Effect of Amphotericin B on Membranes: A Spin Probe Study†

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ABSTRACT: The effect of the polyene antibiotic amphotericin B on the electron paramagnetic resonance spectra of lipid probes intercalated in model membranes was examined. When the antibiotic was added to the aqueous phase, no spectral effects occurred. However, when the antibiotic was incorporated during membrane preparation, changes in spectral parameters suggested the appearance of a new phase. The spectral changes do not necessarily corroborate the pore models proposed previously for amphotericin B in membranes. With a spin probe that partitions between water and membrane, an interaction between the amphotericin B and probe is observed. This interaction does not occur in the membrane, but in the aqueous phase, between the probe and the aggregated antibiotic. Some of the equilibria involving the antibiotic appear to be achieved slowly.

The effects of polyene antibiotics upon biological and artificial membranes have been examined extensively (Hammond, 1977). A large amount of work has dealt with the functional aspects of these interactions, but little is known about their structural mechanisms. Particular interest has been paid to the pore models proposed to account for the permeability changes induced by amphotericin B (Andreoli, 1974; Marty & Finkelstein, 1975; van Hoogevest & de Kruijff, 1978). It has been suggested that the conducting structure is a complex between antibiotic and sterol molecules (de Kruijff & Demel, 1974). Ockman (1974), and Bunow & Levin (1977), concluded that amphotericin B is located at the water–membrane interface in cholesterol-free membranes, whereas it penetrates into the hydrophobic core when the sterol is present.

The molecular details of membrane structural changes resulting from interaction with antibiotics are less well documented. Bunow & Levin (1977) detected changes in Raman active vibrations of methylene and methyl groups in the phospholipid acyl chains of egg phosphatidylcholine (EPC)[1] and cholesterol mixtures. Addition of the methyl ester of amphotericin B to membranes containing a fluorescent analogue of ergosterol affected the fluorescence spectrum (Archer, 1975).

The spin-label technique has been employed to examine the effects of polyene antibiotics on model membranes (Smith, 1971; Flick et al., 1976; Flick & Gelerinter, 1977; Ohki et al., 1979; Oehlschlager & Laks, 1980). In extension of this approach, we have investigated the effects of amphotericin B on the spectra of a variety of spin probes incorporated in model membranes of EPC and DPPC with various cholesterol contents.

Materials and Methods

The sources of chemicals were the following: egg phosphatidylcholine, Lipid Products; dipalmitoylphosphatidylcholine, Sigma Chemical Co.; cholesterol, Steraloids; spin probes CSL, ASL, and 5-SASL, Syva. Amphotericin B was a gift from Squibb-Industria Quimica S.A. All other reagents were of analytical grade. Phosphate buffer (300 mosM) and phosphate–NaCl buffer (300 mosM), pH 7.4, were used. X-band Varian E-4 and E-9 EPR spectrometers, with flat quartz cells for aqueous solutions, were used at room temperature (22 ± 2 °C).

Stock solutions of lipids and spin probes were prepared in $CHCl_3$ and kept at −15 °C. For experiments done in the absence of membranes, the stock solution of spin probe was prepared in ethanol; the final solutions contained 5% ethanol (v/v). The stock solution of amphotericin B was freshly prepared in Me_2SO. Unless stated otherwise, the final concentration of Me_2SO was 2% (v/v). When amphotericin B was added during membrane formation, it was dissolved in methanol.

Oriented multibilayers were prepared as described by Smith & Butler (1976). The films were left under vacuum for no less than 2 h and hydrated with buffer, buffer–Me_2SO (50:1), or buffer–Me_2SO containing amphotericin B. Spectra were run after 1 h, before and after draining.

Multibilayer dispersions (liposomes) were prepared by removing the organic solvent with a flux of nitrogen, exposure to vacuum for at least 2 h, and suspension upon addition of buffer by shaking with a Vortex mixer for 15–20 min.

For experiments performed in the absence of membranes, ethanolic stock solutions of spin probes were diluted with either buffer or solutions containing Me_2SO or amphotericin B in Me_2SO at various concentrations.

