Phase Equilibria and Molecular Packing in the *N,N*-Dimethyldodecylamine Oxide/Gramicidin D/Water System Studied by ^{2}H Nuclear Magnetic Resonance Spectroscopy

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ABSTRACT A partial phase diagram of the system *N,N*-dimethyldodecylamine oxide (DDAO)/water/gramicidin D was determined by ^{2}H-NMR. Both ^2H_2O and perdeuterated DDAO (DDAO-d_{31}) were studied by solid state NMR techniques. Addition of gramicidin D to the micellar (L_1), normal hexagonal (H_I) and cubic (I) phases of DDAO induces phase separations, giving two-phase regions, which all contain a lamellar (L_α) phase. The L_α phase containing gramicidin is characterized by larger order parameters for DDAO-d_{31} compared with the corresponding order parameters in the L_α and H_I phases of DDAO-d_{31}/H_2O. The L_α phase may stay in equilibrium with any other phase in the phase diagram. The DDAO exchange between the coexisting phases is slow on the NMR timescale, which is why the recorded NMR spectrum consists of superimposed spectra from the different phases occurring in the sample. Gramicidin D can be solubilized in appreciable quantities only in the lamellar phase of DDAO-d_{31}. Increasing amounts of gramicidin in the liquid crystalline phases result in a continuous increase in the molecular ordering up to about 5 mol% gramicidin, where a plateau is reached. This is consistent with a recent theoretical model describing the influence on the ordering of lipids by a membrane protein with larger hydrophobic thickness than the lipid bilayer. The solvent used for dissolving gramicidin at the incorporation of the peptide in the lipid aggregates has no effect on the ^{2}H-NMR lineshapes of DDAO-d_{31}. It is concluded that gramicidin is solubilized in the L_α phase and that it always adopts the channel conformation independent of a particular solvent. The channel conformation is also supported by CD studies. In some of the samples, macroscopic orientation of the lipid aggregates is observed. It is concluded that DDAO-d_{31} in the binary system favors an orientation with the long axis of the hydrocarbon chain perpendicular to the magnetic field, whereas when gramicidin D is present the hydrocarbon chain orients parallel to the magnetic field. This is explained by the fact that gramicidin aligns with its helical axis parallel to the magnetic field, thereby forcing also the DDAO-d_{31} molecules to obtain such an orientation.

INTRODUCTION

Surfactants are widely used in processes like extraction of membrane proteins and in reconstitution of peptides and proteins in lipid vesicles (Gibson and Brown, 1990; Helenius and Simons, 1975). In structure determinations by multidimensional NMR methods of membrane-bound proteins and peptides, the result depends critically on the ability to solubilize the macromolecule in micelles, which are small enough to give high resolution NMR spectra (Killian et al., 1994). In the crystallization process of membrane proteins, the choice of detergent can greatly influence the result (Michel, 1982). The zwitterionic surfactant *N,N*-dimethyldodecylamine oxide (DDAO) has often proved to be a good choice for membrane protein work, but there is still a lack in the understanding of how detergent and proteins interact with each other. It is often difficult to predict which detergent should be chosen to obtain the best results. Therefore, it is a prerequisite to characterize the factors governing detergent-protein interactions and their thermodynamic phase behavior. Such studies will most probably also give information about the interactions between membrane proteins and the various lipids present in the membrane. Furthermore, such information would be necessary for an understanding of the influence of different lipid compositions in the membrane on enzyme activity.

To get information about the physico-chemical properties and the mechanisms behind the solubilization process of a large macromolecule like a peptide in a lipid bilayer, we have started a systematic investigation of such systems. In this work we investigate the pentadecapeptid gramicidin D in a detergent often used in the extraction of membrane proteins. This peptide has been used in many previous investigations and has many advantages. It is available in large quantities, the chemical structure is well known, and it has been shown to have large effects on the order and dynamics of lipids. It has also been shown to induce changes in the phase structures in membrane lipid systems. An extensive review on gramicidin in lipid systems has recently been published (Killian, 1992).

This communication reports on changes in the detergent organization by addition of gramicidin D to a DDAO/water system. A ternary phase diagram has been determined mainly by ^{2}H-NMR on deuterated water and perdeuterated DDAO (DDAO-d_{31}). ^{2}H-NMR studies of DDAO-d_{31} show that addition of gramicidin to a DDAO/water system results in the formation of a lamellar (L_α) phase, which forms at a wide range of water contents and may be in equilibrium with any other phase formed in the system.

MATERIALS AND METHODS

DDAO was purchased from SERVA Feinbiochemica GMBH & Co. (Heidelberg, Germany), the perdeuterated DDAO-d_{31} was synthesized

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according to a method described below, deuterium-depleted water was purchased from Fluka (Ronkonkoma, NY), deuterated water was from Dr. Glaser AG (Basel, Switzerland), and gramicidin D was purchased from Sigma Chemical Co. (St. Louis, MO). All of the materials purchased were used without further purification.

NMR

DDAO-d_{31} and gramicidin D were dissolved in chloroform:methanol (C:M), 2:1 or trifluoroethanol (TFE) to the desired mole fraction of gramicidin (X_G) and transferred into glass test tubes. X_G denotes the mole percentage of gramicidin with respect to the total amount of gramicidin and DDAO ($X_G = 100 \cdot n_G/(n_G + n_{DDAO})$. The solvent was removed under vacuum, and the appropriate amount of deuterium-depleted water was weighed into the test tube, which was flame-sealed and thoroughly mixed. The sample was left at room temperature to equilibrate for several days. The test tube was finally cut and sealed with a silicon stopper to fit in the NMR-probe.

The ^{2}H-NMR spectra of DDAO-d_{31} were recorded at 76.78 MHz on a Bruker AM-500 spectrometer equipped with a high power broad band probe for cylindrical samples with horizontal long axis (Cryomagnet Systems Inc., Indianapolis, IN). ^{2}H-NMR on deuterated water was performed at 15.36 MHz on a Bruker MSL-100 spectrometer with a Bruker high power probe. Temperature was controlled by a heated air flow and monitored by means of a thermocouple close to the sample. The signal was collected with a quadrupole echo sequence (Davis et al., 1976) and transferred to an IRIS workstation for data processing with the FTNMR program (Hare Research Inc., Woodinville, WA). The 90° pulse length was 4.7 μs on the AMX-500 and 4.0 μs on the MSL-100. The time between the 90° pulses was 60 μs, and the relaxation delay was 1 s. The resulting DDAO-d_{31} spectra were corrected for incomplete spectral coverage (Bloom et al., 1980), and a depakeing program (Bloom et al., 1981; Sternin et al., 1983) was then used to calculate an oriented sample spectrum from the powder pattern. The resulting depaked spectra correspond to a director orientation perpendicular to the magnetic field.

Low angle x-ray diffraction

The x-ray experiments were performed at Station 8.2 at the Daresbury Laboratory (Cheshire, U.K.) using a monochromatic beam of wavelength 1.5 Å. The repeat distance obtained from the measurements corresponds to the thickness of the bilayer and the water (Luzzati, 1968). If the density of DDAO and the water is approximated to be equal, the bilayer thickness can be calculated simply by subtracting the water contribution. This value has then been multiplied by 0.5 to obtain the thickness of one monolayer, corresponding to the length of the DDAO molecule.

Light spectroscopy

The UV light absorption spectra of the micellar (L_1) phases were collected on a Beckman DU-70 spectrometer after a 10-fold dilution of the samples to obtain an absorption at 280 nm of about 0.5. For the calculations of the gramicidin concentration in the samples, the extinction coefficient of gramicidin was taken to be 22,400 $cm^{-1} M^{-1}$ (J. A. Killian, personal communication).

