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INTERACTION OF AMPHOTERICIN B WITH MEMBRANE LIPIDS AS VIEWED BY ^{2}H-NMR *

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The effects of amphotericin B upon the organization and dynamics of multibilayer membranes of dimyristoylphosphatidylcholine (DMPC) were investigated by means of ^{2}H-NMR. At high amphotericin B concentrations (30 mol% with respect to the lipid) and at temperatures above 25°C, DMPC experiences two different environments which are in slow exchange on the ^{2}H-NMR time scale. In one of these, the lipid is immobilized by the antibiotic, in a molar ratio of approximately 1:1, whereas the lipid unsequestered by amphotericin B is more ordered than in its pure state. This ordering effect is perceived at relatively low antibiotic doses (4%). The local lipid order, and the relative percentage, of sequestered DMPC, are temperature-independent (up to 65°C), whereas the ordering of the unsequestered lipid domain is not. The perturbation induced by amphotericin B is manifest similarly at the edges as well as in the center of the bilayer. Antibiotic addition leads to large decreases in the transverse relaxation time, T_2, of the labelled lipid, but not in the spin-lattice relaxation time, T_1. This indicates an increased density of slow motional modes and little change in rapid motions.

Introduction

Polyene antibiotics are known to mediate changes in the membrane permeability of a number of organisms, thus inducing a leakage of important cellular constituents and ultimately lysis and death of the cell [1–3]. The principal characteristic of these antibiotics is that they apparently require the presence of sterols in the cell membrane to promote such an effect [4–6]. Permeability and calorimetry data on liposomes led to the conclusion that polyene antibiotics and sterols formed molecular complexes to create channels or disrupt the membrane (Refs. 7–11; for a review see Ref. 12). Amphotericin B, the only such antibiotic for which both chemical structure and absolute configuration are known [13,14], has received particular attention. Andreoli [7], and De Kruijff and Demel [10], have proposed that amphotericin B and cholesterol form an 8:8 molecular complex, spanning the membrane vertically and creating a pore of maximum diameter approx. 8Å.

Because the permeability and calorimetric studies mentioned above did not demonstrate the pore structure directly, other methods such as electron spin resonance (ESR) or circular dichroism (CD) have been used to gain insight, at the molecular level, on the effective geometry of the amphotericin B-cholesterol complex. Okhi et al. [15] observed little change in the order parameter, as

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measured by ESR, of PC spin probes in model membranes containing cholesterol upon addition of amphotericin B. Conversely, Aracava et al. [33] found evidence for an immobilized state of a cholestane spin probe in egg phosphatidylcholine in the presence of amphotericin B. Oeshlschlager and Laks [16] studied the interaction between amphotericin B and ergosterol in egg PC using PC spin probes. They concluded that the complex amphotericin B-ergosterol could exist in the 1 : 2 as well as 1 : 1 (molar) forms. Bolard et al. [17] using CD techniques on the Ampho B/cholesterol/DMPC system also invoked multiple forms of the Ampho B-cholesterol complex (1 : 2, 1 : 1, etc.). Although there is evidence that amphotericin B induces permeability changes only when sterols are present in the membrane, there is still no unequivocal demonstration of the postulated pore geometry or its lifetime.

Deuterium (^{2}H) solid state-NMR has proven to be a useful nonperturbing technique to study the organization and dynamics of biological systems [18,19]. We have therefore used it to investigate the possible structure and dynamics of the proposed pore. The results presented herein deal with the action of amphotericin B on pure lipid systems, whereas its effects on cholesterol-containing membranes are reported elsewhere [20].

