Optimizing and characterizing alignment of oriented lipid bilayers containing gramicidin D

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ABSTRACT ^{31}P NMR spectroscopy and optical microscopy have been used to characterize samples of gramicidin D in oriented lipid bilayers. Correlations have been made between the defect structures observed under crossed polarizers by optical microscopy and characteristic features of ^{31}P NMR spectra. The sample preparation protocol has been improved using these techniques to achieve minimal dispersion of the bilayer normal and minimal amounts of unoriented sample. The molar ratio of gramicidin to dimyristoylphosphatidylcholine, the extent of hydration, and the cosolubilizing solvent system were used as the protocol variables. While hydration level and solvent system had profound effects on the sample orientation the molar ratio did not. However, the ^{31}P chemical shift anisotropy is very sensitive to the molar ratio and can be used as an in situ method for determining the molar ratio.

INTRODUCTION

Acquiring knowledge of protein function on an atomic level has been made possible by atomic resolution structural models derived from diffraction data. However, there are many proteins that are not readily amenable to crystallization. Integral membrane proteins are a prime example; very few of these have been crystallized in the presence of lipids (Wallace, 1986; Michel, 1983; Garavito and Rosenbusch, 1986; Ribi et al., 1988). Consequently, our knowledge of membrane proteins is much more limited than, for instance, our knowledge of cytoplasmic enzymes. Solid state nuclear magnetic resonance (NMR) techniques are being developed as an approach for obtaining atomic resolution structural and dynamic information without requiring crystallization (Cross and Opella, 1985; Cross, 1986*a*, *b*; Nicholson et al., 1987; Fields et al., 1988). Instead only uniaxial orientation of the molecules is needed. For reconstituted membrane proteins the lipid bilayers can be utilized as an orientable matrix. Not only can such bilayer preparations be utilized by solid state NMR in a variety of experiments (Peng et al., 1988; Jarrell et al., 1987; Braach-Maksvytis and Cornell, 1988; Seelig and Gally, 1976), but a great many other techniques, such as neutron (Worcester, 1976; Pachence et al., 1984; Buldt et al., 1979) and x-ray (Herbette et al., 1977) diffraction; visible (Mathis et al., 1976) and infrared (Rothschild et al., 1982) linear dichroism; depolarized resonance Raman (Van de Ven et al., 1984) fluorescence depolarization (Adler and Tritton, 1988; Deinum et al., 1988); circular dichroism (Huang and Olah, 1987), and electron spin resonance (Seelig, 1970; Tanaka and Freed, 1984). This paper focuses on optimizing the conditions for uniform alignment of bilayers containing gramicidin and characterizing such samples.

Gramicidin D is a biosynthetic mixture of linear polypeptides composed of 15 amino acid residues which are extremely hydrophobic. As a dimer the polypeptide spans a lipid bilayer forming a monovalent cation selective channel. Models of this structure suggest that the peptide linkages line a pore with the hydrophobic sidechains interacting with the fatty acyl chains of the lipids (Urry, 1971). Furthermore, the models have the linkage planes with the N—H and C═O bonds approximately parallel with the bilayer normal. Many studies have focused on the lipid/polypeptide interactions involving gramicidin D. When the bilayer thickness becomes substantially greater than the length of the channel (fatty acyl chains greater than C_{16}) then gramicidin may induce curvature into the bilayer surface and changes in the lipid phase result (Van Echteld et al., 1982). Recent studies have indicated that the gramicidin conformation is dependent on the solvent history of the sample before incorporation of gramicidin into the lipid bilayer (LoGrasso et al., 1988; Killian et al., 1988). In this study the effect of solvent history on the orientability of the samples is assessed as well as the effects of the gramicidin to lipid ratio chosen for the sample preparation.

Lipid bilayers and membranes have been aligned with varying degrees of success by many different approaches. Since this paper describes conditions for optimzing alignment, a brief review of the various techniques is described here. Four general categories for these approaches can be described. One of the most elegant and technically most

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successful approaches for aligning lipid bilayers is by generating Langmuir/Blodgett films (MacNaughtan et al., 1985; Tamm and McConnell, 1985). However, it would be very difficult to achieve by this approach the necessary number of oriented bilayers that is needed for many of the diffraction and spectroscopic techniques.

Magnetic or electric field orientation of lipid bilayers has only very recently resulted in uniform alignment of specific lipid mixtures (Seelig et al., 1985; Rosenblatt et al., 1987; Speyer et al., 1987). This approach is dependent upon a lipid preparation that has an inherent tendency to form very extensive lipid bilayers as well as an ability to absorb a high weight percentage of water. The later aspect results in a viscosity that is low enough so that the small interaction between the anisotropic diamagnetic susceptibility of the bilayer and the magnetic field can align the bilayers with respect to the field. However, for a variety of reasons related to these two requirements only partial alignment of membrane proteins in reconstituted systems has been achieved by this technique (Van Echteld et al., 1982).

Another category, biological alignment, takes advantage of the natural liquid crystalline arrays of certain biological membranes such as the membranes in retinal rod outer segments (e.g., Yeager, 1976). Fairly uniform alignment has been obtained directly with this technique in rare situations. However, in combination with magnetic orientation more samples such as chloroplasts, purple membranes, and the retinal outer rod segments have been uniformly aligned (e.g., Hong et al., 1971; Michel-Villaz et al., 1979; Mathis et al., 1976; Sadler, 1976; Neugebauer et al., 1977). Despite these successes, this approach has an inherent limited range of applicability.

The fourth category is based upon orientation by shear between glass plates. There are many ways in which this approach can be implemented (for a review see Clark et al., 1980). If hydrated lipids are spread between glass plates then it is necessary to have an extensive annealing process which may include incubation at extreme temperatures (Powers and Clark, 1975; Powers and Pershan, 1977) or repeated application of pressure (Asher and Pershan, 1979*a*) to the glass plates. While this annealing process has often resulted in near perfect alignment of the lipid bilayers, the thermal process restricts samples to <8% by weight water and pressure results in a very small amount of lipid between the glass plates. The most common approach for shear orientation is to dissolve the lipids and other solutes in an organic solvent to achieve uniform mixing followed by drying under vacuum on a flat glass or quartz surface before hydration for forming the bilayers (I.C.P. Smith, 1971; Rothgeb and Oldfield, 1981). Given the right conditions these bilayers can be very well oriented and do not require the extensive annealing process described above unless absolute defect-free preparations are required. The degree of orientation is dependent upon the organic solvent chosen, the level of hydration, and the specific protocol used for preparing the samples. The addition of a solute such as the hydrophobic polypeptide, gramicidin D, further complicates the optimal protocol. Several variations on this later approach are described in this paper. Furthermore, it is possible to combine these various shear approaches with magnetic orientation as was done by Pachence et al. (1984) to achieve a very well-oriented sample of reconstituted sarcoplasmic reticulum.

