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AMPHOTERICIN AND MODEL MEMBRANES

THE EFFECT OF AMPHOTERICIN B ON CHOLESTEROL-CONTAINING SYSTEMS AS VIEWED BY ^{2}H-NMR *

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(1) The interactions between amphotericin B and sterol-containing model membranes were monitored by ^{2}H-NMR of deuterium-labelled dimyristoylphosphatidylcholine (DMPC), cholesterol and epicholesterol. (2) The addition of amphotericin B to a cholesterol/DMPC (3:7) system was perceived differently by the lipid, depending upon the depth in the bilayer: no structural change was manifest in the acyl chain region associated with the plateau in molecular ordering (C4′), whereas the lipid clearly senses two environments near the center of the bilayer (C13′, C14′). The amount as well as the ordering properties of the more ordered antibiotic-induced component, sensed at C14′, increased with decreasing temperature. (3) The structural parameters of deuterium-labelled cholesterol in cholesterol/DMPC mixtures were unchanged upon addition of amphotericin B, regardless of the bilayer depth. (4) Upon addition of amphotericin B, the lipid T_1 values are unchanged, whereas the T_2 values are reduced by a factor of 2. The minimum in T_1 observed for cholesterol in DMPC at 32–35°C was shifted towards 38–40°C in the presence of amphotericin B. (5) Epicholesterol-containing dispersions of DMPC had properties similar to those of their cholesterol-containing analogs; a noticeable difference between the systems was an approx. 10% increase in the segmental order parameters on the addition of amphotericin B to the system containing the α-isomer of cholesterol. (6) The concept of a dynamic complexation between amphotericin B and sterol is discussed.

Introduction

Deuterium nuclear magnetic resonance (^{2}H-NMR) has recently been used to show that both Δ^5-cholesten-3β-ol (cholesterol) [1] and the polyene antibiotic amphotericin B [2] have finite effects on the structure and dynamics of model membranes of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC). Cholesterol acts as a regulatory agent with respect to the amplitude of the C-^{2}H angular fluctuations of the lipid fatty acyl chains: it orders the lipid above the transition temperature, T_c, of the pure lipid membrane and disorders it below T_c. The α-isomer of cholesterol possesses some of these regulatory properties and shows, in addition, a tilted configuration with respect to the director of motion (the bilayer normal), which the β-isomer does not. On the other hand, the antibiotic 'immobilizes' about 30% of the lipids when present at 30 mol% with respect to DMPC, at temperatures above T_c. The interaction between this immobilized portion of the lipids and the antibiotic is so strong that neither the width of the spectral fea-

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Abbreviation: DMPC, dimyristoylphosphatidylcholine.

ture representing these lipids nor its percentage with respect to the total lipid changes with temperature.

In the present study, polyene antibiotic-cholesterol-lipid interactions were followed using both deuterium-enriched lipids and sterols as reporter molecules.

Materials and Methods

We have reported elsewhere on the synthesis of deuterated materials [1,3], preparation of model membranes [2], and ^{2}H-NMR spectroscopy and spectral analyses [1,4]. In order to compare the results with those of our previous studies [1,2], the samples always contained 30 mol% sterol with respect to the lipid; the antibiotic, when present, was in equimolar amounts with the sterol.

Results

1. Lipid viewpoint

Action of amphotericin B on cholesterol-containing DMPC membranes. Deuterium spectra of model membranes of cholesterol/[4′-$^2H_2$]DMPC in the presence and absence of the antibiotic were obtained between 0 and 55 °C. Fig. 1 shows some of these spectra. The temperature dependence of $\Delta\nu_Q$, the separation between the two most intense features of the powder patterns in Fig. 1, is almost identical for both cholesterol/DMPC and cholesterol/DMPC/amphotericin B systems (not shown). The quadrupolar splittings of both systems differ only at low (approx. 5 °C) or high (approx. 55 °C) temperatures where the $\Delta\nu_Q$ of the system containing amphotericin B is slightly higher. At low temperatures (0 and 5 °C) the spectra show evidence of axially asymmetric motion. The appearance of such shapes has already been interpreted, for the system lacking amphotericin B, in terms of a reduction in the rate of motional averaging around the long axis of both the fatty acyl chains and the cholesterol molecules [1]. The nature of this motional reduction appears to be unaffected by the presence of the polyene antibiotic.

A similar study of the center of the bilayer, using [14′-$^2H_3$]DMPC, yields the spectra shown in Fig. 2. The antibiotic has a marked effect on the

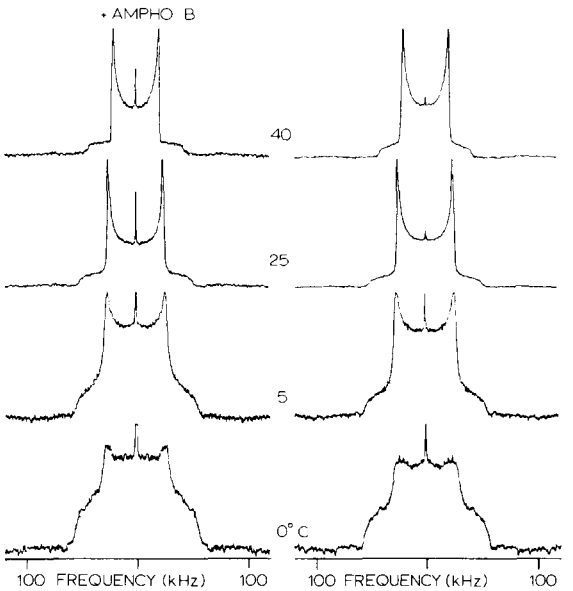

Fig. 1. ^{2}H-NMR spectra of the β-cholesterol: [4′-$^2H_2$]DMPC system in the presence or absence of amphotericin B (Ampho B). Experimental parameters were: π/2 pulse-length, 4 μs; pulse spacing, 60 μs; recycle time, 100 ms; spectral window, 250 kHz, and 30000 accumulations.