Materials and Methods

1-Myristoyl-2-[^{2}H]myristoyl-*sn*-glycero-3-phosphocholine (DMPC) was synthesized from 1-myristoyl-*sn*-glycero-3-phosphocholine (lyso DMPC, Sigma, St. Louis, MO, U.S.A.). according to reported procedures [21,22]. Amphotericin B was purchased from Sigma. Due to the lack of solubility of amphotericin B in the usual organic solvents, multilamellar membranes were prepared as follows. DMPC in methanol/chloroform (1 : 2, v : v), and amphotericin B in dimethylsulfoxide (DMSO) were mixed together and the solvent removed under vacuum at -10°C. The resulting residue appeared as a gel and was dispersed in 5 ml of distilled water. The mixture was vortexed, cooled and warmed ($\leqslant 40$°C) several times and finally lyophilized overnight to give a yellow powder. This cycle was repeated once. The lyophilization step was necessary to obtain reproducible samples. The sample was hydrated with deuterium-depleted water, agitated on a vortex mixer and freeze-thawed until reproducible ^{2}H-NMR spectra were obtained. The samples were protected from light throughout the entire procedure and frozen at -20°C if not used immediately.

The stability of amphotericin B was checked by ultraviolet spectroscopy on either freshly made or one month old samples. The model membranes were lyophilized and a small quantity was weighed accurately and diluted in DMSO. Comparison was made with a standard of pure amphotericin B in DMSO at the same concentration. Over a period of one month there was no detectable change in either extinction coefficient or shape of the ultraviolet spectrum of the sample.

For consistency, all membranes were prepared similarly, even if they did not contain the drug. The samples containing amphotericin B had a pH of approx. 6.5. The pH was found to be unchanged after the NMR experiment.

Nuclear magnetic resonance spectroscopy and data treatment were accomplished as described elsewhere [21]. The frequency of the spectrometer was carefully set at the center of the quadrupolar powder patterns. The spectra were folded about their centers (symmetrized) in order to increase the signal-to-noise ratio by a factor of $\sqrt{2}$. However, symmetrized and unsymmetrized spectra were compared to ensure that no distortions had been introduced by the folding procedure (Fig. 1b).

Results and Discussion

Ordering effect of amphotericin B on pure lipids

Deuterium powder patterns for a sample containing amphotericin B and [4′-$^2H_2$]DMPC (3 : 7 molar ratio), obtained between 5°C and 60°C, are compared in Fig. 1 with the spectra of pure [4′-$^2H_2$]DMPC at the same temperatures. At low temperatures (5°C), the spectra of lipids with and without amphotericin B are almost identical. They exhibit the spectral shape characteristic of the so-called gel phase of the pure lipid with a maximum spectral width of approx. 120 kHz. On increasing the temperature above 23°C (the gel to liquid-crystalline phase transition temperature, T_c), the spectra of the pure lipid show well-defined

