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^{2}H-NUCLEAR MAGNETIC RESONANCE INVESTIGATIONS ON PHOSPHOLIPID ACYL CHAIN ORDER AND DYNAMICS IN THE GRAMICIDIN-INDUCED HEXAGONAL H_{II} PHASE

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ABSTRACT The following results are reported in this paper: (*a*) The interaction of gramicidin with [11,11-$^2H_2$]dioleoylphosphatidylcholine (DOPC) and [11,11-$^2H_2$]dioleoylphosphatidylethanolamine (DOPE) at different stages of hydration was studied by ^{2}H- and ^{31}P-nuclear magnetic resonance. (*b*) In the L_α phase in excess water the acyl chains of phosphatidylethanolamine (PE) are more ordered than phosphatidylcholine (PC) most likely as the result of the lower headgroup hydration of the former lipid. (*c*) In excess water gramicidin incorporation above 5 mol % in DOPC causes a bilayer → hexagonal H_{II} phase change. In the H_{II} phase acyl chain order is virtually unaffected by gramicidin but the peptide restricts the fast chain motions. (*d*) At low water content gramicidin cannot induce the H_{II} phase but it markedly decreases chain order in the DOPC bilayer. Increasing water content results in separation between a gramicidin-poor and a gramicidin-rich L_α phase with decreased order of the entire lipid molecule. Further increase in hydration reverts at low gramicidin contents the phase separation and at high gramicidin contents results in a direct change of the disordered lamellar to the hexagonal H_{II} phase. (*e*) Gramicidin also promotes H_{II} phase formation in the PE system but interacts much less strongly with PE than with PC. The results support our hypothesis that gramicidin, by a combination of strong intermolecular attraction forces and its pronounced cone shape, both involving the four tryptophans at the COOH-terminus, has a strong tendency to organize, with the appropriate lipid, in intramembranous cylindrical structures such as is found in the H_{II} phase.

INTRODUCTION

Gramicidin A is a linear pentadecapeptide produced by *Bacillus brevis* strain ATCC 8185 (1). It is an uncharged, very hydrophobic polypeptide with alternating L and D amino acids. This structural feature permits an organization in β-type of helices. Besides its proposed role in gene regulation during the shift from vegetative growth to sporulation (2), the peptide has two pronounced effects on model and biological membranes. At very low concentrations it can form by NH_2-terminal to NH_2-terminal dimerization (3, 4), possibly in combination with lateral oligomerization (5), hydrophilic channels with an inner diameter of ~4 Å (for a recent review on channel properties see reference 6). At higher concentrations it can strongly influence membrane lipid order, dynamics, and organization (for a recent review see reference 7).

In aqueous media mixtures of the peptide with lysophosphatidylcholines are organized in extended gramicidin-rich bilayers (8, 9) despite the preferred micellar organization of the pure lipid. In these bilayers acyl chain order and dynamics are virtually unaffected by the peptide (10), which is probably highly aggregated under these conditions (9, 10).

In hydrated mixtures with saturated diacylphosphatidylcholines with chain lengths of 16 carbon atoms or less an overall bilayer structure is maintained (11, 12). At low peptide concentrations (gramicidin/lipid < 1:15, molar), the acyl chains are more ordered (13–15), but at higher concentrations (gramicidin/lipid > 1:15, molar), acyl chain order decreases (13–15) in conjunction with peptide aggregation (12, 16).

Most interestingly, the peptide has a pronounced H_{II} phase promoting ability in liquid–crystalline phosphatidylcholine (PC) model membranes in which the acyl chain length exceeds 16 carbons atoms (11, 17). This property is not restricted to PCs but also is observed for phosphatidylethanolamines (PEs) (11, 16, 17), which by themselves already have a strong tendency to adopt this phase (for recent review of lipid polymorphism see reference 18), for the negatively charged phospholipids phosphatidylserine, phosphatidylglycerol, and cardiolipin (19), and even for the total lipid extract and the membrane of the human erythrocyte ghost (Tournois, H., unpublished observa-

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tions). H_{II} phase preferring lipids and the structures they can form appear to be of vital importance for various functional abilities of biological membranes, such as fusion, protein insertion, and translocation, lipid flip-flop, transport of polar compounds across membranes, and activation of membrane-bound enzymes, and for the formation of specific structures such as the tight junction and prolamellar body (for review see reference 18).

Gramicidin can serve as an interesting model to understand the mechanistic details of peptide-induced H_{II} phase formation. A combination of factors has been shown to be of importance for the gramicidin-induced H_{II} phase formation in dioleoylphosphatidylcholine (DOPC) model membranes, a system that has been studied most thoroughly. Concerning the chemical structure of the peptide it appears that the four tryptophans, all localized at the COOH-terminus at positions 9, 11, 13, and 15, play a very important role. These bulky residues govern a pronounced cone shape to the molecule. Formylation of them results in complete loss of H_{II} phase formation (20) and even a single replacement of tryptophan 9 or 11 by phenylalanine results in a drastic reduction in the extent of H_{II} phase formation (20a). Furthermore it was shown that the peptide needs to be in a hydrated conformation for H_{II} phase induction (21).

From these data and the motional behavior of water in this system (21), the thermodynamics of interaction (16), and the results from a theoretical conformational analysis (Brasseur, R., J.-M. Ruysschaert, J. A. Killian, and B. de Kruijff, unpublished observations), a model was proposed in which the strong tendency of the molecule to self associate (in PEs even stronger than in PCs [16]) via inter- and intramolecular tryptophan stacking interactions into multimolecular cylindrical structures was considered to be the main driving force for H_{II} phase formation by gramicidin (7). In other words, it appears that the peptide itself has a strong tendency to organize in tubular structures and that it thus can be considered the first example of a new class of hydrophobic peptides or proteins that together with suitable membrane lipids can form stable intramembraneous cylindrical structures.

To gain further insight into the effect of gramicidin on order and dynamics in the hydrocarbon region of DOPC model membranes, we applied in this study ^{2}H-nuclear magnetic resonance (NMR) techniques to specifically chain ^{2}H-labeled DOPC. With this technique valuable information has been obtained on acyl chain order and dynamics in protein–lipid systems (22) and on lipid polymorphism in pure lipids systems (23).

The results will be compared with those obtained from similar measurements on model membranes of ^{2}H-acyl chain–labeled dioleoylphosphatidylethanolamine (DOPE), which undergoes a temperature-dependent bilayer $\rightarrow$ H_{II} phase transition (24), and will be discussed in light of the proposed model for gramicidin-induced H_{II} phase formation.

MATERIALS AND METHODS

Materials

Gramicidin (from *Bacillus brevis*, consisting for 80% of gramicidin A) and deuterium-depleted water were purchased from Sigma Chemical Co. (St. Louis, MO). Methyl hydrogen nonanedioate, 1.6 M solution of butyl lithium in hexane, BH_3-tetrahydrofuran complex, triphenylphosphine, and nonanal were obtained from Aldrich (Bruxelles, Belgium). ^2H_2O and CaH_2 were obtained from E. Merck (Darmstadt, Federal Republic of Germany). All chemicals were of analytical grade or better. Tetrahydrofuran was dried by refluxing with CaH_2 (2 g/200 ml) for 2 h followed by distillation onto a 4-Å molecular sieve. Dimethylsulfoxide was dried overnight over BaO, followed by distillation over CaH_2 onto a 4-Å molecular sieve.