Results

Planar Multibilayers. Planar multibilayers of EPC or DPPC containing CSL were prepared with 0, 15, 30, and 50 mol % cholesterol. When incorporated during the membrane preparation, amphotericin B was present at various molar ratios with respect to cholesterol, as described in the figures. Figure 1 shows the effect of the antibiotic on the spectra of CSL in EPC- or DPPC–cholesterol films. The order parameter (S) (Figure 2) was measured according to Schreier et al. (1978), and the b/c ratio (Figures 3 and 4) as indicated in Figure 1. Cholesterol increased the order parameter of EPC,

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[1] Abbreviations used: EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; EPC, egg phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; CSL, ASL, and 5-SASL, the 2,2-dimethyloxazolidinyl-*N*-oxy derivatives of cholestan-3-one, 17β-hydroxy-5α-androstan-3-one, and 5-ketostearic acid, respectively; Me_2SO, dimethyl sulfoxide.

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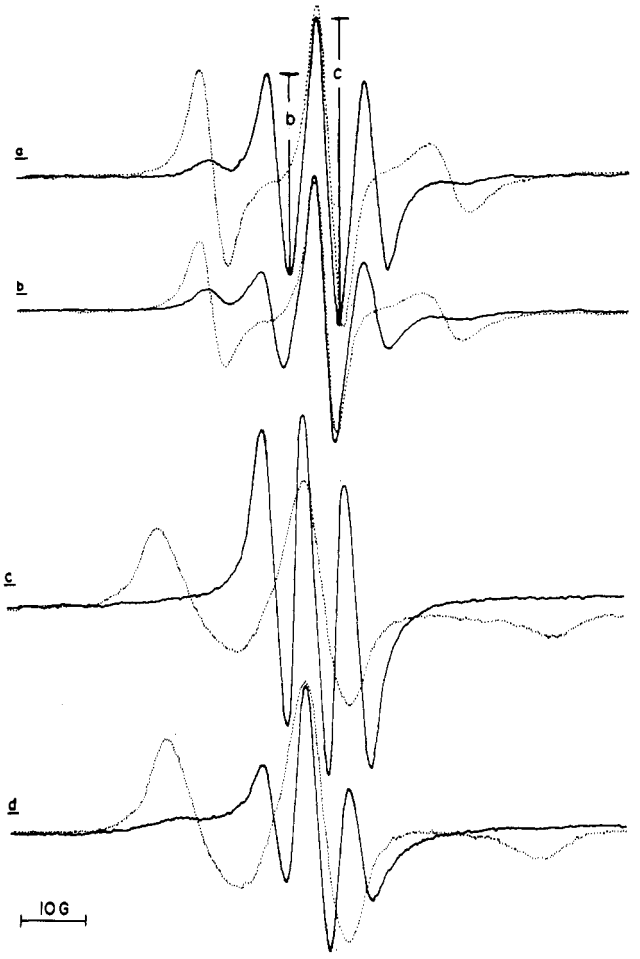

FIGURE 1: EPR spectra of CSL in the following oriented multibilayers: (a) EPC–30 mol % cholesterol; (b) same as (a), prepared in the presence of amphotericin B (antibiotic:sterol mole ratio 1:8.6); (c) DPPC–30 mol % cholesterol; (d) same as (c), prepared in the presence of amphotericin B (antibiotic:sterol mole ratio 1:7.3). Magnetic field parallel (—) and perpendicular (···) to the bilayer normal.

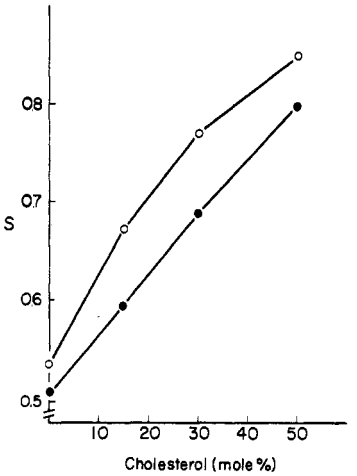

FIGURE 2: Order parameter (S) as a function of sterol content for spectra of CSL in planar multibilayers of EPC prepared in the absence (○) and in the presence (●) of amphotericin B. The antibiotic:sterol mole ratios were 1:3.5, 1:8.6, and 1:20.0 for 15, 30, and 50 mol % cholesterol, respectively.

