 10–15 ^2H_2O molecules per DOPC, which are more loosely associated with the head group but which are also in rapid exchange with the ^2H_2O molecules in the primary hydration shell. Finally, a third pool of free moving unbound ^2H_2O is found in excess of $N \cong 21$. This is due to phase separation between the maximally swollen DOPC/^2H_2O phase and excess ^2H_2O. These latter molecules cannot exchange anymore with the ^2H_2O molecules present in the other pools at least on the time scale of the ^2H NMR experiment.

Gramicidin incorporation has a dramatic influence on the properties of the ^2H_2O molecules as revealed by ^2H NMR. Two effects are most noticeable. At the lowest stages of hydration (N = 3–5) the spectra are broadened, but a single quadrupolar splitting is observed with a value that at N = 5 and up to 4 mol % gramicidin decreases linearly with about 0.2 kHz per mole percent of the peptide. Above N = 5 multicomponent spectra are observed. One component has a quadrupolar splitting very similar to that of the gramicidin-free bilayer (Figure 8). In addition, the spectra show a (broad) isotropic component or a component with a very small value of $\Delta\nu_q$. The intensity of these components increases with increasing gramicidin concentration at any given stage of hydration. These composite spectra can be simulated by using two spectral components as is shown in Figure 9 for a DOPC/gramicidin/^2H_2O (25:1:315 mol/mol/mol; thus N = 12.6) sample. The slight difference is intensity of the two shoulders between the experimental and simulated spectra most likely is caused by some preference for bilayer orientations

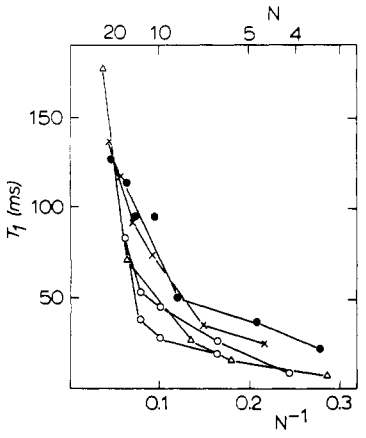

FIGURE 10: Spin–lattice relaxation times (T_1) at 25 °C of ^2H_2O present in DOPC/gramicidin/^2H_2O mixtures of various compositions. In the case where the T_1's of both components in two-component spectra could be resolved both values are given. In these cases the isotropic component or the component with a small $\Delta\nu_q$ had always a smaller T_1 than the component with the larger quadrupolar splitting. Molar ratio gramicidin/DOPC was 0 (●), 1:50 (×), 1:25 (○), or 1:10 (△).

perpendicular to the magnetic field due to ordering of the bilayers during the prolonged centrifugation of the samples.

The T_1 relaxation time of ^2H_2O in pure DOPC/^2H_2O mixtures is like $\Delta\nu_q$ in a biphasic way dependent on the ^2H_2O content (Figure 10), in good agreement with the study of Borle & Seelig (1983). For pure ^2H_2O at 25 °C a T_1 of 420 ms was found, which value shortens upon interaction with DOPC. In those cases tested, T_1 increased with temperature. Arrhenius plots in the 25–45 °C temperature range yielded straight lines with negative slopes. Activation energies were found to be 18.2, 25.6, and 22.0 kJ/mol for pure ^2H_2O, DOPC/^2H_2O (N = 21.8), and DOPC/gramicidin (10:1 mol/mol) for N = 11.1, respectively. This behavior indicates that the motions determining T_1 are in the fast-correlation regime where $\omega_0\tau_c \ll 1$ ($\omega_0 = 2\pi \times 30.7$ MHz; τ_c is the correlation time for the reorientation of the ^2H_2O molecule). Thus, the decrease in T_1 upon interaction of ^2H_2O with DOPC indicates a decrease in the rate of these motions. Gramicidin incorporation further decreases T_1 (Figure 10). The isotropic spectral component or the component with the small $\Delta\nu_q$ displays a shorter T_1 than the component with the larger quadrupolar splitting. It thus appears that there are two types of ^2H_2O molecules in these DOPC/gramicidin mixtures which are not in rapid exchange. This is even more clearly illustrated in a plot of T_1 vs. $\Delta\nu_q$ (Figure 11). In the presence of gramicidin there are ^2H_2O molecules with motional properties that resemble those in the pure DOPC bilayer and there are ^2H_2O molecules that have a reduced T_1 and $\Delta\nu_q$.