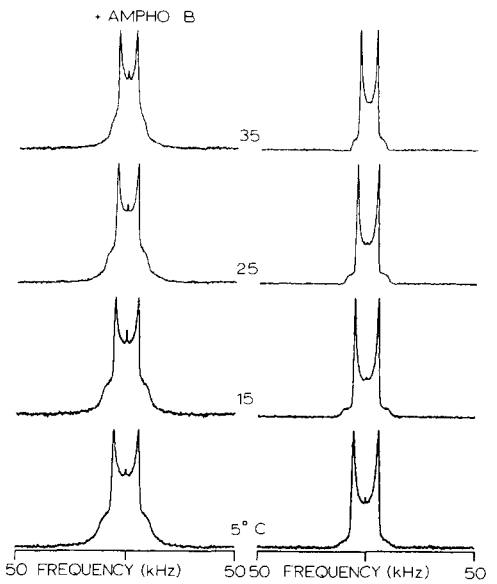

Fig. 2. ^{2}H-NMR spectra of the cholesterol/[14′-$^2H_3$]DMPC system in the presence or absence of amphotericin B (Ampho B). Same experimental parameters as in Fig. 1, except recycle time of 1 s and 5000 accumulations.

spectra of the labelled lipid. A close inspection of Fig. 2 reveals that a second spectral component appears below the shoulders of the major powder pattern in the spectra of the samples containing amphotericin B. The relative areas of these two spectral components may be estimated by simulating the major powder pattern and subtracting it from the experimental spectrum. The remaining spectral feature is thus that of the broad component whose area is integrated and scaled according to the total experimental spectral area. An example of such an analysis is shown in Fig. 3. The simplest model was chosen to simulate the major spectral component obtained for the amphotericin B-containing system, that is, assuming a Lorentzian lineshape and angular-independent linewidth for the orientational components of the powder pattern. Although the method is intrinsically inaccurate, since it is model-dependent, one can rely on the relative integrated areas within ±8%. This procedure yields a broad component with 28% of the total area at 5 °C, and 10% at 55 °C. Despite the inaccuracy involved in estimating the areas, it is clear that the broad component is favoured at low temperatures. From the difference spectrum of Fig. 3, the maximum spectral width of the broad

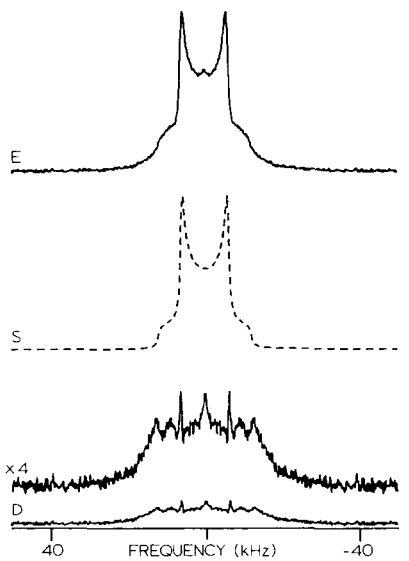

Fig. 3. Determination of the area of the broad component (see text) in the spectra in Fig. 2. E, Experimental spectrum; S, simulated spectrum of the 'major' component (see text); D = E − S, broad component spectrum.

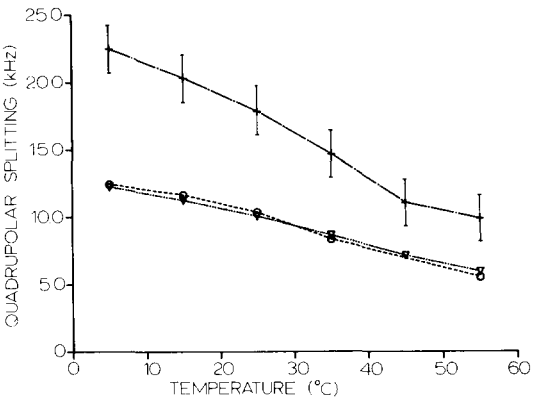

Fig. 4. Temperature variation of the quadrupolar splittings of DMPC labelled at C14'. ▽, DMPC/β-cholesterol/amphotericin B (major component); +, DMPC/β-cholesterol/amphotericin B (broad component), scaled ×0.5; ○, DMPC/β-cholesterol. The bars or symbols give an estimate of the error.

component can be measured. For comparison with the temperature dependence of $\Delta\nu_Q$ for spectra with and without the antibiotic, this measured maximum spectral width was scaled by 0.5, Fig. 4.

If this broad spectral feature represented an 'immobilized' lipid, that is, if the acyl chains were all *trans* and only rotating around the long molecular axis, the maximum observed splitting at that labelled position can be calculated from:

$$\Delta\nu = \tfrac{3}{32}A_Q(3\cos^2\theta' - 1)\overline{(3\cos^2\alpha - 1)}\,\overline{(3\cos^2\gamma_1 - 1)} \times \overline{(3\cos^2\gamma_2 - 1)} \quad (1)$$

with $A_Q = 170$ kHz, $\theta' = 0°$, $\alpha = 0°$, $\gamma_1 = 35.25°$ and $\gamma_2 = 109.5°$, that is, $\Delta\nu_{max} = 42.5$ kHz or $\Delta\nu_Q$ $(\theta' = 90°) = 21.25$ kHz. Eqn. 1 is simply an extension of that derived by Stockton et al. [5] for methyl groups using the formalism of Petersen and Chan [6]. In Eqn. 1, all the coordinate transformations from the principal axis system of the C-^{2}H bond to the axis system linked to the static magnetic field direction were taken to be axially symmetric. It is also assumed that the methyl group rotates around its C_3 axis, i.e., the angle γ_2 transforms from the C-^{2}H principal axis system to the C13-C14 bond axis system. The angle γ_1 represents the transformation from the C13-C14 bond direction to the axis of segmental motion of the methyl unit, α accounts for the angular fluctuations of

that axis of segmental motion with respect to the bilayer normal, and θ' transforms from the bilayer normal axis system to the static magnetic field direction. Such a formalism has proven useful for comparison of segmental order parameters ($\frac{1}{2}\overline{(3\cos^2\alpha - 1)}$) for methyl and methylene groups in fatty acyl chains. From Fig. 4 it can therefore be concluded that the broad component is representative of a lipid in an environment such that there are essentially no angular fluctuations of the methyl group below 25 °C, whereas above that temperature, some angular fluctuations take place. Fig. 4 summarizes in some aspects the action of amphotericin B on cholesterol-containing lipids, at the center of the bilayer. When the drug is present, the lipid methyl group senses two environments: in one of them, its angular fluctuations are highly restricted, whereas in the other, these fluctuations are identical to those of the sample without the drug. It is worthwhile to point out that the percentage as well as the motional restrictions of the so-called broad component decrease on raising the temperature. The latter result is not surprising, since the sterol-antibiotic complex is expected to dissociate as the temperature is elevated.