Lipids

General. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine(DOPC) was synthesized as described (25). 1,2-[11,11-$^2H_2$]dioleoyl-*sn*-glycero-3-phosphocholine ([11,11-$^2H_2$]DOPC) was synthesized from [11,11-$^2H_2$] oleic acid according to Warner and Benson (26) and was partially converted with phospholipase D (27) into 1,2-[11,11-$^2H_2$]dioleoyl-*sn*-glycero-3-phosphatidylethanolamine ([11,11-$^2H_2$]DOPE). All lipids were finally purified by preparative high performance liquid chromatography (HPLC) on straight phase silica (28) and were considered to be pure as high performance thin layer chromatography (HPTLC) of highly loaded plates in the appropriate solvent system revealed a single spot after staining.

The gel–liquid crystalline phase transition properties of hydrated (50% wt H_2O) dispersions of DOPC and [11,11-$^2H_2$]DOPC were determined from heating curves (2.5°C/min) on a DSC 4 calorimeter (Perkin-Elmer Corp., Norwalk, CT) to be similar (transition temperatures $-19°$ and -20°C; transition enthalpies 8.0 and 7.1 kcal/mol, respectively).

Synthesis of [11,11-$^2H_2$]Oleic Acid. The synthesis of oleic acid specifically labeled with two deuterons at the 11-position is a modified version of the procedure described by Farren et al. (29) and involves the coupling, via a Wittig reaction of 9-carboxymethoxynonyltriphenyl phosphonium iodide (compound I) with [2,2-$^2H_2$]nonanal (compound II), to yield the methylester of [11,11-$^2H_2$]oleic acid ([11,11-$^2H_2$]18:1$_c$). Compounds I and II were prepared essentially as described (29). To improve the reproducibility and the yield of the Wittig coupling we used lithium *t*-butylate instead of sodium ethanolate as the catalyst. A typical synthesis goes as follows. To prepare the catalyst 3.2 ml of *t*-butanol and 10 ml of dry tetrahydrofuran are mixed followed by the addition, at 0°C under a stream of argon, of 20 ml of a 1.6 M solution of butyl lithium. The mixture was stirred for 30 min and was then added at 0°C under argon to a solution of 17.5 g compound I dissolved in 70 ml of dry tetahydrofuran/dimethylsulfoxide 6:1 (vol/vol). The solution was stirred an additional 30 min at room temperature and was then cooled back to 0°C. 3.9 g of compound II was added and the mixture was stirred for 1 h at room temperature. 10 ml H_2O was added and the solution was cooled to 0°C, whereafter it was poured into 150 ml ice-cold H_2O followed by two times extraction with 150 ml hexane. The combined extracts were washed with 100 ml H_2O and dried over anhydrous Na_2SO_4. Hexane and traces of dimethylsulfoxide were removed by rotary evaporation under high vacuum. The reaction mixture was dissolved in 100 ml dry hexane and the insoluble residue was discarded. Thin layer chromatography (TLC) analysis on silica gel in hexane/ether (1:1 vol/vol) revealed that the main product was the methyl ester of oleic acid. The free acid was obtained by refluxing in 100 ml 1 N KOH in 95% methanol for 5 min. The solution was cooled to 0°C and 400 ml H_2O was added. The pH was lowered to 1.0 by the addition of 6 N HCl. The free acid was extracted from the aqueous phase with hexane as described above and was purified

to a final yield of 70% on an open silica gel column eluting with hexane with up to 10% diethylether. High resolution gas-liquid chromatography on a capillary glass column (model 438A; Chrompack, Inc., Bridgewater, NJ) of the methylester of [11,11-$^2H_2$]18:1$_c$ and ^{13}C-NMR (WP-200; Bruker Instruments, Inc., Billerica, MA) of the free acid in $CDCl_3$ revealed that 6% of the double bond had a *trans* configuration (elaidic acid). The incorporation of deuterium in methyl-[11,11-$^2H_2$]18:1$_c$ was determined by mass spectrometry (MS80/DS55 mass spectrometer-computer combination; Kratos Analytical Instruments, Ramsey, NJ) to be 95%. The mass spectrum of methyl-18:1$_c$ consisted of peaks with m/e (% intensity): 294 (1.4%), 296 (71.7%), 297 (22.8%), 298 (4.1%); and of methyl-[11,11-$^2H_2$]18:1$_c$: 296 (1.1%), 297 (6.7%), 298 (66.7%), 299 (22%), and 300 (3.5%).

Sample Preparation

Lipid or lipid-gramicidin samples (~50-μmol lipid with appropriate amount of peptide) of controlled content of deuterium-depleted water were prepared for NMR as described before (21). Unless otherwise indicated samples were hydrated with an excess of 50% by weight of H_2O, which corresponds to $N = H_2O$/lipid (molar) of ~45. In experiments with [11,11-$^2H_2$]DOPC this lipid was sometimes, with identical results, 1:1 diluted with nonlabeled DOPC.

NMR

^{2}H-NMR spectra were recorded either at 46.1 MHz on a Bruker Instruments, Inc. MSL 300 spectrometer in a high resolution 10-mm broadband probe head with a 14-μs 90° radiofrequency (rf) pulse or at 30.7 MHz on a Bruker Instruments, Inc. WM 200 spectrometer in a 5-mm high resolution broadband probe head with a 12-μs 90° rf pulse. A $90°_x$–τ-$90°_y$ quadrupolar echo pulse sequence (30) was used with a pulse separation τ of 50–70 μs, a 50-KHz sweep width, 2 K data points, and a 5 × T_1 interpulse time (0.12–0.2 s). An exponential multiplication corresponding to a 100-Hz line broadening was applied to the accumulated free induction decays (obtained from 500–1,000 transients) before Fourier transformation. All ^{2}H-NMR spectra were symmetrized. Deuterium T_1 relaxation times were measured by the inversion recovery method, which was programmed to include the quadrupolar echosequence. T_1 was obtained with an accuracy of ~10% from a least-squares fit of the signal amplitudes. In all cases the data points could be fitted with a single exponential curve. Proton-noise decoupled ^{31}P-NMR spectra were recorded either at 121 MHz on the MSL 300 in a high resolution 10-mm broad band probe with a 14-μs 90° rf pulse or at 81 MHz on a Bruker Instruments, Inc. WP 200 spectrometer in a 10-mm high resolution probe head with a 18-μs 90° rf pulse. The decoupler was gated with an input power of 10, respectively 5 W on during 2, respectively 10% of the pulse cycle. Typically, 500–1,000 transients were recorded using 45° rf pulse using a 38.5 and 25 KHz sweep width, respectively, 4 K data points, and a 2-s interpulse time. The free induction decays were exponentially filtered resulting in a 75-Hz line broadening. The 0 ppm positions in the ^{2}H- and ^{31}P-NMR spectra correspond to the chemical shift positions of isotropically moving [11,11-$^2H_2$]DOPC molecules.