The most prevalent defect structures that are observed in these hemeotropically aligned lipids in the L_α phase are known as "oily streaks" and "parabolic focal conics" (Asher and Pershan, 1979*a*; Tanaka and Freed, 1984). These defects have been extensively described using optical microscopy with polarized light. Oily streaks result from folds in the multibilayer structure (Friedel, 1922; Kleman et al., 1976). These folds may involve a small or large percentage of the bilayers in the hydrated film between two glass plates. Parabolic focal conics result when the surface of the bilayer contracts and expands causing the bilayer surface to buckle (Delaye et al., 1973; Clark and Meyer, 1973; Asher and Pershan, 1979*b*; Rosenblatt et al., 1977). This defect structure is a property of the entire thickness of the film between glass plates and results in a structural deformation over a large region of the sample. By optical microscopy the size of these defects are proportional to the intensity of the birefringence and the size of the optical domains.

The quality of the alignment in these samples has also been characterized by diffraction and spectroscopic techniques by determining the mosaic spread for the bilayer normal with respect to the mean bilayer normal orientation. Mosaic spreads that range from 0.7° to 30° have been reported for oriented lipid bilayers using neutron diffraction (Pachence et al., 1984; Saxena and Schoenborn, 1977; Engelman and Zaccai, 1980), x-ray diffraction (Herbette et al., 1977), and the use of NMR for characterizing the mosaic spread has been suggested (Nicholson et al., 1987). Part of the goal for the study reported here is to correlate the diffraction and spectroscopic characterizations of the lipid alignment with the optical characterizations. Furthermore, the spectroscopic data is used for an in situ approach to evaluate the molar ratio of polypeptide to lipid in the prepared samples and to address issues of polypeptide aggregation.

METHODS AND MATERIALS

Both DMPC and natural abundance gramicidin D were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Uniformly ^{15}N labeled gramicidin D was prepared biosynthetically

from cultures of *Bacillus brevis* as described previously (LoGrasso et al., 1988). All oriented gramicidin/DMPC samples were prepared by cosolubilizing the desired ratio of polypeptide and lipid in one of the three spectroscopic grade solvent systems used in previous studies: methanol (Nicholson et al., 1987), 3% methanol in benzene (Smith and Cornell, 1986), or 5% ethanol in benzene (Cornell et al., 1988). Once the samples were cosolubilized they were allowed to equilibrate at room temperature. The solution was then spread as a thin layer onto 24 glass coverslips (Corning Glass Works, Corning, NY) having dimensions of 5.6 × 12 mm. These coverslips, with the exception of being rinsed in methanol, were not treated in any other way so that the hydrophilic surface provided by Corning Glass Works remained intact. Surfactants were not used because results have shown that better alignment is obtained using an untreated hydrophilic surface. The sample dry-weight used was ~1.2 mg/coverslip. The solvent was evaporated at room temperature, before drying under vacuum (excess drying time was taken to insure complete removal of the solvent). 0.5 μl of HPLC grade H_2O was placed onto each coverslip before stacking the coverslips and placing the stack into a 12-mm section of square glass tubing. The sample was weighed and additional water was added to achieve the desired hydration level before sealing the end with epoxy. The sample was then placed into an oven at 45°C, without agitation, for 48 h before recording spectra. This method is a slight variation of that described previously (LoGrasso et al., 1988; Nicholson et al., 1987).

The ^{31}P NMR spectra were recorded on a homebuilt 62 MHz spectrometer, for phosphorus, utilizing a 10-cm-wide bore 3.5-T magnet. Complete proton decoupling of ^{31}P was obtained with 5 W of power at 151 MHz. A 15-μs 90° pulse width was used along with a 5-s recycle delay and a sweep width of ±25 kHz. ^{15}N NMR spectra were recorded on a modified spectrometer (model WP200 SY; IBM/Bruker Instruments, Inc., Billerica, MA) with a solids package (Nicholson et al., 1987). A homebuilt static (i.e., non–magic angle spinning) probe with variable temperature control was constructed for observing both oriented and unoriented samples. Experimental conditions included a sweep width of 62.5 kHz, a preacquisition delay of 16 μs, and a recycle delay of 7 s. Spectra were obtained by cross polarization with fields generated by 5.0-μs 90° pulse lengths, a mixing period of 1 ms, and increased ^{1}H decoupling field during acquisition of 2.0 mT. ^{15}N chemical shifts are given relative to the resonance of a saturated solution of $^{15}NH_4NO_3$.

Enough coverslips were made during sample preparation so that one could be used for optical microscopy experiments. A coverslip with 1.2 mg of dry-weight sample was removed from the desiccator after extensive vacuum drying. HPLC grade water was added (by weight) to achieve the desired hydration. The coverslip was then placed face down onto a precleaned GoldSeal Microslide (Corning Glass Works, Corning, N.Y.). A bead of quick set epoxy was placed around the coverslip to eliminate evaporation. Once the epoxy hardened, the slide was placed into the oven at 45°C without agitation for at least 48 h, assuring well-hydrated lipid bilayers. Once hydrated, the slide was placed onto a homebuilt heating stage and held constant at 45°C during observations.

The optical microscopy photomicrographs were taken from an Orthoplux II microscope (Leitz, Germany). The polarizers were crossed perpendicular to the optical axis. All pictures were taken at 40× using Orthomat-W fully automagic camera system (Leitz, Germany). The photomicrographs were taken using Polaroid's High Contrast PolaChrome 35-mm instant slide film (HCP-135-12 ISO 40/17).

