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A Spin Label Study of Nystatin and Amphotericin B Action on Lipid Planar Multibilayers

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EPR spectra of a cholestane probe and a stearic acid probe dissolved in egg yolk lecithin and lecithin-cholesterol planar multibilayers were observed as a function of nystatin and amphotericin B dose. Spectral components characteristic of bilayer fragmentation (tilted domains) were most evident in cholesterol-containing samples and increased with drug dose. The spectra indicate that cholesterol-containing films decrease in microscopic order as drug dose is increased. This suggests that cholesterol is involved in the commonly accepted pore-formation model for nystatin and amphotericin, since removal of cholesterol from interaction with lecithin would account for the observed decrease in order. Spectra from the cholestane probe in liposomes (with and without the drugs) were also observed.

INTRODUCTION

Considerable evidence suggests that the polyene fungicides nystatin and amphotericin B (Figure 1) cluster to form aqueous channels through the plasma membrane.[1–3] Many studies also indicate that sterol may be necessary for the action of these drugs.[4–6] Nystatin and amphotericin both have been shown to form complexes with specific sterols *in vitro*.[7]

In order to study these membrane-active drugs, we have used hydrated lipid planar multibilayers as a simple model of the biological membrane. We have observed electron paramagnetic resonance (epr) spectra from nitroxide spin labels dissolved in such multibilayers in the presence and absence of drug. We present evidence which supports the involvement of sterol in the mechanism of action of nystatin and amphotericin B.

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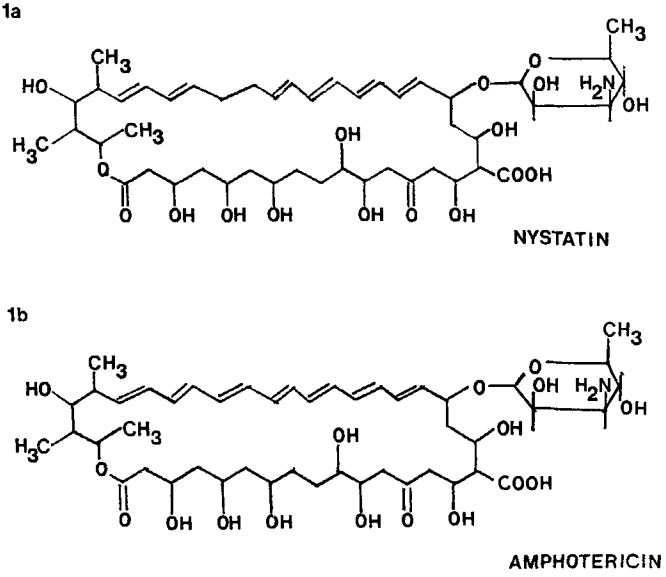


FIGURE 1 Structures of the Polyenes used.

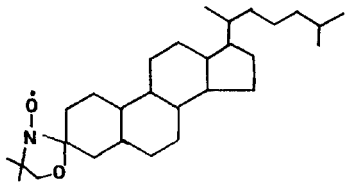
MATERIALS AND METHODS

We used egg yolk lecithin from Sigma (Types III-E and V-E), cholesterol recrystallized from ethanol, and the spin probes 3-doxyl-5α-cholestane (Figure 2a) (synthesized by Dr. C. F. Sheley at Kent[8]) and 12-doxyl-stearic acid from Syva (Figure 2b). Nystatin was kindly supplied by Dr. G. B. Whitfield of Upjohn. Amphotericin B (96.6% pure, A grade) was obtained from Calbiochem.

For the epr planar bilayer samples, a typical method of preparation was the following:

An aliquot of a stock solution of probe in chlorofrom (0.2 mg cholestane probe/ml or 0.3 mg stearic acid probe/ml) was added to an appropriate amount of lipid (egg yolk lecithin with 0%, 30%, or 55% cholesterol) in a small flask to make a final mole ratio of lipid to probe of ≈200 : 1. This solution was heated to ≈40°C for 10–30 minutes to aid the homogeneous incorporation of the spin probe. An aliquot of drug stock in methanol (2 mg filipin/ml, 1 mg nystatin/ml, or 0.16 mg amphotericin B/ml) was then added. Alternatively, sufficient methanol was added to the original lipid chloroform solution to facilitate the dissolution of the drugs, which are more soluble in methanol. Then, after heating, the lipid solution was added to an appropriate amount of drug in a flask.

