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Deuterium Nuclear Magnetic Resonance Studies of the Interaction between Dimyristoylphosphatidylcholine and Gramicidin A'†

David Rice‡ and Eric Oldfield*

ABSTRACT: Deuterium nuclear magnetic resonance spectra of dimyristoylphosphatidylcholines (DMPCs) specifically labeled with deuterium at one of positions 2', 3', 4', 6', 8', 10', 12', or 14' of the 2-chain have been recorded at 34.1 MHz in the presence of varying concentrations of the linear pentadecapeptide antibiotic gramicidin A'. Deuterium quadrupole splittings ($\Delta\nu_Q$) have been used to partially characterize the motion and hydrocarbon chain order of phospholipid in contact with the polypeptide surface. At lipid concentrations below 4 lipids/gramicidin molecule the quadrupole splitting of a terminal methyl-labeled DMPC collapses to a single line. The quadrupole splittings of the methylene labels are decreased, and the line shapes are dominated by large intrinsic line widths. The time constants characterizing the decays of the echo intensity (T_{2e}) are correspondingly reduced in the lipid–polypeptide complexes. At lipid–polypeptide molar ratios of greater than 15:1, the quadrupole splittings of the labels increase linearly with gramicidin concentration to a value ~30% greater than that of pure lipid. Between ratios of 15:1 and 4:1, the splittings decrease slowly. At a ratio of about 4:1 a rather abrupt transition occurs to the high gramicidin phase, and on the time scale of the deuterium NMR experiment (~5.0 μs) lipid adjacent to polypeptide appears disordered (smaller $\Delta\nu_Q$) compared to pure lipid. At all lipid–protein ratios investigated above the gel-to-liquid crystalline phase transition temperature of the pure lipid (T_c), the experimental spectrum is shown to be accurately simulated by using one quadrupole splitting together with a Lorentzian line broadening corresponding to the quadrupolar echo decay rate $(\pi T_{2e})^{-1}$. In some gramicidin–lecithin complexes, T_{2e} values as short as 49 μs are observed. This implies in general for studies of protein–lipid organization in both model and biological membranes that T_{2e} values should be determined routinely in order to eliminate spectral distortions due to relaxation.

In recent years there has been much interest in developing and applying physical methods to study the structure of model and intact biological membranes. Information on the static structures of both types of membrane has been obtained by using X-ray diffraction (Engelman, 1971; Tardieu et al., 1973) and neutron diffraction techniques (Worcester & Franks, 1976; Büldt et al., 1978; D. L. Worcester, M. Meadows, D. Rice, and E. Oldfield, unpublished experiments), while information on the dynamic structures of these systems has been gained predominantly from a variety of magnetic resonance techniques [for example, Chapman & Salsbury (1966), Oldfield et al. (1971, 1972, 1976, 1978a,b) Gaffney & McConnell (1974), Davis et al. (1976), Lawaczeck et al. (1976), Gent et al. (1978), Mantsch et al. (1977), Seelig (1977), Feigenson et al. (1977), Marsh et al. (1978), and Griffin et al. (1978)].

Although proteins normally constitute about one-half of a membrane's dry weight, there have been remarkably few studies of protein–lipid interaction using physical techniques. Electron spin resonance (ESR)[1] studies using nitroxide free-radical spin-labels have suggested that, at least on the time scale of $\sim 10^{-8}$ s, there is a reduction in the rate of motion of a fraction of the hydrocarbon chains in a protein-containing lipid bilayer, the so-called "boundary lipid" or "annulus" (Jost et al., 1973; Hesketh et al., 1976).[2] Calorimetric studies indicate a decrease in the enthalpy of the gel-to-liquid crystalline phase transition of zwitterionic phospholipids with intrinsic protein- or polypeptide-containing bilayers (Chapman et al., 1974; Papahadjopoulos et al., 1975; Curatolo et al., 1977), and Raman spectroscopy has shown a reduction of the number of gauche isomers at low concentrations of the polypeptide gramicidin A' (Chapman et al., 1977), which is in agreement with spin-label results which indicate an increase in chain segmental order parameters (Chapman et al., 1977; Cornell et al., 1978).

More recently, deuterium quadrupole-echo Fourier transform nuclear magnetic resonance (NMR)[1] studies of protein–lipid and polypeptide–lipid interactions in model and intact biological membranes have been reported (Oldfield et

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[1] Abbreviations used: DPPC, 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine; DMPC, 1,2-dimyristoyl-3-*sn*-phosphatidylcholine; PPPC, 1-palmitoyl-2-palmitoleyl-3-*sn*-phosphatidylcholine; NMR, nuclear magnetic resonance.

[2] More recent studies by Jost & Griffith (1978) have interpreted the ESR results as indicating that "boundary lipid" is far more ordered than free bilayer lipid.

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al., 1978a; Kang et al., 1979a,b), and it has been suggested that, on the time scale of the deuterium NMR experiment, one effect of protein (or polypeptide) is to cause a *disordering* of the lipid hydrocarbon chain, at least in the region of the terminal methyl groups. In order to try to characterize the exact nature of the molecular motions occurring in such lipid–protein (or lipid–polypeptide) systems, it is clearly desirable to have as simple a polypeptide constituent as possible. Given a sufficiently low molecular weight species, it should be possible to use ^{2}H-labeling techniques (Oldfield et al., 1978b) combined with neutron, Raman, and NMR methods to precisely characterize the spatial dispositions at ~1-Å resolution and the dynamical behavior of most groups in the system. We have chosen the polypeptide gramicidin A′ for such a study. Gramicidins are peptides of molecular weight ~1800, containing 15 nonpolar amino acid residues, having the sequence (Sarges & Witkop, 1965a,b; Rinehart et al., 1977)

HCO-Val-Gly-Ala-Leu-Ala-Val-
L L D L D
Val-Val-Trp-Leu-Trp-Leu-Trp-Leu-Trp-NHCH$_2$CH$_2$OH
L D L D L D L D L

The amino-terminal valine may be replaced by an isoleucine residue, and in addition in gramicidins B and C, Trp-13 is replaced by Phe or Tyr, respectively. It is known to bind to membranes and to allow the passage of ions through the membrane. Its mechanism of action, established from conductance studies, indicates that the functional form of the peptide is a dimer and that this dimer forms a channel which spans the bilayer (Urry, 1971; Krasne et al., 1971; Hladky & Haydon, 1972; Veatch et al., 1974; Kolb & Bamberg, 1977; Bamberg & Janko, 1977). In addition, gramicidin A′ appears to be one of the most hydrophobic polypeptide sequences known (Segrest & Feldmann, 1974), which together with its commercial availability favors its use as a model for a membrane-penetrating protein segment.