In the simplest case (solely quadrupolar contribution to the lineshape; angular independent linewidth; asymmetry parameter $\eta = 0$) the lineshape of the ^{2}H-NMR powder pattern can be described by reference 31:

$$g(\omega, \Delta\nu_q) = \int_0^{\pi/2} d\theta \sin\theta (\delta/\pi)/\{\delta^2 + [\omega \pm (\Delta\nu_q/2)(3\cos^2\theta - 1)]^2\}, \quad (1)$$

where δ is the halfwidth at halfheight of the Lorentzian broadening, $\Delta\nu_q$ the quadrupolar splitting, ω the resonance frequency relative to the central frequency, ω_o and θ the angle between the principle axis of the electric field gradient tensor at the deuterium nucleus and the magnetic field H_o. Such lineshapes were calculated for various values of δ and $\Delta\nu_q$ on a microcomputer (Apple Computer Inc., Cupertino, CA).

RESULTS

We decided to label both oleic acid chains of DOPC at the 11-position with two deuterons. At this position one single quadrupolar splitting ($\Delta\nu_q$) is observed in the ^{2}H-NMR spectrum of the hydrated lipid (29; see also Fig. 1), which demonstrates that all four deuterons in the molecules are motionally equivalent. The relative small value of $\Delta\nu_q \approx 6$ KHz (32; see also Figs. 1 and 2) makes that the spectrum can be recorded with good sensitivity and minimal instrumental distortion of the lineshape.

The ^{2}H-NMR lineshape is typical for an axially symmetric quadrupolar interaction tensor with an asymmetry parameter $\eta = 0$ (33, 34). Such spectra are commonly encountered for deuterons attached to phospholipid acyl chains in the liquid crystalline L_α phase (33, 34). The peak separation, which is equivalent to $\Delta\nu_q$, is a measure of the segmental order of the C-D bond and can be described in terms of an order parameter (S_{CD}).

$$\Delta\nu_q = \frac{3}{4}\left(\frac{e^2qQ}{h}\right)S_{CD}, \quad (2)$$

where (e^2qQ/h) is the static quadrupolar coupling constant (~170 KHz for a C-D bond). Gramicidin incorporation up to a molar ratio of [11,11-$^2H_2$]DOPC/gramicidin of 20 only results in a small decrease in $\Delta\nu_q$ (Fig. 2). Increasing the gramicidin content results in drastic changes in the ^{2}H-NMR spectrum. Besides the induction of an isotropic component of low intensity (<1% of the total signal) and of unknown origin, a second spectral component appears (Fig. 1) with a smaller value of $\Delta\nu_q$ (Fig. 2). The intensity of this component increases with increasing gramicidin content. These spectra can be reasonably well simulated with a set of two powder patterns with $\Delta\nu_q$ of, respectively, 6 and 1.5 KHz and an isotropic component (Fig. 1).

Insight into the origin of these spectral changes can be obtained from previous structural investigations in the DOPC-gramicidin system (21) and the ^{31}P-NMR spectra of the [11,11-$^2H_2$]DOPC–gramicidin samples shown in Fig. 1. In the absence of gramicidin a lineshape typical of an axially symmetric tensor describing the chemical shift anisotropy (csa) is observed, which is characteristic for phospholipids undergoing fast axial rotation in extended liquid–crystalline bilayers (39).

Incorporation of gramicidin in excess of [11,11-$^2H_2$]DOPC/gramicidin (20:1, molar) results, as in case of the nonlabeled DOPC (21), in the appearance of a second spectral component with a reduced linewidth and a reversal in sign of the residual chemical shift anisotropy ($\Delta\sigma$). This is due to the additional averaging of the csa by lateral diffusion of the phospholipids around the tubes of which the hexagonal H_{II} phase induced by gramicidin under these conditions is composed. Increasing the gramicidin content to a [11,11-$^2H_2$]DOPC/gramicidin ratio of five results in a lineshape that suggests that virtually all lipids are orga-

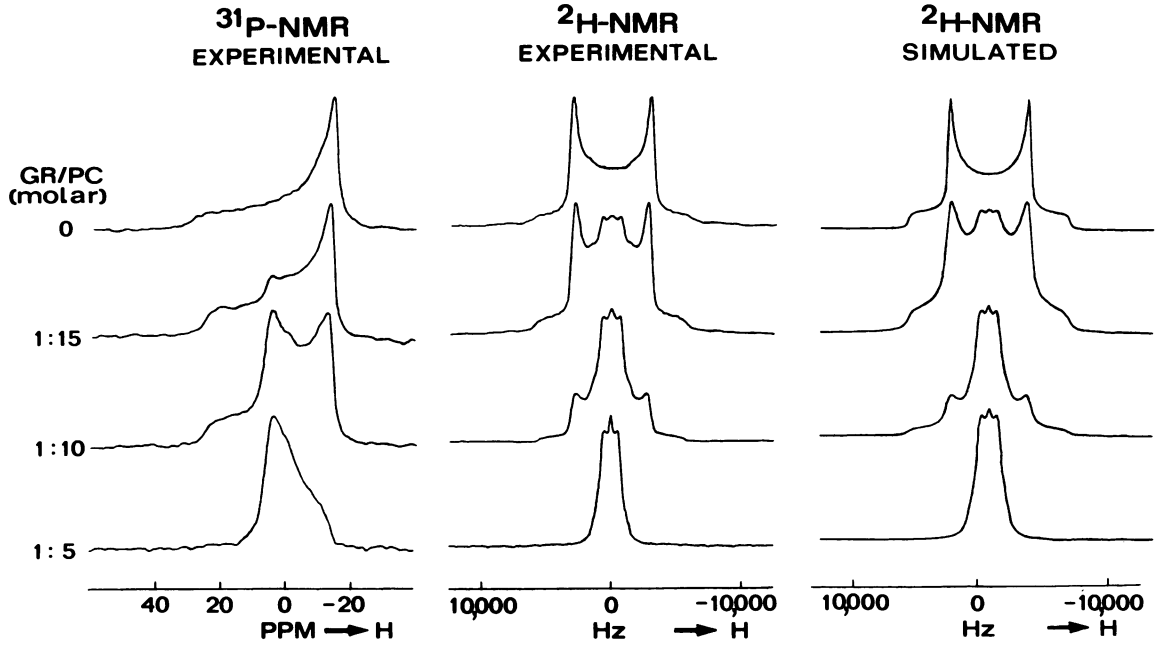

FIGURE 1 Experimental 81.0 MHz ^{31}P- and 30.7 MHz ^{2}H-NMR spectra of hydrated mixtures of gramicidin (GR) and [11,11-$^{2}H_2$]-DOPC (PC) at 30°C and computer simulated ^{2}H NMR spectra of the same samples. The computer simulations were carried out as described in the Materials and Methods section with the following parameters: GR/PC 0, ($\Delta\nu_q$ = 6.0 KHz, δ = 300 Hz); GR/PC 1:15, ($\Delta\nu_q$ = 6.0 KHz, δ = 600 Hz) + 0.12[($\Delta\nu_q$ = 1.5 KHz, δ = 800 Hz) + 0.003($\Delta\nu_q$ = 0 KHz, δ = 800 Hz)]; GR/PC 1:10,($\Delta\nu_q$ = 6.0 KHz, δ = 600 Hz) + [($\Delta\nu_q$ = 1.5 KHz, δ = 800 Hz) + 0.003($\Delta\nu_q$ = 0 KHz, δ = 800 Hz)]; GR/PC 1.5, ($\Delta\nu_q$ = 1.5 KHz, δ = 800 Hz) + 0.003($\Delta\nu_q$ = 0 KHz, δ = 800 Hz).

nized in the H_{II} phase. It thus can be concluded that the ^{2}H-NMR lineshape with the small value of $\Delta\nu_q$ originates from [11,11-$^{2}H_2$]DOPC present in the gramicidin-induced H_{II} phase. In this phase $\Delta\nu_q$ slightly decreases with increasing gramicidin content (Fig. 2) but appears to be temperature independent in the 25°→55°C temperature range, in contrast to $\Delta\nu_q$ of gramicidin-free [11,11-$^{2}H_2$]DOPC in the L_α phase, which decreases with increasing temperatures (Fig. 3). Small angle x-ray diffraction performed as described (16) demonstrated that the tube diameter of the H_{II} phase in DOPC/gramicidin (10:1, molar) in excess water also was virtually temperature independent (20°C, 71.4 Å; 70°C, 69.6 Å).