In order to monitor simultaneously the effect of amphotericin B at several bilayer depths,the above experiments were repeated using DMPC perdeuterated in the acyl chain at the *sn*-2 position. Fig. 5 shows the DePaked [7] spectra with and without the antibiotic, at 25 °C. The unprimed numbers on the DePaked spectra represent $\Delta\nu_Q$ in kHz, whereas the primed numbers stand for the labelled carbon position (assigned by analogy with previously published results [8]). At first sight, there are no changes between DePaked spectra arising from samples with and without amphotericin B. However, a more detailed consideration shows that the resonances of C-^{2}H bonds at C14′ and C13′ make a smaller contribution to the total spectral intensity when the drug is present. This observation correlates well with the fact that a second broad component was observed at C14′ but not at C4′ (vide supra). The area at C14′ is partitioned between the major and broad components; therefore, the major peak at C14′, in the presence of antibiotic, will be less intense relative to those of the plateau positions (C4′ to C10′) (the DePaked broad component cannot be reasonably detected in Fig.

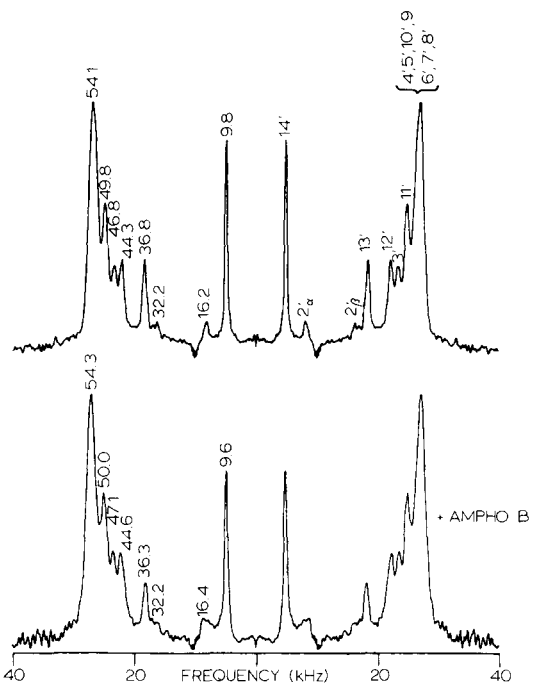

Fig. 5. DePaked ^{2}H-NMR spectra of the [*sn*-2-$^2H_{27}$]DMPC/cholesterol system in the presence or absence of amphotericin B (Ampho B) at 25 °C. Same experimental parameters as in Fig. 1, except spectral window of 500 kHz and recycle time of 1 s. Processing parameters: spectra deconvolution on 600 points, three iterations.

5). Since peaks associated with the plateau positions in dePaked spectra of samples with and without the antibiotic are scaled accordingly, the C14′ peaks appear less intense in the presence of the drug than in its absence. Similarly, the peaks for the C13′ position in the lower spectrum of Fig. 5 appear less intense than the corresponding peaks in the top spectrum; this leads to the conclusion that a broad component appears also at C13′. One should mention here that the linewidths in the spectra with and without amphotericin B are different (vide infra); however, the relative change is similar for both the C4′ and C14′ positions, suggesting that the comparison between relative intensities is reasonable.

It is also interesting to note that the quadrupole splittings at C14′ and C13′ are slightly smaller in the presence of the drug than in its absence, whereas for all other splittings, the converse is true. It appears therefore that although amphotericin B induces small spectral changes on the

lipid deuterium powder spectra of cholesterol-containing membrane lipids, these changes are different in the plateau and central regions of the bilayer.

Relaxation time measurements. The spin-lattice, T_1, and transverse, T_2, relaxation times were measured as described elsewhere [3,9] for lipid labelled at positions C4′ and C14′ for the DMPC/cholesterol system, in the presence or absence of amphotericin B. The results are summarized in Table I. T_1 and T_2 of pure DMPC at C4′ have also been reported in Table I for comparison. At 25 °C, the T_1 values at C4′ are nearly identical for the pure DMPC, DMPC/cholesterol or DMPC/cholesterol/amphotericin B systems; the same is true for the lipid methyl group, C14′. The T_2 values appear, however, to be more sensitive to the presence of amphotericin B. Although T_2 was found to vary across the powder pattern, the values reported in Table I were obtained using the total spectral area. To our knowledge, there is presently no reliable theory to describe variation of T_2 across the powder pattern. However, when there are marked changes in T_2 for different systems, one can discuss in a general way the dynamics involved in the transverse relaxation mechanism. On comparing the T_2 at C4′ and C14′ for systems with and without amphotericin B, one notices (Table I) that the transverse relaxation rates ($1/T_2$) are greatest in its presence. The broad component has a very large relaxation rate compared to that of C14′ in the absence of drug. As a general comment, one can thus state that the presence of amphotericin B in cholesterol/DMPC bilayers influences T_2 but not T_1; that is, the antibiotic modulates the slow motions of the lipids but has no effect on the fast motions (those occurring near the NMR observe frequency). Since the ordering properties are nearly identical for both the DMPC/cholesterol and the DMPC/cholesterol/amphotericin B model membrane systems, one may conclude that the principal effect of addition of amphotericin B is to increase the density of slow motions contributing to the lipid transverse relaxation rate.

