^{2}H NMR Evidence for Antibiotic-Induced Cholesterol Immobilization in Biological Model Membranes

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ABSTRACT: The interaction of the polyene antibiotic filipin with membrane sterols has been studied by deuterium nuclear magnetic resonance of the molecular probes [2,2,3,4,4,6-$^2H_6$]cholesterol and 1-myristoyl-2-[4′,4′,14′,14′,14′-$^2H_5$]myristoyl-*sn*-glycero-3-phosphocholine. At physiological temperatures, there is evidence of filipin-induced cholesterol immobilization in the membrane. The ^{2}H NMR spectra of cholesterol show two domains in which ordering and dynamics are very different. In one of these, cholesterol is static on the ^{2}H NMR time scale, whereas in the other it undergoes rapid axially symmetric motions similar to those it exhibits in the drug-free membrane; this indicates that the jumping frequency of cholesterol between the labile and immobilized domains is less than 10^5 s^{-1}. The distribution of cholesterol between these two sites is temperature dependent; at 0 °C all sterol molecules are immobilized, whereas at 60 °C they are almost totally in the labile site. In contrast to cholesterol, the phospholipids sense only one type of environment, at both the top and center of the bilayer, indicating that cholesterol acts as a screen, preventing the lipids from direct interaction with the antibiotic. At low temperature, the ordering of the lipid in the presence of cholesterol does not change upon filipin addition, whereas at elevated temperatures the local ordering of both the lipid and the labile cholesterol is significantly lower than that in the absence of the drug. Moreover, there is a very important difference between the degree of local ordering as measured by the lipids and by cholesterol at high temperatures. The orientation of cholesterol in the labile site has been found to be *perpendicular* to the bilayer plane. Although the orientation of cholesterol in the immobilized site cannot be calculated, it seems unlikely that a configuration of the complex in which filipin and cholesterol are *parallel* to the membrane surface [de Kruijff, B., & Demel, R. A. (1974) *Biochim. Biophys. Acta 339*, 57–70] would favor the sterol exchange between the two sites. The present data show that the interaction between filipin and cholesterol occurs on a time scale greater than 10^{-5} s; this should be related to our previous ^{2}H NMR observation that amphotericin B interacts with cholesterol on a time scale lower than 10^{-5} s [i.e., near the nanosecond scale; Dufourc, E. J., Smith, I. C. P., & Jarrell, H. C. (1984) *Biochim. Biophys. Acta 776*, 317–329].

In the early 1960s, evidence was found that polyene antibiotics could mediate changes in the cellular permeability of a number of organisms, thus promoting a leakage of important cellular constituents and ultimately lysis and death of the cell (Kinsky, 1961a,b). Permeability studies on model membranes (de Kruijff et al., 1974a,b) indicated that the presence of sterols within the membranes was required for the polyene antibiotics to produce permeability changes. Among the polyene antibiotics investigated, it was found that amphotericin B was size selective in the release of cytoplasmic components whereas filipin was not. On these grounds, de Kruijff & Demel (1974) proposed molecular models for complexes between sterols and amphotericin B or filipin.

In attempts to demonstrate the existence of such complexes, at the molecular level, physical methods such as ESR[1] (Okhi et al., 1979; Oehlschlager & Lacks, 1980; Aracava et al., 1981) and CD (Bolard et al., 1980) were used. The results showed that the complexes between filipin or amphotericin B and the sterol were not necessarily equimolar, as postulated by de Kruijff & Demel (1974), and that in certain cases no evidence existed for such complexes.

We have recently studied by ^{2}H NMR the action of amphotericin B on model membranes in the presence or absence of cholesterol (Dufourc et al., 1984a,b). It was observed that in the absence of cholesterol amphotericin B immobilizes lipids in a 1:1 molecular ratio and increases the degree of local order of the remaining lipids. When cholesterol is present in the membrane the above effect is absent and the evidence for a sterol–antibiotic "complex" is rather subtle. The most important effect of amphotericin B in this case is to reduce the rate of axial motion of cholesterol within the phospholipid membrane.

