# NMR Study of the Interactions of Polymyxin B, Gramicidin S, and Valinomycin with Dimyristoyllecithin Bilayers†

Raphael Zidovetzki,*,‡ Utpal Banerjee,§ Darrell W. Harrington,‡ and Sunney I. Chan§

*Department of Biology, University of California, Riverside, California 92521, and Arthur Amos Noyes Laboratory of Chemical Physics, California Institute of Technology, Pasadena, California 91125*

*Received January 20, 1988; Revised Manuscript Received April 12, 1988*

ABSTRACT: The interactions of three polypeptide antibiotics (polymyxin B, gramicidin S, and valinomycin) with artificial lecithin membranes were studied by nuclear magnetic resonance (NMR). Combination of $^{31}P$ and $^{2}H$ NMR allowed observation of perturbations of the bilayer membrane structure induced by each of the antibiotics in the regions of the polar headgroups and acyl side chains of the phospholipids. The comparative study of the effects of these membrane-active antibiotics and the lipid bilayer structure demonstrated distinct types of antibiotic–membrane interactions in each case. Thus, the results showed the absence of interaction of polymyxin B with the dimyristoyllecithin membranes. In contrast, gramicidin S exhibited strong interaction with the lipid above the gel to liquid-crystalline phase transition temperature: disordering of the acyl side chains was evident. Increasing the concentration of gramicidin S led to disintegration of the bilayer membrane structure. At a molar ratio of 1:16 of gramicidin S to lecithin, the results are consistent with coexistence of gel and liquid-crystalline phases of the phospholipids near the phase transition temperature. Valinomycin decreased the phase transition temperature of the lipids and increased the order parameters of the lipid side chains. Such behavior is consistent with penetration of the valinomycin molecule into the interior of the lipid bilayers.

A considerable amount of information was obtained in recent years about the molecular details of the action of antibiotics. It is known that the primary site of action of certain polypeptide antibiotics is the surface of the target cell. In the present work, we extend our previous study of the interaction of the polypeptide antibiotic alamethicin with dimyristoyllecithin (DML)[1] bilayers (Banerjee et al., 1985) to include three other membrane-active polypeptide antibiotics of different classes: polymyxin B, gramicidin S, and valinomycin.

Polymyxins were originally isolated from cultures of *Bacillus polymyxa* and *Bacillus aerosporus* (Ainsworth et al., 1947; Stansly et al., 1947; Benedict & Langlykke, 1947). They are broad-spectrum antibiotics, active against yeasts, protozoa, Gram-positive, and, especially, Gram-negative bacteria (Newton, 1956; Schwarz et al., 1960; Storm et al., 1977). Other biological effects of polymyxin B, one of the major components of the polymyxins, include induction of histamine release from mast cells (Franzen, 1981; Nishio et al., 1984) and isolated guinea pig heart (Zilleti et al., 1965; Bhide & Gupta, 1967) and increase of histamine concentration in rat muscle tissue (Breene & Fisher, 1986). At high concentration, polymyxin B suppresses the development of tumor cells (Navashim et al., 1961). Recent works showed that polymyxin B can act as an inhibitor of protein kinase C activity (Wise et al., 1982; Kuo et al., 1983; Wrenn & Wooten, 1984) and proliferation of B-lymphocytes (Nel et al., 1985). Therapeutic use of polymyxin B against Gram-negative bacterial infections can be particularly effective since the antibiotic can directly neutralize bacterial endotoxins (Craig et al., 1974; Bannatyne et al., 1977; From et al., 1979), probably by binding to the lipid A portion of endotoxins (Morrison & Jacobs, 1976). The medical use of polymyxin B is, however, limited due to major side effects which include electrolyte abnormalities (Rodriquez et al., 1970; O'Connor & Stone, 1978), renal dysfunction, neurotoxicity, and neuromuscular blockade (Sabawala & Dillon, 1959; Lindesmith et al., 1968; Kock-Weser et al., 1970; Pedersen et al., 1971).

Polymyxin interacts with bacterial membranes (Koike & Iida, 1971) and bacterial phospholipids (Few, 1955; Storm, 1974; Imai et al., 1975), increasing the permeability of cell membranes (Newton, 1953; Hsu Chen & Feingold, 1973; Feingold et al., 1974; Imai et al., 1975), which in turn inhibits bacterial respiration and RNA and DNA synthesis (Wahn et al., 1968; Teuber, 1974). It was shown that in Gram-negative bacteria, polymyxin disrupts the structure of both the outer membrane (Cerny & Teuber, 1971, 1972; Storm et al., 1977) and the cytoplasmic membrane (Newton, 1956; Storm et al., 1977; Klemperer et al., 1979). The earliest effects of the membrane disruption increase its permeability only for low molecular weight compounds, but longer incubation with polymyxin may cause release of periplasmic proteins (Cerny & Teuber, 1971, 1972; Storm et al., 1977; Vaara & Vaara, 1981).

The decisive evidence showing that the interaction of polymyxin with the outer membrane of bacteria is sufficient to produce most of the bactericidal effects, including inhibition of respiration, came from the ability of polymyxin B, covalently attached to agarose beads, to inhibit the respiration and growth of two Gram-negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa* (La Porte et al., 1977).

† Contribution No. 7706 from the Arthur Amos Noyes Laboratory of Chemical Physics. This research was supported by Grant GM22432 from the National Institute of General Medical Sciences, U.S. Public Health Service (to S.I.C.), and by Grant PRF 16919-G4 from the American Chemical Society (to R.Z.). The Southern California Regional NMR Facility is funded by Grants CHE-791634 and CHE-8440137 from the National Science Foundation. A preliminary account of some of this work was presented at the Symposium on NMR in Biology and Medicine, Taipei, Taiwan, Dec 1984.

‡ University of California.

§ California Institute of Technology.

[1] Abbreviations: DML, dimyristoyllecithin; DPL, dipalmitoyllecithin; NMR, nuclear magnetic resonance; DSC, differential scanning calorimetry; DML-$d_{54}$, bis(perdeuteriomyristoyl)lecithin.

