Lipid–Amphotericin B Complex Structure in Solution: A Possible First Step in the Aggregation Process in Cell Membranes†

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ABSTRACT: The interactions between the polyene antibiotic amphotericin B with dipalmitoylphosphatidylcholine were investigated in vesicles (using circular dichroism) and in chloroform solution (using circular dichroism and ^1H, ^{13}C, and ^{31}P nuclear magnetic resonance). The results show that amphotericin B readily aggregates in vesicles and that the extent of aggregation depends on the lipid:drug concentration ratio. Introduction of sterol molecules into the membrane hastens the process of aggregation of amphotericin B. In chloroform solutions amphotericin B strongly interacts with phospholipid molecules to form a stoichiometric complex. The results suggest that there are interactions between the conjugated heptene stretch of amphotericin B and the methylene groups of lipid acyl chains, while the sugar moiety interacts with the phosphate head group by the formation of a hydrogen bond. A model is proposed for the lipid–amphotericin B complex, in which amphotericin B interacts equally well with the two lipid acyl chains, forming a 1:1 complex.

Amphotericin B (amp-B)[1] is a polyene antibiotic that is used widely for treating systemic fungal infections and synergistically in anticancer therapy (Gale, 1984; Bolard, 1986). It has also been reported to play a role in delaying neurodegenerative disease symptoms in animals (Xi et al., 1992), and the methyl ester of amp-B has been shown to block in vitro replication and infection by human immunodeficiency virus (Schaffner, 1984). The antibiotic acts at the plasma membrane level where it increases the permeability to small solutes. Amp-B is toxic to cells that have plasma membranes with sterols. Ergosterol-rich fungal cells are very sensitive (Lampen, 1966; Kobayashi & Medoff, 1977), while sterol-free bacterial cells are quite resistant. Investigations of the antifungal activity as well as attempts to understand the mechanism of amp-B action at the molecular level have been reported (Finkelstein & Holtz, 1973; De Kruijff & Demel, 1977; Van Hoogevest & De Kruijff, 1978).

Amp-B is the only member of polyene antibiotics whose crystal structure has been solved (Mechlinski et al., 1970). Among the models that have been proposed to explain the sterol-dependent action of amp-B, the most accepted one is based on the formation of a channel composed of alternate sterol and antibiotic molecules (Finkelstein & Holtz, 1973; Kleinberg & Finkelstein, 1984). An alternative membrane defect model has recently been proposed (Hartsel et al., 1991). However, the role of the membrane phospholipids in stabilizing the pore (or defect) structure has not been clearly defined. It is thus possible that amp-B can form stable complexes with the membrane phospholipids and that sterol molecules help to organize these complexes into channels. Such a possibility has been proposed on the basis of amp-B interactions with model membrane systems (Cohen, 1992; Hartsel et al., 1988; Whyte et al, 1989; Milhaud et al., 1989).

In this paper we report results based on circular dichroism (CD) measurements, on the interaction of amp-B with L-α-dipalmitoylphosphatidylcholine (DPPC) vesicles in the absence and presence of sterols at different lipid:amp-B concentration ratios. The results show a concentration-dependent aggregation of amp-B when incorporated into bilayer vesicles without sterol. Sterol molecules, when present, induce a faster aggregation of amp-B. We also report for the first time experimental evidence based on CD and ^1H, ^{13}C, and ^{31}P nuclear magnetic resonance (NMR) results, which demonstrates directly the formation of lipid–amp-B complexes in nonpolar solvents like chloroform. The effect of sterols on these complexes in solution is also reported.

MATERIALS AND METHODS

Amp-B, DPPC, ergosterol, and cholesterol were obtained from Sigma Chemical Co. (St. Louis, MO). Amp-B and DPPC were used without further purification. Both ergosterol and cholesterol were recrystallized before use. Deuterated solvents for NMR experiments were from Sigma Chemical Co. Stock solutions of amp-B were prepared in dimethyl sulfoxide (DMSO) and used within 2 days. DPPC vesicles were prepared by hydrating a dry film of the lipid and sonicating for 3 min. Amp-B was added while making the lipid film. Sterol was also added while making the lipid film when required. All spectra were recorded above phase transition temperature and within 20 min. of sonication. Control experiments were carried out using DPPC sonicated vesicles without amp-B in order to check for light scattering within the wavelength range studied. The base line was good, and no light scattering problems were encountered.