Action of amphotericin B on DMPC membranes containing epicholesterol. From permeability studies [10] it has been concluded that the antibiotic induced greater leakage when cholesterol rather than epicholesterol was present in the membrane. This differential efficiency was attributed to the ability of the sterol OH group to form more hydrogen bonds with amphotericin B when located in the β- rather than the α-orientation with respect to the steroid skeleton. In order to verify this hypothesis, the experiments described for

TABLE I

RELAXATION TIMES [a] OF THE CHOLESTEROL/AMPHOTERICIN β/DMPC SYSTEM

System	Labelled carbon position	T_1 (ms)	T_2 [b] (μs)
DMPC	[4′-$^2H_2$]	18.6	280
Cholesterol/DMPC	[4′-$^2H_2$]	21.7	500
	[14′-$^2H_3$]	174	1180
Cholesterol/DMPC/amphotericin B	[4′-$^2H_2$]	20.2	264
	[14′-$^2H_3$] [c]	183	632
	[14′-$^2H_3$] [d]	158	282

[a] At 25 °C and 46.1 MHz. Accuracy ±5% on T_1 and ±10% on T_2.

[b] Estimated from the entire powder pattern.

[c] Major component (see text).

[d] Broad component (see text), accuracy approx. 10% in T_1 and approx. 20% in T_2.

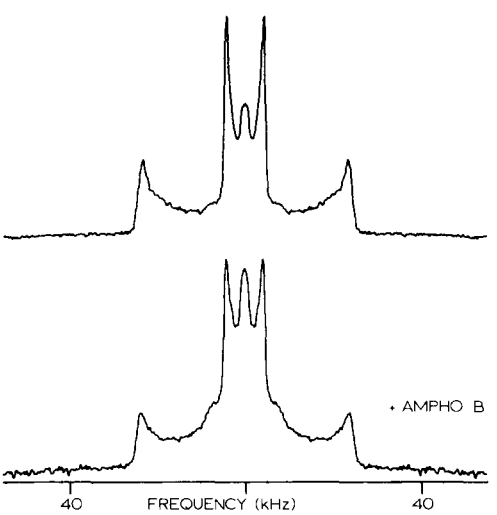

Fig. 6. ^{2}H-NMR spectra of the [4′,14′-$^2H_5$]DMPC/α-cholesterol system in the presence or absence of amphotericin B (Ampho B) at 25 °C. Same experimental parameters as in Fig. 5.

cholesterol were repeated with the α-isomer. The lipid behaviour in systems containing only the sterol, or both the drug and the sterol, was monitored simultaneously at both the plateau region and at the center of the bilayer by means of [4′, 14′-$^2H_5$]DMPC. Fig. 6 shows the spectra of this labelled lipid in the presence of epicholesterol and epicholesterol/amphotericin B (1 : 1 molar ratio), at 25 °C. The broad component found for the C14′ label in the DMPC/cholesterol/amphotericin B system is also present in the bottom spectrum of Fig. 6, whereas no additional component is detected for the C4′ label. The quadrupolar splittings associated with the lipid in these two systems (epicholesterol with and without the antibiotic) were measured at several temperatures and are reported in Table II. In this table, the $\Delta\nu_Q$ values of the DMPC/cholesterol/amphotericin B system have been included for comparison.

It is interesting to notice in Table II that amphotericin B acts so as to give rise to larger values of $\Delta\nu_Q$ for the DMPC/epicholesterol system, especially at C4′ and at high temperatures. Assuming that the average orientation of the C-^{2}H reporter bonds is the same in samples with and without amphotericin B, one can say that the antibiotic induces an increased ordering of the lipid in the DMPC/epicholesterol system. Interestingly, this ordering effect is such that, at high temperatures, the lipid at C4′ in the DMPC/epicholesterol/amphotericin B system almost reaches the same value as in the corresponding system containing the natural cholesterol isomer. Since it has been shown already that epicholesterol induces less ordering of the lipid than does cholesterol, at temperatures above 23 °C [1], it appears that amphotericin B provides some of this 'missing' ordering to the epicholesterol-containing lipid. At C14′, the situation is even more complex. Amphoterican B orders the major lipid component weakly and restricts highly the angular fluctuations of a certain percentage of the lipid, previously described as being the C14′ 'broad component'. The percentage of that broad component, as well as its spectral width, were found to decrease when the temperature of the system was increased. It is interesting that the antibiotic induces similar effects on lipid membranes containing either of the sterols.

2. Sterol viewpoint

Action of amphotericin B on DMPC model membranes containing deuterium-labelled cholesterol. In the previous sections, the structural and dynamical information at the plateau level of the lipid bilayer was recorded by means of the [4′-$^2H_2$]DMPC. In order to follow the sterol properties at the same level, cholesterol labelled in the first two rings ([2, 2, 3, 4, 4, 6-^{2}H]cholesterol) was employed. Fig. 7 shows the deuterium spectra of cholesterol in DMPC in the presence and absence of antibiotic, at several temperatures. The central peak of the powder patterns has been deliberately truncated in order to enhance details of the overall spectral

TABLE II

LIPID QUADRUPOLAR SPLITTINGS[a] IN THE EPICHOLESTEROL/AMPHOTERICIN B/DMPC SYSTEM

System	Labelled carbon position	Temperature (°C)						
		10	20	25	30	35	45	55
Epicholesterol/DMPC	[4′-$^2H_2$]	53.7[b]	50.8	48.3	45.9	43.0	38.3	34.9
	[14′-$^2H_3$]	12.0[b]	10.3	9.3	8.3	7.3	5.9	5.1
Epicholesterol/DMPC/amphotericin B	[4′-$^2H_2$]	54.0[b]	51.7	47.0		45.0	41.0	37.1
	[14′-$^2H_3$][c]	11.7[b]	10.3	9.3		7.5	6.4	5.4
Cholesterol/DMPC/amphotericin B	[4′-$^2H_2$]	54.7[b]	52.4	51.0	49.0	47.0	42.6	38.8
	[14′-$^2H_3$][c]			10.0		8.6	7.1	5.9

[a] From DePaked spectra, in kHz, accuracy 1–2%.
[b] Estimated on the powder spectra, accuracy approx. 5%.
[c] Quadrupolar splitting of the major component (see text).