Experimental Section

Materials. Gramicidin A′ is a mixture of gramicidins A, B, and C and was obtained from Nutritional Biochemicals Co. and used without further purification. Deuterium-depleted water was obtained from Aldrich Chemical Co. (Milwaukee, WI). Benzene and methanol were reagent grade and were obtained from Mallinckrodt Chemical Co. (Paris, KY). Dimyristoylphosphatidylcholines labeled with deuterium at one of the positions 2′, 3′, 4′, 6′, 8′, 10′, 12′, or 14′ in the 2-chain were synthesized by using the methods previously described (Oldfield et al., 1978b). 1,2-[16′,16′,16′-^{2}H$_3$]Dipalmitoylphosphatidylcholine and 1-[16′,16′,16′-^{2}H$_3$]palmitoyl-2-palmitoleylphosphatidylcholine were prepared as described previously (Kang et al., 1979a).

Nuclear Magnetic Resonance Spectroscopy. Deuterium Fourier transform NMR spectra were obtained at 34.1 MHz by using the quadrupole-echo pulse technique (Davis et al., 1976). We used a "home-built" instrument for data acquisition (Oldfield et al., 1978b). Single-phase detection combined with a spectrum reverse (Nicolet Instrument Corp., Madison, WI; software package FT 74) permitted the use of a 100-kHz effective spectral bandwidth. The 90° pulse width was ~6.5 μs, which corresponds to a radio-frequency field strength (γH_1) of about 40 kHz, which is more than adequate to cover the relatively narrow line widths we have observed. Values of τ in the quadrupole-echo experiment varied upward from a minimum of 30 μs. Below 30 μs pulse feed-through distortions dominated the transformed spectra.

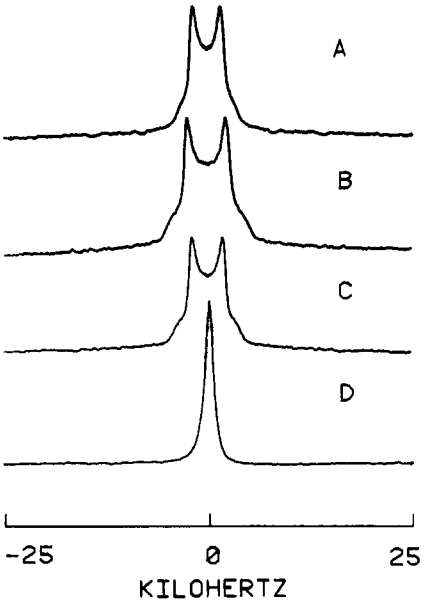

FIGURE 1: Deuterium quadrupole-echo Fourier transform NMR spectra at 34.1 MHz of 2-[14′,14′,14′-^{2}H$_3$]DMPC in the presence of several concentrations of gramicidin A′, in excess deuterium-depleted water. Spectral conditions were as follows: 50.0-kHz spectral width, 0.540-s recycle time, 2K data points, $\tau_1 = \tau_2 = 60$ μs, 7-μs 90° pulse widths, 3000 scans, 150-Hz line broadening, sample volume 300 μL. (A) Pure lipid. (B) 15 wt % gramicidin. (C) 33 wt % gramicidin. (D) 50 wt % gramicidin. All samples were at 30 °C.

Spectral simulations were carried out on a CDC Cyber-175 system by using the laboratory peripherals described previously (Kang et al., 1979a).

Sample Preparation. Both gramicidin and DMPC were dissolved in 95% benzene–methanol (v/v), the two solutions were mixed and frozen at liquid N_2 temperature, and solvent was removed by lyophilization. Within 3 h most solvent had sublimed. Last traces were eliminated by pumping under vacuum at ~54 °C over P_4O_{10} for a period of 2–3 days. Proton NMR spectra were taken in deuterioethanol to confirm the absence of benzene and methanol used in sample preparation. The lipid complex was then mixed with twice its weight of deuterium-depleted water in the NMR sample tube, followed by vortex mixing and stirring at ~40 °C to form a homogeneous lipid–gramicidin dispersion.

Results and Discussion

In Figure 1 we present spectra of 2-[14′,14′,14′-^{2}H$_3$]dimyristoylphosphatidylcholine (DMPC-d_3) bilayers in H_2O, obtained by the Fourier transform method at 34.1 MHz, using the quadrupole-echo pulse technique (Davis et al., 1976), as a function of gramicidin A′ concentration in the bilayer. Up to a lipid–gramicidin mole ratio of ~4:1, the ^{2}H NMR spectrum is a well-resolved doublet, characteristic of the spin $I = 1$ deuterium nucleus in a C–D bond having an asymmetry parameter (η) of zero. The observed "quadrupole splitting" ($\Delta\nu_Q$) of the NMR absorption line, which corresponds to the frequency separation between the singularities in the distribution functions for the $+1 \leftrightarrow 0$ and $0 \leftrightarrow -1$ transitions, may be expressed as

$$\Delta\nu_Q = \frac{3}{2}\frac{e^2qQ}{h}\langle P_2(\cos\theta)\rangle$$

e^2qQ/h is the deuterium quadrupole coupling constant, which has been found to be about 170 kHz for ^{2}H nuclei in aliphatic C–D bonds (Derbyshire et al., 1969; Burnett & Muller, 1971). θ is the angle between the applied dc magnetic field H_0 and