CD experiments were done on a Jasco J500A spectropolarimeter calibrated with *d*-10-camphorsulfonic acid. ^1H NMR and ^{13}C NMR experiments were done on an AMX 400-MHz Bruker spectrometer. ^{31}P NMR experiments were done on AMX 400-MHz Bruker and 80-MHz Varian spectrometers. Two-dimensional (2D) ^1H nuclear Overhauser enhancement spectroscopy (NOESY) experiments were done at 400- and 100-ms mixing times.

^{13}C spin–lattice relaxation times (T_1) were determined by the inversion–recovery method (Vold et al., 1968) with 12

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[1] Abbreviations: amp-B, amphotericin B; CD, circular dichroism; NMR, nuclear magnetic resonance; DPPC, dipalmitoylphosphatidylcholine; DMSO, dimethyl sulfoxide; NOESY, 2D nuclear Overhauser enhancement and exchange spectroscopy; NOE, nuclear Overhauser enhancement; T_1, spin–lattice relaxation time; T_{eff}, effective rotational correlation time.

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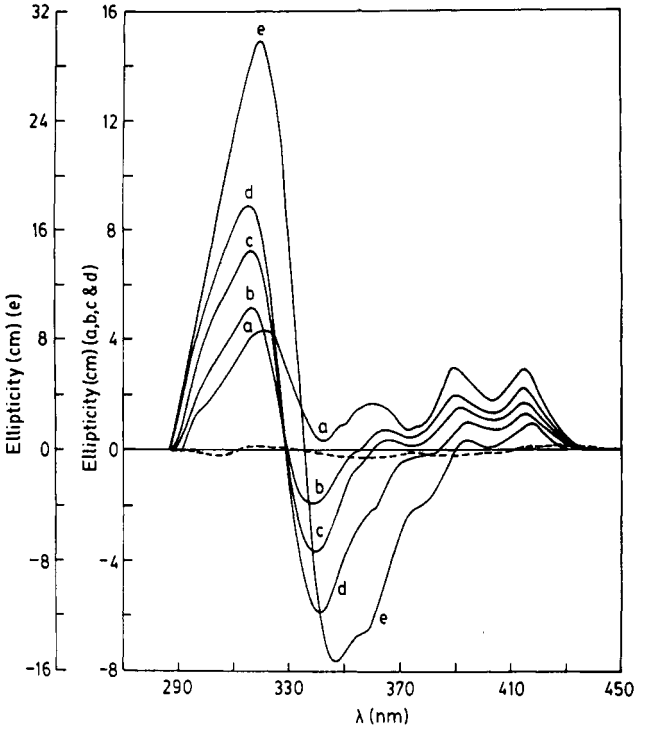

FIGURE 1: CD spectra of amp-B in sonicated DPPC vesicles as a function of concentration. DPPC:amp-B concentration ratios: (a) 150:1, (b) 120:1, (c) 90:1, (d) 60:1, (e) 30:1; (- - -) is the trace of DPPC vesicles without amp-B. Cell length 0.1 cm; sensitivity 1 mdeg/cm; temperature 50 °C. Observed ellipticity values are given.