We report herein similar ^{2}H NMR studies using the polyene antibiotic filipin, which is known to induce rather dramatic effects on biological membranes. As we shall see, these effects are well monitored by ^{2}H NMR, which allows differentiation between the action of filipin and amphotericin B on cholesterol-containing membranes, at the molecular level.

Materials and Methods

[2,2,3,4,4,6-$^2H_6$]-β-Cholesterol was prepared as already described (Dufourc et al., 1984c). [4′,4′,14′,14′,14′-$^2H_5$]-DMPC was prepared by acylation of 1-myristoyl-*sn*-glycero-3-phosphocholine with [4,14-$^2H_5$]myristic acid anhydride, according to Perly et al. (1984). Deuterated myristic acid was a generous gift from Dr. H. C. Jarrell (NRCC, Ottawa, Canada). Filipin was kindly provided by the Upjohn Co.

[1] Abbreviations: ESR, electron spin resonance; CD, circular dichroism; ^{2}H NMR, deuterium nuclear magnetic resonance; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; UV, ultraviolet.

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(Kalamazoo, MI), and deuterium-depleted water was purchased from Aldrich Chemical Co. (Milwaukee, MN).

Model membranes were prepared as described below. DMPC and cholesterol were dissolved in chloroform–methanol (2:1 v/v). Filipin was dissolved in methanol and added to the DMPC–cholesterol solution, and the solvent was evaporated under vacuum at −20 °C. The residue was then dispersed in a few milliliters of distilled water and lyophilized overnight. The resulting fluffy powder was hydrated in excess deuterium-depleted water (1:2 w/w) and agitated on a vortex mixer. Samples were freeze-thawed and mixed until they appeared to be homogeneous and until reproducible ^2H NMR spectra were obtained. This procedure has been shown to yield large, multilamellar vesicles (Westman et al., 1982). The steps described above were followed even for samples without filipin. Samples containing the antibiotic were protected from light, and the polyene antibiotic stability was checked by UV spectroscopy as described for amphotericin B (Dufourc et al., 1984a).

^2H NMR data were acquired at 30.7 MHz on a home-built spectrometer operated by a Nicolet 1280 computer. NMR signals were obtained by means of a modified quadrupolar echo sequence (Dufourc et al., 1983); quadrature detection was used to record the echo signals. Experimental parameters are given in the figure captions. The frequency of the spectrometer was carefully set at the center of the quadrupolar powder patterns. The spectra were symmetrized in order to increase the signal to noise ratio by a factor $\sqrt{2}$. Folded and unfolded spectra were compared to ensure that no distortion had been introduced by the symmetrizing procedure. Samples were enclosed in a glass jacket where the temperature was regulated to ±1 °C.

The NMR data collected on the Nicolet 1280 processor were transferred to an IBM/TSS 370 computer and subsequently to a VAX-11 computer, where they were processed. The axially symmetric quadrupolar powder patterns were "dePaked" according to Bloom et al. (1981) to obtain oriented-sample spectra in which the quadrupolar splittings were easily measured. Spectral interpretation in terms of order parameters and quadrupolar splittings has been treated in detail elsewhere (Seelig, 1977; Davis, 1983).