0006-2960/88/0427-5686$01.50/0 

The membrane activity of polymyxin B correlates with the amphiphilic character of this polypeptide, which includes a hydrophilic heptapeptide ring moiety and the hydrophobic acyl chain, which is thought to penetrate into the lipid membranes (Sixl & Galla, 1982). This extended structure of polymyxin B in solution suggested from dialysis (Craig, 1964) and NMR experiments (Galardy et al., 1974) is consistent with such a mode of action. Chemical modification studies showed that two out of five of the free amino acid groups of polymyxin B, namely, residues 1 and 3 on the side chain, are essential for the bactericidal activity (Srinivasa & Ramachandran, 1978).

Early studies of the effect of polymyxin concentrated on correlating the biological action with the presence of a particular type of lipid in the cell membranes or extracts, and it was suggested that negatively charged lipids act as chemoreceptors for polymyxins (Few, 1955). Specifically, the absence of interaction between polymyxin and zwitterionic lipids was reported (Feingold et al., 1974; Hartman et al., 1978; El Mashak & Tocanne, 1980; Sixl & Galla, 1981). The absence of interaction between polymyxin B and synthetic dipalmitoyllecithin (DPL) was deduced from the absence of a change of the gel to liquid-crystalline phase transition temperature ($T_c$) of DPL in the presence of up to 50 mol % polymyxin B (Hartman et al., 1978). A monolayer study reported the absence of interactions between polymyxin B and dihexadecanoylphosphatidylcholine (Nel et al., 1985). Fluorescence polarization measurements showed the absence of interactions between polymyxin B and distearoylphosphatidylcholine membranes (Sixl & Galla, 1981). Only the presence of weak interactions of polymyxin B with DML headgroups was reported by Sixl and Watts (1985). In conflict with these studies, significant perturbation of DML by polymyxin B was reported by Mushayakara and Levin (1984) from the Raman spectroscopy data.

Gramicidin S, the cyclic decapeptide antibiotic produced by *Bacillus brevis*, was isolated by Gause and Brazhnikova (1944). Gramicidin S was shown to bind to bacterial membranes (Bulgakova & Polin, 1966a) and induce a sharp increase in the permeability of the plasma membranes which results in the release of intracellular low molecular weight compounds into the medium (Bulgakova & Polin, 1966b). Gramicidin S and its analogue were also shown to affect the permeability of plasma cells and protoplasts of the Gram-positive bacteria *Macrococcus lysodeikticus*, *Bacillus subtilis*, *Bacillus megaterium* (Polin et al., 1979; Bulgakova et al., 1982; Petrykina et al., 1984), and *Streptomyces* sp. 26-115 (Bulgakova et al., 1986). In agreement with the suggestion that the biological activity of gramicidin S is due to its interaction with cell membranes, the antibiotic was shown to disrupt and solubilize lecithin liposomes (Finer et al., 1969; Pache et al., 1972; Wu et al., 1978).

The interaction of gramicidin S with DPL membranes was studied by $^{31}P$ NMR, $^{2}H$ NMR of exchangeable sites on the peptide, and differential scanning calorimetry (DSC) by Datema et al. (1986). These authors suggested that gramicidin S interacts with the phospholipid headgroups but not with the hydrophobic portion of the membrane (Datema et al., 1986).

The cyclic dodecadepsipeptide antibiotic valinomycin was isolated from *Streptomyces* fermentation products (Brockmann & Schmidt-Kastner, 1955). The first biological effect of valinomycin was shown to be the uncoupling of mitochondrial oxidative phosphorylation (McMurray & Begg, 1959). Valinomycin was shown to increase the ion permeability of synthetic lipid bilayers by 3–4 orders of magnitude, indicating that the cell membrane may be a target for this antibiotic (Mueller & Rudin, 1967; Lev & Buzhinsky, 1967). This activity of valinomycin was shown by Pressman and co-workers to be due to carrying monovalent cations, especially $K^+$, across lipid bilayers (Pressman et al., 1967). Valinomycin disrupts membrane transport in microorganisms, which is assumed to be the basis for its antibiotic activity (Harold, 1972).

The results from $^{1}H$ NMR study of interactions of valinomycin with DML and DPL suggested that valinomycin penetrates the DML bilayers, while it is interacting only with the headgroups of DPL (Hsu & Chan, 1973). A similar conclusion about the DML–valinomycin interaction was reached using Raman spectroscopy (Susi et al., 1979). Incorporation of valinomycin into the interior of sonicated DML vesicles was reported by Feigenson and Meers (1980).

In the present work, we used the combination of $^{2}H$ and $^{31}P$ NMR to characterize the interactions of polymyxin B, gramicidin S, and valinomycin with bilayers of DML. The results, as discussed below, show that the interactions are unique for each antibiotic studied.

## Materials and Methods

DML was obtained from Sigma (St. Louis, MO) and was checked for purity by thin-layer chromatography, using silica gel 60 F-254 plates (VWR Scientific, Norwalk, CA) in $CHCl_3$/MeOH/$H_2O$ (65:35:5). Lipids were detected with Phospray (Supelco, Bellefonte, PA).

Bis(perdeuteriomyristoyl)lecithin (DML-$d_{54}$) was purchased from Avanti Polar Lipids, Birmingham, AL, and was found to be pure by thin-layer chromatography.

Polymyxin B sulfate was from Sigma. Gramicidin S dihydrochloride (Sigma) was dissolved in $H_2O$/dioxane (5:2 v/v), filtered, and lyophilized prior to use. Valinomycin was purchased from Calbiochem (San Diego, CA).

Multilamellar lipid dispersions were made by first dissolving DML, or a DML–antibiotic mixture, in methanol or chloroform. The solvent was then evaporated off with dry nitrogen, and the sample was kept under vacuum (<1 mTorr) for at least 8 h. The thin film thus formed was hydrated with a 25 mM Tris buffer solution (pH 7.4), prepared in deuterium-depleted $H_2O$ for $^{2}H$ NMR *or* in $D_2O$ for $^{31}P$ NMR experiments. The samples were always fully hydrated and were typically 1:10 w/v in lipid to water. In the experiments with polymyxin B, the antibiotic was added to the dry lipids as a solution in the hydrating buffer.

$^{31}P$ and $^{2}H$ NMR spectra were acquired at 11.74 T (corresponding to 500.13-MHz $^{1}H$, 202.49-MHz $^{31}P$, and 76.78-MHz $^{2}H$ frequencies) on a Bruker WM500 spectrometer. $^{31}P$ spectra were acquired by using a spectral width of 50 kHz, a 75° pulse (30 $\mu$s), a relaxation delay of 1.5 s, and gated broad-band proton decoupling of 10 W. $^{2}H$ spectra were acquired with a high-power home-built probe (Müller & Chan, 1983) using the standard quadrupole echo sequence (Davis et al., 1976). The spectral width was 166 kHz, refocusing time 50 $\mu$s, and 90° pulse of 3.2 $\mu$s.