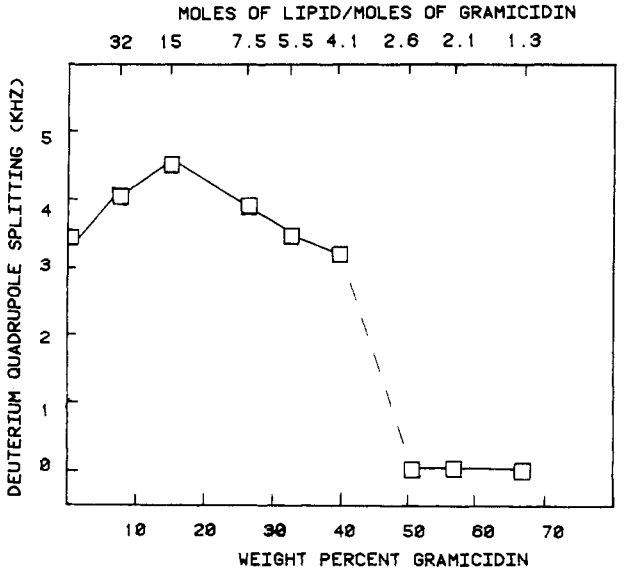

FIGURE 2: A plot of deuterium quadrupole splitting for 2-[14′,-14′,14′-$^2H_3$]DMPC vs. weight percent gramicidin A′. The top horizontal axis shows moles of lipid per mole of gramicidin. Data were recorded at 30 °C. The broken line indicates a region in which two-component (powder pattern plus isotropic peak) spectra are observed.

the principal component of the electric field gradient tensor at the deuterium nucleus, and $\langle P_2(\cos\theta)\rangle$ is the time-averaged value of $1/2(3\cos^2\theta - 1)$ over any motions that are rapid compared with 170 kHz.

As the gramicidin content of the bilayer is increased above ~40 wt % gramicidin, the ^2H NMR quadrupole splitting collapses and a broad line of about 1200-Hz width remains. The spectrum remains unchanged up to the highest gramicidin concentrations we have investigated (67 wt % gramicidin or ~1.3:1 lipid–gramicidin mole ratio). These results are more graphically represented in Figure 2, where we have plotted the quadrupole splittings as a function of the weight percent gramicidin in the lipid bilayer.

The X-ray results of Chapman et al. (1977) support the idea that even at very high mole fractions of gramicidin a bilayer structure persists. These workers showed that at both 20 and 50 °C through the concentration range from pure (dipalmitoyl) lecithin to a concentration of 2 mol of (dipalmitoyl) lecithin/mol of gramicidin A′ the X-ray long spacings were those of a lamellar phase and at all concentrations of gramicidin A′ a single family of lamellar diffraction peaks occurred. At concentrations of gramicidin A′ greater than ~5 phospholipids/polypeptide, the high angle diffraction peaks became progressively broader, indicating a disordering effect on the hydrocarbon chain packing (Chapman et al., 1977).

Scanning calorimetric studies by these same workers show that the enthalpy of the gel-to-liquid crystal transition (of dipalmitoyllecithin) decreased linearly with increasing amounts of gramicidin A′ until a concentration of ~20 lipids/polypeptide was reached. As may be seen from Figure 2, this behavior may correlate with the increase in quadrupole splitting seen from 0 to ~15 wt % gramicidin in Figure 2. At ~15 mol of lipid/mol of gramicidin it has been suggested that an aggregation process which produces localized polypeptide clusters within the membrane occurs (Chapman et al., 1977). This is the region where the quadrupole splitting begins to decrease as gramicidin A′ concentration increases (Figure 2). At concentrations of polypeptide of less than 5 lipid (dipalmitoylphosphatidylcholine) molecules/gramicidin A′ molecule, freeze-fracture electron microscopy shows the presence of liposomes with smooth fracture faces, and at higher polypeptide concentrations sheetlike structures are observed which also have smooth fracture faces (Chapman et al., 1977). From Figure 2 we see that above ~4 mol of lipid/mol of polypeptide the quadrupole splitting rapidly collapses and that the single narrow ^2H NMR line is characteristic of these high gramicidin A′, sheetlike lipid bilayer structures.

Although the studies of Chapman and co-workers have utilized both DMPC and dipalmitoylphosphatidylcholine (DPPC) and we have used in Figures 1 and 2 only dimyristoylphosphatidylcholine, we have carried out additional experiments with 1,2-[16′,16′,16′-$^2H_3$]DPPC and find a similar decrease in quadrupole splitting at high gramicidin concentrations (unpublished experiments). The effect we have observed is thus not restricted to lecithin-containing di-C14 saturated hydrocarbon chains.

Recently, Cornell et al. (1978) have reported monolayer studies of the interaction between gramicidin A′ and DMPC and between gramicidin A′ and egg yolk lecithin. For DMPC (at 23 °C) at surface pressures of 15 dyn cm^{-1} mixing was ideal, while at surface pressures of 30 dyn cm^{-1} there was a "condensing effect". For the egg lecithin–gramicidin A′ system (at 22 °C) the condensing effect was more pronounced and was apparent even at surface pressures of 10 dyn cm^{-1}. These results were interpreted as indicating a "cholesterol-like" condensing effect of the gramicidin A′ molecules on the lecithin hydrocarbon chains which is not reflected in any simple way in our ^2H NMR results. However, an assumption made by these workers was that the gramicidin A′ molecule had the same molecular area in the mixed monolayer as it did in the pure monolayer. Other workers who have examined the same system (Kemp & Wenner, 1976) have reported that gramicidin packing in a pure monolayer becomes staggered and disordered above surface pressures of 10 dyn cm^{-1}, but that in mixed monolayers this disordered packing may be partially prevented. There appears therefore to be no convincing evidence which can accurately quantitate the changes in surface area (or packing) of the lecithin and gramicidin molecules on going from pure to mixed bilayer systems; thus, predictions of ^2H NMR behavior are difficult. The situation for cholesterol is of course far simpler since the steroid has a rigid, fused tetracyclic ring structure and is not known to undergo any dimerization or aggregation processes as has been demonstrated for gramicidin A′ (Urry, 1971; Veatch et al., 1974).