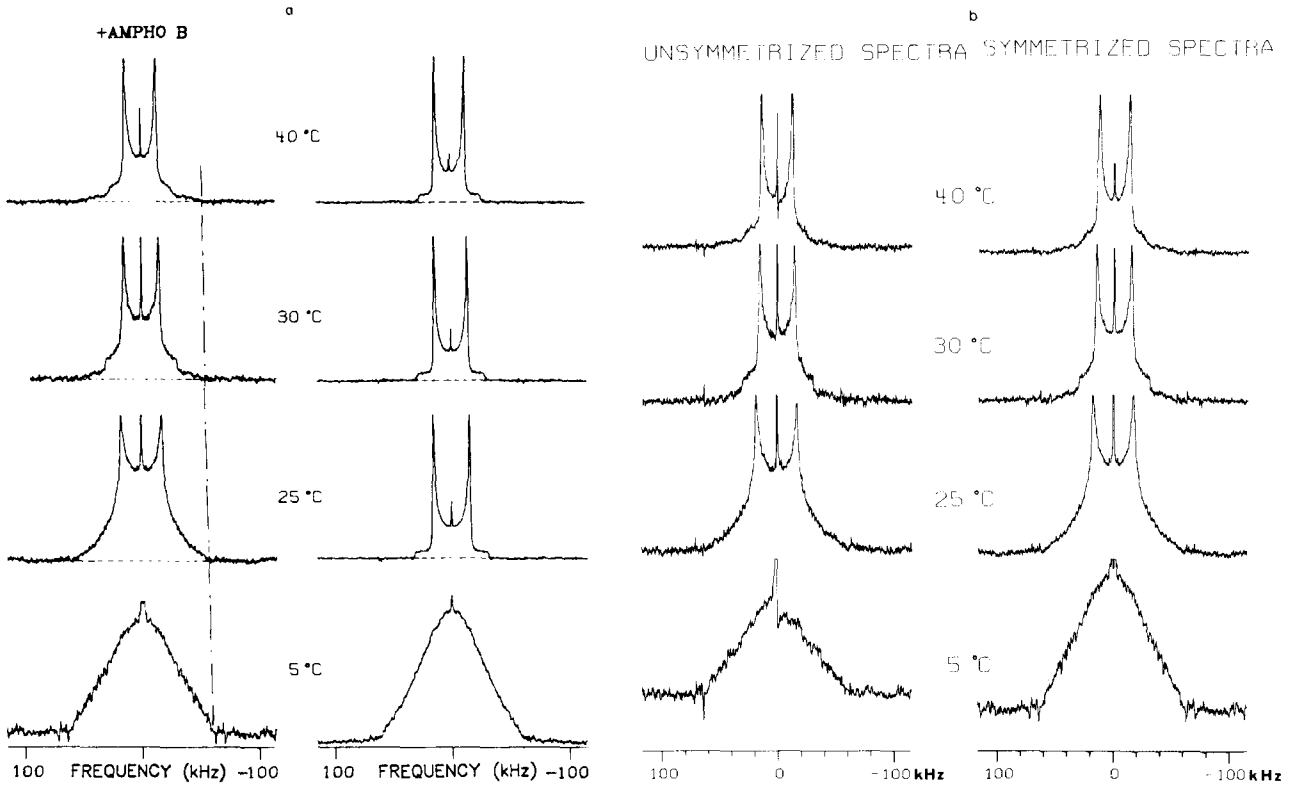

Fig. 1. (a) ^{2}H-NMR quadrupole echo spectra of [4'-$^2H_2$]DMPC multilamellar membranes in the presence or absence of amphotericin B (30 mol%), as a function of temperature. Experimental parameters: $\pi/2$ pulse length 4 μs; pulse spacing 60 μs; recycle time 100 ms; spectral window 25 kHz; 240000 accumulations. (b) Spectra taken in the presence of amphotericin B (Ampho B), as in (a), but showing the influence of folding (symmetrizing) the spectra.

axially symmetric shapes indicative of a lipid in only one average environment. In contrast, those of the sample containing amphotericin B exhibit, in addition to the usual quadrupolar doublet, a broad spectral feature of maximum width approx. 120 kHz. The relative percentages of these two components can be estimated via either spectral simulations or the spectral moments. The major component which appears to be axially symmetric above 25°C was simulated using the quadrupolar splitting of the sharp peaks and a constant Lorentzian linewidth. The simulated spectrum was then subtracted from the total experimental spectrum and the remaining area was integrated and scaled according to the total area of the experimental spectrum. In an alternative approach, the first moment of the total spectrum, M_1^T, can be expressed as [23]:

$$M_1^T = aM_1^{Ax} + (1-a)M_1^{Im} \quad (1)$$

where M_1^{Ax} represents the first moment of the axially symmetric powder pattern (major component in the spectra above 25°C) and can be estimated from the quadrupolar splitting $\Delta\nu_Q$: $M_1^{Ax} = (2/3\sqrt{3})(2\pi)\Delta\nu_Q$ [24]. In Eqn. 1 M_1^{Im} represents the first moment of the broad component and was taken to be the value of the experimental first moment at 5°C and a is the relative amount of the axially symmetric powder pattern. Above 25°C, the relative amount of the broad component estimated from both methods is $30 \pm 6\%$, and invariant to increasing temperature.

The presence of the broad component at all temperatures above 25°C indicates that 30% of the lipids are 'immobilized' by the antibiotic. The temperature-independence of this interaction is indicative of very strong binding. The word 'immobilized' means that, on the time scale of the quadrupolar interaction there are no fluctuations of the C-^{2}H bonds with respect to the molecular