as reported earlier (Lapper et al., 1972; Schreier-Muccillo et al., 1973b). The b/c ratio increased for up to 30 mol % sterol for both EPC and DPPC and decreased with higher sterol content. The order parameter was not calculated for the DPPC systems, since the rate of motion of the probe is too slow to allow the use of the order parameter formalism, as

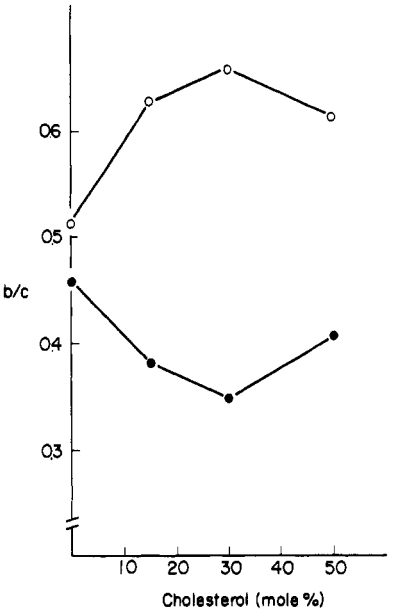

FIGURE 3: b/c ratio as a function of sterol content for the same spectra as in Figure 3. (○) Absence and (●) presence of amphotericin B.

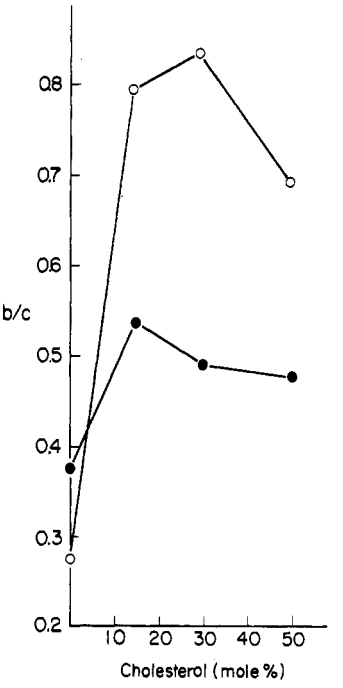

FIGURE 4: b/c ratio as a function of sterol content for spectra of CSL in planar multibilayers of DPPC prepared in the absence (○) and in the presence (●) of amphotericin B. The antibiotic:sterol mole ratios were 1:3.1, 1:7.3, and 1:16.9 for 15, 30, and 50 mol % cholesterol, respectively.

indicated by the large splittings and broad resonances in the spectra taken with the magnetic field parallel to the membrane plane (dotted spectra, Figure 1c,d). This indicates a much slower rate of rotation about the long molecular axis of CSL in DPPC than in EPC. The large increase in b/c upon addition of cholesterol to DPPC (Figure 4) is related to the change in tilt angle of the acyl chains with respect to the bilayer normal (Ladbrooke et al., 1968; Schreier-Muccillo et al., 1973b). The effects of amphotericin B on S and b/c will be analyzed under Discussion. The results obtained with amphotericin B were constant over a period of 24 h.

Experiments were performed by adding the antibiotic to the hydrating solution. The molar ratios of amphotericin B, in the solution, to cholesterol, in the membranes, were 1:1.75,