Since the possibility of a more specific interaction between the unsaturated lipid (egg yolk lecithin) and gramicidin A′ appeared to exist from the monolayer studies (Cornell et al., 1978), we also investigated the interaction between gramicidin A′ and the unsaturated lipid 1-[16′,16′,16′-$^2H_3$]palmitoyl-2-palmitoleylphosphatidylcholine (PPPC-d_3). At 50 wt % gramicidin A′, however, the effect of gramicidin is again to cause a collapse of the ^2H quadrupole splitting $\Delta\nu_Q$, although again, as found with DPPC, the effect is less pronounced (E. Oldfield and D. Rice, unpublished experiments). The ^2H NMR results with the unsaturated lecithin are thus in qualitative agreement with those for DMPC and DPPC at high levels of gramicidin. Presumably, the increased condensing effects observed in the monolayer experiments (Cornell et al., 1978) with the unsaturated lecithin are due to the fluid-expanded nature of the monolayer.

The effects which we have observed of gramicidin A′ upon the terminal methyl label are not restricted to this position of the chain. We show in Figure 3 deuterium NMR spectra of 2-[6′,6′-$^2H_2$]DMPC in the presence of several concentrations

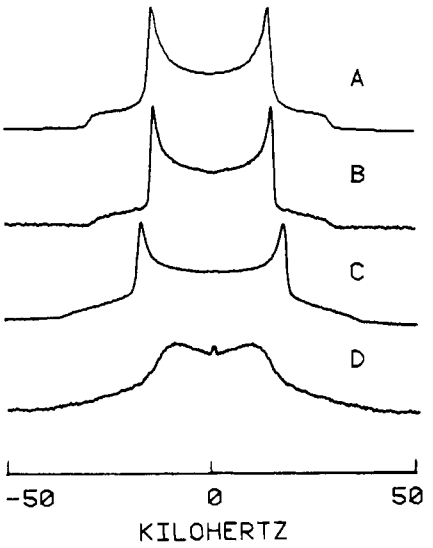

FIGURE 3: Theoretical spectrum and experimental deuterium quadrupole-echo Fourier transform NMR spectra at 34.1 MHz of 2-[6′,6′-$^2H_2$]DMPC in the presence of several concentrations of gramicidin A′ and in excess deuterium-depleted water. Spectral conditions were as in Figure 1 with the noted exceptions. (A) A theoretical deuterium quadrupole powder pattern with a splitting $\Delta\nu_Q$ = 29.0 kHz and a line width δ = 1.42 kHz. (B) Pure lipid with 100.0-kHz spectral width, a recycle time of 0.054 s, and 10 000 scans. (C) 15 wt % gramicidin A′, 100.0-kHz spectral width, a recycle time of 0.054 s, and 180 000 scans. (D) 50 wt % gramicidin A′, 100.0-kHz spectral width, $\tau_1 = \tau_2$ = 40 μs, a recycle time of 0.054 s, and 50 000 scans.

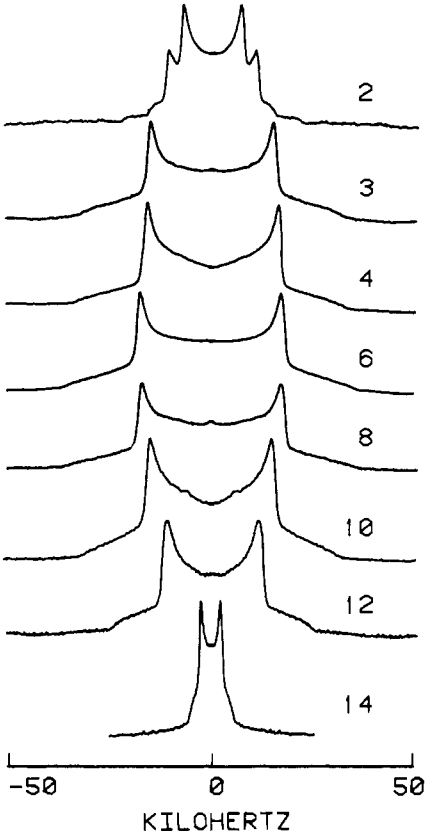

FIGURE 4: Deuterium quadrupole-echo Fourier transform NMR spectra at 34.1 MHz and 30 °C of DMPC labeled at the 2′, 3′, 4′, 6′, 8′, 10′, 12′, or 14′ positions of the 2-chain in the presence of 15 wt % gramicidin and in excess deuterium-depleted water. Spectral conditions for spectra 2′–12′ were as follows: 100.0-kHz spectral width, 0.054-s recycle time, 2K data points, $\tau_1 = \tau_2$ = 60 μs, 7-μs 90° pulse widths, 180 000 scans, 150-Hz line broadening, sample volume 200 μL. The conditions for spectrum 14 were the same except for a 50.0-kHz spectral width, 0.540-s recycle time, 2K data points, and 3000 scans.

of gramicidin A′. In Figure 3A is shown a deuterium NMR powder pattern with a zero asymmetry parameter characteristic of the deuterium nucleus in a C–D bond. In Figures 3B–D we show experimental spectra of 2-[6′,6′-$^2H_2$]DMPC in the presence of 0, 15, and 50 wt % gramicidin A′, corresponding to pure lipid, 15 mol of lipid/mol of gramicidin A′, and 3 mol of lipid/mol of gramicidin A′. A comparison of Figures 3B–D shows that, as in the case of the 14′ label, the spectra of pure lipid and the spectra of lipid plus 15 wt % gramicidin are spin I = 1 powder patterns with modest line broadenings. At a concentration of 50 wt % gramicidin the powder pattern and splitting are no longer resolved. Instead there is a very broad resonance with peak maxima separated by ~20.0 kHz.