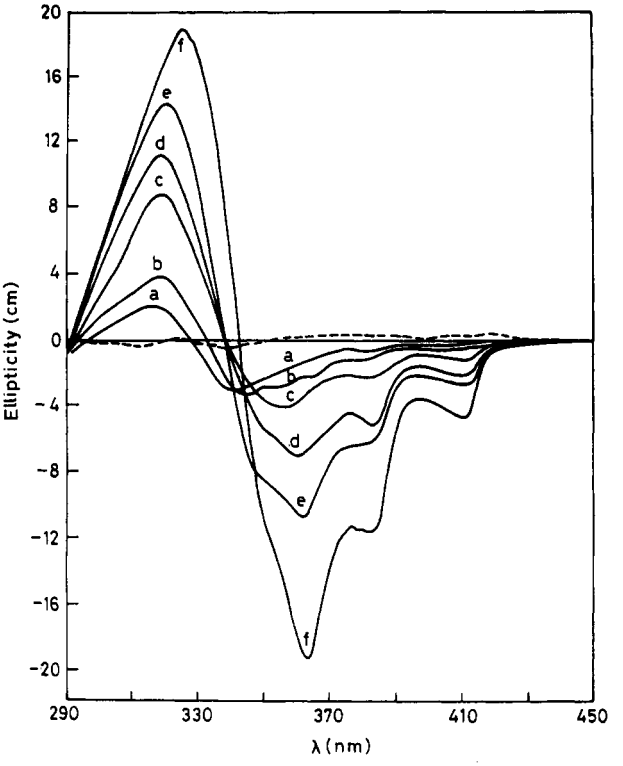

FIGURE 2: CD spectra of amp-B in sonicated DPPC vesicles with ergosterol as a function of concentration. DPPC:ergosterol:amp-B concentration ratios: (a) 180:1:1, (b) 150:1:1, (c) 120:1:1, (d) 90:1:1, (e) 60:1:1, (f) 30:1:1; (- - -) is the trace of DPPC vesicles with ergosterol and without amp-B. Cell length 0.1 cm; sensitivity 2 mdeg/cm; temperature 50 °C. Observed ellipticity values are given. Spectra f has been scaled down by half.

delay times, ranging from 0.1 to 25.0 s. Spectra were collected for free DPPC and for DPPC–amp-B complexes in a $CDCl_3$/DMSO-d_6 (4:1 v/v) solvent mixture, under otherwise identical conditions. ^{13}C T_1's and effective correlation time (τ_{eff}) were calculated for individual carbon atoms coupled to protons. The effects of internal motion in an isotropically tumbling DPPC molecule, in its free and complexed states, on nuclear magnetic relaxation are considered here. Our interest is in the τ_{eff} of each $(CH_2)_n$ group from the glycerol moiety toward the terminal methyl group of the fatty acid chain and the $N(CH_3)_3$ head group. Measuring ^{13}C T_1 of lipid molecules in solution reflects the motional restraint of the molecule when complexed to the antibiotic.

^{31}P (161.98- and 32.39-MHz) data were obtained with broad-band ^1H decoupling, and the chemical shifts are reported relative to external 85% phosphoric acid (H_3PO_4). ^{31}P NMR experiments were performed to estimate the effect of complexation on the ^{31}P resonance of DPPC molecules. Spectra were recorded for both free DPPC and DPPC–amp-B complex under identical conditions.

RESULTS

I. In Vesicles

Figure 1 shows CD spectra of amp-B in sonicated DPPC vesicles at different lipid:amp-B ratios above the lipid's phase transition temperature. At a low lipid:drug ratio (150:1), one sees an intense CD band at around 328 nm. This band becomes increasingly bisignate as the lipid:amp-B ratio is decreased. The other bands corresponding to heptene chromophore however show a fine structure even at maximum drug concentration (30:1). The CD spectra of amp-B in sonicated DPPC vesicles with ergosterol are given in Figure 2. The most pronounced difference from the previous set of spectra (Figure 1) is the increase in ellipticity and inversion

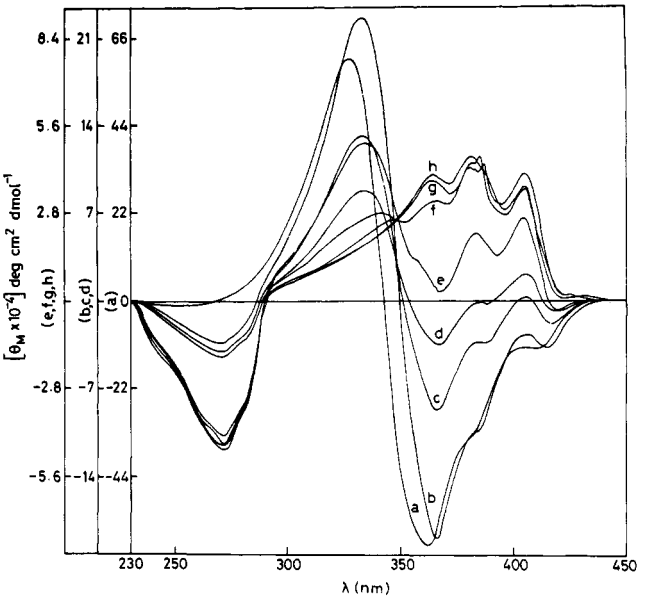

FIGURE 3: CD spectra of amphotericin B as a function of increasing DPPC concentration in chloroform. Concentration of amp-B is 2.5×10^{-4} M. The concentration of DPPC is increased by 27 mM per titration step. (a) Amp-B. (b–h) DPPC titration.

of the bands corresponding to the heptene chromophore. There is, however, also a strong similarity in that the band at around 328 nm becomes increasingly bisignate as the lipid:amp-B ratio is decreased.