^{2}H-NMR spin-lattice relaxation times (T_1) probe the dynamics experienced by the deuterons in the hydrophobic interior of the model membrane (33, 34). Fig. 4 demonstrates that gramicidin incorporation reduces T_1 in two ways. In the L_α phase at low gramicidin concentrations the nonlinear reduction in the T_1 with increasing gramicidin concentration strongly suggests a progressively less efficient interaction between gramicidin and the lipid molecules, possibly as a result of peptide aggregation. In the H_{II} phase T_1 is most strongly reduced, but appears now to be virtually independent of the gramicidin concentration. Fig. 5 shows that T_1 increases with increasing temperature both for the gramicidin-free [11,11-$^{2}H_2$]DOPC bilayer as well as for the hexagonal H_{II} phase of [11,11-$^{2}H_2$]DOPC/gramicidin (5:1, molar) system, demonstrating that the motions determining T_1 fall in the fast correlation regime: $\omega_0{}^2\tau_c^2 \ll 1$, where ω_0 is the Larmor frequency and τ_c the motional correlation time. The activation energy for these motions can be calculated to be 21.5 and 19.2 KJ/mol for DOPC and DOPC/gramicidin (5:1, molar), respectively. The lowering of T_1 by gramicidin can then be understood as a decrease in the rate of motion. For isotropic motion in such systems,

$$\frac{1}{T_1} = \frac{3\pi^2}{10}\left(\frac{e^2qQ}{h}\right)^2\left[\frac{\tau_c}{1+\omega_0^2\tau_c^2} + \frac{\tau_c}{1+4\omega_0^2\tau_c^2}\right]. \quad (3)$$

At 30°C correlation times of 0.11 and 0.18 ns for deuterons in [11,11-$^{2}H_2$]DOPC and [11,11-$^{2}H_2$]DOPC/gramicidin (5:1, molar) could be calculated, respectively.

In a recent study (21) of the hydration dependency of the phase structure of DOPC/gramicidin systems of vari-

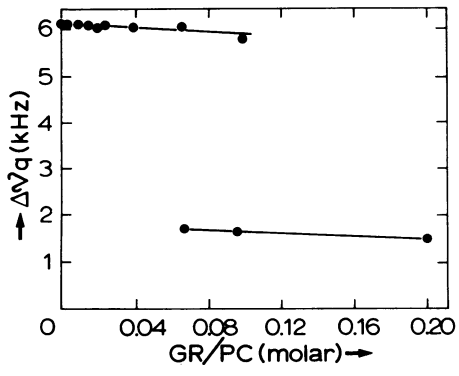

FIGURE 2 Quadrupolar splitting ($\Delta\nu q$ of 30.7 MHz ^{2}H-NMR spectra of hydrated mixtures of gramicidin (GR) and [11,11-$^{2}H_2$]DOPC(PC) at 30°C.

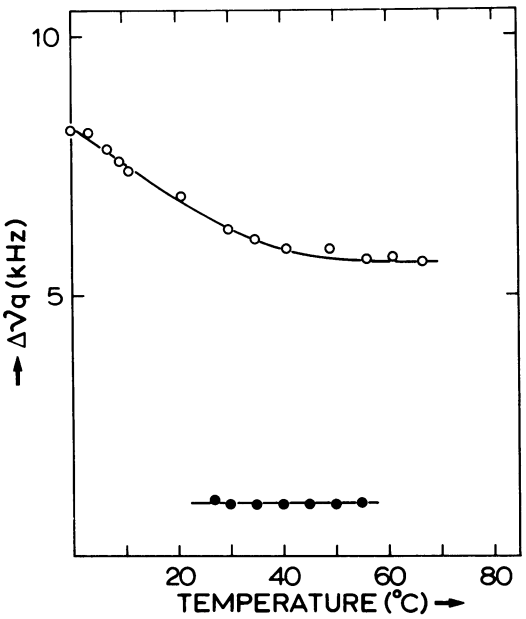

FIGURE 3 Temperature dependency of the quadrupolar splitting ($\Delta\nu_q$) in 30.7 MHz ^{2}H-NMR spectra of hydrated [11,11-$^2H_2$]DOPC in the absence (*open circles*) and presence (*solid circles*) of gramicidin (DOPC/gramicidin = 5:1, molar).

able water content, it became apparent that gramicidin is hydrated in preference over DOPC and that a hydrated conformation of gramicidin is essential for H_{II} phase formation. The formation of the H_{II} phase was preceded by separation in two lamellar phases, one rich in gramicidin with a decreased headgroup order, which converts into the H_{II} phase at higher water content, and one of low gramicidin content with the structural and motional features of the gramicidin poor or free DOPC bilayer. This is also apparent in the 121 MHz ^{31}P-NMR spectra of the [11,11-$^2H_2$]DOPC/gramicidin (10:1, molar) system with increasing H_2O content (Fig. 6). For N=H_2O/PC = 5 a single ^{31}P-NMR lineshape characteristic of a bilayer organization is observed (Fig. 6, *U*). Increasing N to 6 results in the appearance of a second "bilayer" ^{31}P-NMR signal with a reduced $\Delta\sigma$ superimposed on the broader "bilayer" lineshape (Fig. 6, *V*), in agreement with the occurrence of phase separation. Further increasing the water content

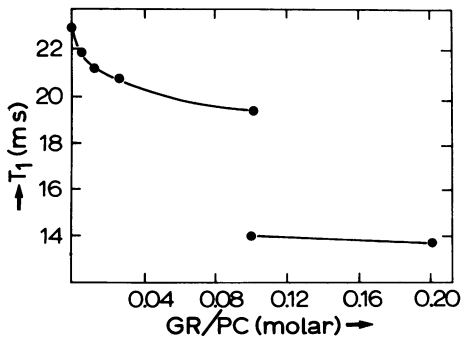

FIGURE 4 30.7 MHz ^{2}H spin-lattice relaxation times (T_1) of hydrated mixtures of gramicidin (GR) and [11,11-$^2H_2$]DOPC(PC) at 30°C.