## Results

*$^{31}P$ NMR Studies.* A typical $^{31}P$ NMR spectrum of DML multilayers above the gel to liquid-crystalline phase transition temperature ($T_c$) in the absence of antibiotics is shown in Figure 1A. The $^{31}P$ spectra of headgroups in phospholipid dispersions allow, in general, the various possible aggregated states of the lipids, such as bilayer, hexagonal, or micellar, to be distinguished. The $^{31}P$ spectrum of the control DML sample (Figure 1A) is characteristic of the multilamellar bilayer conformation of lipid molecules (Seelig, 1978). The useful parameter obtainable from such $^{31}P$ NMR spectra is

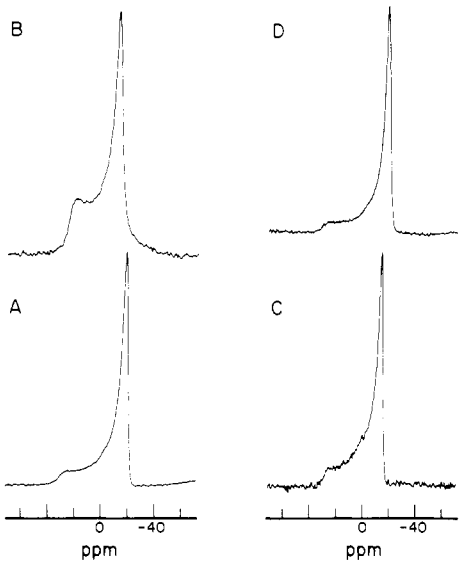


FIGURE 1: $^{31}P$ NMR spectra at 25.3 °C of aqueous dispersions of antibiotic–DML mixtures (1:5.5 mol/mol). (A) DML; (B) DML–gramicidin S; (C) DML–valinomycin; (D) DML–polymyxin B.

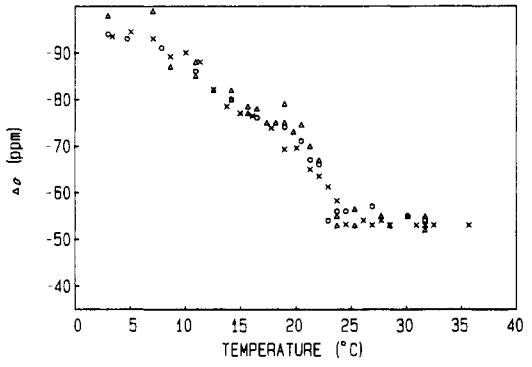


FIGURE 2: Plot of $\Delta\sigma$ vs temperature for aqueous dispersions of polymyxin B containing DML. The experimental errors in $\Delta\sigma$ are ±1 ppm above $T_c$ and ±2 ppm below $T_c$. (×) No polymyxin B; (○) 1:16 polymyxin B:DML molar ratio; (△) 1:5.5 polymyxin B:DML molar ratio.

the chemical shift anisotropy ($\Delta\sigma$). This parameter is a function of the molecular motions of the lipid molecules which average the orientation-dependent chemical shift interactions and the average orientation of the phospholipid headgroups relative to the normal to the plane of the bilayer (director). The motional state of the lipids depends on temperature and undergoes a sharp change at $T_c$. The average orientation of the headgroups may be affected by interactions with membrane-bound compounds, in our case, antibiotics. Thus, $\Delta\sigma$ allows one to determine the change induced in $T_c$ and the average headgroup orientation in the presence of the antibiotics. Our results on antibiotic-induced changes in $T_c$ were in each case confirmed by performing corresponding $^2H$ NMR measurements and observing characteristic broadening of $^2H$ NMR spectra at $T_c$. From the spectra of Figure 1, $\Delta\sigma$ can be determined from the splitting between the edges of the spectrum at the half-height of the low-frequency "foot". $^{31}P$ NMR spectra of DML with added antibiotics are shown in Figure 1B–D. It is clear from Figure 1 that the basic miltilamellar bilayer structure of the lipids is maintained in the presence of each of the antibiotics up to the antibiotic:DML molar ratio of 1:5.5.

Figures 2–4 show the temperature dependence of $\Delta\sigma$ of DML bilayers in the presence of different concentrations of antibiotics, illustrating that the effects of the antibiotic are different in each case.

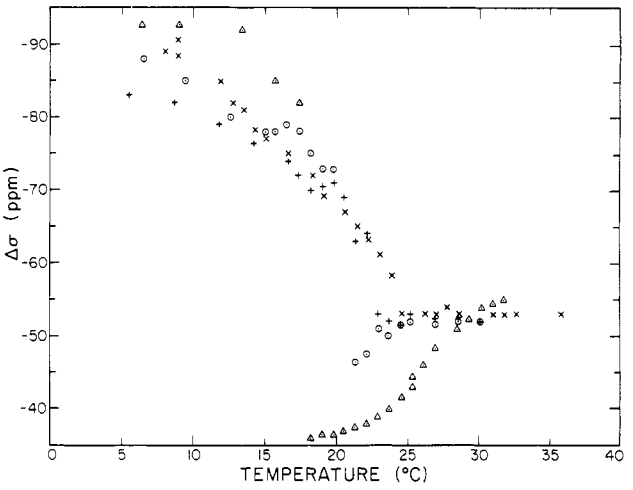


FIGURE 3: Plot of $\Delta\sigma$ vs temperature for aqueous dispersions of gramicidin S containing DML. (×) No gramicidin S; (+) 1:33 gramicidin S:DML molar ratio; (○) 1:16 gramicidin S:DML molar ratio; (△) 1:5.5 gramicidin S:DML molar ratio.