RESULTS

Oriented samples of hydrated DMPC bilayers have been characterized by analyzing the ^{31}P and ^{15}N NMR spectra of these samples. Shown in Fig. 1 is a series of spectral simulations of oriented and unoriented samples. Fig. 1 *A* represents the powder pattern spectrum of a sample undergoing uniaxial rotation at a frequency greater than the frequency width of the spectral pattern. Such a pattern is typical for lipid bilayers where the lipids display axial rotation about the bilayer normal. Hexagonal and isotropic phases display spectral patterns consistent with bi- and triaxial rotation about orthogonal axes. When a bilayer sample is perfectly aligned a single sharp resonance is predicted, provided that all sites being detected by the NMR experiment are identical. This resonance, for a sample with the rotational axis aligned parallel with respect to the field, is shown in Fig. 1 *B*; the resonance is symmetric and its width is dictated by a T_2^* relaxation time. Imperfections in the orientation of the samples are manifested in the NMR spectra in several ways. First, as shown in Fig. 1 *C*, the resonance may become asymmetric. If the rotational axis for part of the sample is not perfectly aligned with the field the resonance frequency shifts toward $\sigma_\perp$ and away from $\sigma_\parallel$. This frequency shift

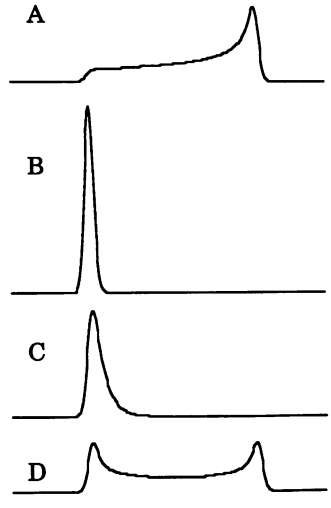

FIGURE 1 Spectral simulations exemplifying sample preparation imperfections for an axially symmetric tensor site in oriented samples. (*A*) Powder pattern representative of a random distribution of orientations with respect to the magnetic field. (*B*) Perfectly oriented sample with $\sigma_\parallel$ aligned with the magnetic field; the linewidth is dictated entirely by T_2 and the resonance is symmetric. (*C*) An asymmetric resonance resulting from a Graussian distribution of $\sigma_\parallel$ orientations with respect to the magnetic field. (*D*) The distribution of intensity expected for a cylindrical distribution of orientations in which the long axis of the cylinder is perpendicular to the field; note the symmetry of this intensity distribution versus the asymmetry of the random powder pattern intensity in *A*. Frequently oriented samples not only show a mosaic spread of orientations but with the protocol described here part of the sample gives rise to powder pattern intensity from randomly dispersed bilayers and from cylindrical defects in which the cylinder axis is perpendicular to the field.

occurs in only one direction and has a specific dependence upon the angle between the rotational axis and the magnetic field. Consequently, an asymmetric lineshape results.

The exact lineshape or distribution of intensity in the "tail" is dependent upon the mechanism by which the orientational imperfections are generated, as well as the limits of the distribution. For the simulations presented in this paper a Gaussian distribution of orientations about the mean rotational axis has been arbitrarily chosen. The magnitude of the orientational distribution is characterized by the mosaic spread, θ, of the sample that can be estimated from a measure of the half width of the resonance at half height. The down field half width of the resonance will be dominated by T_2^* while the upfield half width will have contributions from both T_2^* and mosaic spread. Quantitation of the mosaic spread can be achieved from spectral simulations with a model-defined orientational distribution. Secondly, as shown in Fig. 1 *D*, intensity across the frequency range may arise when bilayers fold back and close on each other. The intensity distribution is not that of a random powder pattern because the orientational distribution is not that over the surface of a sphere but rather over the surface of an extended cylinder. Thirdly, powder pattern intensity, such as that shown in Fig. 1 *A*, may appear in the spectra of oriented samples. If certain parts of the sample have no preferred orientation with respect to the field then powder pattern intensity will be observed. The net result is a spectral lineshape with considerable information for characterizing the alignment of bilayers with respect to the field and conversely a very powerful tool with which to optimize the protocols for orienting samples.

Fig. 2 presents ^{31}P NMR spectra of oriented bilayers of DMPC, with and without gramicidin at the specified molar ratios. All of the spectra are indicative of lipid bilayer preparations with no hexagonal or isotropic phase. Two different hydration levels were studied to determine the sensitivity of the ^{31}P chemical shift anisotropy to the hydration level, as well as our ability to characterize the orientation of the samples by this approach. While these are not pure powder pattern spectra, both $\sigma_\perp$ and $\sigma_\parallel$ can be readily evaluated from these spectra. Enough powder pattern is present to evaluate $\sigma_\perp$ and because the samples are oriented with the rotational axis parallel with respect to the field, the frequency of the intense resonance is that of $\sigma_\parallel$. The anisotropy ($\sigma_\parallel - \sigma_\perp$) is shown by this data to be a function of the ratio of gramicidin to DMPC and it has been suggested that this could result either from increased local dynamics of the headgroup or from an altered orientation of the headgroup with respect to the global rotational axis of the lipid molecule (Rajan et al., 1981). The anisotropy is not significantly affected by the two different hydration levels studied. At hydration levels lower than those studied here, the anisotropy increases as the level of hydration is decreased. The amount of powder pattern is only slightly affected by the molar ratio of gramicidin to lipid, but hydration has a much more pronounced effect. The comparison of spectra from 12 different samples (Fig. 2) shows that 30% hydration (volume of water to weight of gramicidin + lipid) results in far less powder pattern intensity than samples prepared with 60% water. Such an increase in powder pattern intensity is most readily explained by a decrease in the sample's viscosity and resultant loss of the sample from between the glass plates that orient the bilayers. Not only is powder pattern present in these spectra but also "tailing" of the oriented resonance is observable. There are significant variations in both T_2^* and mosaic spread for this set of data, but no obvious or reproducible trend in these parameters as a function of molar ratio or hydration level has been observed. Despite the variations observed in Fig. 2 it is very important that a well-defined protocol be followed in order to achieve even such a set of data. One of the most important factors is a substantial incubation period without agitation to allow for uniform hydration, since nonuniform hydration will appear as broadening of both the powder patterns and oriented spectra. Most of