CD-spectra were obtained using a Jasco 3000. The samples were diluted 100-fold before measurements.

Synthesis of DDAO-d_{31}

Deuterium gas was from ICON Stable Isotopes and purchased from Larodan (Malmö, Sweden). Deuterium oxide (99.9%) was from Sigma, lithium aluminium deuteride (99.0%) and dimethylamine-d_6·HCl (99.5%) from Dr. Glaser AG (Basel, Switzerland). Adam's catalyst (PtO_2 type IV) was obtained from Johnson Matthey (Royston, U.K.).

All solvents applied as reaction media were of analytical grade and dried over molecular sieve for several days before use.

Melting points were determined with a Gallenkamp apparatus and are uncorrected. TLC analyses were performed on 0.25-mm thick, precoated silica plates (Merck DC-fertigplatten Kieselgel 60 F-254) in the following systems: (A) methylene chloride:acetone:acetic acid 40:10:1; (B) chloroform(NH_3-sat):MeOH 97:3; (C) chloroform:methanol:water 65:25:4 or (D) on plates precoated with aluminum oxide 150 (neutral) in 2-propanol. Spots were visualized by exposure to iodine vapors or by inspection under UV light at 254 nm.

The high temperature incubations were carried out in a 400 ml stainless steel autoclave in a shaking jacket-type heater made in the workshop at the Institute of Chemistry, University of Uppsala.

Adam's catalyst was reduced with deuterium gas at 2–4 atm in a Parr apparatus.

The synthesis of DDAO-d_{31} was accomplished via a four-step route from dodecanoic acid (Fig. 1).

Dodecanoic acid-d_{23}: step 1

Compound 1 is commercially available, but was prepared according to Dinh-Nguyen (Dinh-Nguyen and Raal, 1976; Dinh-Nguyen et al., 1972; Thomas, 1971) on a 20 mmol scale. Three exchanges gave an isotopical purity of 98% D by MS in an overall yield of 65%.

N,N-Dimethyldodecanoylamide-d_{29}: step 2

Compound **1** (3.176 g, 14.24 mmol), dimethylamin · HCl-d_6 (1.306 g, 14.92 mmol), *N*-hydroxybenzotriazole (HOBt · H_2O 2.478 g, 14.49 mmol), and CH_2Cl_2 (10 ml) were mixed and the suspension chilled in an ice-water bath. The amine was liberated from its hydrochloride by adding triethylamine (2.05 ml, 14.8 mmol). Dicyclohexylcarbodiimide (2.94 g, 14.27 mmol) dissolved in CH_2Cl_2 (5.0 ml) was added dropwise to the reaction mixture, initially giving a perfectly clear solution. After 5 min, dicyclohexyl urea started to precipitate. The reaction was allowed to take room temperature and was stirred overnight.

The urea was filtered off and washed with CH_2Cl_2 (three portions). The filtrate was evaporated to dryness, and the residue redissolved in ethyl acetate (120 ml) and washed with 1 M aqueous $KHSO_4$ (3 × 50 ml), 1 M aqueous $NaHCO_3$ (3 × 30 ml) and a saturated, aqueous sodium chloride solution (3 × 30 ml). After drying over $MgSO_4$, the organic phase was evaporated to dryness, giving 3.57 g of a colorless oil (13.9 mmol, 98%). TLC (A) showed an almost pure compound containing no traces of dodecanoic acid. Isotopic purity by MS >96% ^{2}H.

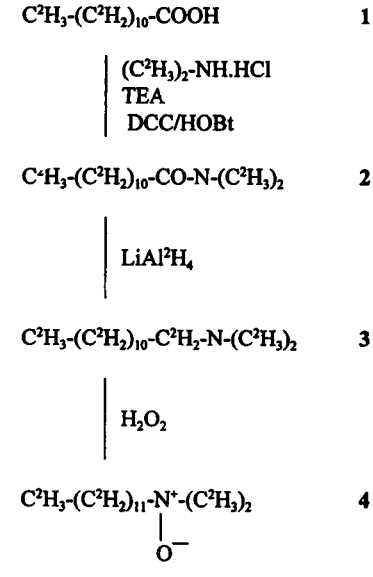

FIGURE 1 Scheme of the synthesis of DDAO-d_{31}.

Dimethyldodecylamine-d_{31}: step 3

The glassware was dried with an open flame before use. $LiAl^2H_4$ (0.498 g, 11.86 mmol) was transferred into a three-necked, round-bottomed flask supplied with a Liebig condenser, a dropping funnel, and an N_2-inlet, and 14.0 ml of tetrahydrofurane was added. A solution of compound **2** (3.55 g, 13.87 mmol) dissolved in 5.0 ml of THF was added dropwise during 10 min under rapid stirring. The funnel was washed with 6.0 ml of THF. The reaction mixture was heated to 75–80°C in an oil bath and refluxed. TLC (A) showed that all amide was consumed after 30 min, and after 1 h, the reaction mixture was chilled on an ice-water bath. Under stirring, water was added (3 × 180 μl) followed by a 15% NaOH-solution (3 × 180 μl) and water (500 μl). The oxides were filtered off and rinsed with three portions of tetrahydrofurane.

The filtrate and washings were dried over Na_2SO_4. Evaporation gave an oil that was used without further purification in the next step.

Dimethyldodecylamine oxide, DDAO-d_{31}: step 4

This synthesis was done essentially according to Applebury et al. (1974). Compound **3** (11.5 mmol) was heated to 55–60°C, and H_2O_2 (1.43 g, 43 mmol) was added over a 15-min period, followed by *t*-butanol (4.0 ml, 43 mmol). The reaction mixture was stirred at elevated temperature for 7 h and was left at room temperature overnight. TLC (B) (Pelka and Metcalfe, 1965) showed that all amine was consumed. Only minor traces of biproducts could be detected (<2%). The reaction mixture was evaporated to dryness and reevaporated from dry ethanol 3 times, giving a semi-solid mass. Recrystallization from toluene (40 ml) gave white needles (1.57 g, 6.1 mmol; 44% yield in two steps, overall yield 30%), pure by TLC (system B, C, and D), m.p. 124–125°C.

Analysis of ^{2}H-NMR spectra

The interaction between the electric field gradient tensor and the nuclear quadrupole moment is characterized by the quadrupole coupling constant, χ, which for paraffinic C-^{2}H bonds has been determined to be 170 kHz (Burnett and Muller, 1971). For a lamellar arrangement of lipids, the ^{2}H NMR signal of a C-^{2}H group consists of a doublet at resonance frequencies $\pm\ \nu_q^{(i)}$, where the quadrupole splitting, $\Delta\nu_q^{(i)}$, is given by (Thurmond et al., 1993)

$$\Delta\nu_q^{(i)} = \frac{3}{2}\chi\left(\frac{3\cos^2\theta - 1}{2}\right)S_{CD}^{(i)}, \tag{1}$$

where i refers to the individual methylene segment and θ is the angle between the director of the lamellae and the main magnetic field. The order parameter, $S_{CD}^{(i)}$, is given by

$$S_{CD}^{(i)} = \tfrac{1}{2}\langle 3\cos^2\beta_i - 1\rangle, \tag{2}$$

where the angle brackets $\langle\ \rangle$ denote a time average and β_i is the angle between the ith C—^{2}H bond and the director.