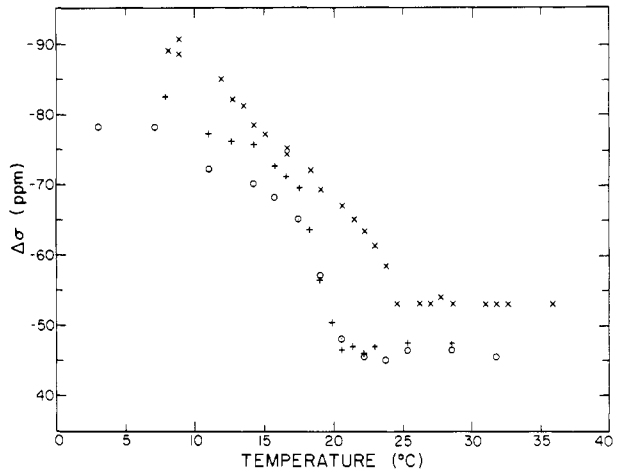


FIGURE 4: Plot of $\Delta\sigma$ vs temperature for aqueous dispersions of valinomycin-containing DML. (×) No valinomycin; (+) 1:16 valinomycin:DML molar ratio; (○) 1:5.5 valinomycin:DML molar ratio.

(*A*) *Polymyxin B.* Polymyxin B does not affect $\Delta\sigma$ or $T_c$ up to the molar ratio of 1:5.5 to DML (Figure 2). This absence of perturbation of the headgroups by polymyxin B makes it unlikely that the antibiotic interacts with DML. This conclusion is further corroborated by $^2H$ NMR experiments (vide infra).

(*B*) *Gramicidin S.* Figure 3 shows the temperature dependence of $\Delta\sigma$ in the presence of different concentrations of gramicidin S. Only a small decrease of the absolute value of $\Delta\sigma$ is observed upon addition of gramicidin S at the molar ratio of 1:33 to DML, without measurable changes of $\Delta\sigma$ below or above $T_c$ (Figure 3). Increasing the concentration of gramicidin S further decreases $T_c$ and induces changes in the $\Delta\sigma$ above $T_c$. The unusual feature of the observed effects is that the magnitude of the gramicidin S induced change in $\Delta\sigma$ is temperature dependent above $T_c$ (Figure 3) while the plots of $\Delta\sigma$ versus temperature are "flat" above $T_c$ in all other cases studied (Figures 2 and 4; Banerjee et al., 1985). At gramicidin S to DML molar ratios of 1:16 and 1:5.5, the change in $\Delta\sigma$ is most pronounced *in the vicinity* of $T_c$. Thus, at the gramicidin S to DML molar ratio of 1:16, the $T_c$ is depressed by 3 °C, and $\Delta\sigma$ is affected only within about 3 °C above this temperature (Figure 3). An increase of the gramicidin S concentration to a 1:5.5 molar ratio to DML enhances the effect further. The phase transition temperature in this case

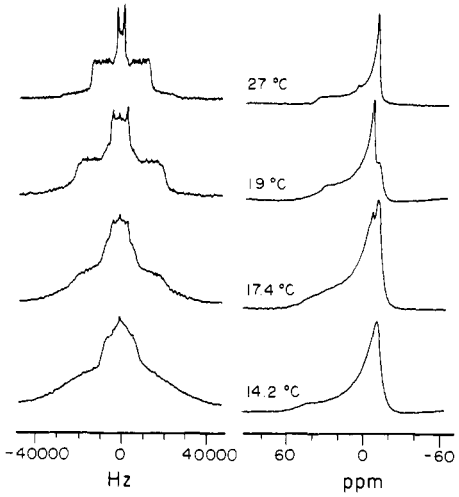


FIGURE 5: $^{31}P$ (right) and $^{2}H$ (left) NMR spectra of gramicidin S–DML mixtures (1:16 mol/mol) at different temperatures.

is decreased by 6 °C to 18 °C, and $\Delta\sigma$ is affected in the range of temperatures of 18–28 °C, decreasing in absolute value monotonously with the decrease of temperature. At 18 °C, just above the $T_c$, $\Delta\sigma$ reaches the value of −36 ppm, which is the lowest absolute value observed by us in the present or previous (Banerjee et al., 1985) work. In contrast, gramicidin S does not affect $\Delta\sigma$ of DML below phase transition temperatures of the antibiotic–lipid mixtures.

Careful examination of $^{31}P$ NMR spectra taken at a 1:16 molar ratio of gramicidin S to DML reveals additional features (Figure 5, right). The spectra taken in the vicinity of the phase transition temperature, at 19 and at 17.4 °C, can be described as weighted superposition of the spectra of the lipids at the liquid-crystalline (27 °C) and gel (14.2 °C) states (Figure 5, right). These results indicate the coexistence of the lipid domains in the liquid-crystalline and gel phases in the vicinity of $T_c$.

The lack of interaction between gramicidin S and the lipids in the *gel* state is perhaps best shown in the following experiment when the concentration of gramicidin S is increased to a 1:2.7 molar ratio to DML. Here, the $^{31}P$ NMR line shape no longer corresponds to the multilayer conformation of the lipids but degenerates into a single peak at a position characteristic of isotropic motions of the phospholipid molecules (Figure 6, right). This result suggests that at a 1:2.7 molar ratio to DML, gramicidin S disrupts the multilayer structure to form micelles or small vesicles above $T_c$. However, even at this high, disruptive concentration of gramicidin S, the cooling of the sample to 13.4 °C results in the formation of bilayers (Figure 6). Upon further cooling to 9.4 °C, most of the intensity of the $^{31}P$ NMR spectrum is in the gel state bilayer powder pattern (Figure 6), and further cooling to 8.5 °C results in complete disappearance of the isotropic peak (not shown).

(*C*) *Valinomycin.* The effect of valinomycin on the $\Delta\sigma$ of DML is different from those of the other two cases presented here (Figure 4). Addition of valinomycin at a molar ratio of 1:16 to DML depresses $T_c$ of the multilayers by 5 °C and causes a decrease in the absolute value of $\Delta\sigma$ (Figure 4). Upon further increase in the valinomycin concentration to a 1:5.5 molar ratio to DML, valinomycin does not further change $T_c$ nor affects $\Delta\sigma$ above $T_c$, though there is possibly a small additional decrease of the absolute value of $\Delta\sigma$ below $T_c$ (Figure 4).