As for the terminal methyl label, low concentrations of gramicidin A′ increase the quadrupole splitting while high concentrations decrease it. Comparison between the results of Figures 3B and 3C shows that addition of 15 wt % gramicidin A′ (15:1 lipid–protein ratio) increases the splitting from 28.0 ± 0.5 to 36.0 ± 0.5 kHz. At a concentration of 50 wt % (a 3:1 lipid–protein ratio) the spectrum is similar to a powder pattern of splitting 22.0 kHz correlated with a line broadening of about 6.6 kHz.

The presence of a splitting of 22.0 kHz for the 3:1 sample indicates that, as in the case of the 14′ label, the quadrupole splitting is reduced from its value for pure lipid. Unlike the terminal methyl label, the splitting is not completely collapsed to a value of zero. This behavior might be observed for all chain positions, as is the case for sonicated phospholipid dispersions containing deuterium-labeled fatty acids (Stockton et al., 1976), if the gramicidin were to allow isotropic rigid body rotations of the lecithin molecule with correlation times of less than ~10^{-6} s (Stockton et al., 1976). This situation would be expected in an isotropic or a cubic phase, or in small lamellar vesicles having a rotational correlation time of less than ~10^{-6} s. However, the freeze-fracture and X-ray results of Chapman et al. (1977) argue against these mechanisms for the decrease in $\Delta\nu_Q$ at high gramicidin A′ concentrations.

The line shape for the 6′ label at 3:1 lipid–protein mole ratios is similar to that of pure methyl-labeled lecithin below the phase transition (Oldfield et al., 1978a; Kang et al., 1979a). Both spectra are similar to a powder pattern spectrum having a large intrinsic line broadening. A number of mechanisms may contribute to the broad line widths. These include proton–deuteron dipole–dipole interactions, deuterium quadrupolar interactions, a static distribution of quadrupole splittings, or relaxation due to exchange. As we show later, it seems that effective quadrupolar relaxation due to slow motions is the main contributor to the broad line width of Figure 3D.

The effect of gramicidin at high mole ratios is thus to decrease the quadrupole splitting at the 6′ position of the 2-chain, in addition to collapsing the quadrupole splitting of the 14′ position. These results thus tend to confirm the idea that there is no drastic change in bilayer structure due to formation of small vesicles or an isotropic phase.

At concentrations of gramicidin A′ up to ~15 mol % with DMPC in excess water at 30 °C, the effect of gramicidin, as viewed by ^2H NMR quadrupole splittings, is similar to that of cholesterol (Oldfield et al., 1971, 1978b) in that there is an increase of $\Delta\nu_Q$ of the lipid with an increase in gramicidin A′ concentration. This increase occurs for DMPC labeled in the 2-chain at each of positions 2′, 3′, 4′, 6′, 8′, 10′, 12′, and 14′, as shown in Figure 4. At these low mole fractions of

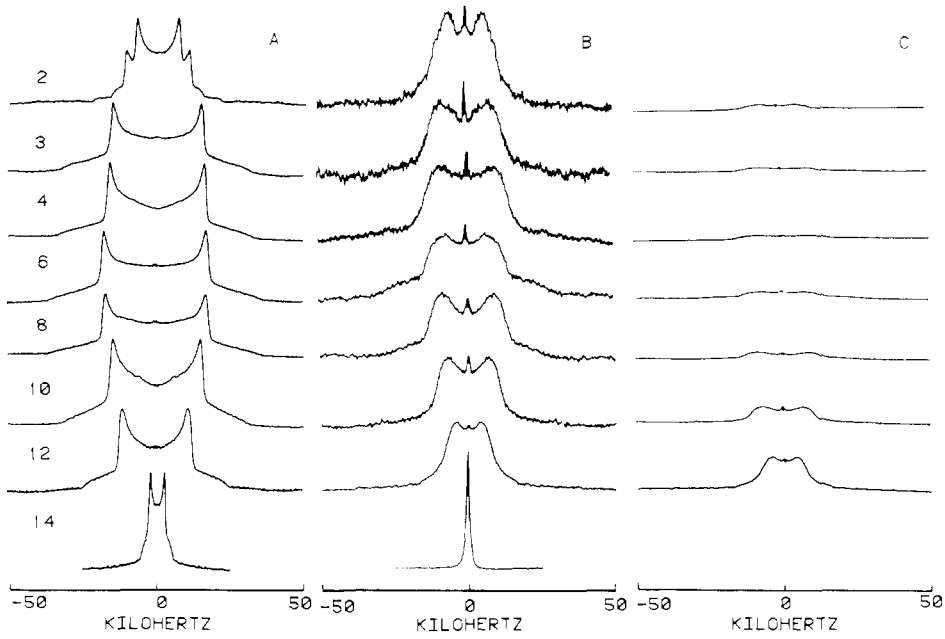

FIGURE 5: Deuterium quadrupole-echo Fourier transform NMR spectra at 34.1 MHz of DMPC labeled at one of the 2′, 3′, 4′, 6′, 8′, 10′, 12′, or 14′ positions of the 2-chain in the presence of 50 wt % gramicidin, with the 15 wt % data for comparison. The spectral conditions were the same as those for Figure 4 for both the 50 and 15 wt % spectra. (A) 15 wt % gramicidin. (B) 50 wt % gramicidin. (C) 50 wt % gramicidin plotted with the same vertical gain as the 15 wt % spectra. Sample temperature was 30 °C in each instance.

gramicidin, it therefore appears that the main effect of incorporating polypeptide into the lipid bilayer is to cause an ordering of the lipid hydrocarbon chains or an increase in $\langle P_2(\cos\theta)\rangle$.