In the normal hexagonal phase (H_I) the translational diffusion of the lipids around the cylinders is fast on the NMR timescale and a further averaging of the static interactions has to be taken into account. The fast lipid diffusion introduces a symmetry axis determined by the cylinder axis. An additional transformation, relating the local director axis to the cylinder axis, gives the following expression for the quadrupole splittings of the H_I phase.

$$\Delta\nu_q^{(i)} = \frac{3}{2}\chi\left(\frac{3\cos^2\theta - 1}{2}\right)\left(\frac{3\cos^2\xi - 1}{2}\right)S_{CD}^{(i)}. \tag{3}$$

Here, ζ is the angle between the local director and the cylinder axis. Because the cylinder axis is perpendicular to the local director, pure geometrical considerations (Wennerström et al., 1974) gives $\frac{1}{2}(\cos^2 x - 1) = -\frac{1}{2}$. Therefore, *if only the change in geometry is accounted for*, the quadrupole splittings observed in the H_I phase are reduced by a factor of two as compared with the corresponding splittings in the L_α phase. In the calculations of S_{CD} from the quadrupole splittings, this factor has to be included.

For a sample consisting of a random distribution of the orientation of liquid crystalline microdomains, the spectrum will adopt a so called "powder pattern," where the intensities at each θ is scaled by the probability density, $p(\theta) = \frac{1}{2}\sin\theta$. A characteristic ^{2}H-NMR spectrum with "peaks" ($\theta = 90°$) and "shoulders" ($\theta = 0°$) is therefore observed.

When analyzing ^{2}H-NMR spectra of perdeuterated molecules, it is advantageous to transform the spectrum of a powder pattern, using the so called dePaking routine (Bloom et al., 1981; Sternin et al., 1983). This transforms the powder pattern into the spectrum one would obtain with the director of the liquid crystalline phase oriented in one direction. The routine used in this work calculates the 90°-oriented spectrum. This greatly improves the resolution of the spectrum and makes it possible to determine the quadrupole splittings for the 90° dePaked spectrum. S_{CD} is calculated from

$$\Delta\nu_q^{(i)} = \tfrac{3}{4}\chi|S_{CD}^{(i)}| \tag{4}$$

for an L_α phase and

$$\Delta\nu_q^{(i)} = \tfrac{3}{8}\chi|S_{CD}^{(i)}| \tag{5}$$

for an H_I phase.

In the assignment of the data to the C^{2}H segments, it is assumed that the order parameter decreases monotonically along the hydrocarbon chain (Sternin et al., 1988). The two methyl groups of the polar head group and the methyl group of the terminal end of the alkyl chain have the smallest quadrupole splittings. The positions of the peaks of the C^2H_3 headgroup are easily distinguished from those of the terminal C^2H_3, because the former have twice as large integrated intensity.

From the order parameters of the individual C^2H_2 segments, it is possible to calculate the average length projected along the director, $\langle L\rangle$, of the hydrocarbon chain in the L_α phase, which is given by (Salmon et al., 1987; Schindler and Seelig, 1975; Thurmond et al., 1993)

$$\langle L\rangle = l_0\left[\frac{n - m + 1}{2} + \sum_{i=m}^{n-1}|S_{CD}^{(i)}| + 3|S_{CD}^{(n)}|\right], \tag{6}$$

where n is the number of carbon atoms in the hydrocarbon chain and m is the number of the first carbon to be included in the calculation. For the calculations performed here, we used $n = 12$ and $m = 1$. $l_0 = 1.25$ Å is the length of the carbon-carbon bond projected along the director.

It is also possible to use the first moment, M_1, of the ^{2}H-NMR spectrum to calculate $\langle L\rangle$. M_1 is defined as

$$M_1 = \int_{-\infty}^{\infty}|\omega| f(\omega)\,d\omega \Big/ \int_{-\infty}^{\infty} f(\omega)\,d\omega \tag{7}$$

and is related to the mean orientational order parameter (Davis, 1979) through

$$M_1 = \frac{\pi}{\sqrt{3}}\chi\langle|S_{CD}|\rangle. \tag{8}$$

It is therefore possible to express $\langle L\rangle$ in terms of the first moment and the quadrupole splitting of the terminal methyl group, $\delta\nu_q^{(n)}$, (Davis, 1979; Thurmond, 1992) as

$$\langle L\rangle = l_0\left[\frac{n - m + 1}{2} + \frac{1}{\chi}\left(\frac{\sqrt{3}(2n - 2m + 3)}{2\pi}M_1 + 2\delta\nu_q^{(n)}\right)\right]. \tag{9}$$

In the derivation of Eqs. 6 and 9, the backfolding of the hydrocarbon chain segments are assumed to be negligible, which is supposed to hold in the L_α phase. The total average length of the DDAO molecule is obtained by adding the length of the headgroup, which from bond lengths and bond angles is estimated to be 3.9 Å.

Analysis of ^2H_2O NMR spectra

Equations 1–5 are valid for the water deuterons as well, with the value of χ replaced by the corresponding value for a O—^{2}H bond (220 kHz). The

quadrupole splittings obtained from deuterated water are usually much smaller than for hydrocarbon chains. They also depend on the water content to a much higher degree than those of lipids and surfactants. Quadrupole splittings of water in anisotropic liquid crystalline phases can be described by a simple two-site model for the water, where the two sites are comprised of one with water bound to the surfactant aggregates and one with "free" water in an isotropic environment (Lindblom et al., 1976). Fast exchange between the two sites is assumed, and the observed quadrupole splittings is a weighted average of the splittings in the two sites. By observing the quadrupole splittings for several water contents, it is possible to determine phase transitions between, e.g., L_α and H_I phases, despite the fact that the two phases are simultaneously present in the sample (Ulmius et al., 1977).

RESULTS AND DISCUSSION

The phase diagram

According to the phase diagram made by Lutton (1966), DDAO forms five different phases in water. At high water content, it forms an L_1 phase, between 33 and 67% w/w water an H_I phase is formed, and between 30 and 33% w/w water a cubic (I) liquid crystalline phase is formed. At low water content, below 30% w/w water, an L_α phase is formed and, finally, at very low water contents, DDAO forms crystals. Addition of gramicidin D to the system results in the ternary phase diagram shown in Fig. 2. It can be noted that the phase boundaries obtained in the present investigation differed somewhat from those reported by Lutton for the binary system, in that we found two-phase regions between the L_1 and the H_I phases as well as between the I and the L_α phases. Note that the phase diagram is given in weight percentage of the nondeuterated DDAO, gramicidin, and H_2O. To make comparisons between samples differing in isotopic content, all sample compositions have been recalculated to the corresponding weight percentage for the nondeuterated compounds.

The phase boundaries in the phase diagram were determined mainly from ^2H NMR data of ^2H_2O. The quadrupole splittings in the L_α and H_I phases varied continuously with the water content for each value of X_G. Fig. 3 summarizes the quadrupole splittings of ^2H_2O obtained for a variation of X_G and water concentration.

In general, only a small shift of the phase boundaries toward the water corner is observed in the phase diagram with increasing temperature.