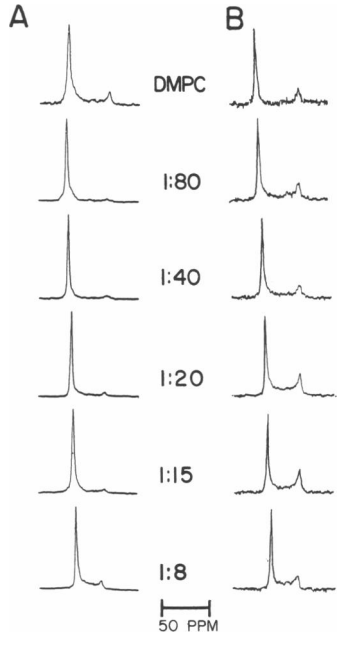

FIGURE 2 Proton decoupled ^{31}P NMR spectra of oriented DMPC bilayers as a function of the gramicidin to lipid ratio and as a function of hydration. The spectra were obtained above the gel to liquid crystalline phase transition temperature. These samples were prepared using 5% ethanol in benzene as the cosolubilizing agent. Approximately 400 acquisitions were averaged for each spectrum. Samples contained ~30 mg total dry weight. (*A*) 30% hydration (vol/dry wt) with HPLC grade water. (*B*) 60% hydration (vol/dry wt) with HPLC grade water.

the spectra presented in Fig. 2 represent mosaic spreads that range from ±6° to ±12° in the variation of the bilayer normal with respect to the magnetic field.

Fig. 3 presents a comparison of ^{31}P NMR spectra as a function of gramicidin to lipid molar ratio for three different cosolubilizing solvent systems. Column *A* of this figure shows data obtained with 100% methanol as the cosolubilizing solvent while column *B* was obtained using 5% ethanol in benzene, and column *C* utilized 3% methanol in benzene. As mentioned for the data in Fig. 2, the anisotropy changes with the molar ratio of gramicidin to lipid. The anisotropy of ~50 ppm for DMPC alone is in agreement with that obtained by Rajan et al. (1981) and the reduced width obtained in the presence of gramicidin is consistent with results from a variety of studies (Rajan et al., 1981; Nicholson et al., 1987; LoGrasso et al., 1988). This change in anisotropy is plotted in Fig. 4 as a function of the mass of gramicidin per milligram of dry-weight sample. The data reported here is consistent with a similar variation in ^{31}P anisotropy using $CHCl_3$ as a cosolubilizing agent (Cornell et al., 1988). Both the data reported here and this previously published data is fit by a single linear relationship, although it could also be fit with the near-linear dependence suggested by the model for gramicidin lipid interactions by Cornell and Separovic (1988). The primary distinction between these sets of data reported here is the amount of powder pattern intensity observed. The use of 5% ethanol instead of 3% methanol in benzene makes a very substantial difference in the quality of the oriented samples while 100% methanol yields spectra of intermediate quality. Furthermore,

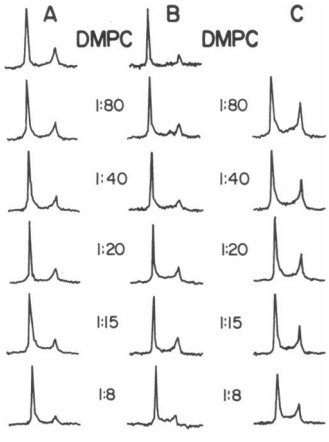

FIGURE 3 Proton decoupled ^{31}P NMR spectra of oriented DMPC bilayers as a function of the gramicidin to lipid ratio for three cosolubilizing solvent systems. Samples were prepared and spectra recorded as described in Fig. 2 using 60% hydration. (*A*) Samples cosolubilized in 100% methanol. (*B*) Samples cosolubilized in 5% ethanol/95% benzene; same spectra as in Fig. 2. (*C*) Samples cosolubilized in 3% methanol/97% benzene.

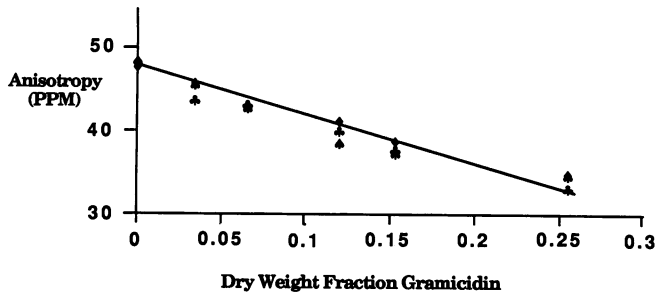

FIGURE 4 ^{31}P chemical shift anisotropy as a function of the fraction gramicidin by weight in the dry sample: (♦) samples cosolubilized in 100% methanol, (♠) samples cosolubilized in 5% ethanol in benzene, (♣) samples cosolubilized in 3% methanol in benzene. These data were obtained from the spectra in Fig. 3 and each measurement has an error of ±2 ppm. The line is a linear least squares fit to all of the data presented.

T_2^* relaxation and "tailing" appear to be substantially worse for the spectra obtained from samples prepared with 3% methanol in benzene. The spectrum of pure DMPC was unobtainable from this solvent system because of the difficulty in spreading a sample of pure DMPC in this solvent onto the polar glass surface. While this was achieved in the presence of gramicidin, the glass plates prepared did not have a uniform distribution of dry solid over the glass surface. When 100% methanol or 5% ethanol in benzene is used as the cosolubilizing solvent a clear film is left on the glass surface after the removal of the solvent; however, when the methanol/benzene solvent system is used a white powdery solid results. This lack of adhesion of the lipid preparation is thought to be responsible for the substantial powder pattern intensity. While both methanol and benzene/ethanol resulted in clear films on the glass plates, the viscosity of the samples from these two solvents, after hydration to 60%, differed substantially. The samples prepared from methanol were much more viscous, which may account for the larger powder pattern intensity in the methanol preparations.