To test the hydration properties of pure gramicidin, some experiments were performed in which a dry gramicidin film was hydrated with ^2H_2O in the same way as was done in the preparation of the mixed DOPC/gramicidin samples. At 25 °C, for 5.2 mol of ^2H_2O per mole of gramicidin a two-component spectrum was observed with 20% of the intensity present in a narrow isotropic component ($\Delta\nu_{1/2}$ = 30 Hz; T_1 = 140 ms) and 80% of the intensity present in a broad symmetrical underlying line shape ($\Delta\nu_{1/2}$ = around 800 Hz; T_1 = 10 ms). For 8.3 ^2H_2O per gramicidin 70% of the intensity was in a narrow isotropic component ($\Delta\nu_{1/2}$ = 32 Hz; T_1 = 410 ms). The T_1 of the broad component ($\Delta\nu_{1/2}$ = approximately 900 Hz) could not be determined. These data suggest that in the absence of DOPC gramicidin decreases the rate of motion of 2–4 mol of ^2H_2O.

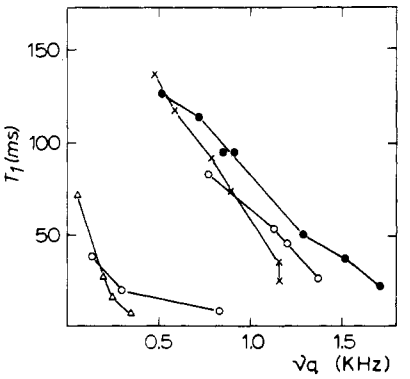

FIGURE 11: Relationship between spin–lattice relaxation time (T_1) and quadrupolar splitting ($\Delta\nu_q$) of ^2H_2O in DOPC/gramicidin/^2H_2O mixtures at 25 °C. Symbols as in legend of Figure 10.

Discussion

The results presented clearly demonstrate the importance of hydration for the formation of the hexagonal H_{II} phase by gramicidin in DOPC model membranes. In analyzing the results it is useful to first consider the effect of gramicidin and hydration on the macroscopic organization and head group structure of DOPC, whereafter a detailed discussion of the state of ^2H_2O in these systems will become possible.

From the combined results obtained from X-ray diffraction and ^{31}P and ^2H NMR (head group label) it can be concluded that at 25 °C DOPC at all stages of hydration forms a lamellar phase. Gramicidin incorporation induces the formation of two new phases, a lamellar one at low hydration and a hexagonal H_{II} phase at higher hydration. Let us first consider the new lamellar phase occurring at low hydration. In the case of a DOPC/gramicidin (10:1 mol/mol) mixture this lamellar phase is characterized by a smaller repeat distance (44 Å vs. 48 Å of the pure DOPC lamellar phase at N = 8) and occurs below N = 12. For lower gramicidin concentrations and down to N = 6 this lamellar phase coexists with a lamellar phase with a d value of pure DOPC at this stage of hydration. The gramicidin-induced lamellar phase occurring between N = 6 and N = 12 has another interesting property in that the head group order of the DOPC molecules is reduced, as can be inferred from the large reduction by approximately 50% of both $\Delta\sigma$ of the ^{31}P nucleus and $\Delta\nu_q$ of the choline methyl deuterons. With the exception of A_1 basic protein–phosphatidylglycerol recombinants (Sixl et al., 1984) no such dramatic changes in $\Delta\sigma$ of the lipid phosphorus have been observed in any protein- or peptide-containing lamellar phospholipid system. Due to insufficient information it is impossible to give a quantitative description of the change in head group order induced by gramicidin. Qualitatively, we can suggest that the increase in motional freedom of the head group could be due to a spacing effect of gramicidin similar to that of cholesterol (Cullis et al., 1976), but to a larger extent possibly in combination with a bending of the head group over the entrance of the channels that gramicidin might form under these conditions. That the two lamellar phases present in the $6 < N < 12$ hydration range both contain gramicidin, but in different concentrations, is indicated by the decrease in $\Delta\sigma$ of both phases. If it is assumed that $\Delta\sigma$ linearly decreases with the gramicidin concentration and that $\Delta\sigma$ only can be affected in one way by gramicidin, then it can be estimated from Figure 6 that for N = 8, N = 12, and 10:1 DOPC/gramicidin (mol/mol) one phase is enriched 4–5 times in gramicidin. The small d value of the gramicidin-induced new lamellar phase is consistent with the high gramicidin content of this phase since the length of the lipid-associated gramicidin dimer is only