Samples of DDAO-d_{31} without gramicidin D

At high water contents (70% w/w water and above) an L_1 phase exists. The ^2H-NMR measurements give narrow and symmetric peaks having about the same relative chemical shifts as obtained by ^1H-NMR of the L_1 phase studied previously (Orädd et al., 1992). The isotropic chemical shifts in the ^1H NMR spectrum with tetramethylsilane as reference are: $C_\alpha H_2$, 3.1 ppm; $(CH_3)_2$ of the head group, 2.9 ppm; $C_\beta H_2$, 1.5 ppm; methylenes of the alkyl chain, 1.1 ppm; and terminal CH_3, 0.7 ppm. The ^2H NMR spectrum at high water contents can be interpreted using the same chemical shifts as for protons. Note the difference of about 2.2 ppm in the isotropic chemical shift between the headgroup methyls and the methyl of the alkyl chain which, at the observing NMR frequency of 76.78 Mhz, will shift the resonance of the headgroup 170 Hz downfield from the terminal methyl group.

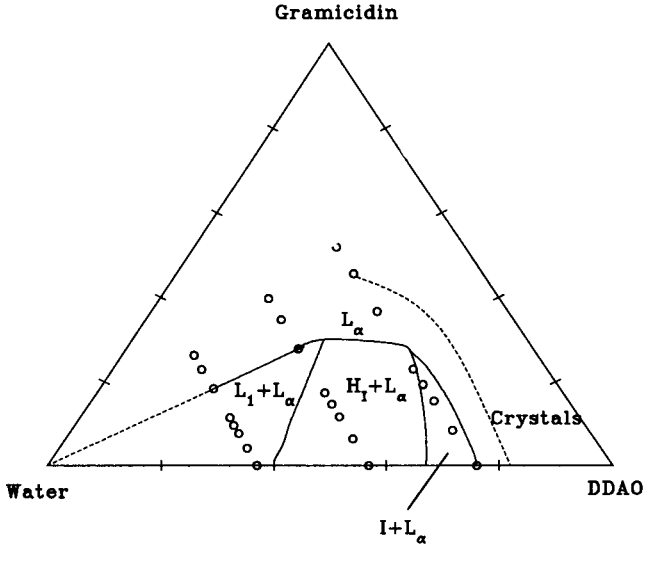

FIGURE 2 Ternary phase diagram of the DDAO/gramicidin/H_2O system. The dashed lines represent rough estimations of phase boundaries. The various phases indicated in the diagram are: L_1, micellar solution phase; H_I, normal hexagonal phase; I, reversed bicontinuous cubic phase; L_α, lamellar phase. Samples with DDAO-d_{31} investigated in the diagram are denoted with circles.

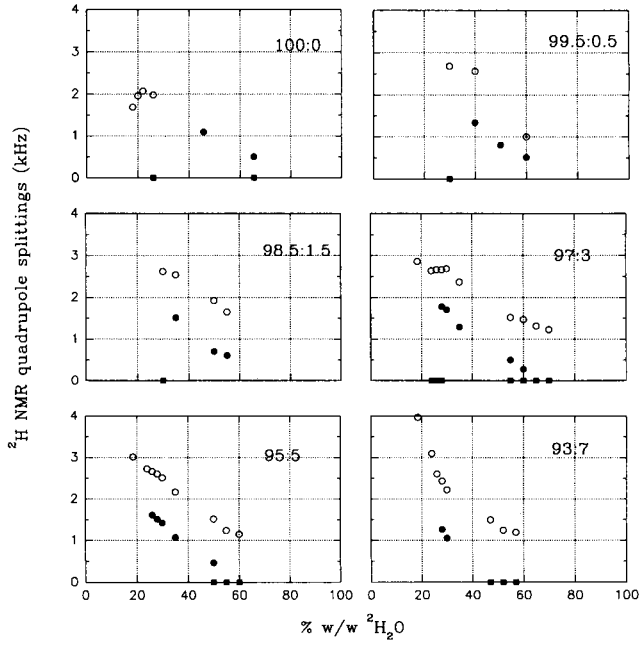

FIGURE 3 Observed ^2H NMR quadrupole splittings in ^2H_2O for various compositions of the DDAO/gramicidin/water. The molar ratio between DDAO and gramicidin is shown in the top right corner of each plot. Filled circles refer to the H_I phase, and open circles refer to the L_α phase. The isotropic phases are indicated by filled squares at zero quadrupole splitting.

This shift causes a slight asymmetry in the NMR lineshapes of the anisotropic phases as can be seen for the central doublets in Figs. 4–7. This effect is also seen for the α-C^2H_2 deuterons with a chemical shift of about 2.0 ppm higher than the chain C^2H_2's. At water contents closer to the H_I phase, the ^2H NMR lines become broadened, indicating a growth in size of the micelles.

At 63% w/w water, the spectrum consists of a component of an anisotropic phase together with two narrow peaks (Fig. 4, *bottom*). These narrow peaks, separated by approximately 2 ppm, and barely resolved in this experiment, are indicative of the presence of an L_1 phase. Thus, this DDAO/water composition represents a two-phase region of L_1 and H_I phases.

The ^2H NMR lineshape of the H_I phase does not show the characteristics of a powder pattern (see Analysis of ^2H-NMR spectra). From the NMR spectra of the pure H_I phase at 40.5% w/w water (vide infra) it can be concluded that this phase at 63% w/w water is highly oriented in the magnetic field. Thus, the axis of the cylindrical aggregates in the H_I phase are aligned parallel with the field. The intensity of the shoulders are enhanced and the apparent lineshape exhibits twice as large splittings as that obtained from a powder pattern of an H_I phase. The same type of lineshape can also be observed for ^2H_2O for samples of this composition.

At a water content of 40.5% w/w, a single H_I phase is observed. No macroscopic orientation effects in the magnetic field is detected and the lineshape is typically that of a powder pattern (Fig. 4, *middle*). The positions of the shoulders in this NMR spectrum match well the peaks of the H_I lineshape at 63% w/w water. The quadrupole splittings obtained from the dePaked spectra are shown in Table 1.

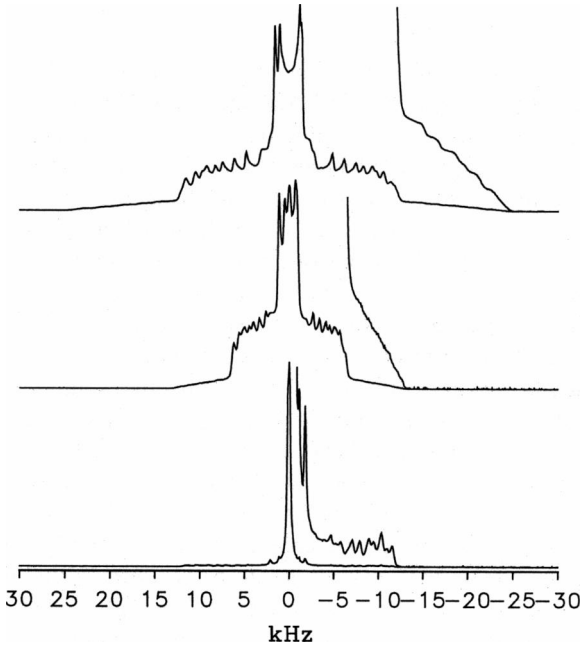

FIGURE 4 ^2H-NMR spectrum of DDAO-d_{31}/H_2O at various water contents. (*top*) 22% w/w water. (*middle*) 40.5% w/w water. (*bottom*) 63% w/w water. To the right of each spectrum a 20 times magnification is inserted.