Fig. 5 illustrates the spectral simulation of the 1:80 molar ratio sample from benzene/methanol solution. The spectral simulation in Fig. 5 *C* assumes no cylindrical defects, but only a mosaic spread of 12° (Fig. 5 *A*) and sample that has seeped from between the glass coverslips (powder pattern, Fig. 5 *B*). Fig. 5 *F* is a somewhat worse simulation assuming no seepage of the sample, but rather the presence of cylindrical defects, (Fig. 5 *E*) and a mosaic spread of 12° (Fig. 5 *D*). For this sample the simulation with powder pattern is significantly better than that with cylindrical defects. When the intensity distribution between $\sigma_\parallel$ and $\sigma_\perp$ is symmetric, cylindrical defects are implicated, but here the intensity distribution

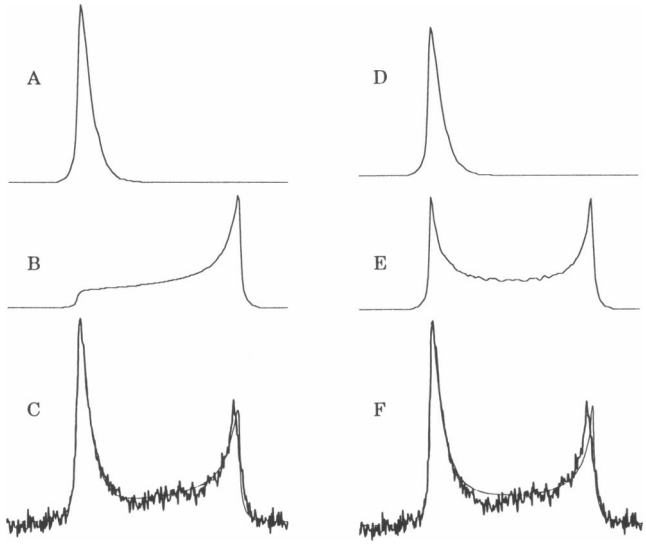

FIGURE 5 Simulation of the ^{31}P NMR spectrum of the 1:80 molar ratio sample cosolubilized in benzene/methanol and hydrated to 60% by weight. The spectrum is the same as one of those shown in Fig. 3. (*A* and *D*) Simulation representing an oriented resonance at $\sigma_\parallel$ with a mosaic spread of 12°. (*B*) Powder pattern intensity representative of a completely random distribution of lipid orientations with respect to the magnetic field. (*C*) Summation of *A*, 37% of the intensity, and *B*, 63% of the intensity, superimposed on the experimental data. (*E*) Intensity distribution associated with cylindrical defects in the bilayer preparation. (*F*) Summation of *D*, 16% of the intensity, and *E*, 84% of the intensity, superimposed on the experimental data.

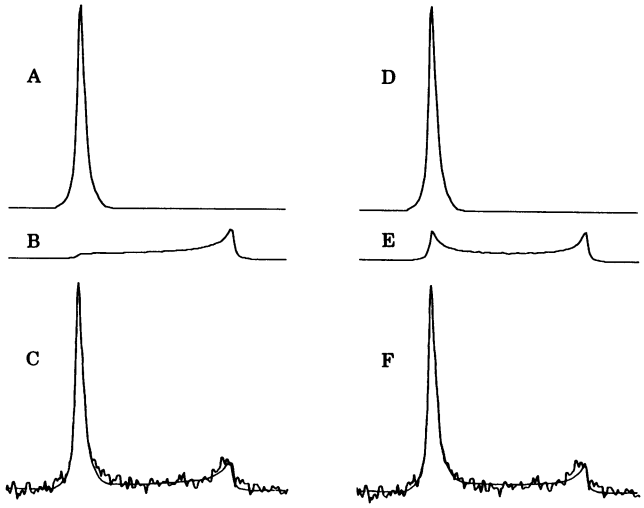

FIGURE 6 Similar spectral simulation as that shown in Fig. 5. This spectrum is obtained from a 1:40 molar ratio sample cosolubilized in benzene/ethanol and hydrated to 60% by weight. The spectrum is the same as one of those shown in Fig. 3. (*A* and *D*) Oriented resonance at $\sigma_\parallel$ with a mosaic spread of 8°. (*B*) Powder pattern intensity. (*C*) Summation of *A*, 62% of the intensity, and *B*, 38% of the intensity, superimposed on the experimental data. (*E*) Cylindrical defect intensity. (*F*) Summation of *D*, 52% of the intensity, and *E*, 48% of the intensity, superimposed on the experimental data.

is asymmetric, implicating a large powder pattern contribution.

Fig. 6 shows similar spectral simulations for the 1:40 molar ratio sample from benzene/ethanol solution. The intensity distribution between $\sigma_\parallel$ and $\sigma_\perp$ is much more symmetric than that in Fig. 5, which suggests a substantial contribution from cylindrical defects. While this defect intensity appears small in the experimental spectrum, 48% of the intensity in Fig. 6 *F* is from cylindrical defects and 38% of the intensity in Fig. 6 *C* results from powder pattern. Protocol development for minimizing this distribution of intensity across the frequency range has the potential for increasing the sensitivity of the experiments dramatically by enhancing the $\sigma_\parallel$ resonance. Many of the spectra obtained from samples prepared from benzene/ethanol solution that were hydrated with 30% by weight water (see Fig. 2) have such a small powder pattern or cylindrical defect intensity that such an analysis is not applicable without much improved signal to noise.

Fig. 7 is a photomicrograph, viewed under crossed polarizers, of an oriented sample of hydrated DMPC bilayers prepared from methanol solution. The black regions (Fig. 7 *A*) represent near perfectly aligned domains in the sample, where the bilayer normal is aligned parallel to the optical axis. The birefringent regions result directly from defects in the sample where the bilayer normal is tilted with respect to the optical axis. Two kinds of defect structures are clearly visible; the "parabolic focal conics" (Fig. 7 *B*) and "oily streaks" (Fig. 7 *C*). Oily streaks result from folds in the multibilayer structure (Friedel, 1922; Kleman et al., 1976). These folds may involve a few or many bilayers in the hydrated film between two glass plates which is $\sim 10^4$ bilayers thick with each hydrated bilayer (at 60% hydration) being on the order of 100 Å thick. The width of these oily streaks, as observed under crossed polarizers, is related to the number of bilayers involved in the fold. In this sample the oily streaks range from 2 to 20 μm in thickness and therefore this image represents a projection of all the oily streaks throughout the sample onto a single plane. Consequently, the near perfectly aligned domains for a single bilayer are much larger than the size of the dark regions in the photomicrograph which represent only those domains in which all of the bilayers throughout the film are perfectly aligned. This defect results in variable orientations for the phosphate head groups which extend from 0 to 90°, generating intensity throughout the chemical shift range. Parabolic focal conics are a property of the entire thickness of the film. This is a regular defect structure that can occur over a large region of the sample,