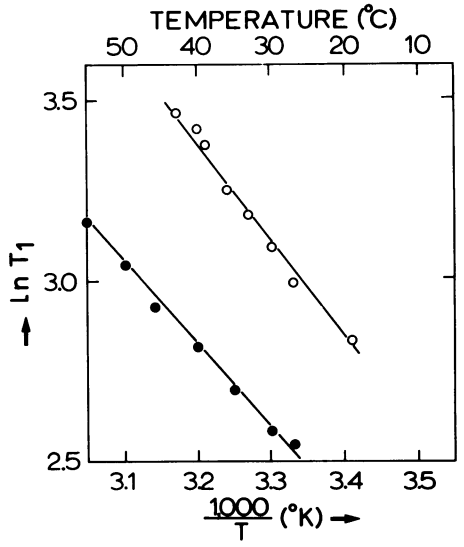

FIGURE 5 Arrhenius plot of the temperature dependency of 30.7 MHz ^{2}H T_1 values of hydrated [11,11-$^2H_2$]DOPC (*open circles*) and gramicidin/[11,11-$^2H_2$]DOPC (1:5, molar) (*solid circles*).

results in a gradual change of this former lineshape into one of an undefined shape, which for $N > 15$, converts to the characteristic "hexagonal" lineshape. With this information the experimental ^{2}H-NMR results of the [11,11-$^2H_2$]DOPC/gramicidin system can be more easily described and understood.

In the absence of gramicidin, $\Delta\nu_q$ increases with decreasing water content (Fig. 6 spectra; Fig. 8 for values of $\Delta\nu_q$) demonstrating that acyl chain order in DOPC increases with decreasing headgroup hydration. In the presence of gramicidin for N = 5, somewhat broadened ^{2}H-NMR lineshapes are observed with a single $\Delta\nu_q$, which decreases in a saturable fashion with increasing gramicidin content (Fig. 7).

In case of the [11,11-$^2H_2$]DOPC/gramicidin (10:1, molar) system, increasing N to 7 results in two overlapping ^{2}H-NMR powder patterns, one with a much reduced value of $\Delta\nu_q$ of 2.6 KHz and one with a value for $\Delta\nu_q$ for 8 KHz, which approaches that of the gramicidin-poor [11,11-$^2H_2$]DOPC bilayer (Fig. 8) in line with the phase separation occurring under these conditions. Further increase in N results in spectra composed of a broad isotropic signal superimposed on a powder pattern with a value of $\Delta\nu_q$, which is now very similar to that of the gramicidin-free [11,11-$^2H_2$]DOPC bilayer (Fig. 8). For $N > 15$ the broad isotropic component changes again in a resolved powder pattern with a much reduced value of $\Delta\nu_q$ (see also Fig. 1), originating from [11,11-$^2H_2$]DOPC in the H_{II} phase. A similar picture is observed for lower gramicidin concentrations. Increasing N from 5 to 7 results in two spectral components: a broad isotropic one and an underlying powder pattern. The intensity of the broad isotropic component increases with increasing gramicidin content. However, in contrast to the 1:10 situation a further increase of N leads to a gradual loss of intensity of the broader

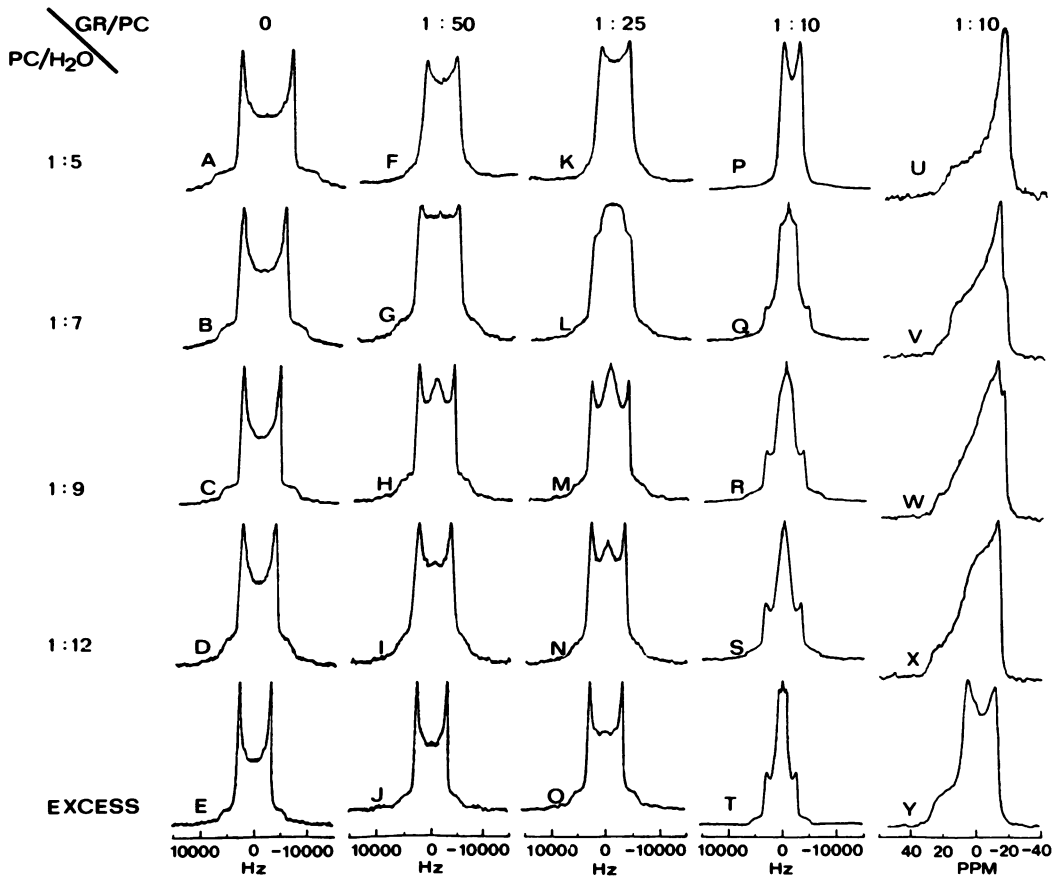

FIGURE 6 Effect of hydration and gramicidin (GR) incorporation on the 46.1 MHz ^2H (*A–T*) and 121 MHz ^{31}P (*U–Y*) NMR spectra of [11,11-$^2H_2$]DOPC(PC) model membranes at 30°C. The indicated numbers are molar ratio's of either GR/PC or PC/H_2O. In this latter case the quoted ratios are approximate. The exact values of H_2O/PC (molar) were: *A*, 4.6; *B*, 7.1; *C*, 9.1; *D*, 12.1; *F*, 4.0; *G*, 6.0; *H*, 9.6; *I*, 12.2; *K*, 4.9; *L*, 5.6; *M*, 9.1; *N*, 12.1; *P*, *U*, 4.8; *Q*, *V*, 7.2; *R*, *W*, 9.2; *S*, *X*, 11.8. Excess (*E*, *J*, *O*, *T*, *Y*) refers to 50% wt H_2O.