*$^{2}H$ NMR Studies.* The $^{2}H$ NMR spectra of DML-$d_{54}$ above $T_c$ in the presence and absence of the antibiotics are

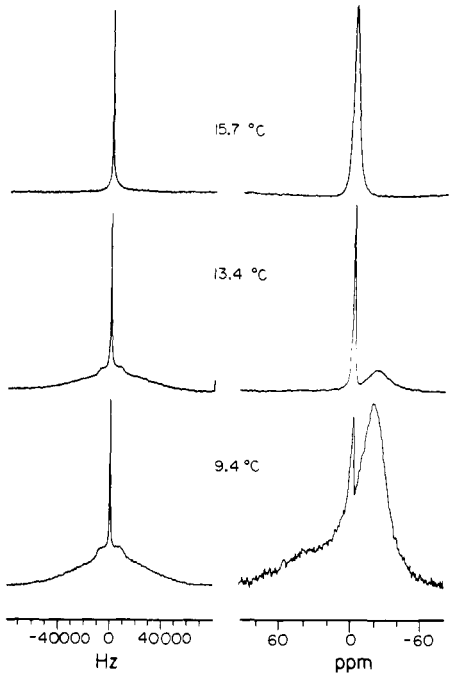


FIGURE 6: $^{31}P$ (right) and $^{2}H$ (left) NMR spectra of gramicidin S–DML mixtures (1:2.7 mol/mol) at different temperatures.

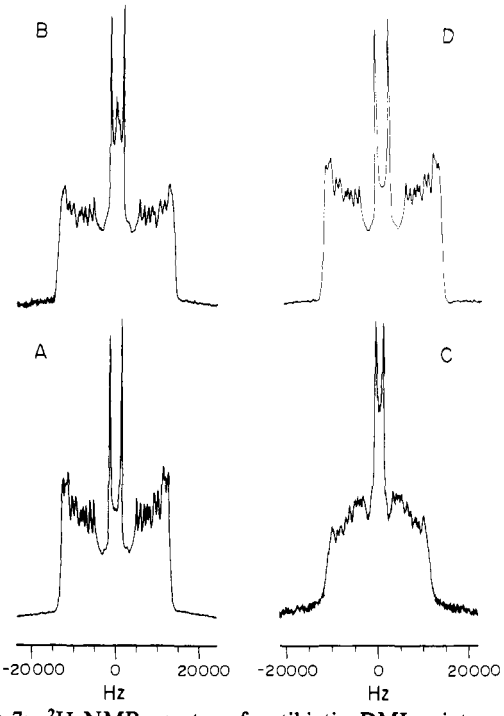


FIGURE 7: $^{2}H$ NMR spectra of antibiotic–DML mixtures (1:5.5 mol/mol) at 32 °C. (A) No antibiotic added; (B) with valinomycin; (C) with gramicidin S; (D) with polymyxin B.

given in Figure 7. An $^{2}H$ NMR spectrum of the fully hydrated chain-perdeuteriated phospholipid represents the superposition of axially averaged powder patterns arising from the different deuterons for the various $CD_2$ segments along the acyl chains, and the terminal $CD_3$ segment. The order parameter ($S_{CD}$) for each segment can be derived from the observed peak to peak quadrupole splitting ($\Delta\nu$), which corresponds to the perpendicular orientation of the bond relative to the external magnetic field, using the equation:

$$\Delta\nu^i = (3/4)(e^2qQ/h)S^i$$

where $e^2qQ/h$ = 167 kHz is the quadrupole coupling constant

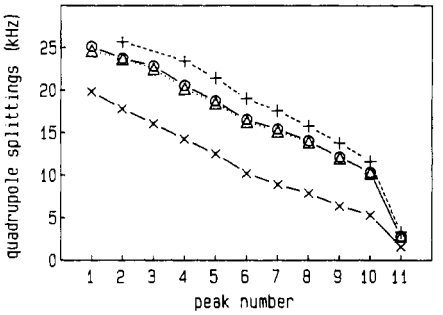


FIGURE 8: Variation of quadrupole splittings with peak position of DML-$d_{54}$ with and without the addition of antibiotics at a molar ratio of 1:5.5 to DML-$d_{54}$ at 32 °C. Peak 1 represents approximately five carbon positions closest to the lipid headgroup ("plateau" region). It is the outermost peak of the spectra on Figure 7. Peak 11 represents the terminal methyl resonance. The size of the symbols corresponds to the experimental error. (○) DML; (△) DML with polymyxin B; (×) DML with gramicidin S; (+) DML with valinomycin.

of a deuteron in a CD bond. Previous $^2$H NMR studies with lipids, deuteriated at specific positions in the side chains, demonstrated the existence of the order parameter profile along the side chains of a lipid molecule in the multilamellar state with a plateau of relatively higher $S_{CD}$ values corresponding to the $CD_2$ segments near the glycerol backbone (Seelig, 1977). This is reflected in the overlapping of the quadrupole splitting which correspond to the first five to six $CD_2$ segments near the edge of a $^2$H NMR spectrum. Further from the glycerol backbone, the side chains become progressively more disordered, resulting in well-resolved peaks at the perpendicular orientation. We were able to resolve up to 11 splittings of segments DML-$d_{54}$ above $T_c$; tentative assignments of these $CD_2$ splittings are given in Oldfield et al. (1978).

Visual examination of the $^2$H NMR spectra in the presence and absence of the antibiotics at a 1:5.5 molar ratio to DML-$d_{54}$ confirms that the phospholipids maintain their basic bilayer structure above $T_c$ (Figure 7) in the presence of the antibiotics under study. Significant perturbation of the $^2$H NMR line shape at this concentration of antibiotics was observed only in the case of gramicidin S, where the intensity of the signal at the edge of the spectrum decreased relative to the center of the quadrupolar powder pattern (Figure 7C).

Further insight into the character of interactions between the antibiotics and DML-$d_{54}$ can be obtained by considering the effects of the antibiotics on the order parameter profile along the hydrocarbon chains. These results are summarized in Figure 8. It is apparent from Figure 8 that the presence of polymyxin B does not significantly affect the order parameters of C–D bonds along the chains. This result is consistent with our $^{31}$P NMR observations and again suggests a lack of interactions between polymyxin B and DML. Significant decrease of the quadrupole splittings of DML-$d_{54}$ is observed, however, in the presence of gramicidin S (Figure 8).