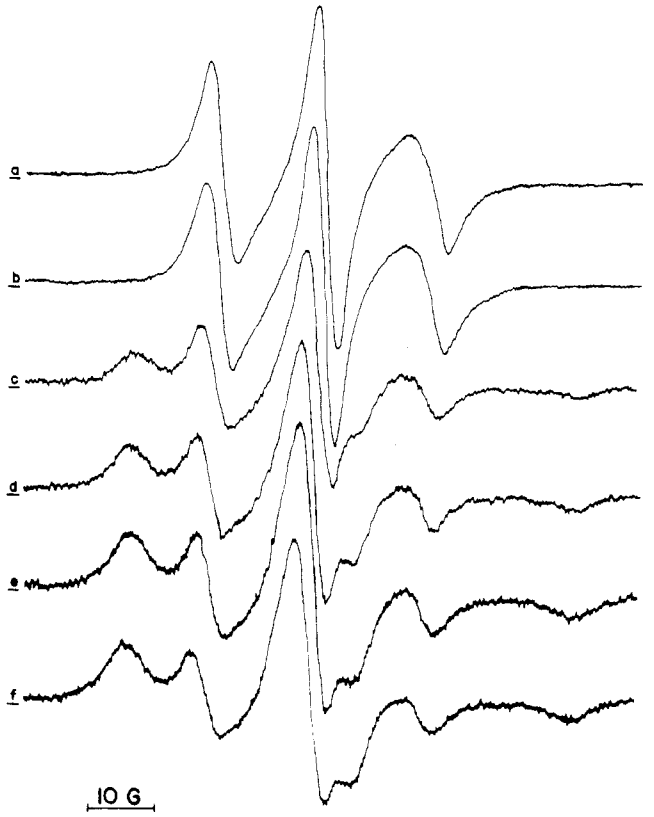

FIGURE 5: EPR spectra of ASL (5×10^{-5} M) in EPC liposomes (5×10^{-3} M) (a) in the presence of Me_2SO (10% v/v) and (b) in the presence of 5×10^{-3} M amphotericin B (in Me_2SO, 10% v/v) after 5 (c), 30 (d), 60 (e), and 90 (f) min.

1:4.3, and 1:10 (in EPC) and 1:1.5, 1:3.7, and 1:8.5 (in DPPC), for 15, 30, and 50 mol % cholesterol, respectively. No changes were detected in the spectra of CSL or 5-SASL under these circumstances up to 24 h after addition of antibiotic.

Liposomes. We have investigated cholesterol-free EPC liposomes where the only available sterol was ASL. Figure 5 shows that 5×10^{-4} M amphotericin B gives rise to a spectral component, due to immobilized spin probe, whose intensity increases with time. A decrease in the ratio of heights of the low-field and mid-field resonances was also observed. Corresponding spectral changes were found with liposomes containing cholesterol. A study of the effect of varying membrane concentration, at fixed spin probe and antibiotic concentrations, indicated a decrease of the more immobile population when the lipid content was increased. When liposomes of EPC, with or without cholesterol, were labeled with CSL [which does not partition into water (Ernandes et al., 1976)], no spectral effects were obtained upon addition of aqueous antibiotic during or after dispersion of the lipids.

Studies in Solution. Experiments were performed in the absence of membranes in order to study possible interactions between the spin probe and amphotericin B in an aqueous medium. The EPR spectra of samples containing a fixed concentration of ASL and increasing concentrations of amphotericin B show an increase in the component due to immobilized ASL as a function of antibiotic concentration (Figure 6). Increasing the Me_2SO content caused the disappearance of the immobilized population and an increase in the spectral component due to ASL free in solution. Similar effects were noted for 5-SASL.

Discussion

Addition of Antibiotic to Aqueous Medium. When amphotericin B was added to the aqueous medium, at concen-

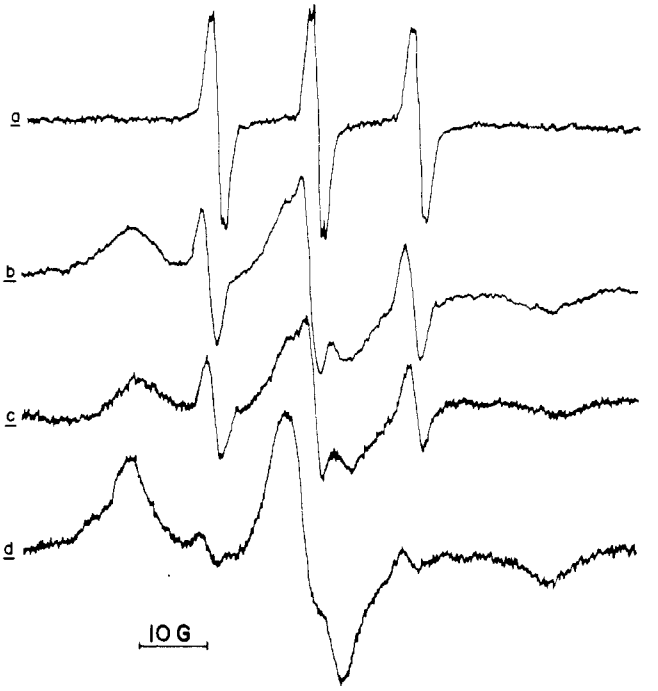

FIGURE 6: EPR spectra of ASL (8×10^{-5} M) in a solution containing Me_2SO (20% v/v) (a) and after addition of 5×10^{-4} M (b), 10^{-3} M (c), and 2×10^{-3} M (d) amphotericin B (in Me_2SO, 20% v/v).