long axis. This can be described in two ways. The chains are 'frozen' in the all-*trans* configuration, the only remaining motion being the rotational diffusion around the long axis of the extended chain. This situation is achieved when $\Delta\nu_{max} = \frac{3}{4} A_Q = 127.5$ kHz (the maximum spectral width is defined as the quadrupolar splitting produced by molecules whose axis of motion is oriented at 0° with respect to the magnetic field direction), where $A_Q = 170$ kHz is the static quadrupolar coupling constant for methylene C-^{2}H segments [25]. The other possibility is that the observed width of the broad component is representative of molecules undergoing no significant motion on the ^{2}H-NMR time scale. This situation would give a $\Delta\nu_{max}$ of 255 kHz (or a $\Delta\nu_Q$ of 127.5 kHz for C-^{2}H bonds oriented at 90° with respect to the magnetic field direction). There is no evidence in the spectra for intensity beyond 130 kHz, although this can be difficult to detect if T_2 values are very short. In view of the similarity in size and amphipathic properties of DMPC and amphotericin B, it seems more likely that one can expect some motion for the antibiotic and therefore the antibiotic-lipid complex, about its long axis, as in the former possibility. Furthermore, the lipids which are not 'immobilized' by amphotericin B manifest larger quadrupolar splittings than in the absence of antibiotic, at corresponding temperatures. Fig. 2 shows

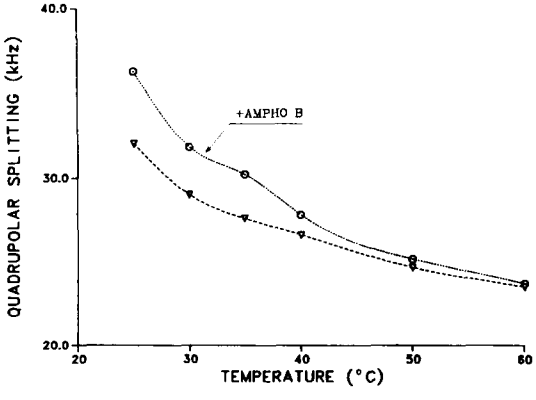

Fig. 2. Temperature dependence of the quadrupolar splittings of DMPC labeled at C4' in the presence (○) or absence (△) of amphotericin B (Ampho B). The quadrupolar splitting in the presence of amphotericin B is that of the 'major' component (see text). The symbols give an estimate of the error.

the temperature variation of $\Delta\nu_Q$ for the 'mobile' lipids, with and without amphotericin B. Assuming that the C-^{2}H units of the lipid (70%) which is not immobilized by the antibiotic have the same average orientations with respect to the director of the motion as those of the pure lipid, one can conclude that from 25°C to 40°C amphotericin B induces ordering in the 'mobile' lipid. However, this ordering action is no longer perceived at high temperatures; above 50°C the lipids of the amphotericin B-containing membranes face two very different physical environments: in one of these domains the lipids are strongly coupled with the antibiotic whereas in the other they are as free as in a pure lipid system. It is interesting to notice that these two domains behave differently with temperature: one of them is temperature-dependent, that is, its local ordering is modulated by temperature, whereas the other is not.

Variation of the Delta 2 parameter with temperature

The Δ_2 parameter has been shown to be sensitive to sample inhomogeneities such as the coexistence of phases [23,24,26]. It has been used for instance to determine the temperature of the gel-to-liquid crystalline phase transition of *Acholeplasma laidlawii* membranes enriched in deuterated oleic acid [27]. This parameter gives the relative mean-square width of the distribution of quadrupolar splittings. A plot of Δ_2 versus temperature is shown in Fig. 3 for [4'-$^2H_2$]DMPC, in the presence and absence of amphotericin B. A system having a unique $\Delta\nu_Q$, that is a single order parameter, gives $\Delta_2 = 0$ (assuming linewidth effects can be ignored). Therefore, any non-zero value of Δ_2 would be indicative of a distribution of order parameters. One notices in Fig. 3 that the Δ_2 value of pure DMPC is virtually zero above 23°C. On cooling pure DMPC below 23°C, Δ_2 undergoes a marked increase due to the appearance of a gel phase whose ordering properties are different from those of the liquid-crystalline phase. The presence of a maximum is due to the coexistence of both phases in almost equal amounts at the same temperature, and hence a maximum distribution of order parameters. It should be mentioned that in the gel phase, around 20°C, the linewidth might play a role in the increase of Δ_2, but this contribution will be at least one order of magnitude lower