2a



2b

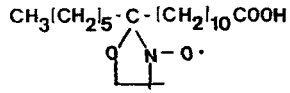


FIGURE 2 (a) Cholestane probe.
(b) Stearic acid probe.

The drug-lipid solution was dropped into glass slides (5 mm × 30 mm, cut from Corning No. 1 cover slips) under a stream of wet nitrogen. The dry film was evacuated for one to two hours to remove excess solvent, and then kept in a closed container over distilled water (flushed with nitrogen) for at least 16 hours. The average sample size was $6 \cdot 10^{-6}$ moles lipid per slide. All samples were fluid enough at room temperature to allow shearing with a top plate to improve alignment before spectra were taken.

Micellar solutions were prepared by drying a lipid-probe (100 : 1) mixture as described above in a small flask under wet nitrogen. The flask was evacuated for 1 to 2 hours, then sufficient distilled water was added for a final concentration of lipid of 0.02 M. The mixture was placed in a Heat Systems-Ultrasonics, Inc. sonic bath for 2 to 3 hours, until a translucent solution was obtained. (The temperature of the bath was kept below 60°C.) Nystatin or amphotericin B [50 g/mole lipid] was then added and the mixture was further agitated for ≈15 minutes to dissolve the drug.

We observed derivative electron paramagnetic resonance (epr) spectra with a Varian V4500-10A X-band spectrometer (≈9.3 GHz) equipped with a Varian dual cavity. The dc field was swept from 3270 G to 3370 G with a 100 KHz modulation of 2 G. The slides were clamped in a slotted aluminum rod and oriented with the static field parallel (0°) and perpendicular (90°) to the slide normal. The rod was contained in a closed quartz tube with a flask of distilled water attached at the bottom. The micellar solutions were placed in a standard quartz aqueous solution cell with the flat cell surface perpendicular to the dc magnetic field. All measurements were taken at room temperature, ≈23–24°C.

SPECTRAL CHARACTERISTICS OF THE SPIN LABELS

We define the order of a lipid bilayer as a measure of the average angular deviation (θ) of the lipid long molecular axes from the bilayer normal, i.e., $\sigma = \frac{1}{2}\langle 3\cos^2\theta - 1\rangle$. We assume that the order parameter of a dissolved probe reflects the order of the lipid host—i.e., that the probe's long axis is generally parallel to the long axis of its neighbors.

1 Cholestane probe

The hyperfine tensor for methyl-protected nitroxides is axially symmetric, with $A_{pp} \approx A_{qq} \approx -6$ G, $A_{rr} \approx -32$ G.[9] The r-axis of the cholestane probe (Figure 2a) is approximately perpendicular to the steroid long axis. Since the nitroxide moiety is rigidly attached to the steroid nucleus, its spectrum is an accurate reflection of the motion and orientation of the entire molecule. Therefore, although we expect that the oxazolidine ring is close to the polar head group region of the bilayer, this probe can effectively test the environment up to approximately one-quarter of the way into the bilayer.

In a perfectly ordered lipid film with all bilayers aligned parallel to the slide surface, we would expect the 0° spectrum for the dissolved probe to show three lines separated by $A_{qq} \approx -6$ G. If the probe were rotating rapidly about its long axis, the 90° splitting would be the average of A_{pp} and A_{rr}: ≈ -19 G. Very slow rotation would result in a superposition of all splittings from 32 G to 6 G. Deviation from perfect (microscopic) order would further complicate the slow-tumbling spectrum and would affect the averaging for the fast-tumbling case: the 0° splitting would increase (due to the increased weight of $A_{rr} \approx -32$ G) and the 90° splitting would decrease (due to increased weight of $A_{qq} \approx -6$ G). Macroscopic misalignment of the bilayers (such as from fragmentation into many domains tilted at various angles) would introduce more complications due to superposition.

The probe dissolved in liposomes would take on all possible orientations relative to the fixed field and (for the fast tumbling case) display a three-line isotropic spectrum (splitting $\approx$ 15 G). As the tumbling rate of the probe decreases, the three-line spectrum becomes more asymmetric due to anisotropy of the hyperfine and g tensors. In the extreme case of very slow ("frozen") motion, a five-line spectrum would result.