trations where large changes in permeability have been measured (de Kruijff et al., 1974a; Singer, 1975; Aracava et al., 1981), no effects on the spectrum of CSL were noted, in agreement with Flick et al. (1976). Ohki and co-workers (Ohki et al., 1979) observed no changes in the spectra of spin-labeled phospholipids when the antibiotic was added to EPC liposomes of varying cholesterol content. In contrast, electron microscopic studies of liposomes (Sessa and Weissmann, 1967) and of vesicles (Bittman et al., 1974) indicated particle enlargement upon addition of amphotericin B to EPC–cholesterol. Gel chromatography and proton NMR data (Gent & Prestegard, 1976) have been interpreted as indicating vesicle destruction and formation of extended bilayers in the presence of the antibiotic at polyene:cholesterol ratios above 1:1.

The latter two studies deal mainly with changes in macroscopic organization. Why are no corresponding changes seen at the molecular level? The most frequently accepted mechanism of action of amphotericin B is formation of aqueous channels consisting of either two half pores (Finkelstein & Holz, 1973; Andreoli, 1974; de Kruijff & Demel, 1974) or one half pore (Marty & Finkelstein, 1975; van Hoogevest & de Kruijff, 1978). The pore has been proposed to contain cholesterol and to have a radius of ca. 0.4 nm (eight–ten molecules of amphotericin B in each monolayer). Molar ratios of cholesterol to antibiotic in the complex of 3.9, 3.3, and 0.7 have been proposed (Norman et al., 1972; de Kruijff et al., 1974a,b; de Kruijff & Demel, 1974). However, the data of van Hoogevest & de Kruijff (1978) indicate that only one molecule of polyene binds per 5000 cholesterol molecules. Holz & Finkelstein (1970) estimated 5×10^9 pores per cm^2. Assuming that eight cholesterol molecules are involved in each pore, on each side of the membrane, we calculate that in the present systems only one out of every 400 cholesterol molecules interacts with the antibiotic. Thus, upon addition of amphotericin B to the aqueous phase, the EPR spectral effects might occur on a very small scale and not be detected in the response of the entire ensemble of membrane components. Optical spectra of the polyenes undergo changes upon interaction with

Table I: Percent Variation of the Order Parameter (ΔS) and of the b/c Ratio [$\Delta(b/c)$] Resulting from the Incorporation of Amphotericin B in Planar Multibilayers of EPC and DPPC Containing Variable Cholesterol Concentrations[a]

	mol % sterol	ΔS theor[b]	ΔS exptl	$\Delta(b/c)$ theor[b]	$\Delta(b/c)$ exptl
EPC	0		−5.6		−10.9
	15	−4.5	−11.4	−4.6	−39.2
	30	−2.0	−10.1	−0.8	−47.5
	50	−0.6	−6.0	+0.7	−34.0
DPPC	0				+36.2
	15			−16.3	−32.2
	30			−0.7	−41.2
	50			+1.2	−31.2

[a] The sterol:antibiotic mole ratios are given in the text and in the captions of Figures 3 and 5. [b] ΔS and $\Delta(b/c)$ theoretical were calculated as described in the text.

membranes; this indicates that a significant portion of the former participates in the interaction (Bolard et al., 1980), although differences observed with and without sterol were small (Bittman et al., 1974).

Addition of Antibiotic during Membrane Preparation. Flick et al. (1976) observed changes in the spectra of CSL in EPC multibilayers containing cholesterol and amphotericin B. Similar results were obtained in the present investigation for planar multibilayers when amphotericin B was added during membrane preparation (Figures 1–4). Although the amphotericin content of these systems is much higher than that required to induce permeability changes in membranes, the present conclusions at least yield insight into the nature of the amphotericin–membrane interaction.