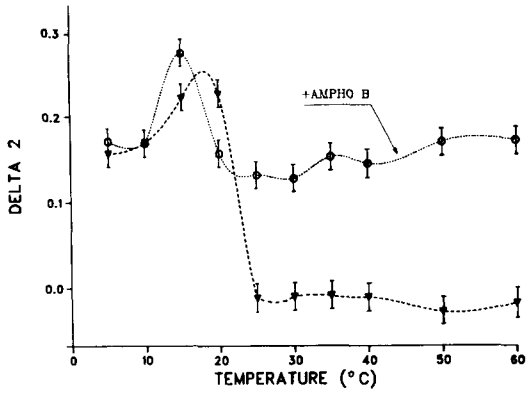

Fig. 3. Temperature variation of the Δ_2 parameter of [4′-$^2H_2$]DMPC powder spectra in the presence or absence of amphotericin (Ampho B). The bars give an estimate of the error.

than that of the distribution of quadrupolar splittings [32]. At low temperatures the Δ_2 profile of the amphotericin B-containing lipid behaves similarly to that of the pure lipid at low temperatures. This correlates well with the finding that at 5°C the gel phase spectra of DMPC with and without amphotericin B were almost identical (Fig. 1). The maximum in Δ_2 of the antibiotic-containing system is shifted towards lower temperatures, Fig. 3, relative to that of the pure lipid. However, the discontinuity is less marked in the presence of amphotericin B. The Δ_2 of the lipid-antibiotic system never reaches zero when the temperature is increased well beyond that of the maximum. This is mainly due to the fact that at high temperatures this system is not homogeneous; it is composed of two domains whose ordering properties are very different. However, the presence of a maximum in the Δ_2 profile of the system plus amphotericin B, indicates that the lipid unsequestered by the antibiotic still undergoes a phase transition, albeit some 5–10 deg. C below the temperature of that of the pure lipid.

Dependence of spectral changes on amphotericin B concentration

Fig. 4 shows the change in the ^{2}H-NMR spectra of DMPC on addition of amphotericin B, at 25°C. Increasing the concentration of antibiotic leads to a progressive loss of the shape characteristic of axially symmetric motion in the liquid-crystalline phase of the pure lipid. When the amphotericin B : DMPC ratio reaches 3 : 7 the axially symmetric shape is lost completely. Although there are several possible explanations for the shape of the bottom spectrum of Fig. 4, it is reasonable to expect that it is the result of the two lipid domains with spectral overlaps such as to mask resolution of individual patterns. The end result would thus be a 'continuum' spectrum from the broad spectral component of maximum width of approx. 120 kHz to the possibly axially symmetric powder pattern of $\Delta\nu_Q$ = 36.3 kHz (Fig. 4, bottom). The quadrupolar splitting of the spectrum for the sample with low amphotericin B concentration (3 : 70) is 33.4 kHz, an increase of approx. 5% relative to the $\Delta\nu_Q$ of the pure lipid at 25°C. Thus, even at low concentrations, the polyene antibiotic orders the membrane lipids detectably. Unfortunately, the low S/N ratio of the central spectrum of Fig. 4

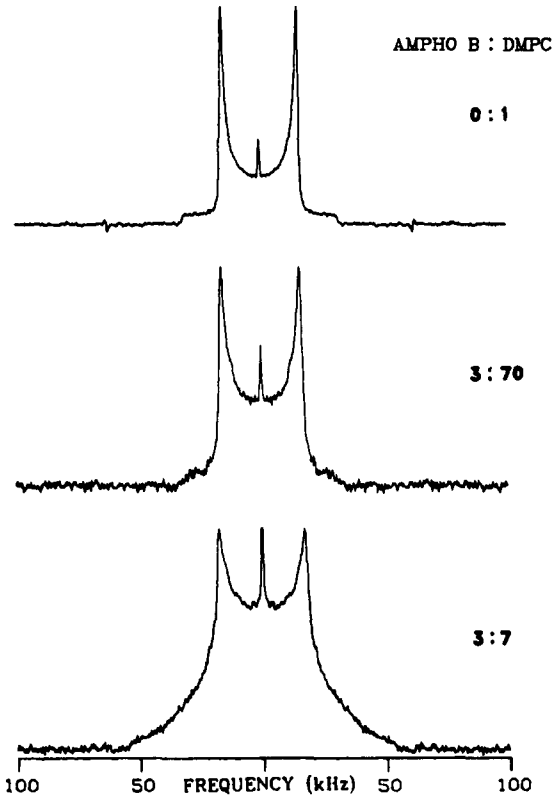

Fig. 4. Effect of amphotericin B (Ampho B) concentration on the ^{2}H-NMR spectral shapes of DMPC labeled at C4′, at 25°C. Same experimental parameters as in Fig. 1.