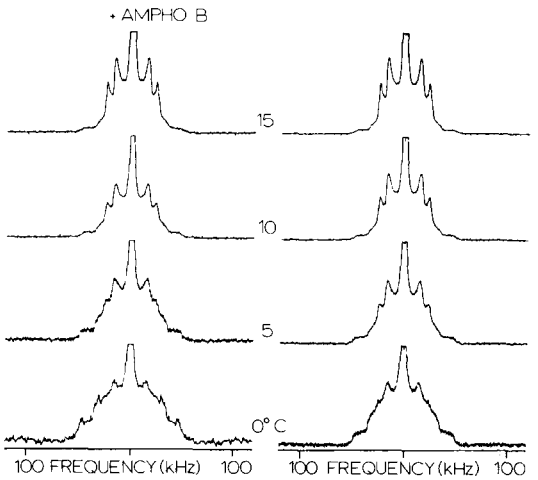

Fig. 7. Temperature dependence of the ^{2}H-NMR spectral shapes of [2,2,3,4,4,6-$^2H_6$]cholesterol in DMPC, in the presence or absence of amphotericin B (Ampho B). Same experimental parameters as in Fig. 1, except spectral window of 500 kHz, recycle time of 50 ms and 50000 accumulations.

shapes. Inspection of Fig. 7 reveals that the only noticeable changes in spectral shape occur at low temperatures (0 and 5 °C). It has been shown previously [1] that at these temperatures, similar changes in the spectral shape, observed for cholesterol in DMPC, could be explained by the onset of axially-asymmetric motions. The same phenomenon also occurs for cholesterol in the presence of the antibiotic. However, the loss of the axially symmetric lineshapes seems to occur at higher temperatures in the presence of amphotericin B than in its absence; one notices indeed that the spectral shape for cholesterol with amphotericin B at 5 °C is almost identical to that of the sterol alone at 0 °C. It appears therefore that the presence of amphotericin B in the model membrane restricts the axial motions of cholesterol at low temperatures.

At temperatures above 10 °C, the spectra with and without the drug do not exhibit marked dif-

TABLE III

QUADRUPOLAR SPLITTINGS [a] OF α- AND β-CHOLESTEROL

System	Labelled carbon [b] position	Temperature (°C)		
		25	40	65
β-Cholesterol/ DMPC	[6-^{2}H]	3.4	3.2	2.2
	[3-^{2}H]	51.5	50.8	46.4
	[2-^{2}H]$_{eq}$ [c]	34.2	33.6	30.3
	[4-$^2H_2$]$_{eq}$ [c]	32.0	31.4	30.3
	[2,4-$^2H_2$]$_{ax}$	48.2	47.0	43.4
β-Cholesterol/ DMPC/amphotericin B	[6-^{2}H]	3.6	3.3	3.0
	[3-^{2}H]	51.8	50.2	47.4
	[2-^{2}H]$_{eq}$ [c]	34.4	33.8	31.2
	[4-$^2H_2$]$_{eq}$ [c]	31.8	31.4	31.2
	[2,4-$^2H_2$]$_{ax}$	48.4	47.4	44.6
Epicholesterol/ DMPC	[6-^{2}H]	5.8	7.8	10.2
	[2-^{2}H]$_{ax}$	54.7	52.2	46.6
	[2-^{2}H]$_{eq}$	24.9	20.5	13.1
	[4-^{2}H]$_{ax}$	54.7	52.2	46.6
	[4-^{2}H]$_{eq}$	24.9	23.0	18.6
Epicholesterol/ DMPC/amphotericin B	[6-^{2}H]	4.2	4.9	7.5
	[2-^{2}H]$_{ax}$	54.8	54.2	49.3
	[2-^{2}H]$_{eq}$	26.4	24.2	19.0
	[4-^{2}H]$_{ax}$	54.8	54.2	49.3
	[4-^{2}H]$_{eq}$	26.4	24.2	19.0

[a] From DePaked spectra, in kHz, accuracy 1–2%.

[b] Assignment according to calculations described in ref. 1.

[c] The assignment of these deuterons is arbitrary and could be reversed.

ferences. The variation of the cholesterol quadrupolar splittings with temperature has been followed up to 65 °C. Some representative values are reported in Table III for systems with and without amphotericin B. One notices that the only significant differences in quadrupolar splitting occur at high temperatures, for example, at 65 °C where the sample containing the drug exhibits larger quadrupolar splittings than the system lacking it. The segmental (or molecular) order parameter of cholesterol was calculated for the sample containing amphotericin B, as described in Refs. 1 and 11, and compared with that of cholesterol in DMPC, without the antibiotic (Fig. 8). It appears in Fig. 8 that up to 40 °C, both order profiles are identical. The two profiles diverge at high temperatures, with the order parameter for cholesterol in the sample containing the drug slightly higher. This shows that amphotericin B extends the range of temperature-independent 'wobbling' of cholesterol [1] by some 20 °C, such that there is almost no change in the angular fluctuations of cholesterol from 10 to approx. 60 °C. The motional axis of the sterol, and thereby the orientation of the cholesterol molecule with respect to the bilayer normal, was calculated using the method described in Refs. 1 and 11. The orientation of cholesterol within the bilayer membrane was found to be identical for systems with and without the antibiotic, that is, if the complex formed between amphotericin B and cholesterol exists, it must be

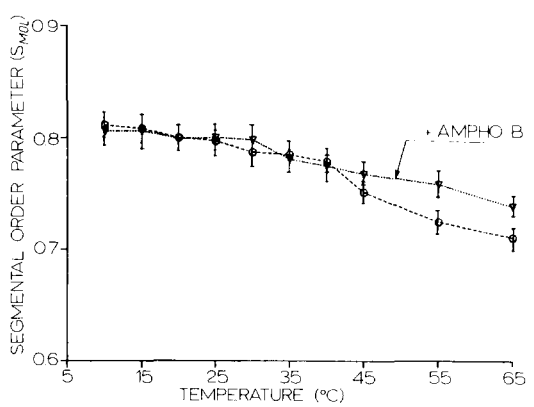

Fig. 8. Temperature dependence of the segmental order parameter, S_{mol}, of the four rings of cholesterol, in DMPC, in the presence and absence of amphotericin B (Ampho B).