Figure 8 summarizes the quadrupole splitting profiles of DML-$d_{54}$ in the presence and absence of valinomycin. Unlike the other antibiotics studied, the presence of valinomycin causes a small but significant increase of the order parameters of DML-$d_{54}$ (Figure 8). The valinomycin-induced increase of order parameters is most prominent for the outermost peaks, which correspond to about five methylenes near the bilayer surface and gradually decrease to zero at terminal methyl groups (Figure 8).

Figures 5 (left) and 6 (left) show the $^2$H NMR spectra of DML-$d_{54}$ at different temperatures and concentrations of gramicidin S. These spectra confirm the $^{31}$P NMR observations that at a 1:16 gramicidin S to DML molar ratio the gel and liquid-crystalline phases of DML coexist (Figure 5, left). At the higher gramicidin S concentration of 1:2.7 molar ratio to DML, the phospholipids are in micellar or small vesicles form at ≥15.7 °C, with bilayer phase re-forming below 13.4 °C (Figure 6, left).

## Discussion

The present work was initiated in order to provide comparative information on interactions of three membrane-active polypeptide antibiotics with model lipid membranes. We found that the addition of polymyxin B to DML multilayers neither affects the orientation of the headgroups, as evidenced by $^{31}$P NMR, nor perturbs the order parameter profile of deuteriated side chains of the lipid. We also did not observe broadening of the various $^2$H resonances upon addition of polymyxin B, the effect which was observed upon incorporation of proteins into lipid bilayers (Seelig & Seelig, 1978; Rice et al., 1979). Thus, results with polymyxin B indicate the lack of interactions between this antibiotic and DML. Our results support the previous works which report the absence of interactions between polymyxin and zwitterionic lipids (Feingold et al., 1974; Hartman et al., 1978; El Mashak & Tocanne, 1980; Sixl & Galla, 1981). However, our results are in direct contradiction with the conclusions of Mushayakara and Levin (1984), who used Raman spectroscopy to show that both C–C skeletal stretching and C–H stretching modes of DML acyl chains are significantly perturbed by the presence of polymyxin B at the molar ratio of 1:5 to DML. In the present work as well as in the earlier studies of Mushayakara and Levin (1984), the same supplier of DML and polymyxin B is used, and similar methods of sample preparation were employed, with the only difference being the hydration level of the lipids, which was 10% w/v of the lipids to $H_2O$ in our work and 50% in the work of Mushayakara and Levin (1984). This difference in the hydration level is not expected to alter the structure of DML; however, we emphasize here that our $^2$H and $^{31}$P NMR results provide direct measurements of conformation and ordering of the lipids while the Raman spectra obtained by Mushayakara and Levin (1984) required corrections for the spectral contribution of polymyxin B molecules.

From the combination of $^{31}$P and $^2$H NMR measurements of interactions of gramicidin S with DML, we detected the presence of strong interactions of the antibiotic with the bilayers at the molar ratio higher than 1:16 to DML. Our work shows that the interaction between gramicidin S and DML takes place only *above* $T_c$, with gramicidin S apparently being excluded from DML bilayers below $T_c$ even at the high disruptive molar ratio of 1:2.7 of gramicidin S to DML. The perturbation of the $^2$H NMR spectrum of DML in the presence of gramicidin S, namely, the broadening of resonances and the decrease of the order parameters of CD bonds of the side chains, is compatible with the gramicidin S molecule *penetrating* into the bilayer, in agreement with the general conclusions of Wu et al. (1978), who reported full incorporation of gramicidin S into DML bilayers. We did not observe the broadening of the thermal phase transition reported in the latter work (Wu et al., 1978) on the basis of lateral diffusion measurements of fluorescent gramicidin S analogues. No such phase transition broadening was noted in DSC studies of gramicidin S–DML mixtures (Wu et al., 1978). While it is possible that the lateral diffusion measurements are more sensitive than DSC and NMR to small subtle changes in the bilayer structure, the discrepancy can also be due to the possibility that the fluorescent analogues of gramicidin S used in the work of Wu et al. (1978) involved neutralization of ornithine residues, which are thought to be important for the

interaction with phospholipids (Pache et al., 1972; Semenov et al., 1977). Our $^{31}P$ NMR results on the temperature dependence of $\Delta\sigma$ at a gramicidin S to DML molar ratio of 1:5.5 show a progressive decrease of the absolute values of $\Delta\sigma$ upon decrease of temperature within the range of 10 °C above $T_c$, closely corresponding to the high-temperature tail of the thermograms obtained from DSC experiments with gramicidin S–DML mixtures at a 1:5 molar ratio of antibiotic to lipid (Wu et al., 1978). In our previous work (Banerjee et al., 1985), we demonstrated that above $T_c$, $\Delta\sigma$ of the $^{31}P$ NMR spectra is sensitive primarily to the orientation of the phospholipid headgroups relative to the director. Another possibility, namely, the decrease of the size of the multilamellar vesicles (Burnel et al., 1980), can be excluded here, as such a decrease would also produce a decrease of the quadrupole splitting of $^2H$ NMR spectra with a decrease of temperature. We observed, instead, an *increase* of the quadrupole splittings under these conditions, a phenomenon caused by the increased ordering of the lipid side chains upon lowering the temperature. We can thus conclude that the change in $\Delta\sigma$ observed here and the high-temperature tail of the thermograms in Wu et al. (1978) of the gramicidin S–DML mixtures are caused by a gramicidin S induced temperature-dependent change in the orientation of the headgroups of DML.

Our $^2H$ and $^{31}P$ NMR results at the gramicidin S to DML molar ratio of 1:16 are compatible with the coexistence of the laterally separated lipid domains in gel and liquid-crystalline phases in the vicinity of $T_c$. Such lateral phase separation was previously demonstrated by $^2H$ NMR in DPL bilayers with incorporated synthetic amphiphilic peptides (Davis et al., 1983; Huschilt et al., 1985). The fact that both states are resolved on the NMR time scale indicates that the lipid diffusion between the domains is negligible and places lower limits for the sizes of gel domains as $1 \times 10^4$ Å$^2$ and liquid-crystalline domains as $1 \times 10^6$ Å$^2$ (Huschilt et al., 1985).