30 Å (Wallace et al., 1981).

In the presence of higher amounts of water (N = 10–15, depending on the gramicidin concentration) a hexagonal H_{II} phase is induced by gramicidin. Thus, hydration is essential for gramicidin-promoted H_{II} phase formation in DOPC model membranes. For pure phospholipid systems hydration will mitigate against H_{II} phase formation (Seddon et al., 1984); therefore, it can be concluded that the hydration of gramicidin is the driving force behind the structural rearrangements of the phospholipid molecules. Since the hydration of gramicidin most likely will change its structure, possibly into a channel conformation, we can suggest that for H_{II} phase formation the hydrophobic sequence of the peptide alone is insufficient but that in addition a particular (hydrated) structure of the peptide has to be formed.

From the decrease in $\Delta\sigma$ in excess ^2H_2O we can estimate a limiting gramicidin/DOPC ratio of 1:45 to occur in the bilayer. For the sample with a gramicidin/DOPC ratio of 1:10 it can be calculated from this bilayer solubility that the H_{II} phase is approximately 6-fold enriched in gramicidin, in agreement with the preceding analysis of the $\Delta\sigma$ in the coexisting lamellar phase. It can be suggested that the H_{II} phase most likely forms from the gramicidin-rich lamellar phase occurring at intermediate water concentrations.

In the sample with a molar ratio of peptide to lipid of 1:25 from the stoichiometry of H_{II} phase formation in excess water a maximum gramicidin/DOPC ratio of 1:2.5 in the H_{II} phase can be calculated, and from the decrease in $\Delta\sigma$ a limiting ratio of 1:5 can be calculated. Since already at a 1:50 molar ratio H_{II} phase formation occurs in excess water, the solubility of gramicidin in the bilayer at these low gramicidin concentrations must be much lower than its limited solubility of 1:45, and therefore it is more likely that the DOPC/gramicidin ratio in the H_{II} phase will be close to 2.5.