2 Stearic acid probe

The r-axis of the stearic acid probe (Figure 2b) is approximately parallel to the molecular long axis (considering the hydrocarbon chain as fully extended). Thus the 0° and the 90° spectra would be reversed relative to the

discussion above for the cholestane probe. Also since the oxazolidine ring is attached at only one point of a more or less flexible chain, its spectrum reports on the motion and orientation only in that locality. The flexibility of the chain will partially average the anisotropies in $\bar{\bar{A}}$ and $\bar{\bar{g}}$, resulting in three lines separated by greater than $A_{pp} \approx A_{qq} \approx -6$ G in the 90° orientation and less than $A_{rr} \approx -32$ G in the 0° orientation.

EPR RESULTS

Planar multibilayer samples were made as described above. Nystatin was studied at doses of 0 g/mole lipid, 5 g/mol, 25 g/mole, 50 g/mole, 100 g/mole, and 242 g/mole in systems of egg yolk lecithin, cholestane or stearic acid probe, and 0%, 30%, or 55% cholesterol. Amphotericin B was studied at doses of 0 g/mole lipid, 10 g/mole, and 25 g/mole in systems of egg yolk lecithin, cholestane or stearic acid probe, and 0% or 55% cholesterol. Results are summarized in Table I and Table II. Some representative spectra are shown in Figure 3.

In the following discussion of the spectra, it will be useful to define the ratio of the heights of the $m_I = -1$ and $m_I = 0$ lines (b/c, as indicated in

TABLE I

Spectral parameters as a function of nystatin dose: cholestane probe

% Cholesterol	Grams nystatin /mole lipid	$A_{\parallel}$	$A_{\perp}$	b/c
	g/mole	G	G	
0%	0	9.35*	16.8*	0.588*
	5	9.35 ± 0.15	17.5 ∓ 0.1	0.445 ∓ 0.006
	25	9.2 ± 0.1	17.3 ∓ 0.05	0.481 ∓ 0.002
	50	9.05 ± 0.05	17.0	0.340 ± 0.014
	100	9.35 ± 0.6	16.3*	0.134 ± 0.022
30%	0	7.15 ± 0.07	18.7	0.669 ± 0.019
	5	7.45 ± 0.05	18.4	0.526 ± 0.009
	25	8.2 ± 0.1	17.8 ∓ 0.1	0.322 ∓ 0.011
	50	8.7 ± 0.1	17.8 ∓ 0.2	0.217 ∓ 0.008
	100	9.0 ± 0.2	17.3 ± 0.3	0.184 ± 0.007
50%	0	6.9 ± 0.1	20.4 ∓ 0.5	0.796 ± 0.214
	5	7.45 ± 0.2	20.0 ∓ 0.5	0.440 ∓ 0.141
	25	7.45 ± 0.2	19.5	0.346 ∓ 0.001
	50	7.7 ± 0.35	18.9 ∓ 0.15	0.319 ∓ 0.050
	100	7.8	18.6*	0.207 ± 0.054

All values are the average of two samples (made from same stock solutions, spectra run the same day) unless marked by an asterisk (*). Sample variations from average are indicated by limits given.

TABLE II

Spectral parameters as a function amphotericin B dose

% Cholesterol	Grams amphotericin/mole lipid	$A_{\parallel}$	$A_{\perp}$	b/c
	Cholestane probe[a]			
	g/mole	G	G	
0%	0	8.95	17.4	0.449
	10	8.8	17.5	0.598
	25	8.9	17.2	0.404
55%	0	7.0	19.05	0.611
	10	7.5	17.2	0.386
	25	7.85	15.8	0.0485(?)
	Stearic acid probe[a]			
0%	0	19.1	11.45	—
	25	19.0	11.4	—
55%	0	22.3	10.3	—
	25	21.4	10.4	—

[a] Data from samples made from same stock solutions, spectra all run the same day.

Figure 3a) in the 0° spectrum of the cholestane probe as a measure of the degree of alignment. It has been shown that this ratio can be related directly to the width of the distribution of probe orientations if the cholesterol probe is allowed to rapidly rotate about the long molecular axis.[10] Qualitatively, since the central line positions are close for all probe orientations, misalignment will increase the height of the central line and so the ratio b/c will decrease. In these samples, the line indicated by an asterisk in Figure 3c becomes more pronounced as b/c decreases. Such a line has been observed in thermotropic liquid crystals and explained by non-uniform alignment.[11,12] Several observations indicate that this ratio b/c is a good measure of the alignment in our samples, i.e., that the motion of the probe about its long axis is not appreciably slowed by drug dose.