Our results with valinomycin show that this peptide interacts with the headgroups of DML, the interaction resulting in changes in the orientation of headgroups and decrease in the absolute values of $\Delta\sigma$ both above and below $T_c$. Only minimal effects of valinomycin are observed on the order parameters of the side chains of DML; a small *increase* of the order parameters is observed upon addition of valinomycin. Such an increase of the order parameters, without a significant effect on the overall profile of the order parameters, has been reported in the case of cholesterol (Oldfield et al., 1978), the polypeptide antibiotic gramicidin A (Rice & Oldfield, 1979), and, very recently, $\alpha$-tocopherol (Wassal et al., 1986), and is compatible with the incorporation of valinomycin into the interior of DML bilayers. This conclusion is an agreement with the laser Raman spectroscopy results of Susi et al. (1979) and $^1H$ NMR results of Hsu and Chan (1973). However, we did not detect the *disordering* effect of valinomycin reported by Susi et al. (1979). The reason for this discrepancy between our results and those of Susi et al. (1979) is not obvious, but we again point out the direct nature of $^2H$ NMR measurements of lipids as compared to the Raman studies that rely on sorting out the peptide and lipid contributions. Decrease of the molecular motions of the lipid side chains was reported in the case of the valinomycin–egg yolk lecithin system and the valinomycin–ox brain phosphatidylserine system (Finer et al., 1969). In our present work, valinomycin caused a *decrease* of the absolute values of $^{31}P$ NMR $\Delta\sigma$ and an *increase* of $^2H$ NMR quadrupole splittings of DML, emphasizing once again that changes in $\Delta\sigma$ above $T_c$ reflect primarily the orientation of the phospholipid headgroups and not their mobility (Banerjee et al., 1985).

*Conclusions.* (1) This comparative study of interactions of three polypeptide antibiotics with DML bilayers demonstrated that straightforward application of $^{31}P$ and $^2H$ NMR measurements is very effective in obtaining information on drug–lipid interactions using nonperturbing probes. (2) The work demonstrated a lack of interaction between polymyxin B and DML, clarifying confusion existing in the literature. This finding points out that the presence of the hydrophobic region of a polypeptide is by itself not sufficient to assume that such a polypeptide would be able to intercalate into PC membranes. (3) Gramicidin S intercalates into the interior of DML bilayers above $T_c$. In the vicinity of $T_c$, this antibiotic induces lateral phase separation of lipids into gel and liquid-crystalline domains, the latter being probably enriched in gramicidin S, since it gets excluded from the bilayers below $T_c$. (4) Valinomycin intercalates into the DML bilayer, causing increased ordering of the lipid side chains.

## Added in Proof

Results obtained recently by using Raman spectroscopy showed that polymyxin B does not perturb DML-$d_{54}$ bilayers at a molar ratio of 1:5 to the lipid and 90% lipid hydration (M. Pézolet, personal communication).

## References


Ainsworth, G. C., Brown, A. M., & Brownlee, G. (1947) *Nature (London) 160*, 263.

Banerjee, U., Zidovetzki, R., Birge, R. R., & Chan, S. I. (1985) *Biochemistry 24*, 7621.

Bannatyne, R. M., Harnett, N. M., Lee, K. Y., & Biggar, D. W. (1977) *J. Infect. Dis. 136*, 469.

Benedict, R. G., & Langlykke, A. F. (1947) *J. Bacteriol. 54*, 29.

Bhide, N. K., & Gupta, I. (1967) *J. Pharm. Pharmacol. 19*, 58.

Brockmann, H., & Schmidt-Kastner, G. (1955) *Chem. Ber. 88*, 57.

Bulgakova, V. G., & Polin, A. N. (1966a) *Antibiotiki (USSR) 6*, 518.

Bulgakova, V. G., & Polin, A. N. (1966b) *Antibiotiki (USSR) 9*, 714.

Bulgakova, V. G., Novozhilova, T. Yu., Orlova, T. I., & Polin, A. N. (1986) *Antibiotiki Med. Biotek. (USSR) 31*, 411.

Burnel, E. E., Cullis, P. R., & De Kruijff, B. (1980) *Biochim. Biophys. Acta 603*, 63.

Cerny, G., & Teuber, M. (1971) *Arch. Microbiol. 78*, 166.

Cerny, G., & Teuber, M. (1972) *Arch. Microbiol. 82*, 361.

Craig, L. C. (1964) *Science (Washington, D.C.) 144*, 1093.

Craig, W. A., Turner, J. M., & Kunin, C. M. (1974) *Infect. Immun. 10*, 287.

Datema, K. P., Pauls, K. P., & Bloom, M. (1986) *Biochemistry 25*, 3796.

Davis, J. H., Jeffrey, K. R., Bloom, M., Valic, M. I., & Higgs, T. P. (1976) *Chem. Phys. Lett. 42*, 390.

Davis, J. M., Calre, D. M., Hodges, R. S., & Bloom, M. (1983) *Biochemistry 22*, 5298.

El Mashak, E. M., & Tocanne, J. F. (1980) *Biochim. Biophys. Acta 596*, 165.

Feigenson, G. W., & Meers, P. R. (1980) *Nature (London) 283*, 313.

Feingold, D. S., Hsu Chen, C. C., & Sud, I. J. (1974) *Ann. N.Y. Acad. Sci. 235*, 480.

Few, A. V. (1955) *Biochim. Biophys. Acta 16*, 137.

Finer, E. G., Houser, H., & Chapman, D. (1969) *Chem. Phys. Lipids 3*, 386.