TABLE 1 ^2H quadrupole splittings (kHz) of DDAO-d_{31} at $X_G = 0$

Carbon #	H_I(40.5% w/w H_2O)	L_α(22.4% w/w H_2O)
Head group C^2H_3	2.2	3.1
Terminal $C^{(12)2}H_3$	1.4	2.8
Chain methylenes, $C^{(i)2}H_2$		
i = 11	5.6	9.8
10	7.1	12.5
9	8.5	15.1
8	9.3	16.8
7	9.9	18.6
6	10.5	19.5
5	11.1	20.8
4	11.5	21.4
3	12.1	22.7
2	12.5	23.5
1	12.8	24.3

At 22.4% w/w water in the DDAO/water system, an L_α phase is present. The ^2H-NMR spectrum of this phase shows a powder pattern with approximately twice as large splittings as the H_I phase (Fig. 4, *top* and Table 1). This is in agreement with the expected behavior of a ^2H-NMR lineshape for the L_α phase (see Analysis of ^2H-NMR).

Samples containing gramicidin D

Fig. 5 shows the dePaked spectra for different molar ratios (X_G) between DDAO-d_{31}:gramicidin D at 63% w/w H_2O. Addition of small amounts (X_G = 1.5) of gramicidin results in the disappearance of the H_I phase (Fig. 4, *bottom*). A new lineshape, corresponding to a gramicidin-rich L_α phase, characterized by a maximal splitting of 33 kHz appears in the spectrum (Fig. 5, *bottom* spectrum and Table 2). The intensity of this part of the NMR spectrum increases with increasing X_G up to X_G = 10, where the isotropic component has disappeared and only the lineshape of an L_α phase is observed. A further increase in X_G induces only minor changes in the NMR spectra. The additional peaks in the spectrum with X_G = 10 are due to artifacts introduced by the dePaking program, because this sample is partially oriented in the magnetic field and the intensities, therefore, do not correspond to a powder pattern. This is discussed further below.

Addition of gramicidin D to the DDAO sample with 40.5% w/w H_2O results in the appearance of new quadrupole splittings from the L_α phase superimposed on the spectrum of the H_I phase (Fig. 4, *middle*). The intensity of the NMR peaks of the L_α phase increases up to X_G = 5. For a gramicidin content above X_G = 5, the H_I phase has disappeared completely from the spectrum (Fig. 6).

Adding gramicidin to the DDAO sample with 22.4% w/w H_2O, the following phase transitions occur with increasing gramicidin content (Fig. 7): first a two-phase region of L_α and I phases (three lowest spectra) is observed and then a two-phase region of L_α and H_I phases (fourth spectrum from the bottom) occurs and finally an L_α single phase is formed (top three spectra). The quadrupole splittings of the L_α phase increase with increasing X_G up to about X_G = 5, where the splittings reach a maximal value (Fig. 8).

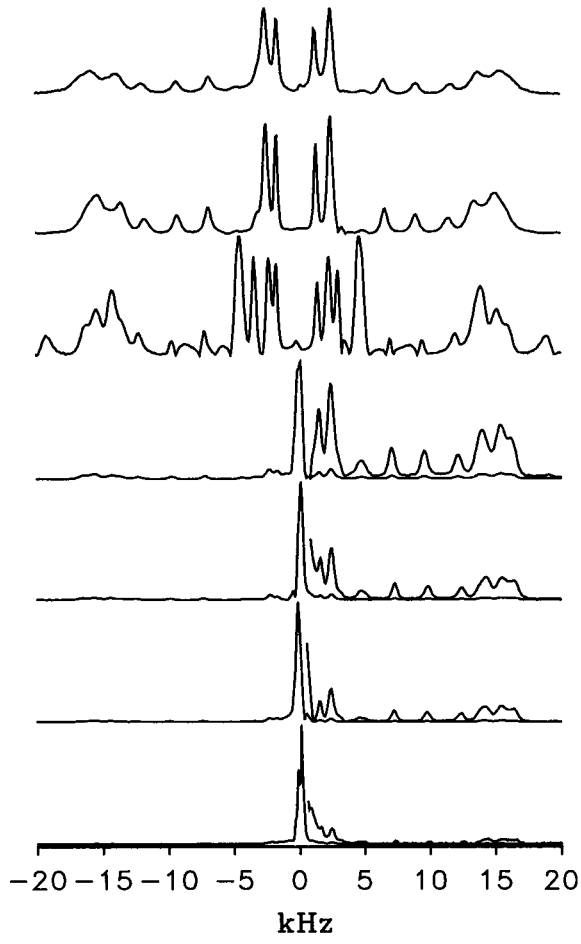

FIGURE 5 DePaked ^{2}H-NMR spectrum of DDAO-d_{31}/gramicidin/H_2O at 63% w/w water. The mole percentage of gramicidin (X_G) is from bottom to top: 1.5, 3, 4, 5, 10, 15, and 20. The magnifications shown are 10 times of the original spectra.

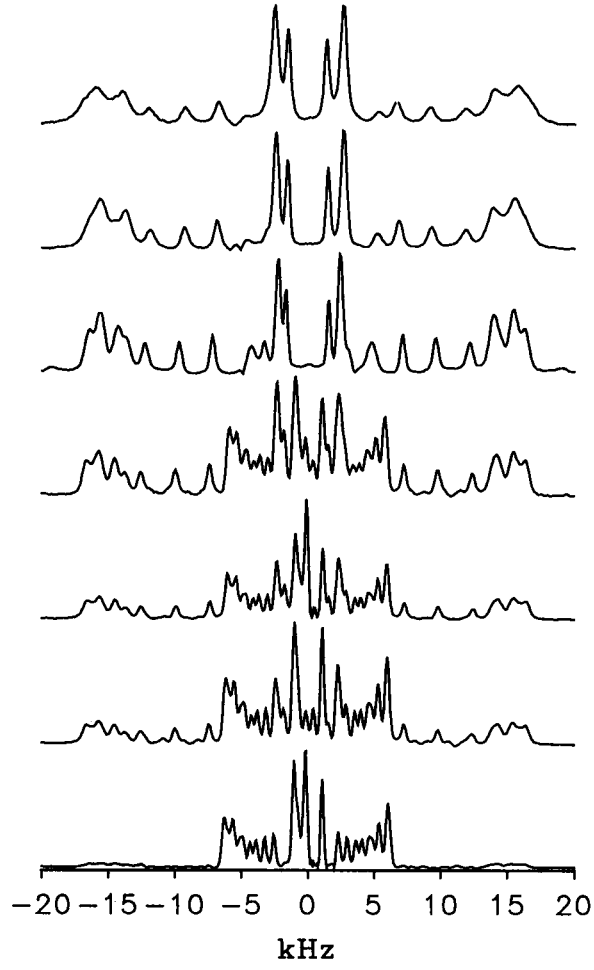

FIGURE 6 DePaked ^{2}H-NMR spectrum of DDAO-d_{31}/gramicidin/H_2O at 40.5% w/w water. The mole percentage of gramicidin (X_G) is from bottom to top: 1.5, 3, 4, 5, 10, 15, and 20.

An increase in the temperature has a rather small but observable effect on the quadrupole splittings. The smaller splittings corresponding to the C^2H_2 groups near the end of the hydrocarbon chain get even smaller, whereas the large splittings from the C^2H_2 groups close to the polar headgroup are enhanced. This indicates that the ordering in the headgroup increases, whereas it decreases close to the terminal end of the hydrocarbon chain.