Results

Labeled Cholesterol. Deuterium NMR spectra of model membranes of DMPC and [2,2,3,4,4,6-$^2H_6$]cholesterol (7:3 molar ratio) were obtained in the presence and absence of filipin (Figure 1). When present, the antibiotic was in equimolar amounts with cholesterol. The bottom spectrum of Figure 1 shows the characteristic powder pattern of labeled cholesterol in DMPC, whose features led to the calculation of a segmental order parameter[2] of 0.8 for the sterol in this phospholipid membrane under identical conditions of temperature and concentration (Dufourc et al., 1984c). The central resonance, which is due to the C6–^2H bond oriented close to the magic angle (Taylor et al., 1981; Dufourc et al., 1984c), has been deliberately truncated in order to enhance details in the shoulders of the powder spectra. When filipin is added to the sample containing labeled cholesterol, one obtains the top spectrum of Figure 1. On comparing top and bottom spectra, one notices that a new quadrupolar powder pattern of maximum width ca. 250 kHz appears in the spectrum arising from the sample containing the antibiotic. This new spectral component is superimposed on a spectrum similar to that of cholesterol in DMPC (bottom spectrum) and exhibits an axially symmetric shape; the peaks of quadrupolar splittings ca. 125 kHz are clearly distinguished.

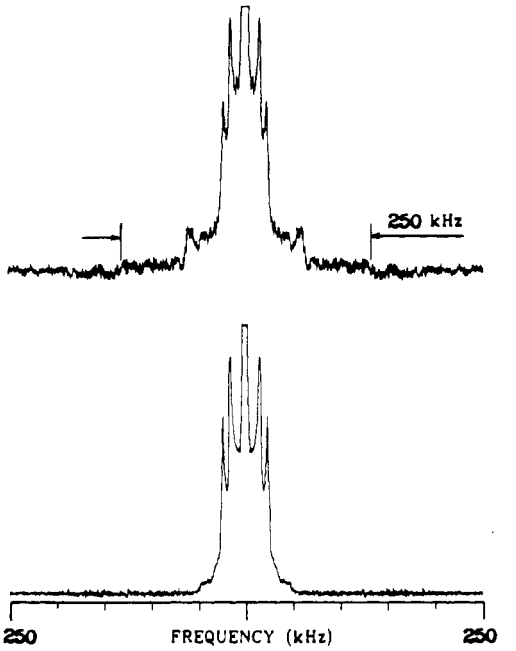

FIGURE 1: ^2H NMR spectra of [2,2,3,4,4,6-$^2H_6$]cholesterol in DMPC (3:7 ratio) in the presence (top) and absence (bottom) of filipin, at 25 °C. The antibiotic, when present, is equimolar to cholesterol. Experimental parameters were as follows: $\pi/2$ pulse length, 3 μs; pulse spacing, 40 μs; recycle time, 100 ms; spectral window, 500 kHz; 18 000 accumulations.

Since the appearance of such a wide powder pattern is crucial to our conclusions, the experimental conditions used to obtain it should be detailed. Experiments were at first performed on a Bruker CXP300 spectrometer with a 90° pulse width of 6 μs. The large subspectrum ($\Delta\nu$ = 125 kHz) was very evident, but the 250-kHz shoulders were more difficult to discern. Experiments were therefore repeated on another spectrometer having a short 90° pulse width, 3 μs, capable of irradiating a very wide frequency range; this gave a better definition of the 250-kHz shoulders (Figure 1, top). The small bumps of ca. 310-kHz width, which are ruled out theoretically, are artifacts resulting from not starting the Fourier transformation exactly at the top (Davis, 1983) of the narrow spike (representing the wide spectrum in the time domain) superimposed on the echo originating from narrower spectral components (Paddy et al., 1981).

The interpretation of the subspectrum of width 250 kHz is straightforward; cholesterol molecules giving rise to this NMR pattern no longer undergo motions that average the quadrupolar interaction; i.e., they behave like a powder of monocrystals and, therefore, yield a spectrum of the maximum possible width (Seelig, 1977). Thus, in the presence of filipin, at 25 °C, cholesterol senses two very distinct environments. In one of these it is immobilized, whereas in the other it undergoes axially symmetric motions similar to those in the membrane lacking the drug.