Franzen, L. (1981) *Acta Pathol. Microbiol. Scand., Sect. A 89A*, 57.
From, A. M. L., Fong, J. S. C., & Good, R. A. (1979) *Infect. Immun. 23*, 660.
Galardy, R. E., Craig, L. C., & Printz, M. P. (1974) *Biochemistry 13*, 1674.
Gause, G. F., & Brazhnikova, M. G. (1944) *Nature (London) 154*, 703.
Greene, S. M., & Fisher, H. (1986) *Life Sci. 38*, 539.
Harold, F. M. (1972) *Bacteriol. Rev. 36*, 172.
Hartman, W., Galla, M.-J., & Sackmann, E. (1978) *Biochim. Biophys. Acta 510*, 124.
Hsu, M.-C., & Chan, S. I. (1973) *Biochemistry 12*, 3872.
Hsu Chen, C. C., & Feingold, D. S. (1973) *Biochemistry 12*, 2105.
Huschilt, J. C., Hodges, R. S., & Davis, J. H. (1985) *Biochemistry 24*, 1377.
Imai, M., Inoue, K., & Najima, S. (1975) *Biochim. Biophys. Acta 375*, 130.
Klemperer, R. M., Gilbert, P., Meier, A. M., Cozens, R. M., & Brown, M. R. W. (1979) *Antimicrob. Agents Chemother. 15*, 147.
Kock-Weser, J., Sidel, V. W., Federman, E. B., Kanarek, P., Finer, D. C., & Eaton, A. E. (1970) *Ann. Intern. Med. 72*, 857.
Koike, M., & Iida, K. (1971) *J. Bacteriol. 108*, 1402.
Kuo, J. F., Raymor, R. L., Mazzel, G. J., Schatzman, R. C., Turner, R. S., & Kern, W. R. (1983) *FEBS Lett. 153*, 183.
La Porte, D. C., Rosenthal, K. S., & Storm, D. R. (1977) *Biochemistry 16*, 1642.
Lev. A. A. & Buzhinsky, E. P. (1967) *Tsitologiya 9*, 102.
Lindesmith, L. A., Baines, D. R., Jr., Bigelow, B. D., & Petty, T. L. (1968) *Ann. Intern. Med. 68*, 318.
McMurray, W., & Begg, R. W. (1959) *Arch. Biochem. Biophys. 84*, 546.
Morrison, D. C., & Jacobs, D. M. (1976) *Immunochemistry 13*, 813.
Mueller, P., & Rudin, D. O. (1967) *Biochem. Biophys. Res. Commun. 26*, 398.
Müller, L., & Chan, S. I. (1983) *J. Chem. Phys. 78*, 4341.
Mushayakara, E., & Levin, I. W. (1984) *Biochim. Biophys. Acta 769*, 585.
Navashim, S. M., Fomina, I. P., & Koroleva, V. G. (1961) *Antibiotiki (USSR) 6*, 912.
Nel, E. A., Wooten, M. W., Goldschmidt-Clermont, P. J., Miller, P. J., Stevenson, C. H., & Galbraith, R. M. (1985) *Biochem. Biophys. Res. Commun. 128*, 1364.
Newton, B. A. (1953) *J. Gen. Microbiol. 9*, 54.
Newton, B. A. (1956) *Bacteriol. Rev. 20*, 14.
Nishio, A., Ishiguro, S., Matsumoto, S., & Miyao, N. (1984) *Jpn. J. Pharmacol. 36*, 1.
O'Connor, D. T., & Stone, R. A. (1978) *Arch. Intern. Med. 138*, 478.
Oldfield, E., Meadows, M., Rice, D., & Jacobs, R. (1978) *Biochemistry 17*, 2727.
Pache, W., Chapman, D., & Hillaby, R. (1972) *Biochim. Biophys. Acta 255*, 358.
Pedersen, F. M., Pedersen, F. J., & Madsen, P. O. (1971) *Invest. Urol. 9*, 234.
Petrykina, Z. M., Bulgakova, V. G., & Polin, A. N. (1984) *Antibiotiki (USSR) 4*, 253.
Polin, A. N., Bulgakova, V. G., & Silaev, A. B. (1979) *Mikrobiologiya 4*, 699.
Pressman, B. C., Harris, E. J., Jagger, W. S., & Johnson, J. H. (1967) *Proc. Natl. Acad. Sci. U.S.A. 58*, 1949.
Rice, D., & Oldfield, E. (1979) *Biochemistry 18*, 3272.
Rice, D. M., Hsung, J. C., King, T. E., & Oldfield, E. (1979) *J. Am. Chem. Soc. 18*, 5885.
Rodriquez, V., Green, S., & Bodey, G. P. (1970) *Clin. Pharmacol. Ther. (St. Loius) 11*, 106.
Sabawala, P. B., & Dillon, J. B. (1959) *Anesthesiology 20*, 659.
Schwartz, B. S., Warren, M. R., Barkley, F. A., & Landis, L. (1960) *Antibiot. Ann. 7*, 41.
Seelig, A., & Seelig, J. (1978) *Hoppe-Seyler's Z. Physiol. Chem. 359*, 1747.
Seelig, J. (1977) *Q. Rev. Biophys. 10*, 353.
Seelig, J. (1978) *Biochim. Biophys. Acta 515*, 105.
Semenov, S. N., Mel'nik, E. I., Snezhkova, L. G., Miroshnikov, A. I., & Ivanov, V. T. (1977) *Bioorg. Khim. 3*, 1055.
Sixl, F., & Galla, H. J. (1981) *Biochim. Biophys. Acta 643*, 626.
Sixl, F., & Galla, H. J. (1982) *Biochim. Biophys. Acta 693*, 466.
Sixl, F., & Watts, A. (1985) *Biochemistry 24*, 7906.
Srinivasa, B. R., & Ramachandran, L. K. (1978) *Indian J. Biochem. Biophys. 15*, 54.
Stansly, P. G., Shepard, R. G., & White, M. J. (1947) *Bull. Johns Hopkins Hosp. 81*, 43.
Storm, D. R. (1974) *Ann. N.Y. Acad. Sci. 235*, 387.
Storm, D. R., Rosenthal, K. S., & Swanson, P. E. (1977) *Annu. Rev. Biochem. 46*, 723.
Susi, H., Sampugna, J., Hampson, J. W., & Ard, J. S. (1979) *Biochemistry 18*, 297.
Teuber, M. (1974) *Arch. Microbiol. 100*, 131.
Vaara, M., & Vaara, T. (1981) *Antimicrob. Agents Chemother. 19*, 578.
Wahn, K., Lutsch, G., & Rockstroh, T. (1968) *Arch. Microbiol. 63*, 103.
Wassal, S. R., Thewalt, J. L., Wong, L., Gorrissen, H., & Cushley, R. J. (1986) *Biochemistry 25*, 319.
Wise, B. C., Glass, D. B., Jen Chou, C.-H., Raynor, R. L., Katou, N., Schatzmany, R. C., Turner, R. S., Kibler, R. F., & Kuo, J. F. (1982) *J. Biol. Chem. 257*, 8489.
Wrenn, R. W., & Wooten, M. W. (1984) *Life Sci. 35*, 267.
Wu, E. S., Jacobson, K., Szoka, F., & Portis, A., Jr. (1978) *Biochemistry 17*, 5543.
Zilleti, L., Guidotti, A., & Mannaioni, P. F. (1965) *Arch. Hal. Sci. Farmacol. 15*, 113.