Effect of solvent on gramicidin conformation

It has been shown that the conformation of gramicidin in lipid and detergent systems depends on the solvent used in the preparation of the samples (Killian et al., 1988). The gramicidin channel consists of dimers in trifluoroethanol (TFE), whereas a nonchannel double-helix of two gramicidin molecules forms with chloroform/methanol mixtures. Upon extensive heating, this double-helix transforms into the more stable channel form. To investigate the effect of the solvent used in the preparation of the samples in the DDAO/water system, two different solvents are used. It is found, however, that the choice of solvent has no effect on the ^{2}H-NMR lineshapes, neither did incubation of the samples at 60°C for several days change the ^{2}H-NMR spectrum. This is not surprising, because similar investigations on perdeuterated dimyristoylphosphatidylcholine bilayers indicate that the deuterium lineshape is essentially unchanged by the conversion from the nonchannel to the channel form (Morrow et al., 1991). Measurements of circular dichroism (CD) have been used to investigate the conformation of gramicidin (Killian (1992) and references cited therein), and the CD spectra obtained from the two conformations are distinctly different. CD measurements on the micellar solutions of DDAO containing gramicidin give spectra characteristic of the channel form. This shows, at least for the small fraction of gramicidin incorporated in DDAO micelles, that it quickly adopts a channel conformation, irrespective of the solvent used. Studies of the dependence of the chain length of the hydrocarbon on the conversion rate from a nonchannel to a channel conformation of gramicidin have shown that the shorter the chains, the faster the conversion takes place (Cox et al., 1992; Killian et al., 1988). Therefore, it is reasonable to assume that gramicidin in the present study adopts the channel conformation. This assumption is further supported

TABLE 2 ^2H quadrupole splittings (kHz) of DDAO-d_{31} in the L_α phase at three water contents

X_G	Head C^2H_3	$C^{(12)2}H_3$	$C^{(11)2}H_2$	$C^{(10)2}H_2$	$C^{(9)2}H_2$
1.5	4.6/ * /3.3	3.2/ * /2.4	14.7/14.7/10.1	19.8/19.8/13.2	25.1/24.9/16.4
3	4.8/4.7/3.7	3.3/3.4/3.0	14.6/14.7/12.6	19.7/19.8/16.6	24.9/24.9/21.2
4	4.7/4.7/4.0	3.3/3.3/3.3	14.6/14.5/13.7	19.8/19.8/18.2	24.9/25.0/22.5
5	4.8/4.7/4.3	3.2/3.3/3.5	14.3/14.6/15.0	19.4/19.8/19.8	24.5/25.0/24.3
10	4.6/4.7/4.9	3.2/3.2/3.3	14.2/15.1/14.2	19.2/19.3/19.0	24.2/24.5/23.7
15	5.0/5.1/5.0	3.1/3.1/3.3	13.5/13.7/14.0	18.2/18.6/18.8	23.3/23.8/23.7
20	5.1/5.2/5.2	2.9/2.9/3.1	13.4/13.4/13.7	18.4/18.5/18.9	23.6/23.9/24.3

The water contents for the three entries are 63/40.5/22.4% w/w H_2O
* Not observed

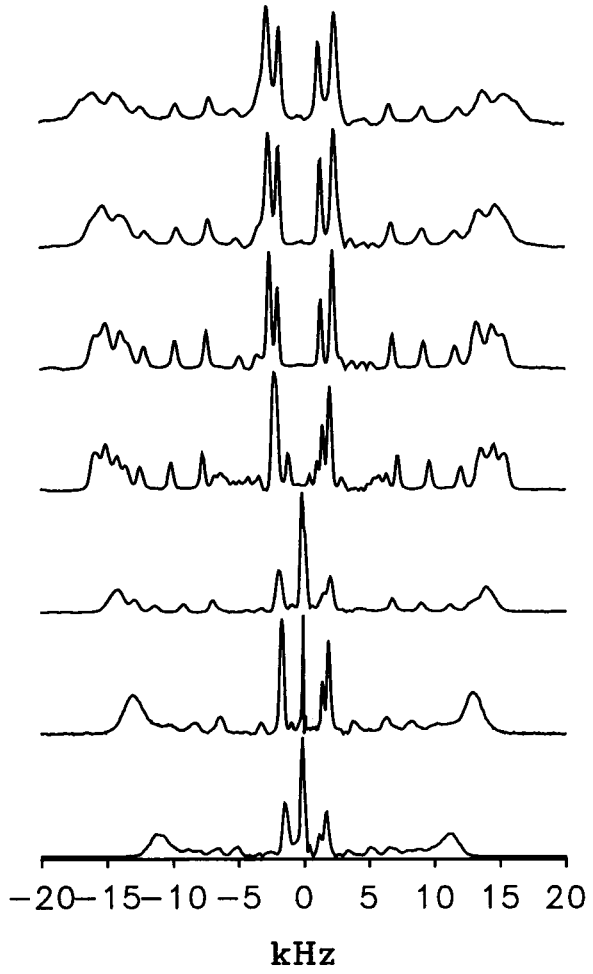

FIGURE 7 DePaked ^2H-NMR spectrum of DDAO-d_{31}/gramicidin/H_2O at 22.4% w/w water. The mole percentage of gramicidin (X_G) is from bottom to top: 1.5, 3, 4, 5, 10, 15, and 20.

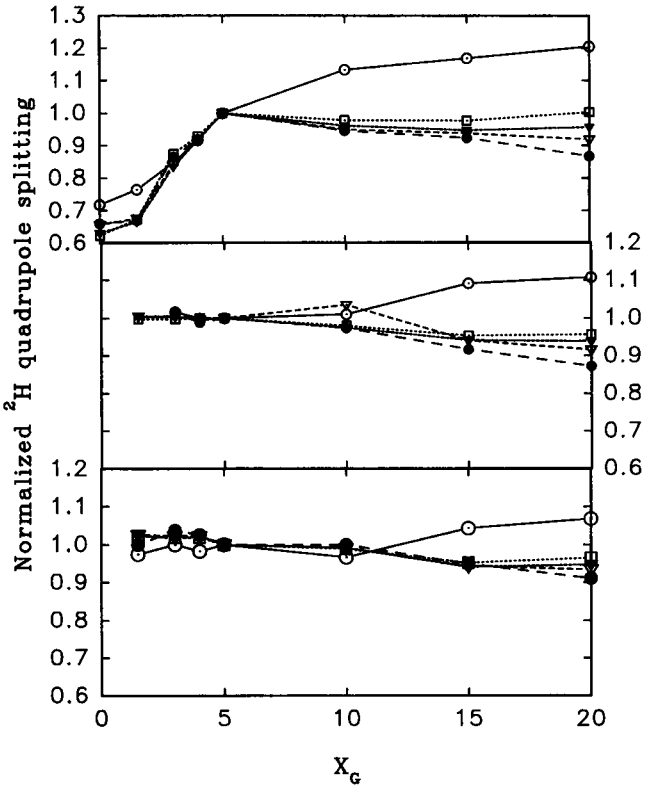

FIGURE 8 Normalized ^2H NMR quadrupole splittings of the L_α phase at various water contents. (*top*) 22.4% w/w water. (*middle*) 40.5% w/w water. (*bottom*) 63% w/w water. The splittings at (X_G = 5 have been set to 1. The symbols correspond to: (○) head group C^2H_3, (□) $C^{(9)}{}^2H_2$, (▼) $C^{(10)}{}^2H_2$, (▽) $C^{(11)}{}^2H_2$, (●) $C^{(12)}{}^2H_3$.

by IR measurements of the L_α phase of DDAO with gramicidin, in which the characteristic bands of the channel form were observed (P.-O. Westlund, personal communication).

Macroscopic alignment of the H_I and L_α phases

For partially oriented samples, the molecular orientations are no longer random but are weighted toward some specific orientation. This leads to deviations from the powder pattern of the ^2H NMR lineshape. A dePakeing of such a lineshape leads to artifacts with dips in the baseline at the positions of diminished intensity and artificial peaks at positions of enhanced intensity.