Our spectral analysis was performed in terms of the order parameter, and the ratio b/c (Figure 1). The order parameter is a measure of the degree of lipid organization *within* the bilayer (Schreier et al., 1978). The b/c ratio can also reflect this property (Neal et al., 1976) (compare the control curves of Figures 2 and 3). The b/c ratio is, however, much more sensitive than S to macroscopic disordering or the presence of other phases. The decreased b/c ratio on going from 30 to 50% cholesterol in Figures 3 and 4 is probably due to exceeding the solubility limit for cholesterol in EPC and DPPC (Lapper et al., 1972). For the samples containing amphotericin B, large differences in behavior are observed: S increases with increasing cholesterol content, while b/c decreases and reaches a minimum value at 30 mol % sterol.

Figure 1 reveals a component with a peak at a field lower than that for the usual $I = +1$ line in the spectrum taken with the field parallel to the bilayer normal. If this component were due to a wide distribution of orientations of the probe long molecular axis within the bilayer, the variation of b/c should parallel that of S (Neal et al., 1976). This component could arise from sample imperfections such as a small population of liposomes formed from bilayers removed from the cell wall, or from the presence of a new phase (Schreier–Muccillo et al., 1973a). Since the extra resonances persisted in the spectra of the drained cells, the latter explanation is more probable. The angular independence of this extra spectrum implies a high degree of disorder and immobility in the new phase. The spectrum of the probe in such a phase would affect the apparent b/c parameter to a larger extent than it would the order parameter.

Table I gives the percent variation of S and b/c [ΔS and $\Delta(b/c)$ experimental]. The changes in b/c greatly exceed those of S, in agreement with the coexistence of more than one phase. The changes caused by amphotericin B are largest for 30 mol % cholesterol. The 15 and 30 mol % cholesterol samples of EPC contained, per 100 mol of phospholipid, 12.6 and 37.9 mol of cholesterol, respectively, and 5.0 mol of a (hypothetical) 1:1 antibiotic–sterol complex. If such a complex were responsible for the decrease in S and b/c, one would expect the largest effect to be for the 15 mol % cholesterol sample. The largest effect on b/c occurs at 30 mol % sterol, suggesting a cholesterol:antibiotic ratio in the complex greater than 1:1.

Amphotericin B also alters the structure of sterol-free membranes (Table I). With DPPC alone, an increase in b/c is observed. A similar effect has been observed with cholesterol (Schreier-Muccillo et al., 1973b), carcinogenic aromatic hydrocarbons (Sanioto & Schreier, 1975), and cholesterol hydroperoxides (Schreier, 1978). It is suggested that all these compounds act upon gel phase phospholipids as impurities, decreasing the degree of cooperativity, and changing the tilt angle of the DPPC molecules with respect to the bilayer normal (Ladbrooke et al., 1968).

Our results are in basic agreement with those of Flick et al. (1976), who studied the effect of nystatin and amphotericin B on EPC–cholesterol systems. However, our data do not support the models for a highly structured pore that have been proposed for the polyene–sterol complex. If complex formation with antibiotic reduced the cholesterol content of the bilayer, a lower degree of ordering would result in this phase, as observed. However, if the spin probe also senses the complex, and if the components of the complex were organized with their long molecular axes parallel to the bilayer normal, the EPR spectrum of the extra component would be strongly angular dependent, which it is not.

Our results do provide evidence for the presence of a second phase, which is disordered and immobile relative to the bilayers, on incorporation of amphotericin B.

Oehlschlager & Laks (1980) examined the effect of amphotericin B on EPC–ergosterol membranes, using spin-labeled phospholipids. For 8.1 and 15 mol % ergosterol, S was essentially unaffected up to an amphotericin B:ergosterol molar ratio of 0.7:1. Thereafter, S decreased to values smaller than those obtained for EPC alone. The results were interpreted as due to amphotericin B residing at the bilayer surface at low antibiotic:ergosterol ratios, and in disordering complexes at ratios higher than 0.7:1. The results agree with the present work for the higher amphotericin B concentrations where a strong disordering effect is observed. We take the lower values of S obtained by Oehlschlager & Laks (1980) as an indication that the labeled phospholipid is probing the new phase we have detected in our experiments. The lack of change in S at amphotericin B:ergosterol ratios lower than 0.7:1 is not in agreement with the decreases in S and b/c obtained in our studies at similar ratios. A possible source of this difference is a lesser sensitivity of the flexible phospholipid probes to the membrane-perturbing effects of amphotericin B.