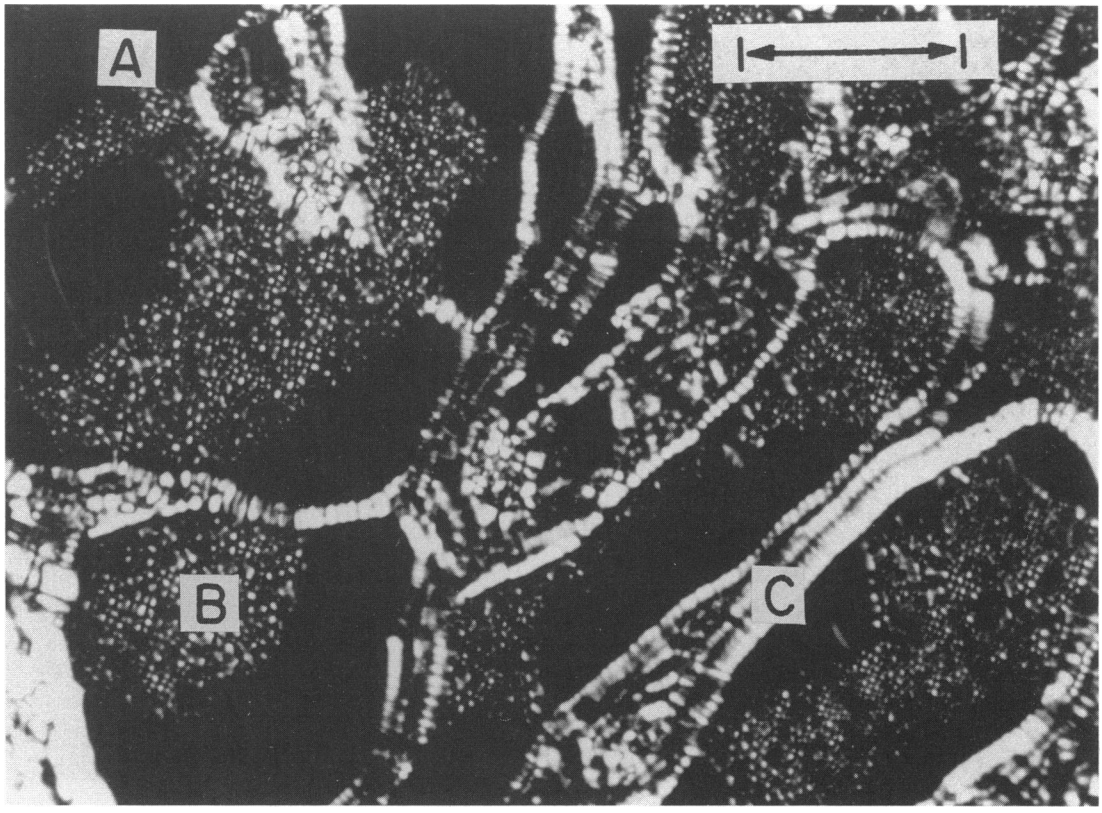

FIGURE 7 Photomicrograph of an oriented lipid preparation obtained under crossed polarizers, 40× and at a temperature of 40°C, which is above the gel to liquid crystalline phase transition temperature. The bar length is 100 μm. This sample of DMPC alone was solubilized in methanol and hydrated to ~60% by weight water. This figure illustrates both near perfectly oriented domains (*A*) as well as two types of defect structures: parabolic focal conics (*B*) and oily streaks (*C*).

resulting in a range of bilayer normal orientations with respect to the optical axis. Therefore, this defect will generate an asymmetric peak at $\sigma_{\parallel}$ that extends in the direction of $\sigma_{\perp}$. The magnitude of this defect can vary substantially, resulting in a variable range of chemical shift frequencies.

In Fig. 8 *A* a photomicrograph is presented of a hydrated, oriented sample prepared with a 1:8 molar ratio of gramicidin to lipid, which was cosolubilized in 3% methanol in benzene. The photomicrograph shows very extensive oily streaks and nodes, which result from the fusion of separate oily streak defects. By comparison a photomicrograph of a sample prepared with a 1:15 molar ratio of uniformly ^{15}N labeled gramicidin to lipid which was cosolubilized in 5% ethanol in benzene is shown in Fig. 8 *B*. The oily streaks are much narrower and the nodes are much further apart. Some fine parabolic focal conic defects are seen as well as large regions of near perfect alignment. These typical photomicrographs clearly document the superior orientation of the preparations from benzene/ethanol. The molar ratio of gramicidin to lipid has relatively little effect on the appearance of the photomicrographs.

Fig. 9 shows the ^{15}N NMR spectrum of oriented uniformly ^{15}N labeled gramicidin D in hydrated (30%) lipid bilayers (1:15 molar ratio). This spectrum represents the $\sigma_{\parallel}$ components of 20 different ^{15}N sites, 16 of which are in the polypeptide backbone and the remaining four are from the indole nitrogens of tryptophan. Many of the resonances in this uniformly labeled sample are multisite resonances and only half of these resonances have been specifically identified by single site isotopic labeling (Fields et al., 1988; Nicholson et al., 1989; Nicholson and Cross, 1989; and unpublished results). However, as in the ^{31}P NMR spectra, the ^{15}N chemical shifts in oriented samples are very sensitive to conformation and therefore, this spectrum can be used as a fingerprint for the channel state which has been previously characterized by circular dichroism, ^{23}Na and ^{15}N NMR in similar preparations (LoGrasso et al., 1988). The amount of broad powder pattern intensity is substantially less in this spectrum obtained from a sample cosolubilized with 5% ethanol in