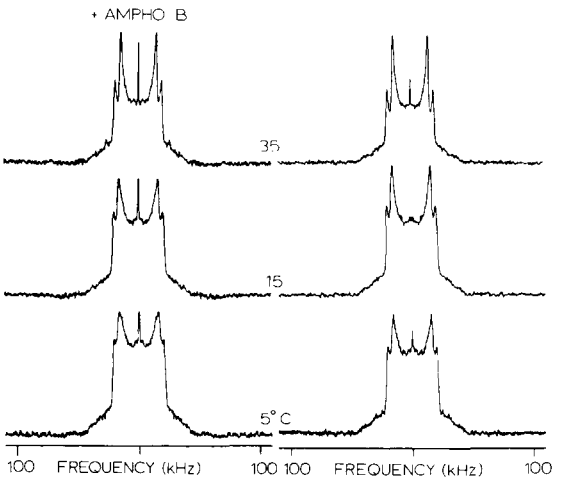

Fig. 9. ^{2}H-NMR spectra of [24-$^2H_2$]cholesterol, in DMPC, with and without amphotericin B (Ampho B). Same experimental parameters as in Fig. 7, except recycle time of 100 ms.

such that the sterol long axis is essentially normal to the membrane surface.

[24-$^2H_2$]Cholesterol and [26, 27-$^2H_6$]cholesterol were also used to monitor the influence of amphotericin B on cholesterol-containing model membranes. The spectra of these labelled sterols are shown in Fig. 9 and 10, respectively, as a function of temperature. The two superimposed powder patterns of Fig. 9 (without the amphotericin B) are the results of two different average orientations, with respect to the axis of motion, of the two C-^{2}H bonds at C24 [1]. Notice in Fig. 9 that the deuterium spectra, for samples with and without the antibiotic, are very similar. Table IV gives some representative values of quadrupolar

TABLE IV

QUADRUPOLAR SPLITTINGS[a] OF [24-$^2H_2$]CHOLESTEROL WITH AND WITHOUT AMPHOTERICIN B

System	Temperature (°C)		
	15	45	65
Cholesterol/DMPC	33.2	27.8	23.7
	42.0	38.1	34.4
Cholesterol/DMPC/amphotericin B	33.4	28.3	23.4
	42.2	38.1	34.0

[a] From DePaked spectra, in kHz, accuracy 1–2%.

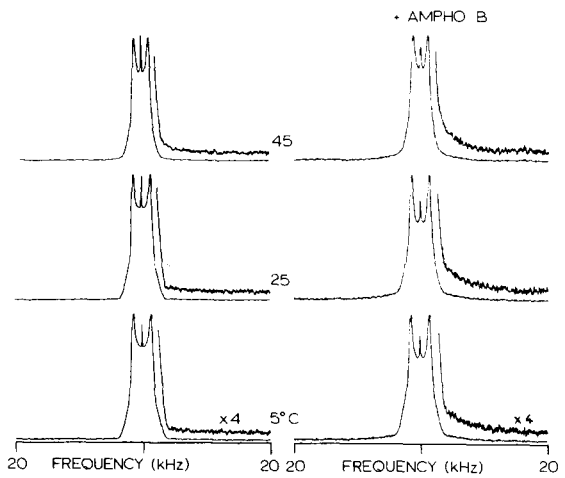

Fig. 10. ^{2}H-NMR spectra of [26,27-$^2H_6$]cholesterol, in DMPC, with and without amphotericin B (Ampho B). Same experimental parameters as in Fig. 1, except recycle time of 300 ms; spectral window of 125 kHz; and 5000 accumulations.

splittings for both sets of spectra. The presence of amphotericin B causes very small changes in $\Delta\nu_Q$. Fig. 10 exhibits the ^{2}H-NMR response of the two methyl groups (at C26 and C27) of cholesterol in DMPC, in the presence or absence of amphotericin B. Although the shapes of both spectral sets are very similar, the spectra of the sample containing the antibiotic show a broad component in addition to the major powder spectrum. Although this may be compared with the occurrence of a similar spectral feature in the spectra of [14'-$^2H_3$]DMPC for the same model membrane system (vide supra), the relative amount of the broad component (not more than 5%), at C26 and C27, is very small in comparison to that found at C14' in DMPC. The major component in ^{2}H-NMR spectra of the sample containing amphotericin B has values of $\Delta\nu_Q$ identical to those found for positions C26 and C27 in the absence of the antibiotic, at corresponding temperatures. One can therefore conclude that for the DMPC/cholesterol/amphotericin B model membrane system, the antibiotic has little effect on the ordering properties of the cholesterol tail.

Relaxation times, T_1, T_2. The spin-lattice, T_1, and transverse, T_2, relaxation times have been measured for cholesterol in DMPC containing amphotericin B and are reported in Table V. It is interesting to compare the T_1 of cholesterol in DMPC (Dufourc, E.J. and Smith, I.C.P., unpublished data) with those of Table I. The spin-lattice relaxation times for position C6 have been plotted in Fig. 11 for systems with and without amphotericin B. Although both sets of data yield a minimum in T_1, at which the effective correlation time, τ_{eff}, of the motion (assuming exponential autocorrelation functions) is equal to approx. $0.62(2\pi\nu_0)^{-1} = 2.1 \cdot 10^{-9}$ s, the minimum of T_1 is shifted towards higher temperatures in the presence of amphotericin B. The minimum occurs

TABLE V

RELAXATION TIMES [a] OF CHOLESTEROL AT 46.1 MHz

System	Labelled carbon position	T_1 (ms)					T_2 (μs)	
		15 °C	25 °C	35 °C	45 °C	60 °C	25 °C	25 °C
Cholesterol/DMPC/amphotericin B	[6-^{2}H]	5.8	3.8	3.3	3.4	4.7	200	260 [c]
	[3-^{2}H] [d]	9.3	6.4	6.6	6.7	10.1	200	260 [c]
	[2,4-$^2H_2$]$_{ax}$	9.2	6.0	5.6	6.2	8.3	200	260 [c]
	[2-^{2}H]$_{eq}$ [b]	7.1	4.5	4.1	4.1	6.0	200	260 [c]
	[4-^{2}H]$_{eq}$ [b]	7.1	4.6	4.0	4.4	6.0	200	260 [c]
Epicholesterol/DMPC/amphotericin B	[6-^{2}H]		4.4					
	[2,4-$^2H_2$]$_{ax}$		7.3					
	[2,4-$^2H_2$]$_{eq}$		5.4					

[a] T_1 in ms, accuracy ±5%; T_2 in μs, accuracy approx. 20%.
[b] The assignment of these positions is arbitrary and could be inverted.
[c] T_2 of cholesterol in DMPC, without amphotericin B.
[d] Accuracy approx. 10%.