One of the arguments in favor of a pore mechanism was based on the size selectivity of permeating species. Amphotericin B caused small ions such as K^+ to leak readily from EPC–cholesterol liposomes, while glucose permeated much more slowly (de Kruijff et al., 1974a). However, similar phenomena occur during the phase transition of phospholipids, in the absence of amphotericin B. Papahadjopoulos et al. (1973) observed that the permeability at the phase transition is higher than that above or below the transition temperature and that the leakage of $^{22}Na^+$ is faster than that of [^{14}C]sucrose. The enhanced permeability at the phase transition could be due either to microscopic regions of disorder in the membrane plane or to boundaries between discrete coexistent solid

and liquid-crystalline domains. The extra phase detected in the present study could give rise to the same type of discontinuities. Electron microscopy of biological membranes evince particle segregtion caused by the antibiotic (Nozawa et al., 1974; Meyer, 1979).

Studies with ASL. EPC liposomes containing ASL as the only sterol yield, in the presence of aqueous solutions of amphotericin B, spectra displaying a component due to immobilized probe (Figure 5). ASL is detectably soluble in water (Ernandes et al., 1976). Interactions are possible between the probe and the antibiotic aggregates known to form in the aqueous phase (Bolard et al., 1980). If the ASL–antibiotic interaction were taking place in the membrane, increasing cholesterol should compete for the antibiotic and reduce this interaction. This was not the case. If the interaction occurred in the aqueous phase, increasing the membrane concentration would decrease the effect, due to decreased partitioning of the probe into water, as observed.

Additional evidence for an interaction *outside* the membrane comes from studies in the absence of membrane. An immobilized population appeared upon adding amphotericin B to an aqueous solution of ASL (Figure 6). This population disappeared gradually with increasing amounts of Me_2SO, which dissolves, and thereby disaggregates, the antibiotic. The interaction between polyene antibiotics and sterols in aqueous solution has been investigated (Bittman & Fischkoff, 1972; Norman et al., 1972; Bittman et al., 1974).

The intensity of the component due to immobilized ASL increases with time (Figure 6). This could be due to a continuous shift due to a slow equilibrium whereby ASL is continually removed from water into amphotericin B aggregates. In the presence of membranes, ASL could be slowly removed from the lipid phase into the amphotericin aggregates. We suggest that the results obtained by Ohki and co-workers (Ohki et al., 1979), using a sterol spin probe similar to ASL, are also due to interaction between the probe and amphotericin B aggregates outside the membrane. Chen & Bittman (1977) concluded from fast kinetic studies that it is the monomeric antibiotic that associates first with the membrane. However, if the equilibrium between monomer and aggregate is achieved slowly, this conclusion may not be valid. Bolard et al. (1980) have also observed time-dependent effects in their studies of the amphotericin B–membrane interaction.

A reviewer has pointed out that the effects observed with ASL and sterol-free membranes could be due to formation of a complex between ASL and membrane-bound amphotericin. The lack of competition of cholesterol with ASL in the complex argues against this possibility, as does the absence of such effects with the water-insoluble probe CSL.

Conclusions

When amphotericin B is added to membranes from the aqueous phase, no spectral effects are observed under conditions that cause large changes in permeability. It is suggested that the interaction between membrane and antibiotic occurs to too slight an extent to be detected by spectroscopic techniques sampling the entire ensemble of membrane components.

When amphotericin B is added during membrane preparation, the EPR spectra of spin probes indicate the appearance of a new phase which also contains phospholipid. This phase yields an angular-independent spectral component, in disagreement with the presence of a highly structured pore. The mechanism of action of the antibiotic may involve discontinuities at phase boundaries.