Figure 2 shows spectra resulting from a temperature variation performed on the sample giving rise to the top spectrum of Figure 1. At 0 °C the spectrum is a single powder pattern, indicating that all cholesterol molecules are immobilized; the central isotropic line in that spectrum is attributed to residual $^1H^2HO$, which cannot be totally avoided during sample

[2] The segmental order parameter, S_α, is defined as in Dufourc et al. (1984c) and represents a measure of the angular fluctuations of the axis of motional averaging of a given rigid subunit (here the four rings of cholesterol) with respect to the optical axis of the mesophase (the bilayer normal).

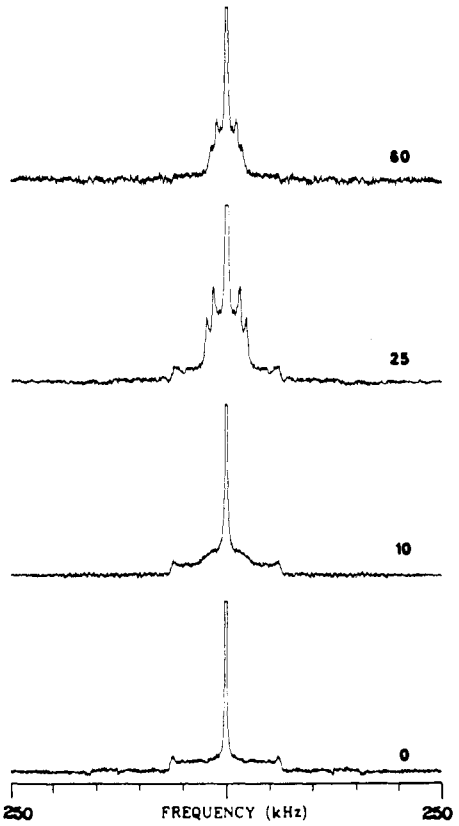

FIGURE 2: Temperature dependence of the ^2H NMR spectra of labeled cholesterol in the DMPC–cholesterol–filipin model membrane (7:3:3 molar ratio). Temperatures are indicated on each spectrum. The experimental parameters are as in Figure 1. The 250-kHz shoulders are less distinguishable than those in Figure 1; this is due to different scalings (e.g., the 25 °C spectrum is that of Figure 1, top).

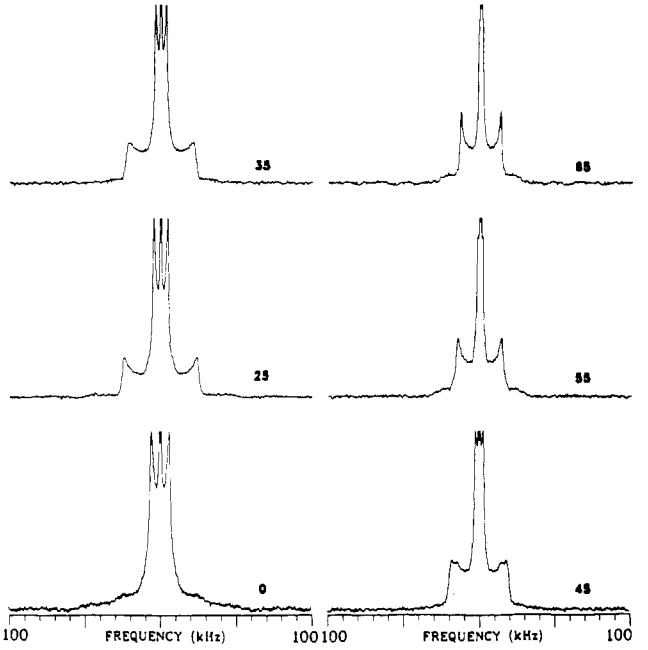

FIGURE 3: Temperature dependence of the ^2H NMR spectra of [4′,4′,14′,14′,14′-$^2H_5$]DMPC in the DMPC–cholesterol–filipin system (7:3:3 molar ratio). Temperatures are indicated on each spectrum. The experimental parameters are as in Figure 1, except the recycle time was 200 ms.