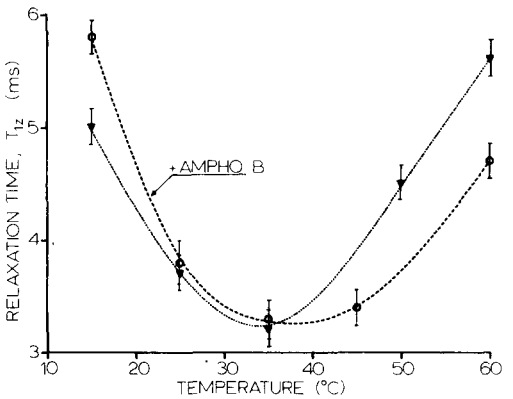

Fig. 11. Temperature dependence of the relaxation time, T_1, of [6-^{2}H]cholesterol in DMPC, in the presence or absence of amphotericin B (Ampho B). The bars give an estimate of the error.

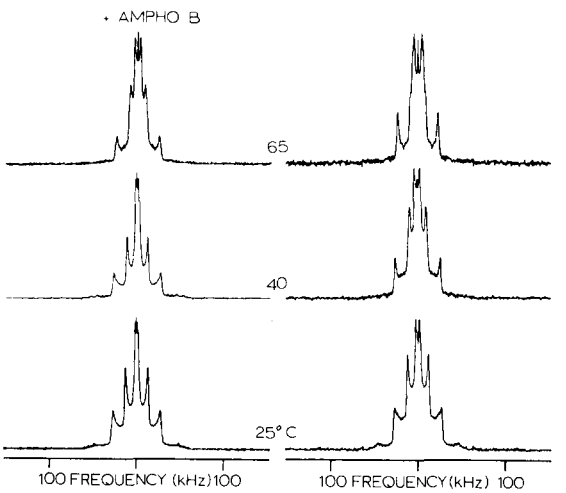

Fig. 12. ^{2}H-NMR spectra of [2,2,3,4,4,6-^{2}H]epicholesterol in DMPC, with and without amphotericin B (Ampho B). Same experimental parameters as in Fig. 7.

around 32–35 °C when antibiotic is absent, and around 38–40 °C when it is present. This indicates that the system containing antibiotic has to be warmed by approx. 5 °C in order to relax with the same efficiency as the system without the drug. In other words, the motions of cholesterol in DMPC are slowed down by the presence of amphotericin B. Unfortunately, the two minima are not separated sufficiently to allow a quantitative characterization of the motional reduction induced by amphotericin B.

The transverse relaxation time of cholesterol has also been measured with and without the antibiotic (Table V). It was impossible to measure T_2 for each deuteron (T_2 was found to vary across the powder patterns); the values reported in Table V stand for the entire spectrum of cholesterol. Unlike the results found for the deuterated lipids (vide supra), there is little change in the cholesterol T_2 on addition of amphotericin B.

Action of amphotericin B on DMPC model membranes containing epicholesterol. The experiments carried out with cholesterol labelled in the first two rings (A, B) (vide supra) were repeated with [2, 2, 3, 4, 4, 6-^{2}H]epicholesterol for temperatures between 20 and 65 °C. Some representative spectra are shown in Fig. 12.

The spectra of deuterated epicholesterol in DMPC, with and without amphotericin B, are quite similar at 25 °C, whereas they differ markedly at higher temperatures. Some representative values of $\Delta\nu_Q$ are reported in Table III for systems with and without the antibiotic. It has been shown [1] that the collapse of the central part of the powder pattern of epicholesterol in DMPC, at high temperatures, was due to the reorientation of the axis of motional averaging of epicholesterol within the bilayer membrane. This reorientation is characterized by a marked decrease of the quadrupolar splittings at the equatorial positions C2 and C4 with a concomitant increase of $\Delta\nu_Q$ at C6. The spectra of the antibiotic-containing sample show similar behaviour. However, it appears that the variation of splittings for the C2, C4 and C6 positions is less pronounced in the presence of amphotericin B, over the same temperature range; the antibiotic retards the temperature-induced reorientation of epicholesterol in DMPC.

The molecular order parameter of epicholesterol in the DMPC/epicholesterol/amphotericin B system was calculated according to the procedure described in Ref. 1. The presence of amphotericin B leads to an increase of 10% relative to the value in its absence [1], which persists over the temperature range 20–65 °C. This finding agrees well with the increase in the segmental order parameter of DMPC labelled at C4′, induced by the addition of amphotericin B to the

DMPC/epicholesterol model membrane system (vide supra).