It is of interest to consider the origin of these phase separations. The two most likely mechanisms are direct gramicidin–gramicidin interactions or clustering of gramicidin/DOPC "complexes". Insight into the occurrence of gramicidin–gramicidin interactions can be obtained from geometrical arguments as applied originally by Chapman et al. (1977). If it is assumed that gramicidin and DOPC have circular cross sections, then it can be estimated from monolayer data (Van Echteld et al., 1982) that the cross-sectional diameter of DOPC is 9.4 Å and from space-filling models that the cross-sectional diameter of gramicidin in the $\pi^6_{L,D}$ helix (the most probable channel conformer in a lipid bilayer; Urry et al., 1971) at the C-terminal (most bulky, tryptophan-rich) site is approximately 18 Å (Veatch et al., 1974). The lowest ratio in number of small (9.4-Å) to large (18-Å) circles to be two-dimensionally closest packed without contact between the large circles was found to be 4.25. Thus, the stoichiometry of $\cong$2.5 DOPC/gramicidin in the H_{II} phase suggests direct gramicidin–gramicidin interaction. In alternating linear arrays of small and large circles the stoichiometry is 2. Interestingly, gramicidin–gramicidin interactions involving tryptophans in linear arrays of gramicidin molecules have been described in a liquid-crystalline lamellar phase with lysophosphatidylcholine (Pasquali-Ronchetti et al., 1983). This suggests to us that interactions between gramicidin molecules with a particular (hydrated) conformation play an essential role in H_{II} phase formation, possibly because these aggregates prefer to organize in a tubular structure. The biological relevance of this suggestion is that tubular structures found in biological systems and resembling the tubes present in the H_{II} phase such as the tight junction (Kachar & Reese, 1982; Pinto da Silva, 1982) could be stabilized by hydrophobic polypeptides (or aggregates) organized in a tubular conformation. We expect polypeptides to be involved in relatively stable tubular structures as the tight junction because of the very low energy barrier between the lamellar and H_{II} phase in pure phospholipid systems (Cullis et al., 1983).

With these structural data in mind let us now discuss the state of water in the gramicidin/DOPC system. Below N = 6, thus in the case of only one lamellar phase present, the ^2H NMR spectra of DOPC/gramicidin/^2H_2O mixtures are characterized by a single quadrupolar splitting. This indicates that either there is one type of ^2H_2O binding site (the DOPC head group) or there are two types, e.g., DOPC and gramicidin, in each of which the motion of the bound ^2H_2O molecules is differently affected, but with rapid exchange between the two sites (time constant $\cong$ 1 ms). The quadrupolar splitting rapidly decreases with increasing gramicidin concentration. This can now be explained in two ways. First, gramicidin does not influence the ^2H_2O binding sites of the DOPC head group, but it increases head group motion thereby causing a decrease in $\Delta\nu_q$ of the bound ^2H_2O molecules. Second, the DOPC head group becomes dehydrated, and the ^2H_2O becomes associated with gramicidin. To account for the decrease in $\Delta\nu_q$, we have to postulate that in this gramicidin-associated form the ^2H_2O molecules would move more isotropically and that there is rapid exchange with the head group bound DOPC. There are several arguments against the first possibility.

Although the decrease in ^{31}P chemical shift anisotropy indicated decreased head group order in the phosphate region, we consider this to be insufficient to cause the much larger relative change in $\Delta\nu_q$ of the ^2H_2O molecules (for N = 5, decrease in $\Delta\sigma$ and $\Delta\nu_q$ per mol percent of gramicidin incorporated was 2 and 17%, respectively). Moreover, the choline methyl part of the head group becomes even motionally restricted at this stage of hydration by the presence of gramicidin. Further evidence against this possibility comes from quantitative arguments on the extent of head group perturbation by gramicidin. If it is assumed that the head group moves isotropically [$\Delta\sigma$ = 0, $\Delta\nu_q$ (choline methyl) = 0] by the interaction with gramicidin, then it can be calculated from the linear part of the curve in Figure 11 that 15 DOPC molecules will have to be affected per gramicidin monomer to give rise to the observed decrease in $\Delta\nu_q$ of the ^2H_2O signal. Since the head group does not move isotropically but still has a restricted motion, this number will be considerably increased. By use of the geometrical approach discussed before, it can be calculated that maximally nine DOPC molecules can be in direct interaction with gramicidin.