The samples of DDAO with 63% w/w water are located in the two-phase region of the L_1 and H_I phases. The ^2H NMR lineshape of both DDAO-d_{31} and ^2H_2O shows that the cylindrical aggregates in this phase region orients with the cylinder axis parallel with the main magnetic field (Fig. 4, *bottom*). Because of the magnetic susceptibility of the hydrocarbon chain the DDAO molecules prefer an orientation with the molecular long axis perpendicular to the magnetic field. When gramicidin is incorporated in the lipid aggregates, an L_α phase is formed and the H_I phase disappears. The lineshape of this L_α phase does not show any orientation

effects. At $X_G = 10$, however, the lineshape again is typical of a macroscopic oriented liquid crystal (Fig. 9, *top*). The bilayers favor the 0° orientation, i.e., the bilayer normal is parallel with the magnetic field. This means that the DDAO molecules now orient with their molecular long axis parallel with the magnetic field in contrast to the case without gramicidin. By vortexing the sample for about 2 min, the powder pattern was restored when the NMR signal was collected immediately after the sample had been placed in the magnet (Fig. 9, *bottom*). If the sample was left in the magnetic field for a couple of hours, the liquid crystal again started to become oriented, and after one night in the magnet it regained the previous orientation.

Influence of gramicidin on the lipid bilayer

This study shows that DDAO does not favor incorporation of gramicidin into any other phase but the L_α phase. Down to gramicidin concentrations as low as $X_G = 0.5$, an L_α phase is formed at all water contents studied. This is in agreement with investigations performed on mixtures of gramicidin and lyso-phosphatidylcholine (LPC), where lamellar aggregates were induced by addition of gramicidin (Killian et al., 1983). Gramicidin has also been solubilized in micelles of sodium dodecylsulphate (SDS). However, to obtain small micelles suitable for high resolution NMR studies, it was necessary to add small amounts of TFE to the solutions (Arseniev et al., 1985). The findings in this work thus give further support for the somewhat surprising observation that gramicidin will not be solubilized in detergent micelles. However, with a well defined lyophilization technique recently developed, this is possible (Killian et al., 1994).

The stoichiometry of the L_α aggregates of DDAO and gramicidin most probably represents an ideal packing of these molecules in a bilayer. From the phase diagram it can be concluded that the ratio between DDAO and gramicidin is 9:1 in the L_α phase at a water content above 40.5% w/w and that this ratio increases at lower water contents. These stoichiometric values can be compared with the value of 7:1 for LPC bilayers (Killian et al., 1988). Raman and ESR studies on phospholipids indicate ratios of about 5:1–10:1, representing 10–20 hydrocarbon chains/gramicidin (Chapman et al., 1977).

The quadrupole splittings at 22.4% w/w water are much larger in the L_α phase containing gramicidin than those for the L_α phase in the DDAO/H_2O system (Tables 1 and 2). This is most probably because the DDAO molecule has to stretch to incorporate gramicidin into the bilayer. Watnick et al. (1990) have shown by ^2H NMR that the ordering of the acyl chains in phosphatidylcholine (PC) bilayers increases with increasing contents of gramicidin for a chain length of less than 18 carbons. The fact that the order of the acyl chains in distearoyl-phosphatidylcholine (DSPC) did not change with the gramicidin content in the L_α phase indicates that the molecular length of gramicidin best fits the length of the DSPC acyl chains. The length of the stearoyl hydrocarbon chains can be estimated to be ≈15 Å, and this can be thought of as an "ideal chain length" for incorporation of gramicidin into the lipid bilayer. Raman spectroscopic studies have also shown an increase of the *trans* conformation in the acyl chains of PC's on incorporation of gramicidin (Chapman et al., 1977). The stretching of the hydrocarbon chain of DDAO results in a longer plateau region in the order parameter plot (Fig. 10). A large number of the individual C^2H_2 signals close to the maximal splitting makes it difficult to resolve the individual C^2H_2 groups (Figs. 5–7). In this work only five individual signals are resolved: the C^2H_3 of the head group, the terminal $C^{(12)2}H_3$ of the acyl chain (carbon #12), and the three C^2H_2-groups closest to the terminal C^2H_3, i.e., carbons #9–11 (see Table 2). For the unresolved peaks, an estimation of the splittings has been made by assigning a

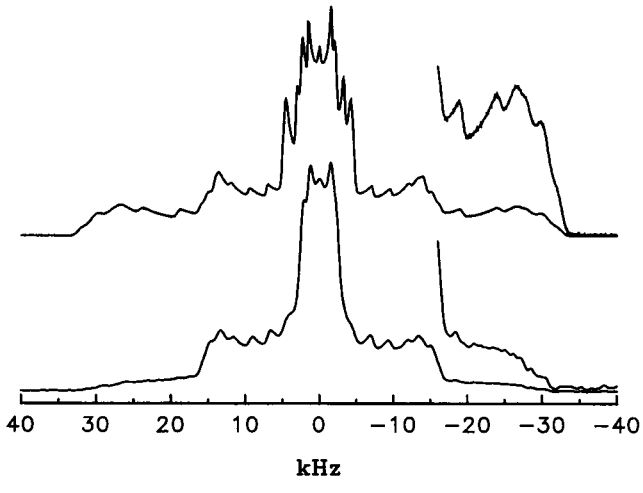

FIGURE 9 ^2H NMR spectrum of DDAO-d_{31} of a sample with 63% w/w water and a mole percentage of gramicidin of 10. Top spectrum is from a sample partially oriented in the main magnetic field. Bottom spectrum was recorded immediately after vortexing the sample. The magnifications shown are 5 times of the original spectra.

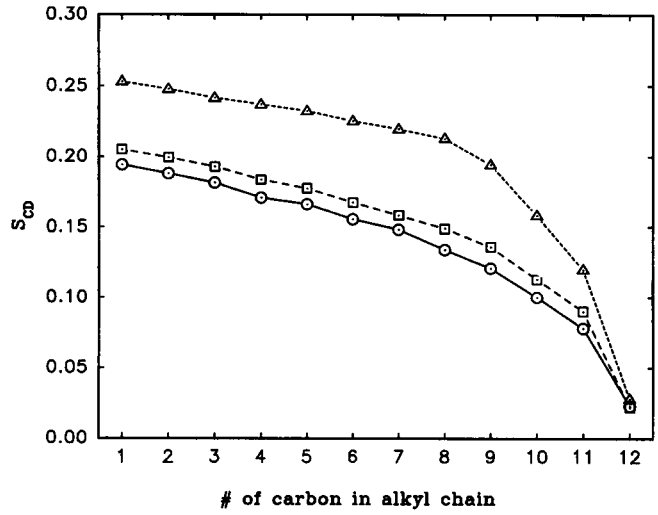

FIGURE 10 Order parameter profiles obtained from ^2H-NMR studies of DDAO-d_{31}. (○) 22.4% w/w H_2O, $X_G = 0$; (□) 40.5% w/w H_2O, $X_G = 0$; (△) 22.4% w/w H_2O, $X_G = 5$.

certain area under the curve to each C^2H_2 group, thereby "slicing" the spectrum into segments corresponding to the individual C^2H_2 groups.