The samples from which the results of Figure 4 were taken contained a concentration of polypeptide considerably lower than that found in a normal functioning biological membrane, and this concentration is less than that used in our model protein–lipid interaction studies (Oldfield et al., 1978a; Kang et al., 1979a). We have therefore obtained deuterium NMR spectra for the same 2-chain positions in the presence of 50 wt % gramicidin A′, a concentration similar to the previous studies. At this concentration, as was seen for the 14′ and 6′ labels, nearly all of the positions of deuterium substitution show a quadrupole splitting less than that of pure lipid, with the disordering effect most pronounced at the terminal methyl end of the chain and smallest at the 2′ position. In Figure 5 we present spectra of DMPC labeled at each of the positions 2′, 3′, 4′, 6′, 8′, 10′, 12′, and 14′ in the 2-chain for samples containing 50 wt % gramicidin A′, and for comparison for samples containing 15 wt % gramicidin A′. The spectra in Figure 5C are those of the 50 wt % sample, plotted with a vertical gain comparable to the 15 wt % samples. It is clear from the figure that all the spectra at 50 wt % gramicidin show large line broadenings and that the total $\Delta\nu_Q$ values of most of these spectra are less than those of the pure lipid or 15 wt % gramicidin samples. Shown in Figure 6 is a plot of quadrupole splitting vs. carbon position labeled at 30 °C for pure lipid, for samples containing 15 wt % gramicidin A′, and for samples containing 50 wt % gramicidin A′. The quadrupole splittings for all samples were determined from the spectral simulations (by using a single Lorentzian line broadening) which gave a best visual fit to the experimental data. Figure 6 shows that except for the 2′ position, all of the 50 wt % samples have splittings less than those of pure lipid. We conclude then that the hydrocarbon chain is increasingly disordered by the presence of gramicidin A′ (at the 50 wt % level) in a direction toward the terminal methyl and that the 2′ position is unaffected.

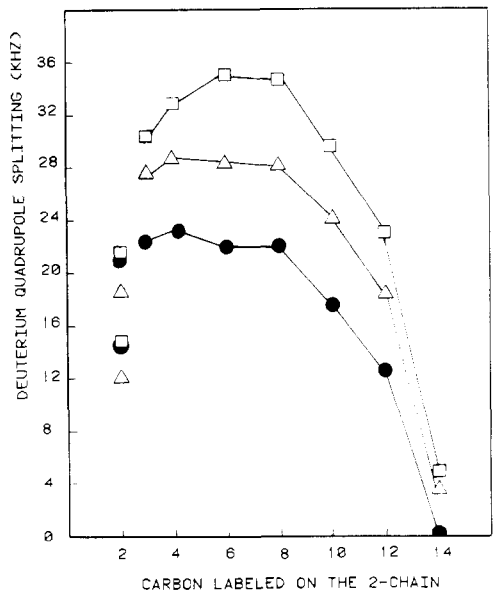

FIGURE 6: A plot of deuterium quadrupole splitting in kilohertz for DMPC vs. deuterium position on the 2-chain. The splittings were derived from the best theoretical fit by using a single splitting and Lorentzian line broadening. (△) Pure lipid; (□) 15 wt % gramicidin; (●) 50 wt % gramicidin. Sample temperature was 30 °C.

A second notable feature of the 50 wt % samples of Figure 5C is that the spectral intensities are considerably less than those for the 15 wt % samples (Figure 5A). Samples at both concentrations contain the same mass of lipid and are acquired by the two-pulse quadrupole-echo pulse sequence (Davis et al., 1976). The signal intensity ratios between 50 and 15 wt % samples vary between 0.25 for the 12′-labeled DMPC to 0.06 for the 6′-labeled DMPC. The 14′-labeled species have within experimental error the same intensities in both Figures 5A and 5C.

The reduced quadrupole-echo intensities of Figure 5C could have at least three causes. One possibility is that the observed signal could represent only a fraction of the lipid which is present, with the remainder of the lipid having under the data

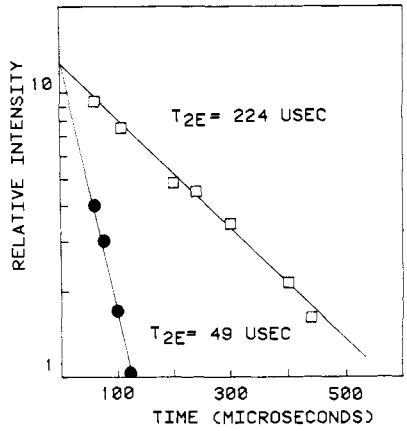

FIGURE 7: A semilog plot of echo intensity vs. time in microseconds of 2-[6′,6′-$^2H_2$]DMPC in the presence of (●) 50 and (□) 15 wt % gramicidin at 30 °C. The intensities were determined directly from the echo height. Spectral conditions were as follows: 10-μs dwell time, 0.054-s recycle time, 2K data points, 7-μs 90° pulse widths, 10 000 scans.

acquisition conditions used a spectrum indistinguishable from the base line (for example, a 100-kHz quadrupole splitting). A second cause could be the presence of relaxation due to random molecular motion during the interval between pulse excitation and signal refocusing. Finally, there could be large differential T_1 effects between the two data sets.

The electron spin resonance spectra of nitroxide-labeled lipids in the lecithin–gramicidin system show line shapes which are characteristic of totally immobile spin-labels on the time scale of the ^{14}N hyperfine interaction (Chapman et al., 1977; Cornell et al., 1978). A reasonable possibility then is that the lipid hydrocarbon chains are immobile on the deuterium time scale as well and that the resultant broad resonances were not detected in our ^2H NMR experiments.

However, if the low signal intensities are due to T_2 relaxation, then at $\tau = 0$ the echo intensity of the 50 wt % sample should equal that of the 15 wt % sample. We have therefore carried out a careful study of the quadrupole-echo intensity as a function of pulse spacing and find that the reduced signal intensity in the 50 wt % complexes is in fact a result of relaxation, and at a limiting value of $\tau = 0$ the signal intensities of both the 50 and 15 wt % samples extrapolate to about the same value. Figure 7 shows a semilog plot of the echo intensity vs. time for 2-[6′,6′-$^2H_2$]DMPC in the 50 and 15 wt % samples. Both plots are within experimental error linear and both extrapolate to the same intensity at $2\tau = 0$. In addition, we have shown that there are no large T_1 differences between the two sets of samples. Spectra representative of the signal-to-noise ratios used in Figure 7 are shown in Figure 8.