For these reasons we favor the second possibility that gramicidin is preferentially hydrated over the DOPC molecule. Assuming that head group order is not affected, it can be estimated from the experimentally observed relationship between $\Delta\nu_q$ and N for pure DOPC (a decrease of 0.14 kHz per H_2O molecule added; Figure 8), the relationship between $\Delta\nu_q$ and the gramicidin concentration (a decrease of $\pm$0.2 kHz per mole percent of gramicidin), and a two-site, e.g., DOPC and gramicidin (with $\Delta\nu_q$ = 0), model that at N = 5 approximately 140 ^2H_2O molecules are associated with one gramicidin monomer. From the decrease in T_1 of the ^2H NMR signal of ^2H_2O it can be concluded that gramicidin causes some restriction in the rapid isotropic motions experienced by the ^2H_2O molecules.

The hydration properties of gramicidin are poorly known. Our preliminary data on gramicidin/^2H_2O mixtures in the absence of lipids indicate that two to four molecules of ^2H_2O are motionally restricted per gramicidin molecule. From energy calculations, Fornili et al. (1984) suggested that in the $\pi^6_{L,D}$ ($\beta^{6,3}_{3,3}$) helical head-to-head dimerized channel conformation [for nomenclature, see Urry et al. (1971)] some 20 H_2O molecules occupy the entire channel. However, these calculations revealed that the entrance of the channel is very attractive for water, which could account for the much higher hydration number we observed. However, it should be realized that the exact conformation of gramicidin in the mixtures with DOPC and ^2H_2O is unknown. It could be argued that some of the ^2H_2O could become involved in exchange with the 17 potential exchange protons in gramicidin, e.g., the 13 amide protons in the peptide bonds, the 4 indole ring protons (tryptophans), and the hydroxyl proton in the C-terminal ethanolamine group. Such exchange could, depending on the exchange rate, lead to an apparent motional restriction of the ^2H_2O molecules. However, this is unlikely in view of the slow exchange of these protons in different solvents (Glickson et al., 1972) and the observation of separate signals from these deuterons present in gramicidin incorporated in model membranes and from deuterons in excess ^2H_2O (Datema, K. P., Pauls, K. P., and Bloom, M., unpublished observations). It thus appears that at low hydration gramicidin becomes preferentially hydrated over DOPC. On further hydration phase separation occurs in two lamellar phases. This is also very evident from the ^2H NMR results in the ^2H_2O/DOPC 5–12 range which indicate that two types of ^2H_2O molecules occur, one with motional properties resembling pure DOPC and another which shows a much smaller $\Delta\nu_q$ and a decrease in T_1. These latter ^2H_2O molecules must be present in the gramicidin-rich phase. From the values of $\Delta\nu_q$ we can conclude that the time constant of the exchange rate for ^2H_2O between the two structures must be 2 ms or more. Assuming the length of the diffusion path of H_2O in PC systems of $\cong$200Å/10^{-6} s (Ulmius et al., 1977) to be applicable in our systems, it can be estimated that the phase separation must be macroscopic in that the structures are separated by at least 40 μm. Also, for $N > 12$, where gramicidin induces the H_{II} phase, ^2H_2O molecules with two types of NMR characteristics are present, one with a $\Delta\nu_q$ and T_1 resembling the pure DOPC system and one with a much smaller $\Delta\nu_q$ and T_1, which most likely originates from the hexagonal H_{II} phase. This latter conclusion is supported by the following quantitative analysis. For the case 25:1 DOPC/gramicidin and N = 12.6 the ^2H NMR spectrum can be simulated by using two components with $\Delta\nu_q$ of 0.14 and 0.96 kHz, respectively, and relative intensities of 30 and 70%, respectively (Figure 9). From ^{31}P NMR it can be concluded that $\cong$10% of the DOPC molecules are in the H_{II} phase. If it is assumed that the 0.14-kHz signal originates from ^2H_2O molecules associated with the H_{II} phase, then 38 ^2H_2O/PC are present in the H_{II} phase and 10 ^2H_2O/PC in the lamellar phase. Assuming that in the H_{II} phase in the head group bound state (primary hydration shell) up to N = 10 the quadrupolar splitting of the ^2H_2O molecule is half of that in the lamellar phase due to fast lateral diffusion around the tubes of the H_{II} phase, as is experimentally observed in the ^2H NMR spectra of the head group labeled DOPC, then it can be calculated from $\Delta\nu_{q(calcd)} = {}^1/_2 \times {}^{10}/_{38} \times \Delta\nu_q$ (DOPC, N = 10) that $\Delta\nu_{q(calcd)}$ = 0.16 kHz, which is close to the observed value of 0.14 kHz. This calculation holds for a number of other experimental cases in the N = 12–15 concentration range. From these data information on the composition of the H_{II} phase can be calculated. Taking the 15:1 DOPC/gramicidin (N = 12.6) case again as an example and assuming a DOPC/gramicidin ratio of 2.5 in the H_{II} phase, this will lead to a gramicidin/DOPC/^2H_2O molar ratio of 1:2.5:95, which translates into a 1:1.08:0.91 weight ratio.