II. In Solution

Circular Dichroism Studies. Figure 3a shows CD spectra of amp-B (2.5×10^{-4} M) in chloroform. Figure 3b–h are CD

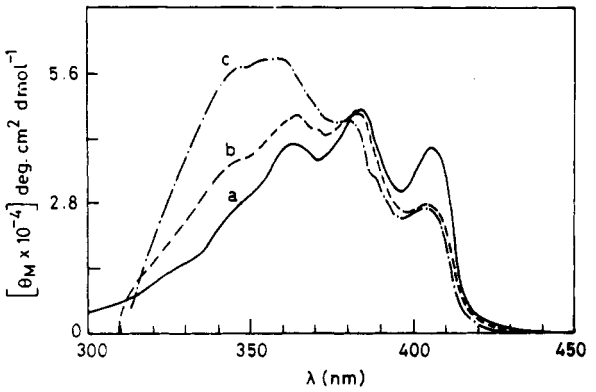

FIGURE 4: CD spectra of amp-B when complexed to DPPC and effects of ergosterol on this complex in chloroform. (a, —) DPPC–amp-B complex; (b, - - -) ergosterol–DPPC (0.5:1); (c, – • –) ergosterol–DPPC (1:1). Concentration of DPPC 0.16 M, concentration of amp-B 2.5 × 10^{-4} M.

spectra of amp-B in chloroform to which increasing amounts of DPPC were added. Amp-B in chloroform is an aggregated structure (Rinnert et al., 1977; Balakrishnan & Easwaran, 1993). With the addition of lipid there are continuous changes in the CD spectra. The 328-nm positive band red shifts to 346 nm with a decrease in intensity and, finally, becomes undetectable. The negative bands at 361, 385, and 412 nm begin to show increasing vibrational fine structure and a reversion to the positive side. The spectra in Figure 3g,h, which were recorded at high lipid concentrations, are very similar to the spectra one observes of amp-B in hydrophilic solvents like DMSO where the molecule exists as a monomer at similar concentrations and as a dimer at higher concentrations. Vibrational fine structure is seen in the spectrum when amp-B molecules are undergoing electronic transitions in a monomeric or oligomeric state. Usually for conjugated systems, aggregation dampens the fine structure and the bands broaden out as seen in the initial spectrum (Figure 3a). Figure 4 shows CD spectra of the effect of ergosterol upon DPPC–amp-B complex (concentration of DPPC is 0.16 M and of amp-B is 2.5 × 10^{-4} M). Both ergosterol and cholesterol when added to the solution containing lipid–amp-B complexes begin to show the intense CD band. Ergosterol induces the band at around 346 nm and cholesterol at around 328 nm.

^{1}H NMR Studies. 1D ^{1}H NMR experiments were done for DPPC and DPPC–amp-B in $CDCl_3$/DMSO-d_6 (4:1 v/v). Spectra were recorded at different concentration ratios of lipid:amp-B (6:1, 4:1, and 2:1). Panels a, b, and c of Figure 5 show spectra of DPPC, DPPC–amp-B (6:1), and DPPC–amp-B (2:1), respectively. The solubility of amp-B is poor in chloroform but in the presence of lipid the solubility of amp-B increases, implying that the lipid–amp-B complex is more soluble in the medium. This enhanced solubility also makes it possible to work with the complex at NMR concentrations. All lipid resonances show up between 0 and 5.3 ppm. The drug resonances extend from 0 to 6.4 ppm. Resonances of the heptene chromophore (a highly coupled set) appear between 6 and 6.4 ppm. These resonances are well separated from the lipid signals. Line broadening was observed in all the lipid resonances except the terminal CH_3 resonance. Corresponding line broadening was observed in amp-B resonances (seen between 6 and 6.4 ppm). Even at a 2:1 lipid:drug ratio the terminal CH_3 resonance of the lipid remained a well-resolved triplet.