1) The ratio b/c generally tended to increase if the samples were allowed to remain an extra day in the humidity chamber (with no significant change in the microscopic order as judged by $A_{\parallel}$ and $A_{\perp}$).

2) Duplicate samples sometimes had large differences in b/c with no significant differences in $A_{\parallel}$ and $A_{\perp}$.

3) The micellar solutions involving nystatin and amphotericin showed no change in spectral lineshape or splittings. This suggests no change in the motion of the probe.

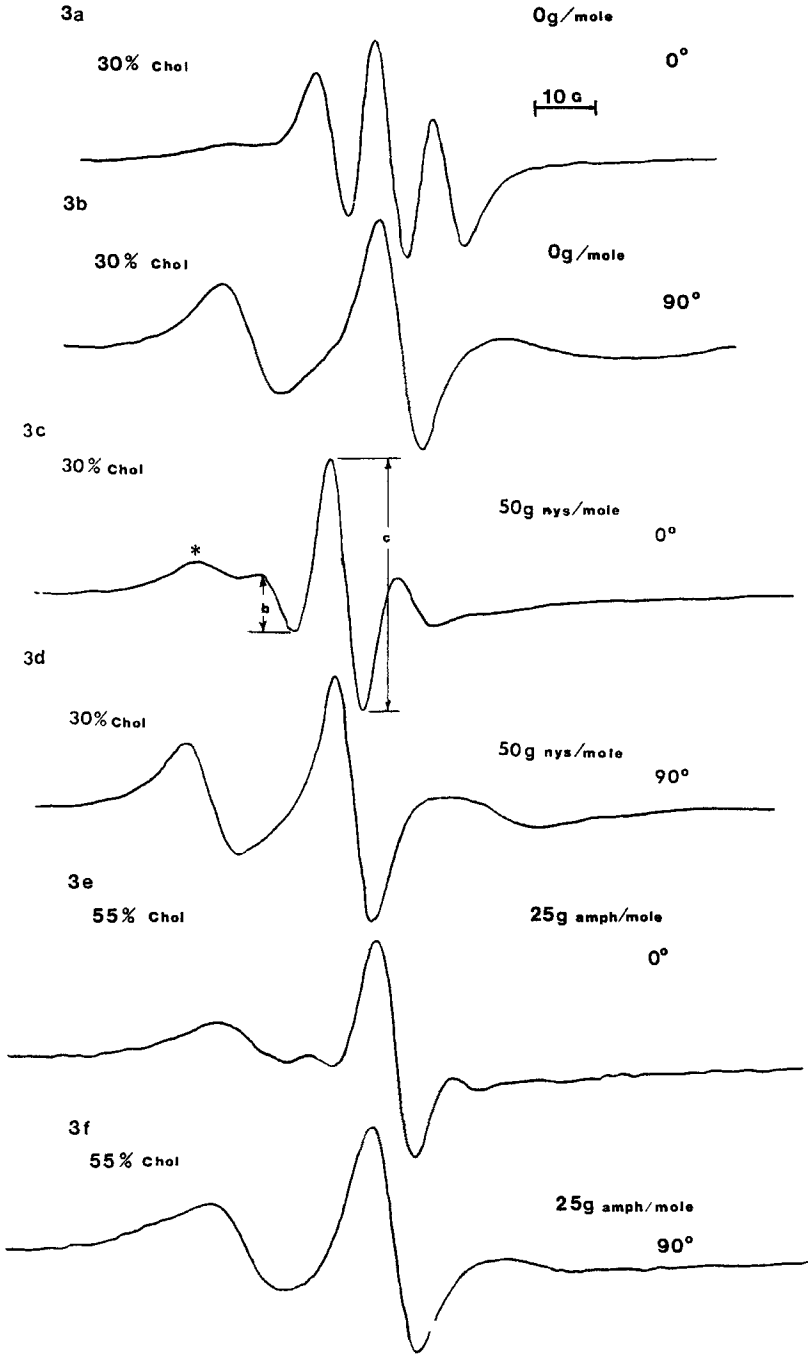


FIGURE 3 Representative paramagnetic resonance spectra.