component to that of the powder pattern of which $\Delta\nu_q$ is again very similar to that of the [11,11-$^2H_2$]DOPC bilayer (Fig. 8). We next investigated the effect of gramicidin incorporation on acyl chain order and dynamics in PE systems. Unsaturated PEs show temperature-dependent bilayer → H_{II} phase transitions (18). For DOPE this transition occurs in the 5°–10°C temperature range (24, 32; this study). For in excess water hydrated [11,11-$^2H_2$]DOPE the ^{31}P-NMR spectrum of 8°C is indicative of a coexisting bilayer and hexagonal H_{II} phase (Fig. 9). In the ^2H-NMR spectrum also two spectral components are present with quadrupolar splittings of 11 KHz and 2.7 KHz originating from the L_α and hexagonal H_{II} phase, respectively. It is of interest to note that $\Delta\nu_q$ of [11,11-$^2H_2$]DOPE is ~5 KHz larger than $\Delta\nu_q$ of [11,11-$^2H_2$]DOPC under these conditions but is very similar to $\Delta\nu_q$ of [11,11-$^2H_2$]DOPC at low water content (compare Figs. 8 and 10). Increasing temperature results in a gradual decrease in $\Delta\nu_q$ of [11,11-$^2H_2$]DOPE in the H_{II} phase (Fig. 10). Gramicidin incorporation in PE model membranes also strongly promotes H_{II} phase formation (16, 17), which is illustrated in Fig. 9 by the much larger H_{II} component in the ^{31}P- and ^2H-NMR spectra of [11,11-$^2H_2$]DOPC/gramicidin (25:1) at 8°C. Fig. 10 shows that gramicidin incorporation has no significant effect on $\Delta\nu_q$ in the lamellar phase but causes a slight reduction of $\Delta\nu_q$ in the H_{II} phase in the 10°–25°C temperature range. Above 25°C gramicidin has virtually no effect on $\Delta\nu_q$. The deuterium T_1 values of [11,11-$^2H_2$]DOPE are similar in the bilayer and H_{II} phase of the lipid. In marked contrast to [11,11-$^2H_2$]DOPC gramicidin incorporation does not affect deuterium T_1 of [11,11-$^2H_2$]DOPE (compare Table I and Fig. 4).

DISCUSSION

The results of this study not only provide new information on acyl chain order and dynamics in the gramicidin/DOPC H_{II} and the molecular mechanism of the gramicidin-induced L_α → H_{II} transition but also illustrate some interesting aspects of lipid acyl chain order and dynamics in peptide-free hydrated unsaturated PC and PE systems, which we will discuss first.

DOPE and DOPC, when hydrated in excess water, both adopt the L_α phase below 10°C. Despite the fact that the acyl chains of both lipids under these conditions are similar, the $\Delta\nu_q$ of the [11,11-$^2H_2$]deuterons in DOPE is substantially larger than in DOPC (11 and 7.5 KHz, respectively, at 10°C; compare Figs. 3 and 10). This

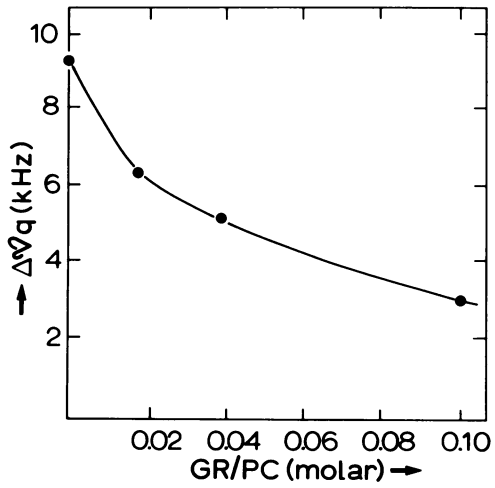

FIGURE 7 Effect of gramicidin incorporation on the quadrupolar splitting ($\Delta\nu_q$) at 46.1 MHz of mixtures of gramicidin (GR) and [11,11-$^2H_2$]DOPC(PC) at H_2O/PC ratios of N = 4.6–4.9. The exact H_2O/PC ratios of the various data points are: GR/PC = 0 (N = 4.6), GR/PC = 0.02 (N = 4.9), GR/PC = 0.04 (N = 4.9), GR/PC = 0.10 (N = 4.8).

demonstrates that in a bilayer acyl chain (at least the 11-position) order is sensitive to headgroup structure and increases going from the PC to the PE headgroup. Although detailed information on headgroup structure in unsaturated liquid crystalline PCs and PEs is very limited, extrapolation from crystal structure data and spectroscopic measurements on saturated analogues (both reviewed in reference 36) suggests that the two most important differences in the headgroup lattice of PE and PC bilayers are the stronger intermolecular polar (electrostatic and hydrogen bonding) interactions between the PE headgroups and the lower state of hydration of the PE headgroup. Since $\Delta\nu_q$ of [11,11-$^2H_2$]DOPC strongly increases upon decreasing headgroup hydration (Fig. 8), we conclude that differences in PC and PE headgroup hydration in the lamellar phase in excess water are the main reasons for the difference in acyl chain order between the lipids. An interesting consequence of this observation for membrane fusion could be that (protein-induced) lipid headgroup dehydration, which appears to be an important step in the fusion process, could result in local changes in lipid order at the site of fusion.

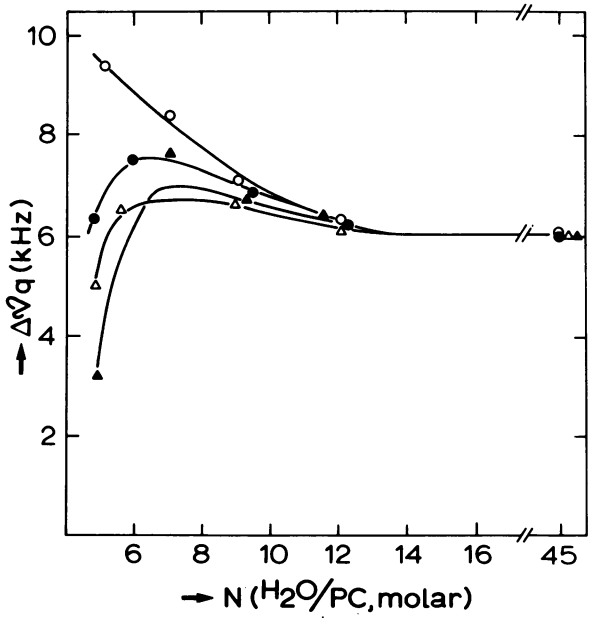

FIGURE 8 Quadrupolar splitting ($\Delta\nu_q$) of 46.1 MHz ^{2}H-NMR spectra of mixtures of gramicidin (GR)-[11,11-$^2H_2$]DOPC-H_2O at 30°C. In multicomponent spectra, the value of $\Delta\nu_q$ is obtained from the outermost splitting. GR/PC = 0 (*open circles*); GR/PC = 1:50 (*solid circles*); GR/PC = 1:25 (*open triangles*); GR/PC = 1:10 (*solid triangles*).

The $L_\alpha \rightarrow H_{II}$ transition of DOPE is accompanied by a large reduction (76%) of $\Delta\nu_q$ of the [11,11-$^2H_2$]deuterons, in agreement with previous observations on this (32) and other PEs (37). This is the result of a combination of further averaging of the nuclear quadrupolar interaction by lateral diffusion of the lipids around the H_{II} tubes and changes in chain order accompanying the $L_\alpha \rightarrow H_{II}$ transition.

The lateral diffusion contribution results in a reduction of $\Delta\nu_q$ by 50% (34). Thus, in the H_{II} phase,

$$\Delta\nu_q = \frac{3}{8}\left(\frac{e^2qQ}{h}\right) \cdot S_{CD}. \quad (4)$$

From Eqs. 2 and 4 the values of S_{CD} in the L_α and H_{II} phase of [11,11-$^2H_2$]DOPE can be calculated to be 0.086 and 0.042, respectively, demonstrating a 51% reduction in C-D bond order parameter on going from the L_α to the H_{II} phase. Such a decreased chain order is in agreement with electron spin resonance (ESR) results (38) and can be attributed to the inverted geometry of the H_{II} phase with its highly curved lipid–water interface. In this respect it is of interest to recall that the value of $\Delta\sigma$ in the ^{31}P-NMR spectrum of PEs is reduced by only 50% by the $L_\alpha \rightarrow H_{II}$ transition, demonstrating that local headgroup structure (at the level of the phosphate) is similar for both phases and that only the lateral diffusion of the lipids around the H_{II} tubes further averages the ^{31}P-csa (39, 40). $\Delta\nu_q$ of the deuterons in [11,11-$^2H_2$]DOPE in the H_{II} phase decreases with increasing temperature, which agrees well with the parallel decrease in tube diameter (16), both reflecting the reduction in overall length of the oleic acid chain in the DOPE H_{II} phase on increasing the temperature.