It may be of some interest to remark at this point that our early studies (unpublished) with 2-[6′,6′-$^2H_2$]DMPC in the presence of 50 wt % gramicidin A′ by using conventional 90° pulse excitation and standard Fourier transform NMR techniques, which included an 80-μs computer delay, resulted in no detectable signal. This suggested to us that the hydrocarbon chains were essentially crystalline and that the splitting was much greater than our 50-kHz spectral width. This we have seen is not the case, and this example illustrates the importance of detecting the entire ^2H spectrum without the effects of computer delay or echo relaxation before drawing any conclusions about chain motion.

The short relaxation times for the 50 wt % complexes are surprising, and similar results have not previously been seen in other lipid or lipid–protein systems. The interpretation of the decay of the quadrupole-echo intensity is in some ways

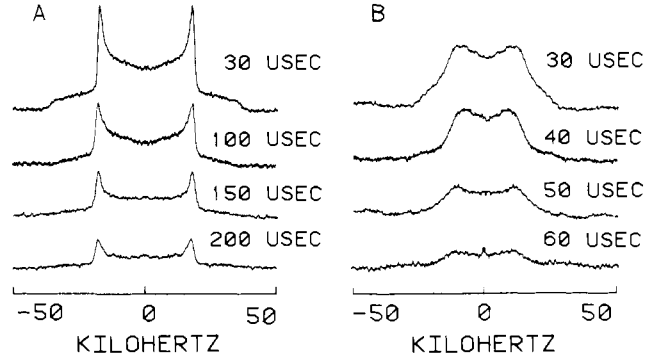

FIGURE 8: Deuterium quadrupole-echo spectra of 2-[6′,6′-$^2H_2$]DMPC in the presence of 50 and 15 wt % gramicidin with several different values of pulse spacing. (A) 15 wt % gramicidin; spectral conditions were as follows: 100.0-kHz spectral width, 0.054-s recycle time, 2K data points, 7.5-μs 90° pulse widths, 10 000 scans. (B) 50 wt % gramicidin. Sample temperature was 30 °C.

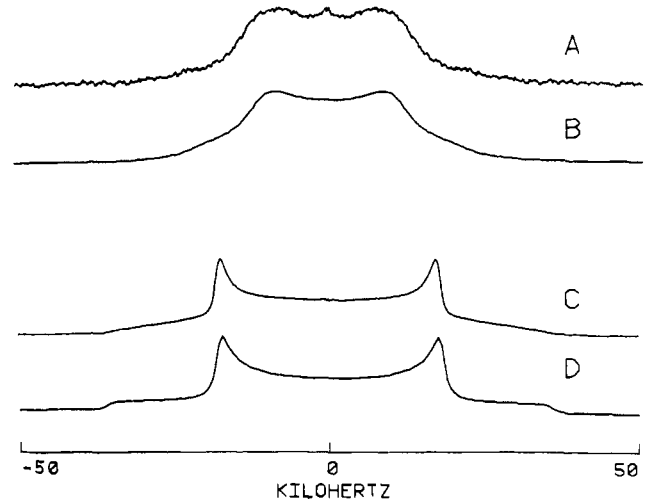

FIGURE 9: Theoretical and experimental spectra of 2-[6′,6′-$^2H_2$]DMPC at 34.1 MHz in the presence of 15 and 50 wt % gramicidin at 30 °C. Spectral conditions were as in Figure 4, and the theoretical spectra have a line broadening equal to $1/\pi T_{2e}$, plus 150 Hz from exponential multiplication. (A) 50 wt % gramicidin A. (B) Theoretical spectrum with a splitting $\Delta\nu_Q$ = 22.0 kHz and a line width δ = 6.6 kHz. (C) 15 wt % gramicidin. (D) Theoretical spectrum with a splitting $\Delta\nu_Q$ = 36.0 kHz and a line width δ = 1.6 kHz.

similar to the interpretation of the decay of an echo after a two-pulse Carr–Purcell sequence. The quadrupole-echo decay measures a "relaxation" caused by processes other than the static distribution of quadrupole splittings, as the decay of the Carr–Purcell echo measures relaxation by processes other than chemical-shift distribution. For deuterium this may be relaxation caused by random fluctuations among different quadrupole splittings with a frequency close to that of the quadrupole interaction ($\sim 10^5$ Hz). The exact nature of the decay is dependent upon the rate and nature of the exchange, whether it be two-site, many-site, or due to a rotational diffusion process. The decay constant (T_{2e}) for the decay can, however, be related to the intrinsic line broadening in the Fourier transformed spectrum. If the broadening is assumed to be Lorentzian (the decay exponential), then the intrinsic line width is $\simeq 1/\pi T_{2e}$ and a rough comparison can be made between the line width expected from the T_{2e} and the experimental line width. For the 15:1 lipid–gramicidin A′ mole ratio sample (Figure 9A) the T_{2e} obtained from Figures 7 and 8 is 224 ± 30 μs, which corresponds to a line broadening of $\sim$1.6 kHz after taking into account a 150-Hz contribution from exponential multiplication, and this value gives an excellent fit to the experimental spectrum, as shown in Figure

9B. For the 2.6:1 lipid–gramicidin sample (Figure 9C) the experimental T_{2e} is 49 ± 5 μs (Figures 7 and 8), which corresponds to a line broadening of ~6.6 kHz. Again, this value gives an excellent agreement with the experimental line shape, as shown in Figure 9D. Thus, although the broad line shape of Figure 9C could be explained by a large static spread of quadrupole splittings, due to the presumed heterogeneous state of the lipid–polypeptide complex, it appears to be unnecessary to invoke such a structure. The agreement between experimental and theoretical line shapes is worst in the area of the wings of the experimental spectra. Here we expect a decrease in intensity of the experimental spectrum since our radio-frequency field strength (γH_1) is only ~40 kHz. However, there may be additional complications due to frequency-dependent line widths arising from two-site (or more) exchange. Here, we would expect the line widths to be largest (and thus peak intensities reduced) in the wings of the experimental spectra.