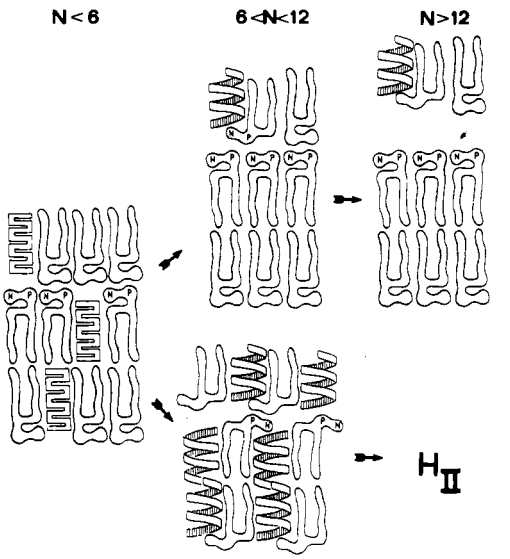

FIGURE 12: Schematic representation of the effect of hydration on the phase properties of DOPC/gramicidin mixtures. The scheme depicts the situation for a DOPC/gramicidin ratio of 25:1. For N (^2H_2O/PC molar ratio) less than 6, one lamellar phase exists with similar structural properties as the pure DOPC system. The gramicidin molecules (represented by the zigzags) are thought to be randomly oriented in the hydrophobic part of the bilayer in a condensed anhydrous conformation. Increasing H_2O concentration will cause preferential hydration of the gramicidin molecules. Above N = 6 phase separation occurs, resulting in a lamellar phase that is highly enriched in the hydrated gramicidin molecules (shown here in the $\pi^6_{L,D}$ head-to-head dimer channel conformation). This bilayer is relatively thin, and the head groups of the lipid molecules have obtained increased motional freedom. The other lamellar phase behaves like the pure DOPC lamellar system but contains some hydrated gramicidin. Above N = 12 the gramicidin-rich lamellar phase converts to a gramicidin-rich hexagonal H_{II} phase, possibly as the result of an interbilayer fusion event involving linear arrays of gramicidin molecules. The gramicidin-poor lamellar phase swells upon further H_2O addition to a limiting value above which the phase separates from an excess H_2O phase.

The H_{II} phase is thus extremely rich in ^2H_2O at this stage of hydration: 38 ^2H_2O/PC vs. 15 ^2H_2O/PE as the maximum hydration for hexagonally H_{II} phase organized PE (Seddon et al., 1984). The water content of the H_{II} phase probably increases upon further increasing the ^2H_2O content, since its tube diameter is still further increasing (Figure 2B). The high water content of the H_{II} phase must be due to the gramicidin present in the phase since the repeat distance of the H_{II} phase in DOPC/gramicidin mixtures in excess H_2O of 72 Å is identical with that of 18:1_c/18:1_c-PE at 25 °C (Killian & de Kruijff, 1985). Quantitative analysis of the ^2H NMR spectra at higher hydration becomes impossible due to the dominant isotropic ^2H_2O signals. Assuming that 10 ^2H_2O molecules are present per DOPC head group in the H_{II} phase, then 70 molecules of ^2H_2O are associated per gramicidin molecule at this stage of hydration. In view of the assumptions and errors involved, this hydration number is of the same magnitude as its value independently observed at low hydration.