Now we would like to discuss the consequences of gramicidin-induced H_{II} phase formation in DOPC model membrane for acyl chain order and dynamics of the lipid. In the gramicidin/DOPC H_{II} phase $\Delta\nu_q$ of the [11,11-$^2H_2$]deuterons is reduced by 72%. From the data depicted in Fig. 2 and Eqs. 2 and 4, it can be calculated that $S_{CD}(L_\alpha)$ = 0.047 and $S_{CD}(H_{II})$ = 0.027. Thus, the reduction in S_{CD} by 43% accompanying the $L_\alpha \rightarrow H_{II}$ transition induced by gramicidin is similar in magnitude to the decrease in S_{CD} caused by the temperature-dependent $L_\alpha \rightarrow H_{II}$ transition in DOPE, demonstrating that the main effect determining the reduction in $\Delta\nu_q$ is the change in motion and order associated with the phase change itself (i.e., lipid diffusion

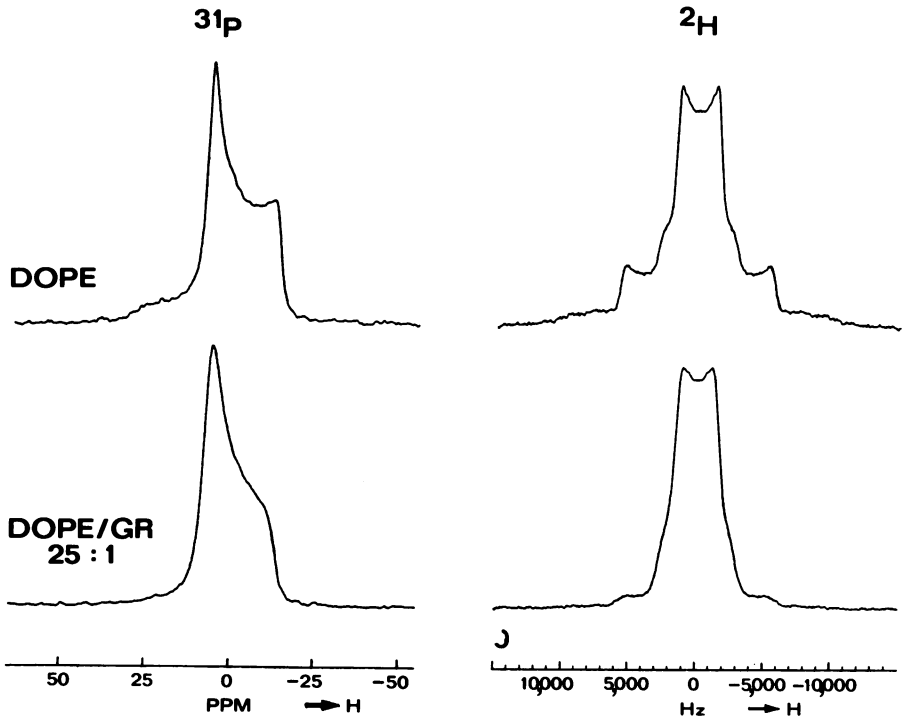

FIGURE 9 121 MHz ^{31}P- and 46.1 MHz ^{2}H-NMR spectra of hydrated [11,11-$^{2}H_2$]DOPE at 8°C in the absence and presence of gramicidin (DOPE/GR 25:1).

around the H_{II} tubes and change in local order due to the inverted character of the phase) and that it is not due to significant changes in chain order induced by the gramicidin–DOPC interaction. In marked contrast to the temperature-dependent decrease of $\Delta\nu_q$ and tube diameter of the [11,11-$^{2}H_2$]DOPE H_{II} phase, $\Delta\nu_q$ and the tube diameter of [11,11-$^{2}H_2$]DOPC in the H_{II} phase with gramicidin is temperature independent. This strongly suggests that the structure of the DOPC/gramicidin H_{II} phase is determined primarily by gramicidin and not by DOPC. That the extent of H_{II} phase formation as well as the H_{II} tube diameter is the same for DOPC and the negatively charged DOPS and DOPG (19) further supports our conclusion that gramicidin forms the structural backbone of the H_{II} phase. The $L_\alpha \rightarrow H_{II}$ phase change induced by gramicidin in DOPC is causing a decrease in T_1 of the [11,11-$^{2}H_2$]deuterons, which as described in the Results section translates into a 64% increase in the correlation time for the rapid motions that determine T_1. Thus, in the H_{II} phase the gramicidin–DOPC interaction results in a decrease in the rate of acyl chain motion while chain order is virtually unaffected.

With respect to the mechanism of gramicidin-induced H_{II} phase formation we first restrict the discussion to the situation in which the samples are hydrated with excess water. At this stage of hydration incorporation of gramicidin into the L_α phase of DOPC causes a slight reduction in $\Delta\nu_q$ and most clearly a nonlinear decrease in T_1 of the [11,11-$^{2}H_2$]deuterons (Fig. 4). These data together with calorimetric measurements on mixed dipalmitoylphosphatidylcholine/gramicidin bilayers (12, 16) demonstrate that at low concentrations gramicidin dissolves in the liquid crystalline bilayer, causes a (slight) decrease in chain order and motion, and interferes with gel state formation. Increasing the gramicidin concentration in the bilayer results in progressively more (lateral) gramicidin–gramicidin interactions, leading to a reduction in the molecular efficiency of the gramicidin–lipid interaction. This process of self-association results at a molar ratio of lipid to gramicidin between 15 and 20 in a separation between a lamellar phase saturated with gramicidin and an H_{II} phase containing the excess gramicidin. This H_{II} phase is highly enriched in gramicidin (20a, 21). Further support for this interpretation comes from the gramicidin concentration dependency of H_{II} phase formation. From the simulated ^{2}H-NMR spectra of Fig. 1 the amount of H_{II} phase can be calculated as a function of the gramicidin content. The results that are depicted in Fig. 11 indicate that for the first 5 mol % of gramicidin no significant H_{II} phase formation is observed. Above this value there is an approximately linear increase in H_{II} phase formation with increasing gramicidin content. From the slope of the curve a stoichiometry of 7 PC per gramicidin in the H_{II} phase can be calculated. Excess gramicidin can be incorporated into this phase (20a). Very recently density gradient centrifugation experiments unambigously established the formation of a separate gramicidin rich H_{II} phase with a PC/gramicidin ratio of 7 or lower (20a).