does not allow a positive statement regarding the existence of an 'immobilized' lipid domain at low antibiotic concentrations.

Dependence of antibiotic-induced perturbation on bilayer depth

In order to monitor the ordering and sequestering action of amphotericin B at several bilayer depths, the system previously described (30% amphotericin B in DMPC) was prepared using a DMPC whose *sn*-2 fatty acyl chain was perdeuterated ([*sn*-2-$^2H_{27}$]DMPC). In view of the inequivalence of the *sn*-1 and *sn*-2 chains [28] only one acyl chain was labelled to simplify the analysis. Fig. 5 shows the resulting spectra for [*sn*-2-$^2H_{27}$]DMPC with and without amphotericin B at 35°C. One notices the presence of the 120 kHz broad spectral feature on the bottom spectrum of Fig. 5, which has already been attributed to the 'immobilized' lipid. The DePaked [29] analogs of the spectra of Fig. 5 are shown in Fig. 6; the

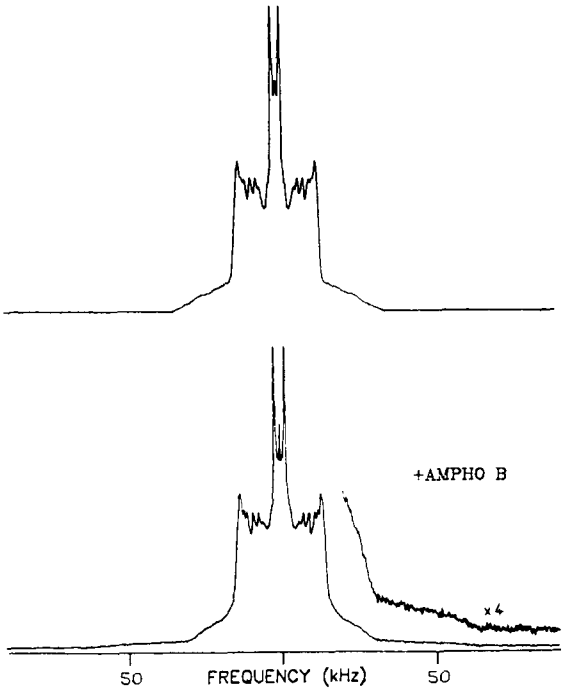

Fig. 5. ^{2}H-NMR spectra of [*sn*-2-$^2H_{27}$]DMPC in the presence or absence of amphotericin B (30 mol% Ampho B) at 35°C. Same experimental parameters as in Fig. 1 except recycle time of 1 s and spectral window of 500 kHz.