The short values of T_{2e} suggest that the lipid molecules may undergo some sort of low-frequency motion with a correlation time of $\sim 10^{-5}$–10^{-6}. A determination of the exact nature of this motion would require a more precise determination of the echo decays and the frequency dependence of the intrinsic line widths. The change in the splitting and line width of the 14′ label suggests an abrupt change in the structure of the complex at a ratio of ~4 lipids/molecule of gramicidin A′. Samples containing less lipid than a 4:1 ratio were observed to be physically different from those containing more than a 4:1 ratio. They were more viscous, and the lipid–polypeptide mixture adhered to itself rather than dispersing evenly as do pure lipid samples. Chapman et al. have examined similar mixtures by X-ray diffraction, optical microscopy, and electron microscopy and have found that they form sheets rather than liposomes, but nevertheless the X-ray data indicated multilayered structures up to a lipid–polypeptide mole ratio of 1:1 (Chapman et al., 1977). The sheetlike appearance of the samples makes it unlikely that the decreased splittings are due to slow overall rotation of gramicidin–lipid structures such as vesicles, although the line shapes are in general appearance similar to those calculated for slowly rotating vesicles (Stockton et al., 1976). The possibility exists that there is slow rotational diffusion of individual lipid molecules, which would cause line shapes similar to those caused by slow rotation of vesicles, although the X-ray results of Chapman et al. would require that these motions occur within a conventional lipid bilayer.

Chapman et al. have studied the electron spin resonance spectra of nitroxide-labeled egg lecithin in gramicidin A′–lipid mixtures (Chapman et al., 1977; Cornell et al., 1978). Above a 5:1 lipid–polypeptide ratio the spectra have been simulated by using two components, one due to a completely immobilized label and the other due to a label in a fluid lipid bilayer. Spectral simulations indicated that the immobilized lipid varied from 0 to 90% between lipid–polypeptide ratios of 5:1 and 1:1. Our samples at a ratio of 2.6:1 would have had a composition of 65% immobilized lipid based upon the electron spin resonance determination. However, in contrast to the ESR results, our deuterium NMR spectra show no evidence of an immobile component since the zero time integrated echo intensities in the 15:1 and 2.6:1 mole ratio complexes have the same values. If there are "immobile" or "bound" lipid molecules and "free" lipid molecules, they must be in intermediate to fast exchange on a time scale of $(\Delta\nu_{Qb} - \Delta\nu_{Qf})^{-1}$ seconds, where $\Delta\nu_{Qb}$ and $\Delta\nu_{Qf}$ are the quadrupole splittings of the bound and free species, respectively, at least above 4 and below 2.6 mol of lipid per mol of gramicidin (Figure 2). The exchange between bound and free lipid may also contribute to the line broadening seen in the 2.6:1 lipid–gramicidin A′ samples.

We have described previously a model which explains the disorder of lipids in the presence of high concentrations of several membrane proteins and polypeptides. The hydrocarbon chains of the lipid adjacent to the protein are disordered by the irregular surface of the polypeptide. Conformational transitions occur at a slower rate than in the pure lipid bilayer (10^5–10^7 s^{-1}) because of the hindrance of these side chains. This accounts for the observation of a rigid lattice ESR spectrum. However, $\langle P_2(\cos\theta)\rangle_{^2H}$ decreases because the angular fluctuations of the C–D vector are larger than those in a pure lipid bilayer and are still fast on the time scale of the reciprocal of the quadrupole coupling constant, which is the parameter being motionally averaged (Oldfield et al., 1978a; Kang et al., 1979a,b). The spectra of the chain-labeled lipid in the presence of high concentrations of gramicidin exhibit this disordering, and the broad line widths suggest a reduction in the rate of conformational transitions to values in the range 10^5–10^7 s^{-1}.

A model for lipid behavior in the presence of gramicidin must take into account both the ordering effect at high concentrations of lipid and the disordering effect at low concentrations of lipid. One model that this might lead to is one in which boundary lipid itself is disordered relative to the bulk lipid; however, the ordering effect of boundary lipid–polypeptide particles in the membrane dominates at high lipid–polypeptide ratios. Hydrocarbon chains of lipid adjacent to polypeptide are disordered so as to fill spaces between the amino acid side chains. For the same reason, this lipid is slowed in its motion and gives an ESR spectrum in its rigid limit. The presence of boundary lipid, however, provides a smooth surface for the next layer of lipid. At high lipid concentration, where the boundary lipid is a small portion of the total lipid, the polypeptide and its boundary lipid layer cause an increase in order of the bulk lipid. The increase in order could result from either a reduction of bond reorientation motion or a reduction of cooperative tilt motions. The variation of the quadrupole splitting of the labels then results from two opposing effects. At high lipid concentrations, the bulk of the lipid is not boundary lipid, and the ordering effect of the lipid–polypeptide complexes dominates the observed spectrum. At lower lipid concentrations, the boundary lipid becomes a significant proportion of the total lipid, and as the relative amount of disordered lipid increases the splitting decreases. However, we note that in the cytochrome oxidase–lecithin system the decrease in $\Delta\nu_Q$ with increase in protein level is monotonic, indicating that this model cannot be completely generalized (Kang et al., 1979a).

Alternatively, one cannot rule out significant structural changes in the DMPC–gramicidin A′ bilayer above ~50 wt % gramicidin A′ concentration which may account for the dramatic change in the ^2H NMR spectra, but which are not manifest in the DPPC–gramicidin A′ X-ray or freeze-fracture results (Chapman et al., 1977) even though we obtain similar ^2H and ^{31}P NMR results with DMPC–gramicidin and DPPC–gramicidin systems. It is clear that additional experimental (neutron, Raman scattering of ^2H-labeled species, X-ray diffraction) studies will have to be carried out in order to more fully characterize the "simple" lecithin–gramicidin system.

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