preparation. As the temperature of the sample is increased, one notices that the spectrum characteristic of axially mobile cholesterol "grows" on the powder pattern of immobilized cholesterol. At 60 °C, the spectrum is due mainly to cholesterol undergoing fast axial rotation in a uniaxial mesophase; the contribution from the immobilized component has diminished significantly. For each temperature the total spectral area was integrated; a 20% decrease was observed on going from 60 to 0 °C. Estimations of the echo decay time, T_2, showed a decrease in T_2 as the temperature was lowered from 60 to 0 °C. Such behavior has already been encountered in lipid systems at and below the phase transition (Davis, 1983) and is thought to reflect the rate of lipid exchange between domains (Rance et al., 1980). Since all experimental parameters were kept identical for all temperatures, the decrease in total spectral area can be acounted for by a concomitant decrease of T_2. In other words, after the decrease in T_2 is accounted for, the overall spectral area can be considered unchanged over the temperature range 0–60 °C. This implies that cholesterol is in slow exchange between the immobile and labile sites. At 0 °C the immobile site is stabilized whereas at 60 °C cholesterol is almost entirely in the labile site. At intermediate temperatures, e.g., 25 °C, one can distinguish cholesterol in the two sites, which indicates that the exchange rate is less than the frequency difference (ca. 10^5 s^{-1}) between the powder patterns associated with each domain. These two regions, in which cholesterol possesses different properties, have thus been characterized in terms of local order and dynamics but not localized with respect to the phospholipid membrane. In the following section, using labeled lipids, we shall try to assess the position of the labile and immobile sites mentioned above. Note that the observation of uncomplexed cholesterol in significant quantities indicates that filipin binds tightly less than one molecule of cholesterol per molecule of antibiotic.

Labeled Lipids. Experiments similar to those performed with labeled sterol were repeated with 1-myristoyl-2-[4′,4′,14′,14′,14′-$^2H_5$]myristoyl-*sn*-glycero-3-phosphocholine as the reporter molecule. Figure 3 shows some spectra of lipid–cholesterol model membranes (7:3 molar ratio) in the presence of filipin (equimolar with cholesterol) for temperatures from 0 to 65 °C. All spectra, except that at 0 °C, possess the shape characteristic of fast uniaxial motion of the phospholipid molecules. We emphasize here that down to 5 °C there is no indication of gel-type spectra. Although interacting strongly with filipin, cholesterol still produces its disordering action on lipids below T_c (23 °C), the temperature of the so-called gel to liquid crystal phase transition of the pure lipid dispersion. The spectra comprise two superimposed powder patterns originating from labeling at positions 4′ and 14′ on the DMPC *sn*-2 chain. Assignment of the spectral components due to the two labeled positions is straightforward, on the basis of previous single-labeling experiments [e.g., Oldfield et al. (1978) and Dufourc et al. (1984c)]. This labeled lipid provides a powerful and unequivocal way to monitor changes near the glycerol backbone (position 4′) and near the center of the bilayer (position 14′), in a single experiment. It is interesting to notice on Figure 3 the absence of the rigid component spectrum such as was observed with labeled cholesterol (vide supra), and this, for any temperature between 5 and 65 °C. Spectra were dePaked (see Materials and Methods), and the corresponding quadrupolar splittings are reported in Table I. Data from labeled DMPC–cholesterol membranes (7:3 molar ratio) (Dufourc et al., 1984a,c) and from pure labeled DMPC are also reported in Table I for comparison. There is practically no difference in quadrupolar splitting between systems lacking or containing the drug, for temperatures ranging from 5 to 35 °C. Above 35 °C the lipid quadrupolar splittings of the DMPC–cholesterol–filipin system tend to those of the pure lipid; the ordering action of cholesterol above T_c is partially