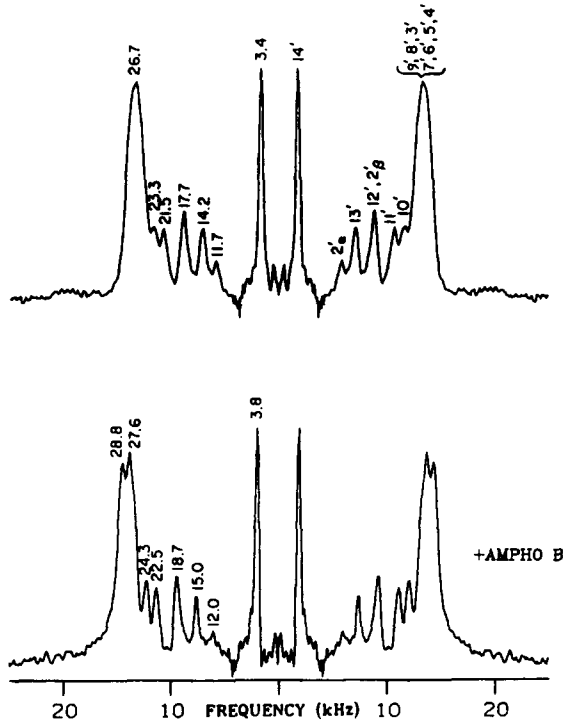

Fig. 6. Same as Fig. 5, but DePaked [29]: processing parameters, spectral deconvolution on 600 points, 3 iterations; the numbers are the quadrupolar splittings in kHz, primed numbers indicate carbon atom assignments (see text). Ampho, B amphotericin B.

numbers represent the values of the quadrupolar splittings, $\Delta\nu_Q$ (in kHz), and the primed numbers designate the labelled carbon positions. Assignment of individual splittings was made by analogy with those reported for specifically deuterated DMPC [30]. The shapes of the DePaked spectra in the presence and absence of amphotericin B are very similar. The $\Delta\nu_Q$ values are increased by 5–10% for all positions. Assuming that this increase is monotonic for all positions, that is, there is no inversion in the labeled carbon assignment, one can conclude that the entire $S_{C\text{-}^2H}$ order parameter profile is shifted towards higher ordering by 5–10%. In other words, a similar perturbation is induced by the antibiotic at all positions of the lipid chains. This is the behaviour expected for the influence of an amphotericin B : lipid complex in which the acyl chains are all-*trans* and the only available motion is rotation about the long molecular axis.

Relaxation time measurements

Using standard techniques described elsewhere [31], spin-lattice, T_{1z}, and transverse, T_2, relaxation times have been measured for [4′-$^2H_2$]DMPC in the presence and absence of the antibiotic, at 25°C. The T_{1z} values were 18.6 ± 0.5 ms and 19.1 ± 1.0 ms in the absence and presence of amphotericin B (30% in DMPC), respectively. Within the experimental error, the T_{1z} were orientationally independent in both cases; the reported relaxation times were constant across the powder pattern. The 'mobile' and 'immobilized' lipids apparently had the same T_{1z}. The spin-lattice relaxation time reported for the sample containing amphotericin B is that of the entire powder pattern. The echo amplitude decay was found to be monoexponential. The T_2 values exhibit anisotropy across the powder pattern; however, since there is, to our knowledge, no simple and reliable interpretation of such behaviour, the reported T_2 values stand for the averages across the powder spectra. The powder-average transverse relaxation time of the lipid has a value of 280 ± 30 μs in the absence of the drug and 140 ± 20 μs in its presence. Thus, T_2 of the lipid is altered by the presence of the antibiotic whereas T_{1z} is not. Since T_2 is much more sensitive to slow motions than T_{1z} [18], the effect of amphotericin B on membrane lipid may be due to reduced rates of the slow motions (lateral diffusion, director fluctuations, slow tumbling of the entire vesicle, etc), or to exchange between antibiotic-associated and non-associated lipid. The changes in T_2 are reminiscent of those for lipid systems containing protein [27,30].

Conclusion

When added to pure lipid membranes, amphotericin B has an overall ordering effect on the lipid fatty acyl chains. The presence of a broad spectral component of approx. 120 kHz width, even at high temperatures, indicates that 30% of the lipids (relative area of the broad component) no longer undergo angular fluctuations to time-average the quadrupolar interaction. The temperature variation of the Δ_2 parameter indicates that the amphotericin/lipid system undergoes a phase transition some 5–10 deg. C below that of the pure DMPC. Since increasing the temperature above 25°C affects neither the maximum spectral width nor the relative percentage of the broad spectral component, it seems reasonable to think that the observed phase transition occurs for only the mobile 70% of total lipid. It appears, therefore, that at temperatures higher than 25°C amphotericin B is in a lipid-aggregated form, in which the lipid is all-*trans*, and for which the only motion observable by ^{2}H-NMR is rotation about the long molecular axis. The drug : lipid ratio in the aggregate has to be approximately 1 : 1. At high temperatures, these aggregates are embedded in a lipid matrix whose properties are similar to those of the pure lipid system.

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