Additional information on the mechanism of gramicidin-induced H_{II} phase formation can be derived from the

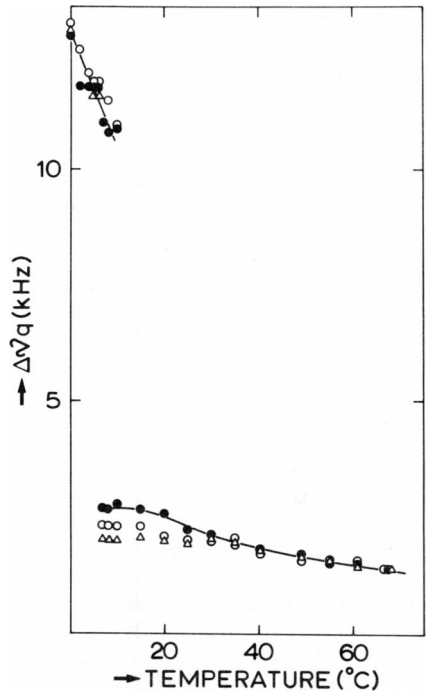

FIGURE 10 Effect of temperature and gramicidin content on the quadrupolar splitting ($\Delta\nu_q$) of 46.1 MHz ^{2}H-NMR spectra of hydrated [11,11 $^2H_2$]DOPE in the absence or presence of gramicidin. DOPE/gramicidin (molar): ∞ (*solid circles*); 50:1 (*open circles*); and 25:1 (*open triangles*).

results obtained from samples of varying water content (Figs. 7–9). Water is essential for the gramicidin-induced H_{II} phase formation in DOPC systems (21). Thus at a low stage of PC headgroup hydration ($N = 5$) a single lamellar phase is observed for all gramicidin concentrations tested. In this lamellar phase there is a drastic reduction in $\Delta\nu_q$ of the [11,11-$^2H_2$]deuterons, which is remarkable in view of the very small changes reported in $\Delta\nu_q$ upon incorporation of different intrinsic membrane proteins and peptides (reviewed in reference 41) into phospholipid bilayers. Since normally at a low water content H_{II} phase formation is favored (42), we previously suggested that apparently gramicidin at low hydration is organized in a particular conformation (which recently for a similar anhydrous lipid system was suggested to be the antiparallel double helix [43]; for terminology of gramicidin structure see reference 44) in which it is unable to induce the H_{II} phase. Apparently in this conformation it is in strong interaction with the lipid acyl chains, thereby decreasing chain order at the 11-position. The ^{31}P residual chemical shift anisotropy is only decreased to a very small extent by gramicidin incorporation for $N = 5$ (21), demonstrating that the disordering effect is restricted to the acyl chain part of the bilayer. The nonlinear saturable decrease in $\Delta\nu_q$ with increasing gramicidin concentration for $N = 5$ (Fig. 7) suggests a progressively extensive peptide aggregation, thereby causing a less efficient lipid–peptide interaction. Upon increasing the water content, gramicidin becomes preferentially hydrated over PC (21) and in this hydrated new conformation phase separation occurs between a gramicidin-poor lamellar phase with its usual structural and motional characteristics and a gramicidin-rich lamellar phase. In this latter phase both headgroup (21) and acyl chain order (Fig. 6) are strongly decreased, suggesting that the entire lipid molecule is highly disordered. The ^{31}P- and ^{2}H-NMR lineshapes originating from this phase at intermediate gramicidin or water contents (Fig. 6) indicate complex motional behavior that at present is not fully understood but could be caused in part by lateral diffusion limitations of PC molecules due to the presence of ordered gramicidin aggregates. For a DOPC/gramicidin ratio of 10:1, upon further increasing the water content, the disordered gramicidin-rich lamellar phase directly converts to the H_{II} phase. At lower gramicidin concentrations, i.e., below the threshold concentrations of gramicidin for H_{II} phase formation in excess H_2O, the broad isotropic component in the ^{2}H-NMR spectra vanishes with increasing water content. This suggests that upon further hydration the phase separation occurring at lower water contents vanishes and that the now fully hydrated gramicidin (possibly in the $\beta_{6.3}$ N-N terminal dimer [44] which is the channel conformation) and DOPC molecules are again fully miscible in a lamellar phase. A detailed structural model of the gramicidin-induced H_{II} phase formation, which integrates all the present knowledge on the system, will be presented elsewhere.

TABLE 1
EFFECT OF GRAMICIDIN (GR) INCORPORATION ON T_1 OF ^{2}H AT 46.1 MHZ AND 8°C OF HYDRATED [11,11-$^2H_2$]DOPE

DOPE/GR	$\Delta\nu_q$	T_1
mol	*KHz*	*ms*
∞	10.8 (L_α)	13 ± 1
	2.7 (H_{II})	13 ± 1
50	2.3 (H_{II})	13 ± 1
25	2.0 (H_{II})	13 ± 1

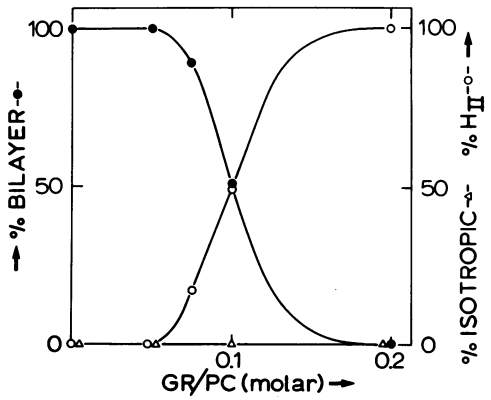

FIGURE 11 Quantification of the "bilayer," "hexagonal H_{II}," and "isotropic" components in the ^{2}H-NMR spectra of different gramidicin-DOPC mixtures. The data are obtained from the spectral simulations described in Fig. 1.

Finally, we would like to compare the interaction of gramicidin with DOPE and DOPC. Previous differential scanning calorimetry (DSC) studies (12, 16) demonstrated that in the lamellar gel state of PE there is virtually no interaction between the peptide and the lipid. From ^{31}P-csa measurements of PE in the H_{II} phase it could be concluded that only headgroup order was slightly decreased by gramicidin incorporation (16).

Our present ^{2}H-NMR data on [11,11-$^2H_2$]DOPE demonstrated that the incorporation of gramicidin does not significantly affect $\Delta\nu_q$ and T_1 of the [11,11-$^2H_2$]deuterons or the temperature dependency of $\Delta\nu_q$. These results contrast the behavior of PC for which gramicidin–lipid interactions in the three different phases are observed (16, 21; and this study).

We suggest that the less efficient PE–gramicidin interaction is the result of a combination of the intermolecular attractive forces between gramicidin molecules (involving the tryptophans) and the strong intermolecular attractive forces between the PE headgroups. The H_{II} phase promoting ability of gramicidin in DOPE and other PE systems then most likely is the result of the tendency of the gramicidin molecules to self-associate and, due to their conical shape, to organize in cylindrical structures that act as nucleation sites for the H_{II} phase formed by the PE.

The skillful contributions of W. S. M. Geurts van Kessel and J. W. Timmermans in the synthesis of the lipids used in this study are gratefully acknowledged.

We would like to thank the mass spectrometric division of the analytical chemistry department of the University of Utrecht for their help in the analysis of [11,11-$^2H_2$]oleic acid. Some of the NMR measurements were carried out on the WM 200 spectrometer of the SON hf-NMR facility in Nijmegen.

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Note Added in Proof. In a recent ^{2}H-NMR study of ^{2}H-labeled oleic acid chain containing phospholipids (Perly, B., I. C. P. Smith, and H. C. Jarrell, 1985, *Biochemistry,* 24:1055–1063) also a more ordered state of the acylchains in PE as compared to PC bilayers was reported.

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