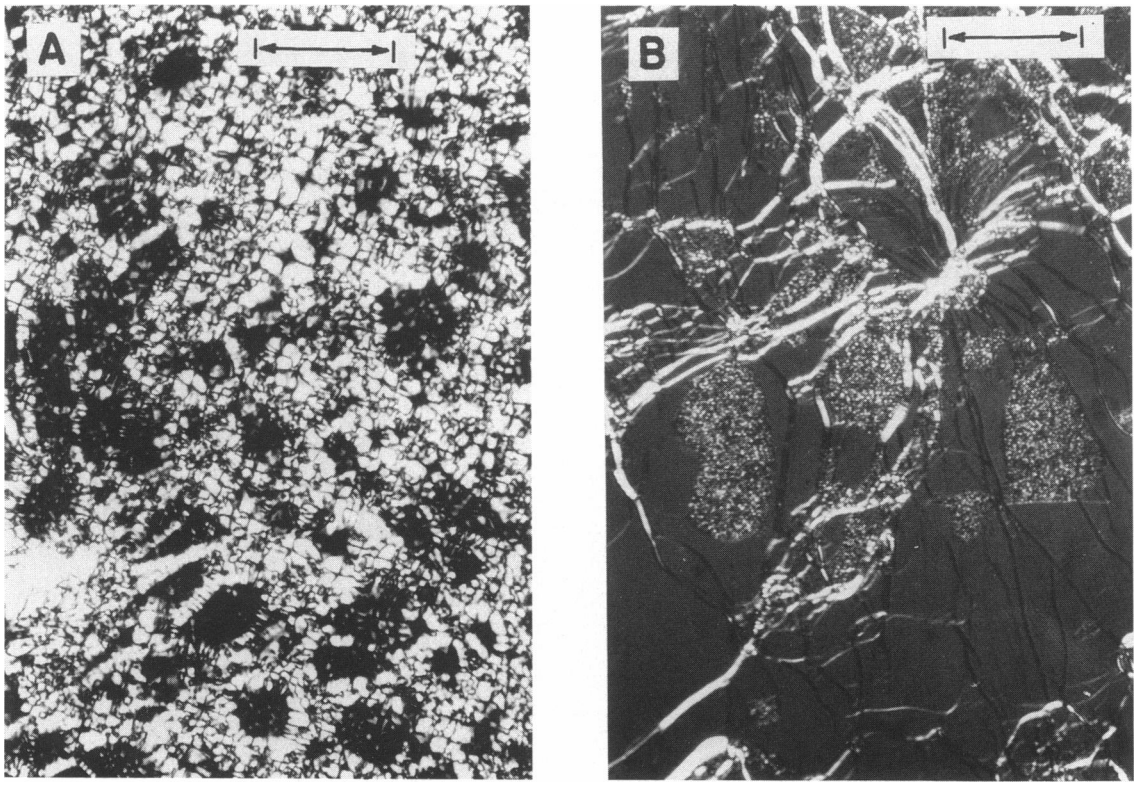

FIGURE 8 Photomicrographs of two preparations containing gramicidin obtained under the same conditions described in Fig. 7. (*A*) The sample is a 1:8 molar ratio of gramicidin to lipid, cosolubilized in 3% methanol/97% benzene and was hydrated with 60% by weight water. (*B*) This sample is a 1:15 molar ratio of gramicidin to lipid, cosolubilized in 5% ethanol/95% benzene and was hydrated with 30% by weight water.

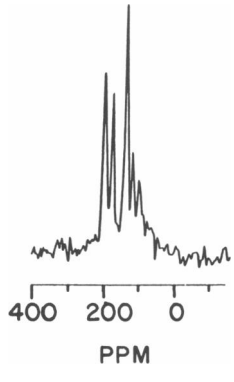

FIGURE 9 ^{15}N NMR spectrum of oriented uniformly ^{15}N labeled gramicidin D biosynthesized by *Bacillus brevis*. This sample is a 1:15 molar ratio of gramicidin to lipid, cosolubilized in 5% ethanol/95% benzene and was hydrated with 30% by weight water. Total mass of gramicidin in the sample is ~4 mg and the signal averaging time was 44 h.

benzene than in previous published spectra of uniformly ^{15}N labeled gramicidin prepared from different solvent systems.

DISCUSSION

^{31}P NMR has been extensively used for a variety of studies of model membranes (Seelig, 1978; Cullis and de Kruijff, 1979). The phase of hydrated lipid preparations is routinely characterized by observing the motionally averaged chemical shift anisotropy as are the dynamics of the phospholipid headgroups (Kohler and Klein, 1977). In particular for this study, lipid preparations containing gramicidin have been characterized (Rajan et al., 1981; Nicholson et al., 1987; Killian and de Kruijff, 1986). Because phospholipids rotate about an axis parallel to the bilayer normal the magnitude of the reduced ^{31}P chemical shift anisotropy (characterized by two elements, $\sigma_{\parallel}$ and $\sigma_{\perp}$) is dictated by three factors: the magnitude of the static principal elements of the chemical shift tensor (σ_{11}, σ_{22}, and σ_{33}) and their orientation with respect to the molecular frame; the orientation of the phosphate group

with respect to the motional axis; and a description of local motions (axis, amplitude, frequency, etc.). For samples aligned with the bilayer normal parallel with respect to the magnetic field, the observed chemical shift, σ_{obs}, equals $\sigma_{\parallel}$ and hence is directly affected by the same factors that affect the chemical shift anisotropy.

For the purposes of this discussion it is reasonable to expect that the magnitude of the static principal elements of the phosphate tensor are a constant for these different preparations of gramicidin and DMPC. The static elements have been studied for DMPC: $\sigma_{11} = -97$, $\sigma_{22} = -34$, $\sigma_{33} = 133$ ppm relative to external 85% H_3PO_4 and their orientation with respect to the molecular frame has been modeled with barium diethyl phosphate through a single crystal study (Herzfeld et al., 1978). Even if local motions were assumed to be insignificant, the chemical shift from an oriented lipid preparation is not enough to determine the orientation of the phosphate group with respect to the global motional axis, which is the bilayer normal for DMPC. Each chemical shift is consistent with a range of possible tensor orientations, each of which can be described by a set of torsion angles. Thayer and Kohler (1981) demonstrated theoretically for the lipid, phosphatidylethanolamine, that rotation about one of these torsion angles (the one connecting the phosphate with the glycerol backbone) by just 16.6° would reduce the 47 ppm anisotropy to zero by generating an orientation that resulted in the isotropic average. It is, therefore, possible to explain the reduction in anisotropy with increasing molar concentration of gramicidin as resulting from a modest change in the phosphate group orientation. As the molar concentration of gramicidin increases the density of the lipid headgroups at the bilayer surface decreases, since the extremely hydrophobic polypeptide is expected to be almost entirely within the hydrophobic domain of the bilayer. It is reasonable to anticipate that the headgroup may tip toward the plane of the bilayer, which would be consistent with the calculations of Thayer and Kohler (1981).