Discussion

The interpretation of the ^{2}H-NMR data of systems composed of lipids, sterols and amphotericin B is not straightforward in terms of the existence of a cholesterol-polyene antibiotic complex. The authors postulating such a complex indicated that the antibiotic was segregating the sterol [12]. If such a situation occurred in the present model membrane system, two physical events would be detectable by ^{2}H-NMR: the first would be a decrease in the local order of the bulk lipid and the second would be the restoration of the lipid phase transition. It is easily understandable that if the antibiotic complexed the sterol tightly, the ordering effect of the latter on the lipid would be removed or modified and the lipids would be free from the sterol interaction and thus capable of undergoing a gel-to-liquid crystalline phase transition. ^{2}H-NMR results show clearly that none of these events occur with either epicholesterol or cholesterol in the presence of amphotericin B in DMPC. Therefore, if the complex exists, it has to be dynamic with respect to the ^{2}H-NMR timescale; that is, the cholesterol has to be in exchange between a site in which it is associated with the antibiotic (site A) and a site in which it is not (site NA) and thereby able to order the lipid. Prior to characterizing these sites, the existence of the cholesterol-amphotericin B complex needs to be clarified. The ^{2}H-NMR data obtained for the lipid indicate that DMPC is sensing only one environment at C4′ (the plateau level) and two different regions at C14′ (the center of the bilayer). It was shown earlier [2] that amphotericin B sequesters approx. 30% of the total lipid (when present at 30 mol%) in the absence of sterols, and that the width as well as the amount of the spectral feature representing the 'immobilized' lipid were temperature-independent. When the sterol is present, this broad spectral component was not detected at C4′, whereas at C14′, both the width and the amount of the observed broad component were diminished on raising the temperature. One must mention here that if the T_2 associated with a hypothetical broad component at C4′ were short compared to the delay between the two $\pi/2$ pulses used to form the quadrupolar spin-echo, this component might not be detected due to the intrinsic limitations of the echo technique. However, the moments and the intensities (scaled according to the measured T_2) of spectra at C4′ with and without amphotericin B were calculated and gave essentially the same figures, within the experimental error, at corresponding temperatures. Since the moments, especially the higher order moments, are sensitive to the presence of high frequencies relative to the center of the powder pattern, it therefore appears that there is no broad component for DMPC labelled at C4′. These findings indicate clearly that there is no region in the bilayer membrane where the antibiotic interacts freely with the DMPC when sterol is present. Furthermore, the shift in the T_1 minimum of cholesterol upon addition of amphotericin B indicates that the antibiotic slows down the motions of cholesterol with rates near the Larmor frequency. It seems reasonable that some form of amphotericin B-sterol 'complex' is present in these systems.

The ^{2}H-NMR results using labelled sterols showed that in the presence of amphotericin B, the sterols were sensing only one environment at the four-ring structure level, and possibly two regions at the terminal methyl groups of the sterol side-chain. These findings agree well with the results obtained from the labelled lipids and lead to the conclusion that the amphotericin B-sterol 'complex' has ordering properties which depend upon depth within the bilayer. As pointed out above, and in order to satisfy the experimental observations, the cholesterol molecule must be in exchange between two sites, namely sites A and NA. The data presented in sections 1 and 2 of Results suggest that site A and site NA possess the same ordering properties, at the plateau level, that is, the sites in which the cholesterol is associated with the antibiotic and freely interacts with the lipid are indistinguishable at the level of the rigid four-ring skeleton. Near the center of the bilayer, the situation appears different. The lipids sense clearly two environments at C14′ (and probably C13′) of the lipid, whereas at C24, the sterol does not perceive these different regions and shows only an indication of a very small (not more than 5%) second spectral component for C26 and C27. Cholesterol

was shown in a previous study [1] to be a 'rigid' structure up to carbons 22–24. The molecular or segmental order parameter of either the four-ring skeleton or the cholesterol tail (up to C24) was 0.7–0.8, which indicates that little motional averaging of the quadrupolar interaction is taking place. Bearing this in mind, one can think of cholesterol as a 'rigid cylinder', very insensitive to external ordering such as that possibly induced by amphotericin B. With this peculiar property, the cholesterol molecule could act as a 'screen', preventing the amphotericin B from 'seeing' the lipid, up to position 14′ (and 13′). This would explain why cholesterol appears to sense only one environment at the plateau level, as does DMPC at C4′, that of the site in which cholesterol is not associated with the amphotericin B (site NA). At the center of the bilayer, the two environments are differentiated. This differential regional sensitivity can be rationalized in two possible ways. Firstly, the terminal methyl groups of the cholesterol tail are considerably less 'rigid' than the rest of the molecule [1], and therefore amphotericin B can reduce their angular fluctuations as well as those of DMPC at C14′, when the sterol is in site A. Alternatively, the methyl groups of the cholesterol tail are almost as intrinsically 'rigid' as the rest of the molecule, but the length of cholesterol mismatches that of amphotericin B, thus allowing the antibiotic to restrict the motions of DMPC at C14′ when cholesterol is in site A. The latter possibility would explain why the second broad component is more important at C14′, on the lipid, than at C24 and C27, on the cholesterol tail. However, a definite answer cannot be given based on the available data.

The interactions of epicholesterol with amphotericin B may be described as for cholesterol, since the spectral features of model membranes containing either the α- or the β-isomer of cholesterol and the antibiotic were similar. The noticeable difference between systems containing epicholesterol or cholesterol resides in an approx. 10% increase in local order induced by amphotericin B in the model membrane containing epicholesterol, especially at the plateau level. It has to be remembered that there was no change in ordering, at the plateau level, of either the lipid or cholesterol when amphotericin B was added to the system. The ordering effect of amphotericin B is dependent on the configuration of the sterol OH group, suggesting that the antibiotic may attenuate the amplitude of the α-cholesterol 'wobbling' through a direct interaction with the hydroxyl group. The interaction of amphotericin B with epicholesterol might appear to contradict what has been found in the permeability studies: amphotericin B induced greater permeability when cholesterol, rather than epicholesterol, was present in the membrane. However, the structural nature of the sterol-antibiotic 'complex' may be different for the two sterols so that only the dynamic association between cholesterol and amphotericin B leads to a permeability change. This structural difference may be due to different sterol-antibiotic amphiphatic interactions depending on whether the sterol hydroxyl is α or β, or may result from the tilted configuration [1] of epicholesterol in the bilayer membrane.

It was shown that at 25 °C, the orientation of epicholesterol in DMPC was not modified by the addition of amphotericin B. The same conclusion was obtained for cholesterol. It appears therefore that the antibiotic molecule adopts essentially the orientation of the sterol within the bilayer. This could explain why the sterols are essential to the amphotericin B-induced permeability. Indeed, due to their character of 'rigid cylinders', the sterols may provide the framework of the antibiotic-sterol complex and thereby the orientation of the complex. The antibiotic, on the other hand, may play the role of conducting cement. However, it must be emphasized that the complex is, on the ^{2}H-NMR time-scale, dynamic. Its lifetime, which certainly depends on the strength of the sterol-polyene antibiotic interaction, cannot be estimated from the experiments presented herein; however, a measure of the rate of lateral diffusion of cholesterol in the bilayer could give an idea of its magnitude.

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