2D NOESY spectra were recorded for free DPPC and DPPC–amp-B (6:1 ratio) in $CDCl_3$/DMSO-d_6 (4:1 v/v) at

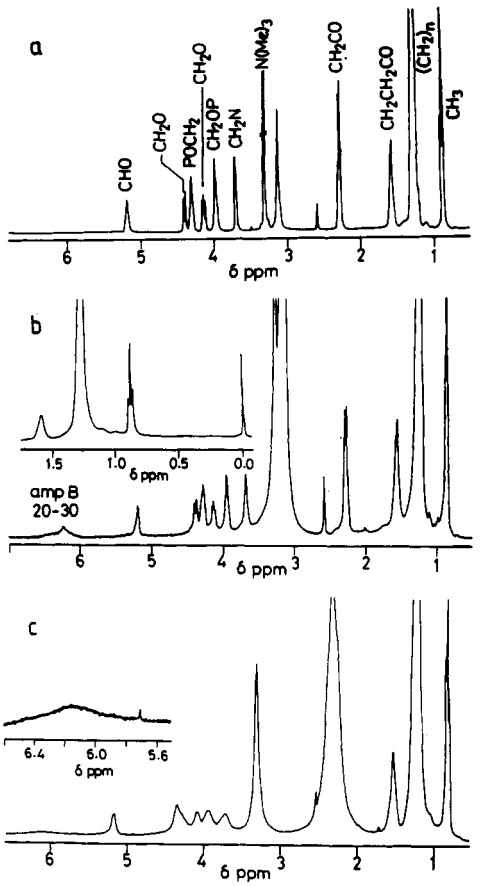

FIGURE 5: 400-MHz NMR spectra of DPPC–amp-B in $CDCl_3$/DMSO-d_6 (4:1 v/v). (a) Free DPPC; (b) DPPC–amp-B (6:1 concentration ratio); (c) DPPC–amp-B (2:1 concentration ratio).

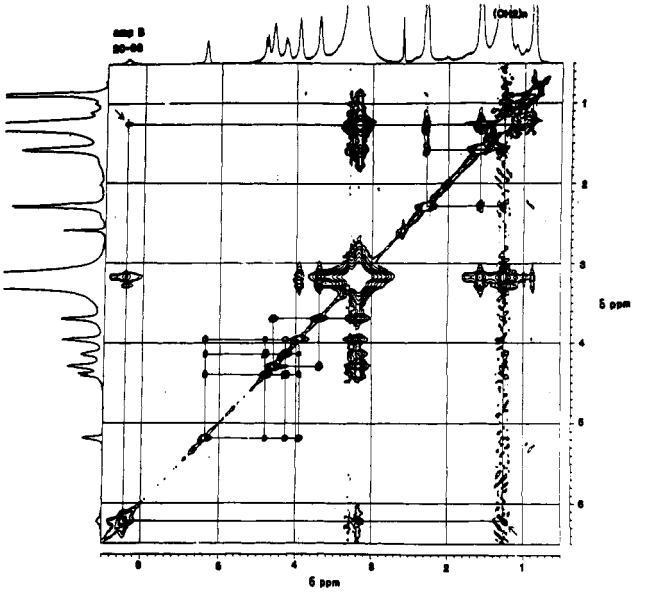

FIGURE 6: 400-MHz NOESY spectrum of DPPC–amp-B (6:1 concentration ratio) in $CDCl_3$/DMSO-d_6 (4:1 v/v) (100-ms mixing time).

400- and 100-ms mixing times. Figure 6 shows the spectra of DPPC–amp-B (6:1) recorded at 100-ms mixing time. NOESY of amp-B at 100-ms mixing time was also recorded to verify the cross peaks. The nuclear Overhauser enhancement (NOE) cross peak of interest is the one seen between the amp-B heptene resonances around 6.2 ppm and the $(CH_2)_n$ resonances of the lipid. A 1D NOE experiment was done to