In Figure 12 a summarizing structural model is presented that is compatible with all experimental details in DOPC/gramicidin/H_2O systems so far.

The next step of investigation will be to get insight into the structure of gramicidin and its aggregation state in relation to the chemical structure of the peptide and the macroscopic

organization of the peptide–lipid complex.

ACKNOWLEDGMENTS

We thank Dr. A. Wieslander for his advice in the preparation of the partially hydrated lipid samples and Dr. P. Allegrini for his help in the simulation of the ^2H NMR spectra. We also thank Dr. K. P. Datema for a preprint of his ^2H NMR study on ^2H-labeled gramicidin.

Registry No. DOPC, 4235-95-4; gramicidin, 1405-97-6.

REFERENCES

Borle, F., & Seelig, J. (1983) *Biochim. Biophys. Acta 735*, 131–136.

Burnell, E., Cullis, P. R., & de Kruijff, B. (1980) *Biochim. Biophys. Acta 603*, 63–69.

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

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

Cullis, P. R., de Kruijff, B., & Richards, R. E. (1976) *Biochim. Biophys. Acta 426*, 433–446.

Cullis, P. R., de Kruijff, B., Hope, M. J., Verkleij, A. J., Nayar, R., Farren, S. B., Tilcock, C., Madden, T. D., & Bally, W. R. (1983) *Membr. Fluid. Biol. 2*, 40–79.

de Kruijff, B., Van den Besselaar, A. M. H. P., & Van Deenen, L. L. M. (1977) *Biochim. Biophys. Acta 465*, 443–453.

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. (1985) in *Progress in Protein–Lipid Interactions* (Watts, A., & de Pont, J. J. H. H. M., Eds.) pp 89–142, Elsevier, Amsterdam.

Fornili, S. I., Vercauteren, D. P., & Clements, E. (1984) *Biochim. Biophys. Acta 771*, 151–164.

Glickson, I. D., Mayers, D. F., Settine, I. M., & Urry, D. W. (1972) *Biochemistry 11*, 477–486.

Kachar, B., & Reese, T. S. (1982) *Nature (London) 296*, 464–466.

Killian, J. A., & de Kruijff, B. (1985) *Biochemistry* (preceding 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.

Luzzati, V. (1968) in *Biological Membranes* (Chapman, D., Ed.) pp 71–123, Academic Press, New York.

Pasquali-Ronchetti, I., Spisni, A., Casali, E., Massoti, L., & Urry, D. W. (1983) *Biosci. Rep. 3*, 127–133.

Pinto da Silva, P., & Kachar, B. (1982) *Cell (Cambridge, Mass.) 28*, 441–450.

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

Seelig, J. (1977) *Q. Rev. Biophys. 10*, 353–418.

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

Shipley, G. G. (1973) *Biol. Membr. 2*, 1–89.

Siminovitch, D. J., Rance, M., & Jeffrey, W. R. (1984) *J. Magn. Reson. 58*, 62–75.

Sixl, F., Brophy, P. J., & Watts, A. (1984) *Biochemistry 23*, 2032–2039.

Södermann, P., Arvidson, G., Lindblom, G., & Fontell, K. (1983) *Eur. J. Biochem. 134*, 309–314.

Ulmius, J., Wennerström, H., Lindblom, G., & Arvidson, G. (1977) *Biochemistry 16*, 5742–5745.

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

Van Deenen, L. L. M., & De Haas, G. H. (1964) *Adv. Lipid Res. 2*, 168–229.

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*, 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.

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

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