Alternatively, local dynamics may be enhanced by the same reduction in headgroup density at the bilayer surface. The consequences for the anisotropy of such motion are dependent upon the axis of the motion with respect to the shielding tensor, whether the motion is continuous or discontinuous, the amplitude of the motion, and its frequency. Such a description has been achieved (Nicholson et al., 1989) for a highly constrained site in the backbone of gramicidin in a lipid bilayer. The lipid headgroup has many more degrees of freedom and therefore, a quantitative description of local motions will be much more difficult. For this discussion it is only important to realize that a motional model for the reduction of the anisotropy is dependent on the same reduced headgroup density that could also allow for a possible change in the orientation of the headgroup. This description of the gramicidin/lipid interactions is consistent with the model recently published by Cornell and Separovic (1988).

Consequently, factors that would reduce the effect of gramicidin on the local headgroup density can be evaluated via observation of the anisotropy. One such concern is the possible aggregation of gramicidin at high molar ratios. Fluorescence studies of gramicidin incorporated into lysophosphatidylcholine bilayers have indicated that intermolecular tryptophan–tryptophan interactions exist (Cavatorta et al., 1982; Spisni et al., 1983). This suggests that the gramicidin is aggregated, and models for the aggregation have been proposed which show hexagonally packed arrays of the channels. If the gramicidin aggregated, the lipid/polypeptide contacts would be reduced and the lipid/lipid contacts would be increased. Averaging of the phosphate shielding tensor would be reduced, as if there were less gramicidin present in the sample. The linear or near-linear dependence of anisotropy on the gramicidin content of the bilayers is a very clear indication that in these preparations of diacyl-lipids significant aggregation is not present.

The determination of the chemical shift anisotropy ($\sigma_{\parallel} - \sigma_{\perp}$) can be used quantitatively as an in situ method for determining the gramicidin/DMPC ratio as it exists in the hydrated lipid bilayers. Reference data such as those presented in Fig. 4 can be used for such a determination where the molar ratio is not known. A tool like this is important in model membrane studies where the final preparation for study often involves a centrifugation step resulting in fractions that may have different molar ratios. The preparation of lysolipid bilayers mentioned above requires such a protocol and the fractions do indeed have different molar ratios. For this tool to be effective it is important that it not be sensitive to the subtleties of sample preparation. While it is sensitive to the choice of lipid it does not appear to be sensitive to a range of cosolubilizing solvents and hydration levels. The channel state of gramicidin is sensitive to the cosolubilizing solvent (LoGrasso et al., 1988; Killian et al., 1988), but the insertion of gramicidin into the bilayer such that the headgroup density of the lipid is reduced, in a consistent way, is not sensitive to the choice of solvent. This further supports the contention that all of these solvent systems incorporate the polypeptide into the bilayer and that the gramicidin is not left in an aggregated state at the bilayer surface, as suggested for different incorporation protocols (Masotti et al., 1980).

The orientation of these bilayer samples as characterized by optical microscopy agrees with the results of the ^{31}P NMR spectra presented in Figs. 5 and 6. The samples prepared for optical microscopy and ^{31}P NMR are very similar, however, the differences are important. The ^{31}P NMR sample consists of many coverslips stacked

together and packed tightly into a container. Lipid that seeps from between the coverslips will be observed as powder pattern intensity. For the microscope preparations only sample between the coverslip and slide is observed. The parabolic focal conic defects yield a distribution of lipid orientations about the bilayer normal and is consistent with the observation of a mosaic spread of orientations in the ^{31}P NMR spectra. Oily streak defects are cylindrical defects and hence the observation of a large powder pattern and/or cylindrical defect intensity in the benzene/methanol spectra of Fig. 5 is consistent with oily streaks observed from the same solvent in the photomicrograph of Fig. 8 *A*. Similarly, the reduced mosaic spread and defect intensity for the 30% hydrated benzene/ethanol preparation in column *A* of Fig. 2 is consistent with the faint parabolic focal conic defects and fine oily streaks shown in the photomicrograph of Fig. 8 *B*.

While the anisotropy is insensitive to the cosolubilizing solvent and extent of hydration, the degree of orientation is not. Clearly, the solvent of choice from those used in this study is 5% ethanol in benzene with the bilayer preparation hydrated to 30% by weight. For these conditions the photomicrographs show minimal oily streak and parabolic focal conic defects while the ^{31}P NMR spectra show minimal powder pattern intensity and cylindrical defect intensity as well as a small or insignificant mosaic spread of the $\sigma_{\parallel}$ resonance. This optimal choice of solvent has resulted in a dramatic improvement in the quality of the oriented samples. In fact, when the samples are hydrated at 30% the underlying powder pattern has virtually disappeared. This high degree of orientation is very important in ^{15}N NMR studies, because a near perfectly aligned sample produces a well-resolved peak in a shorter signal averaging time. The decrease in mosaic spread results in better resolution, and improved sensitivity results when the powder pattern and tailing intensities are minimized forcing all of the intensity into the narrow frequency range of the oriented resonance. This is illustrated by the well-resolved resonances seen in Fig. 9 and the very low intensity of the underlying powder pattern. This spectrum also shows by virtue of the similarity in the chemical shift frequencies that the backbone conformation of gramicidin D cosolubilized in benzene/ethanol is the same as that out of a variety of other solvent systems which have been previously documented (LoGrasso et al., 1988).

The consistent nature of the NMR and microscopy results provides a very useful characterization tool for assessing the orientation of liquid crystalline samples. Because of the small amount of sample needed and the ease with which samples can be prepared, optical microscopy will be very helpful in the continuing effort to optimize oriented samples for spectroscopy. For now it has been an aid for determining that 5% ethanol in benzene is a very good solvent system for achieving highly oriented samples with minimal powder pattern intensity.

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