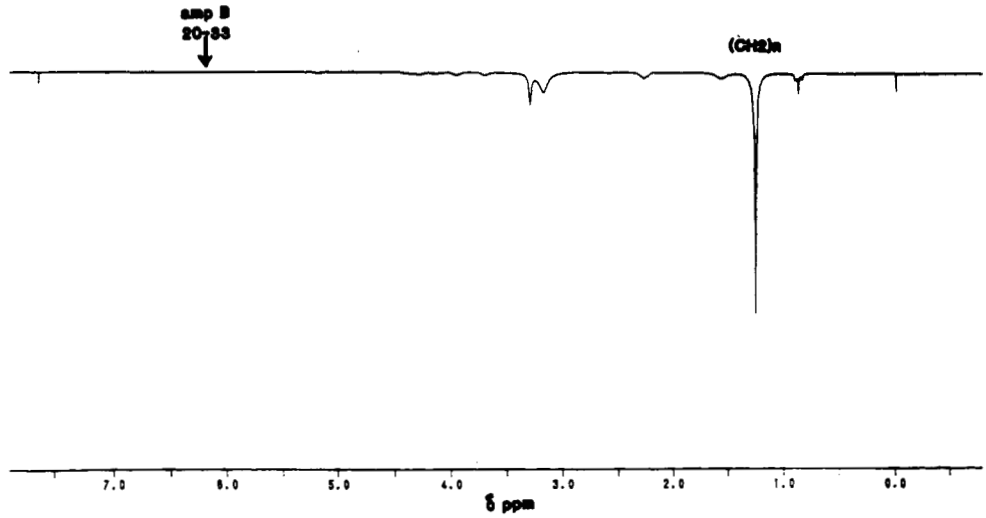

FIGURE 7: 200-MHz 1D NOE difference spectrum of DPPC–amp-B (6:1 concentration ratio) in $CDCl_3$/DMSO-d_6 (4:1 v/v).

Table I: Spin–Lattice (T_1) Relaxation Times (s) and Rotational Correlation Times (s) of DPPC and DPPC–Amp-B Complex

	DPPC			DPPC–amp-B complex			
	T_1	NT_1	τ_{eff} (10^{-12})	T_1	NT_1	τ_{eff} (10^{-12})	τ_{eff}
acyl chain							
C2	0.38 ± 0.02	0.77	3.55	0.34 ± 0.03	0.68	4.00	0.45
C3	0.62 ± 0.03	1.25	2.17	0.55 ± 0.05	1.11	2.45	0.28
C4–13	1.29 ± 0.10	2.58	1.05	1.08 ± 0.09	2.17	1.26	0.21
C14	2.56 ± 0.10	5.12	0.53	2.08 ± 0.10	4.16	0.65	0.12
C15	3.42 ± 0.14	6.85	0.40	2.78 ± 0.14	5.56	0.49	0.09
CH_3	4.27 ± 0.15	12.81	0.48	3.93 ± 0.19	11.80	0.52	0.04
head group							
$POCH_2$	0.1 ± 0.01	0.20	13.59	0.08 ± 0.01	0.16	16.98	3.39
CH_2N	0.15 ± 0.01	0.30	9.06	0.12 ± 0.01	0.24	11.32	2.26
$N(Me)_3$	0.20 ± 0.01	0.60	10.18	0.19 ± 0.01	0.58	10.54	0.36

Table II: ^{13}C Line Widths (Hz)

	DPPC	DPPC–amp-B complex	line width
acyl chain			
C2	5.8	7.6	1.8
C3	4.0	5.6	1.6
C4–13	9.8	10.8	1.0
C14	8.0	9.0	1.0
C15	2.0	3.0	1.0
CH_3	2.0	2.4	0.4
head group			
CHO	6.4	8.0	1.6
CH_2O	7.8	8.8	1.0
CH_2OP	3.8	5.0	1.2
$POCH_2$	6.0	6.8	0.8
CH_2N	6.0	6.8	0.8
$N(Me)_3$	2.0	2.4	0.4

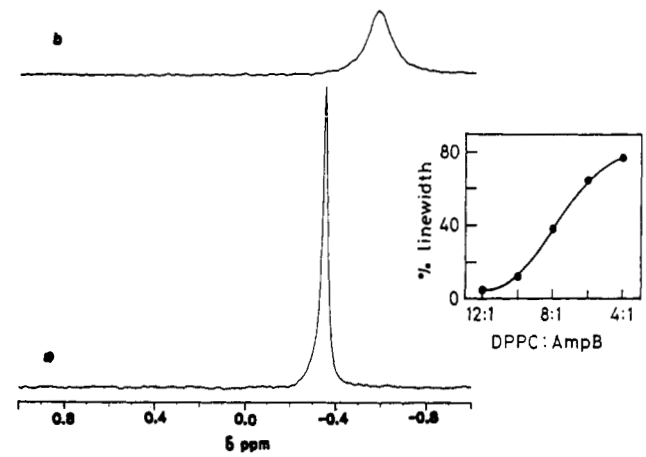

FIGURE 8: 161.98-MHz ^{31}P NMR of (a) DPPC and (b) DPPC–amp-B (6:1 concentration ratio) in $CDCl_3$/$DMSO_{d6}$ (4:1 v/v). Inset: Plot of line widths from titration spectra at different DPPC: amp-B concentration ratios.