Equation 9 is used to calculate the chain length of the DDAO molecule from M_1 of the DDAO-d_{31} spectra. The results are shown in Table 3 for some values of X_G. $\langle L \rangle$ increases from 13.1 Å for $X_G = 0$ to 13.6 Å for $X_G \geq 5$. This is in agreement with the suggestion of an "ideal" chain length of 15 Å. The DDAO molecule is shorter than the ideal chain length and therefore has to stretch when gramicidin is solubilized in the bilayer. From x-ray diffraction experiments, it was also possible to calculate the chain length from the bilayer thickness obtained in such measurements (Table 3). Although the values from the two methods differ by approximately 1 Å, they both show an increase in the chain length as the gramicidin content is increased in the bilayer. The difference in the results from the two methods, however, is not surprising considering the assumptions made in both methods.

The effect of incorporating proteins into bilayers has recently been investigated by computer simulations of proteins having different hydrophobic thicknesses than the bilayer (Fattal and Ben-Shaul, 1993). The calculations of the order parameter profile were done for lipids in bilayers with integral proteins of both positive and negative mismatch (i.e., both thicker and thinner than the nonperturbed bilayer) as well as for proteins with a thickness that matched the bilayer. The effect of the protein on the surrounding lipids in the bilayer was found to extend to about 10 Å, which approximately corresponds to two layers of lipids around the protein. For a positive mismatch of 3.5 Å, similar to gramicidin in a DDAO-bilayer, they find a significant increase in the order parameters of the hydrocarbon chain segments near the polar headgroup for the closest neighbors of the protein. This situation would correspond to the experimental observation, where the L_α phase with $X_G \geq 5$ is assumed to have all DDAO molecules adjacent to a gramicidin molecule. A comparison between our experimental order parameters and those calculated by Fattal and Ben-Shaul, however, is somewhat difficult to make. The chain length used in the calculations is 14 carbons, whereas DDAO has only 12 carbons. A further complication is that in the calculations, the first two CH_2 segments are assumed to protrude into the aqueous region resulting in a reduction in the order parameters of these segments. This is not compatible with the experimental order parameters, and we believe that this kind of protrusion is very rare in DDAO bilayers. To compare the experimental and theoretical values of the order parameters, we have therefore deleted the order parameters of the first two CH_2 segments obtained theoretically. This serves both to remove the protrusion effect and to make the total chain length the same in both systems, thereby facilitating the comparison. It is now possible to compare the profiles of the theoretical order parameters at close and long distances from the protein with the corresponding ^{2}H-NMR order parameters for bilayers with $X_G = 0$ and $X_G = 5$. Fig. 11 shows that the two order parameter profiles agree reasonable well. However, the experimental and calculated order parameters deviate from each other as we go down the hydrocarbon chain. For carbon numbers 7–11, the experimental values are larger than the calculated ones. This deviation possibly arises from the fact that the chains in the calculation were longer than that of DDAO. It is therefore difficult to make an absolute comparison between separate CH_2 segments along the chain. However, the trends of the change in the order parameter profiles are the same in both the experiment and the theoretical model.

Further addition of gramicidin above the "ideal" stoichiometry of the L_α phase results in changes of the order parameter profile of DDAO. These changes can be understood in terms of the shape of the gramicidin molecule. The cross sectional area of gramicidin is larger at the C-terminus than at the N-terminus (Brasseur et al., 1986). Thus, the gramicidin molecule has a shape of an truncated cone with a larger area at the polar-apolar interface and a smaller area in the interior of the bilayer, thereby increasing the pressure on the headgroup methyls. This pressure cannot be reduced simply by diminishing the bilayer thickness, because the length of the gramicidin dimer has to be covered by DDAO molecules. The result is an increase in the packing at the interface of the bilayer and a corresponding decrease in the molecular packing of the hydrocarbon chains within the interior of the bi-

TABLE 3 Calculated average molecular length of DDAO in the L_α phase

X_G (mol%)	Average molecular length (Å) obtained from Eq. 9	Average molecular length (Å) obtained from x-ray diffraction
0	13.1	12.0
1.5	12.9	*
3	13.5	12.4
4	13.2	*
5	13.5	12.8
10	13.8	13.1
15	13.6	13.1
20	13.6	13.1

* Not measured.

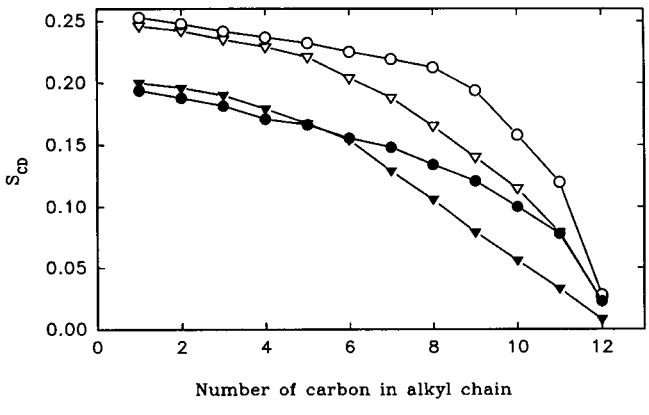

FIGURE 11 Experimental (*circles*) and calculated (*triangles*) order parameter profiles for an unperturbed bilayer (*filled symbols*) and a bilayer containing proteins with positive hydrophobic mismatch (*open symbols*). The values of the calculated order parameters are from Fattal and Ben-Shaul, 1993.

layer. This is reflected in an increased ordering of the headgroup and a decrease in the ordering of the terminal part of the chain. Similar arguments have been used to explain changes in the quadrupolar splitting and the chemical shift anisotropy of ^{13}C and ^{31}P in NMR studies of the effect of gramicidin content on DMPC bilayers (Cornell and Separovic, 1988). There exists an important difference though, between the DMPC and the DDAO molecules, because for the former there is an increased ordering observed for the acyl chains and a decreased ordering in the headgroup (see Figs. 3 and 4 in Cornell and Separovic, 1988). The choline headgroup of DMPC is relatively large and situated in the polar water phase and it may cover the top of the gramicidin molecule. This results in a reduction of the lateral pressure in the interfacial region of the bilayer and a corresponding increase in the interior of the bilayer. Solubilization of gramicidin in the DMPC bilayer will therefore not result in a decrease in the headgroup area, leading to an increase in the headgroup ordering. On the other hand, the headgroup of the DDAO molecule is rather small and situated deeper in the interface between polar and apolar regions and cannot interact in the same way as DMPC with the polar part of the gramicidin molecule. The ^2H NMR quadrupole splittings of DDAO-d_{31} in the L_α phase are summarized in Fig. 8, normalized to those observed at $X_G = 5$ to be able to compare the effects of increasing gramicidin content in the bilayers. The splittings of the headgroup methyls behave differently than those from the remaining part of the DDAO-d_{31} molecule in that their splittings in the L_α phase increase monotonically with X_G. This finding is compatible with a closer packing of the headgroups of DDAO with increasing gramicidin concentration. The effect of the gramicidin content on the hydrocarbon chain is not as marked as for the headgroup, but there is a small decrease in the order parameter of the terminal part of the hydrocarbon chain.

CONCLUSIONS

The peptide gramicidin D largely effects the organization and the ordering of DDAO in bilayers. Gramicidin induces an L_α phase at all water contents studied. Because the DDAO molecule has to stretch to cover the hydrophobic surfaces of the peptide, the order parameter of the hydrocarbon chain is increased. Upon solubilization of large amounts of gramicidin in the bilayer, the difference in cross sectional area of gramicidin between the C-terminus and the N-terminus causes a considerable tightening in the headgroup packing, and a smaller decrease in the molecular ordering of the terminal part of the hydrocarbon chain of DDAO.

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