When a probe is finitely soluble in water and an aggregate other than the membrane is present, as in the case of ASL and amphotericin B, caution should be exercised in the spectral analysis since the observed effects may not be due to events occurring in the membrane.

Further studies should focus on the relationship between the state of aggregation of the antibiotic and its mechanism of action, and on the rates of attainment of the possible equilibria for the antibiotic in water, between water and membrane, and in the membrane.

Acknowledgments

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Characterization of a Third Phase Transition in Multilamellar Dipalmitoyllecithin Liposomes†

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ABSTRACT: The thermotropism of dipalmitoyllecithin in fully hydrated multilamellar dispersions has been reexamined by differential scanning calorimetry, X-ray diffraction, and ^{31}P nuclear magnetic resonance. Apart from the well-known pretransition and main transition, there exists a third transition at about 11 °C with a transition enthalpy of approximately 3.7 kcal/mol. Both adjoining phases are lamellar, but they differ in the lateral acyl chain packing of the lecithin molecules and in the dynamics of the polar head groups. The kinetics of this third phase transition are extremely slow in comparison with those of the other two transitions.

Different lamellar phases are spontaneously formed by pure synthetic phospholipids in aqueous dispersions. Their structural and dynamic properties have been investigated in detail (Tardieu et al., 1973; Janiak et al., 1976; Inoko & Mitsui, 1978; Seelig, 1977, 1978; Davis, 1979; Cameron et al., 1980; Marsh, 1980; Kawato et al., 1977), since phospholipid bilayers are believed to be a basic structure of many biomembranes. Within the context of liquid-crystal physics, purely physicochemical reasons have also promoted the study of lipid polymorphism.

For dipalmitoyllecithin (DPL)[1] and other structurally related lipids in excess water, two thermotropic phase transitions are well characterized by their thermodynamic (Ladbrooke & Chapman, 1969; Jacobson & Papahadjopoulos, 1975; Mabrey & Sturtevant, 1976; Albon & Sturtevant, 1978) and kinetic parameters (Tsong & Kanehisa, 1977; Lentz et al., 1978; Teissie, 1979). These are the so-called "main transition" between a "liquid-crystalline" phase and a "gel" phase at T_m = 41 °C (DPL) and the "pretransition" between two gel phases at T_p = 35 °C (DPL). On a molecular level, the main transition is better understood (Marcelja, 1974; Jähnig, 1979) than the pretransition, the nature of which is still under discussion.

This paper describes another thermotropic phase transition between two gel phases at T_s = 11 °C (DPL). It will be referred to as the "subtransition" in line with the nomenclature of a recent DSC study (Chen et al., 1980).

Materials and Methods

Commercially available DPL (obtained from Fluka, Buchs, Switzerland) was chromatographed on CM-cellulose (Comfurius & Zwaal, 1977) and recrystallized twice from acetone. Its purity was checked by TLC and ^1H and ^{31}P NMR. The homogeneity of the fatty acids was assured by cleaving them and by analyzing their methyl ester derivatives with gas chromatography (Eibl & Lands, 1970).

Sample Preparation. Multilamellar liposomes were prepared by incubating the dry lipid in doubly distilled water (ca. 10 mg/mL) at 45 °C for 1–2 h. During the incubation time, the dispersion was vortexed several times for about 1 min at the elevated temperature. The liposomes were then centrifuged at low *g* forces (1000–15000*g*, 10 min, 4 °C) to give lipid concentrations in the pellet between 30% and 60% (w/w).

Calorimetry. A differential scanning calorimeter (Perkin-Elmer DSC 2 with Intracooler I) was used for the calorimetric measurements. The sample pans (stainless steel, hermetically sealed) usually contained 8–12 mg of the liposomal pellet; an appropriate amount of water was taken as reference. Each sample was scanned several times at a heating/cooling rate of 0.313 °C/min and at a sensitivity of 1 mcal/s (full scale).

The lipid content of the samples was determined gravimetrically after completion of the measurements. Also, comparison of the initial and final total weights of the sample pans ensured that no water loss had occurred.

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[1] Abbreviations used: DPL, dipalmitoyllecithin; DSC, differential scanning calorimetry; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; FID, free induction decay.

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