double check this observed intermolecular NOE. The drug resonance was irradiated at 6.2 ppm. The difference spectrum recorded is given in Figure 7. The observed negative NOE is in good agreement with that observed in the NOESY experiment. Calculated NOE is 5.08%. The heptene group of amp-B stretches from the sugar moiety to the tail end of the drug molecule, hence the observed small NOE on the polar head group of DPPC resonance (Figure 7).

^{13}C T_1 Measurements. Table I lists T_1 and τ_{eff} calculated for DPPC and DPPC–amp-B complex (6:1 ratio). Table II lists the line widths at half-maxima for both sets of experiments. There is a significant decrease in T_1 and a corresponding increase in τ_{eff} values for the carbons from the glycerol moiety to the terminal CH_3 group of the lipid acyl chain. The head group carbons also showed a similar trend; from the glycerol moiety upward there was a significant decrease in T_1. τ_{eff} showed significant changes until the C14 carbons. C15 and the terminal CH_3 carbon were affected to a lesser extent. Line widths at half-maxima showed significant increases in all resonances except for the CH_3 and $N(CH_3)_3$ peaks.

^{31}P NMR Studies. Spectra of DPPC and DPPC–amp-B under identical conditions are shown in Figure 8. The ^{31}P signal of DPPC–amp-B observed shows line broadening and a small upfield chemical shift. The inset in Figure 8 gives the plot of line widths measured from titration spectra at different DPPC:amp-B concentration ratios. A progressive increase

in line widths and a corresponding upfield shift of the resonance were observed.

DISCUSSION

From the overview of the extensive work which has been done on amp-B (Lampen, 1966; Bolard, 1986) it is clear that the biological effects of amp-B are brought about by its interaction with both lipid and sterol molecules of membranes. CD results (Figure 1) of amp-B incorporated into DPPC bilayer vesicles (without sterol) as a function of concentration showed a concentration-dependent aggregation of the antibiotic. In DPPC vesicles with sterol the aggregation of amp-B is promoted and occurs at lower drug concentrations, as seen by the changes in ellipticity (Figure 2). The inversion of the CD bands corresponding to the heptene chromophore by the sterol indicates that the interaction between the sterol molecule and amp-B most likely is at the heptene stretch of the antibiotic. The bisignate shape of the CD band centered at around 342 nm is an indication of a multimolecular structure in apolar solvents (Rinnert et al., 1977). The vibrational fine structure observed in the bands between 412 and 361 nm coupled with the bisignate shape of the spectra at higher concentrations of the drug in sterol-free vesicles, as well as that observed upon interaction with sterol, suggests the formation of an organized multimolecular structure within the bilayer.

Amp-B in chloroform (2.5×10^{-4} M) is an aggregated structure (Figure 3a). When DPPC is added to amp-B in chloroform (Figure 3b–h), the multimolecular structure begins to disaggregate and assumes a monomeric structure at high lipid concentrations, a finding that implies complexation between lipid and amp-B. In addition, the ^{1}H NMR studies (Figure 5b,c) showed line broadening in both DPPC as well as amp-B resonances, which also indicates formation of a complex between the two molecules. The well-resolved CH_3 resonance of DPPC in a completely line-broadened spectrum indicates that the selective line broadening is due to complexation with the antibiotic and not other effects. The resolved CH_3 resonance (a triplet) further reflects that this group is not in close interaction with the drug molecule. The intermolecular NOE cross peak (Figure 6) observed between the $(CH_2)_n$ of DPPC and amp-B (20 ^{1}H to 33 ^{1}H) resonances gives the spatial proximity of the two groups. 1D NOE results (Figure 7) further confirm this dipolar interaction. Other lipid drug NOEs cannot be looked at as there is an overlap of both resonances. Estimated distance from the calculated % NOE (5.08% negative NOE) is in the range of 3.6–4.0 Å. ^{1}H NMR observations confirm formation of DPPC–amp-B complex and give a rough idea of the location of the drug molecule with respect to the lipid molecule. The spatial proximity of the heptene transconjugated double bond system with the lipid acyl chains has been confirmed.