Table I: Temperature Dependence of Quadrupolar Splittings of [4′,4′,14′,14′,14′-$^2H_5$]DMPC in Various Model Membrane Systems[a]

temp (°C)	quadrupolar splittings (kHz)					
	[4′,4′-$^2H_2$]DMPC			[14′,14′,14′-$^2H_3$]DMPC		
	alone[b]	+chol[c]	+chol–fil[d]	alone	+chol[c]	+chol–fil[d]
5		54.7	55–50		12.4	12.4
10		54.2			12.0	
15		53.2	54–52.5		11.6	11.2
20		52.5			11.0	
25	31.9	50.8	49.2	4.3	10.3	9.8
30	28.9	48.8		3.7	9.3	
35	27.5	46.9	45.4	3.3	8.3	7.8
40	26.6	44.4		3.1	7.3	
45		42.1	37.1			5.6
50	24.8			2.7		
55		37.9	29.8		5.6	3.4
60	23.8					
65			26.8	2.2		2.9

[a] Accuracy on quadrupolar splittings is 1%–3%. [b] Data from Dufourc et al. (1984a). [c] Data from Dufourc et al. (1984c). DMPC–cholesterol in 7:3 molar ratio. [d] This study. DMPC–cholesterol–filipin in 7:3:3 molar ratio.

removed. This decrease is perceived at both the glycerol backbone level and at the center of the bilayer (Table I). Furthermore, it is interesting to notice that the relative decrease in quadrupolar splitting, in the presence of filipin, is greater at the center of the bilayer than near the top (e.g., 20% decrease at position 4′ and 40% decrease at position 14′, at 55 °C).

Discussion

The experiments performed with labeled cholesterol demonstrate unequivocally that filipin interacts strongly with the steroid skeleton. Two environments are distinguished for cholesterol, one in which the quadrupolar interaction is time independent (i.e., the sterol is immobile on the ^2H NMR time scale) and another is which this quadrupolar interaction is time averaged by the motions of the molecules bearing the deuterium nuclei. These states have been designated immobilized and labile, respectively (vide supra). Moreover, the association between the antibiotic and the sterol is temperature dependent. Figure 2 shows that as the temperature is increased, the amount of filipin-bound cholesterol decreases. At temperatures up to 10 °C, there is no exchange; cholesterol is immobilized by the antibiotic, on the ^2H NMR time scale. However, the lipid "feels" the effect of cholesterol as if the latter were alone in the phospholipid membrane and does so at both the top and/or the center of the bilayer (labeled positions 4′ and 14′, respectively, Table I). This observation implies that all the cholesterol molecules are embedded within the membrane (and therefore are all the antibiotic–sterol complexes) and that the filipin–cholesterol complex has a disordering action on lipids similar to that of cholesterol alone, below T_c.

Above ca. 10 °C the exchange of cholesterol between sites is slow on the ^2H NMR time scale, much less than 10^5 s^{-1} (vide supra); these two regions are therefore observed in ^2H NMR spectra of labeled cholesterol (e.g., Figure 1, top). In the labile site, cholesterol has a segmental order parameter identical with that of cholesterol in DMPC (3:7 molar ratio, respectively) at corresponding temperatures, e.g., 0.8 at 25 °C (Dufourc et al., 1984c). The labeled lipids reveal only one environment in which they are less ordered than in the DMPC–cholesterol system. Using a cholestane spin probe in a similar system, Okhi et al. (1979) observed two distinct environments whereas only one was revealed by a spin-labeled lipid. In previous studies (Dufourc et al., 1984b), we proposed that cholesterol acts as a screen, preventing the polyene antibiotic amphotericin B from aggregating the lipids (Dufourc et al., 1984a). This seems to be also the case with filipin.

The ordering action of cholesterol on lipids, above T_c, is known to increase with concentration (Oldfield et al., 1978). Since it is observed from the spectrum in Figure 1 (top) that at least 30%–40% of cholesterol is tightly bound to filipin, and therefore not available to order the lipid directly, it is not surprising that the quadrupolar splittings (ordering) of the fatty acyl chains are lower in the presence of the antibiotic. However, at higher temperatures, the spectral component due to filipin-bound cholesterol has almost disappeared (e.g., top spectrum in Figure 2), and one might expect the molecular ordering to resemble more closely that of DMPC–cholesterol in the absence of the antibiotic (neglecting in this case any ordering/disordering effects of the liberated antibiotic). The absolute value of the segmental order parameter of the lipid at position 4′ is found to be 0.42 ± 0.01, in the presence of filipin and cholesterol, at 65 °C [$S_\alpha = |2S_{C\text{-}^2H}|$, according to Seelig & Niederberger (1974)]. This is significantly lower than that expected from the behavior of the system without filipin, Table I. The disordering of the lipid matrix is also sensed by cholesterol. The segmental order parameter of the steroid nucleus was calculated according to Dufourc et al. (1984c), at 60 °C in the presence of filipin (Figure 2, top spectrum), and found to be equal to 0.60 ± 0.03. This value is well below that of 0.72 ± 0.03 calculated for cholesterol in DMPC under identical conditions of temperature and concentration of sterol with respect to the lipid (Dufourc et al., 1984c). These disordering effects must be due to the presence of the antibiotic.

On comparing the absolute values of the segmental order parameter, at 60–65 °C, as probed by the lipid at position 4′ or by the four rings of cholesterol, in the presence of the antibiotic, one finds different answers although the two probes monitor events occurring at approximately the same bilayer depth. This is due to the fact that one measures the ordering of a given molecular species in a system, rather than an average value for the entire system. The very important difference in local ordering of the lipid ($S_\alpha = 0.42$) compared to that of cholesterol ($S_\alpha = 0.60$) indicates that the filipin–sterol-containing model membrane may be pictured as an assembly of very mobile domains (lipids) and very rigid domains (macromolecular aggregates of filipin–cholesterol that lose their sterol molecules as the temperature is increased).

At 25 °C, the spectrum of cholesterol in the labile site (Figure 1, top) is identical with that of cholesterol in DMPC (Figure 1, bottom), i.e., labile cholesterol has the same ordering and average orientation in this lipid membrane, in the presence or absence of filipin. This implies that the steroid skeleton, in the labile site, is normal to the membrane surface (Dufourc et al., 1984c). The orientation of cholesterol bound to the antibiotic cannot be calculated as for the labile site; however, it seems reasonable to imagine that the sterol has the same average orientation with respect to the membrane surface in the labile and immobilized sites in order to facilitate the exchange. A configuration of the complex in which filipin and cholesterol are parallel to the membrane surface, as postulated by de Kruijff & Demel (1974), seems an unlikely way to favor this exchange. However, due to the motional heterogeneity of the system and to the immobility of filipin-bound cholesterol, this latter possibility cannot be completely ruled out.

Conclusions

The interaction between polyene antibiotics and membrane cholesterol is clearly monitored by ^2H NMR. Previous studies (Dufourc et al., 1984b) showed that the powder spectra of

deuterium-labeled cholesterol in a lipid membrane were slightly or not modified by the addition of amphotericin B (i.e., the interaction occurs on a time scale lower than 10^{-5} s). However, the T_{1Z} minimum of cholesterol in DMPC was shifted toward high temperatures by the presence of the antibiotic. This revealed that the interaction is perceived on the time scale of 10^{-9} s at physiological temperatures (T_{1Z} is sensitive to motional rates occurring near the Larmor frequency). This study shows that at comparable temperatures the interaction between filipin and cholesterol occurs on a time scale greater than 10^{-5} s. Moreover, a filipin-induced two-phase region in the lipid membrane can be observed by means of deuterium-labeled cholesterol. The structural and dynamical properties of cholesterol in these two regions, as well as a limit on its jumping rate between these two sites, can be determined.

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