^{13}C T_1 analysis (Table I) showed a significant decrease in T_1 down the acyl chain as well as up the head group region with respect to the glycerol moiety. The calculated τ_{eff} for carbons coupled to protons showed the expected inverse relation to the T_1 trend. This analysis maps sequential reduction in motion down the acyl chain of DPPC due to the complex formation with amp-B. The τ_{eff} values looked at show a small difference in the terminal methyl group rotational correlation time after complexation. This small difference is attributable to the overall reduction of the isotropic tumbling of the DPPC–amp-B complex. The observed increase in τ_{eff} of the head group carbons is again due to the reduced effective motion about each proton–coupled carbon. From these results one can deduce that the amp-B molecule interacts with carbon from position 15 or 14 upward and that the interaction spans the length of the lipid molecule right up to the head group region.

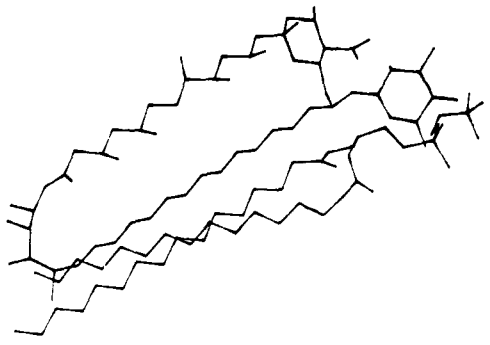

FIGURE 9: Proposed model for a 1:1 DPPC–amp-B complex.

The ^{31}P chemical shift of DPPC in $CDCl_3$/DMSO-d_6 is similar to that reported for phosphatidylcholine from solubilized sarcoplasmic reticulum membranes (London & Feigenson, 1979). The observed line broadening (Figure 8) in the ^{31}P resonance of DPPC–amp-B reflects motional constraint of the phosphodiester group. The small upfield shift can be attributed to the following: first, that an increased electron density on phosphorus leads to increased shielding (Gorenstein & Kar, 1975); second, small changes in O–P–O bond angles which have been suggested to play a role in ^{31}P chemical shifts (Prudela, 1971; Blackburn et al., 1971). ^{31}P analysis supports our other inferences about the complex formation between DPPC and amp-B. The upfield shift indicates a likely formation of a hydrogen bond between oxygen atoms on the phosphorus and the drug molecule. The sugar moiety and the glycosidic oxygen of the drug molecule are likely candidates for hydrogen bond formation. The sugar OH group at position C4 (equatorial) can form a good hydrogen bond with the oxygen on the lipid phosphorus. The glycosidic oxygen can do the same with the oxygen connecting glycerol CH_2 and the phosphorus atom. According to this picture the role of the sugar moiety, a β-pyranose 3-aminomannose, is to orient the drug molecule to complex with the lipid molecule in such a way that the polar head groups of the two molecules interact favorably. Further, this leaves the carboxyl group free to take part in other interactions that may be required to change permeability properties of membranes. These structural features can be incorporated into a 1:1 lipid–amp-B complex (Figure 9). Such a 1:1 lipid–amp-B complex takes into consideration the fact that only one set of resonances appear in ^{1}H and ^{13}C spectra after complexation. According to this model the drug molecule is positioned equidistant from the two acyl chains of the lipid molecule, the sugar interacting favorably with the head group and the terminal methyl group not being involved in the interaction.

The effect of sterol upon the lipid–amp-B complex in solution (Figure 4) seems to induce oligomeric aggregates of the complex, perhaps by the formation of a ternary complex. Relating these results with that reported in the literature for amp-B in model membrane systems and also from our own observations (Figure 2), we deduce the following. Amp-B shows concentration-dependent aggregation when incorporated into bilayer membrane systems, and sterols promote drug aggregation.

In conclusion, the results obtained establish the formation of lipid–amp-B complexes in solution and in lipid bilayers. The formation of lipid–amp-B complex is modulated by sterols, which are responsible for the formation of membrane pores which alter the membrane permeability.

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