The cholesterol-free samples for both drugs showed no significant change in $A_{\|}$, $A_{\perp}$ up through 100 g nystatin/mole lipid. At this dose misalignment became much more pronounced for the cholestane probe (the stearic acid probe spectrum was unchanged): b/c dropped from an average of ≈ 0.4 for the lower doses to ≈ 0.13. It should be noted that 100 g/mole corresponds to $\approx 10:1$ mole ratio of lipid to nystatin. At 242 g nystatin/mole lipid, the sample looked powdery and was barely shearable. An almost glassy spectrum resulted from the cholestane probe, with only slight differences in the 0° and 90° spectra.

The 30% cholesterol samples (all with the cholestane probe) showed an increase in $A_{\|}$ and a corresponding decrease in $A_{\perp}$ over the range 0 g nystatin/mole lipid to 100 g/mole [Figure 3a–d]: from $A_{\|} = 7.15$ G, $A_{\perp} = 18.7$ G for 0 g/mole to $A_{\|} = 9.0$ G and $A_{\perp} = 17.3$ G for 100 g/mole. The ratio b/c decreased steadily with increasing nystatin dose from ≈ 0.7 to ≈ 0.2. The change in $A_{\|}$ is large enough to indicate a real decrease in microscopic order of the cholestane probe despite overlap problems due to misalignment. Samples were also prepared with the stearic acid probe at doses of 25 g nystatin/mole lipid and 100 g/mole; misalignment made accurate measurement of the hyperfine parameters difficult (the splittings are not included in Table I). However, $A_{\|}$ did not seem to vary significantly from the 0 g/mole value of ≈ 23 G. This suggests that the order of the stearic acid probe was unchanged.

The 55% cholesterol samples with the same doses of nystatin showed a steady but smaller increase in $A_{\|}$ and decrease in $A_{\perp}$ for the cholestane probe: from $A_{\|} = 6.9$ G, $A_{\perp} = 20.4$ G for 0 g/mole to $A_{\|} = 7.8$ G, $A_{\perp} = 18.6$ G. Overall the ratio b/c showed a tendency to decrease. It should be noted that large differences in b/c for the same drug dose was sometimes observed together with very close values of $A_{\|}$. This suggests that overlap of lines due to misalignment has not so seriously interfered with measurement of the hyperfine parameters, so that the decrease in $A_{\|}$ indicates a real decrease in the microscopic order as nystatin dose is increased.

The 55% cholesterol samples with amphotericin B (0 g/mole, 10 g/mole, and 25 g/mole) showed a definite decrease in alignment from spectra of both the cholestane and stearic acid probes. (This is indicated by the decreasing b/c for the cholestane probes, and by the increasing prominence of extra lines in the stearic acid probe spectra.) In particular, the 25 g amphotericin/mole lipid spectrum of the cholestane probe [Figure 3e, f] exhibited the same features characteristic of pronounced misalignment as the 100 g nystatin/mole spectrum. The cholestane probe spectra show a possible slight decrease in order ($A_{\|} = 7.0$ G increases to $A_{\|} = 7.86$ G) but overlap obscures reliable measurement of $A_{\|}$ (particularly in the 25 g/mole spectra). As with the nystatin samples, no change in order of the stearic acid probe was

observed. It should be noted that our results seem consistent with those reported by Smith[11] on the influence of amphotericin on the cholestane probe spectrum of a hydrated ox brain lipid film (including cholesterol) formed in an epr flat cell.

Micellar solutions of egg yolk lecithin and 1% cholestane probe with 0% and 55% cholesterol were also prepared. Drug was added (50 g/mole lipid) to these dispersions. Both the 0% and 55% cholesterol samples displayed an asymmetric three-line spectrum with splitting of $\approx$16–17 G (i.e., approximately the isotropic value); the lowfield and highfield lines of the 55% cholesterol samples were significantly broader than the 0% lines. However, the addition of drug produced no change in the spectra. This suggests that the motion of the probe was unchanged by the addition of nystatin and amphotericin.

DISCUSSION

The spectra are certainly consistent with the currently accepted pore mechanism for nystatin and amphotericin. Large numbers of pores might be expected to hinder the macroscopic alignment. At the lower drug doses, often the sheared samples increased their b/c ratio after standing for an extra day in the humidity chamber, suggesting that the hindering mechanism is not irreversible. The fact that amphotericin B causes greater misalignment at lower doses than nystatin is compatible with the greater toxicity of amphotericin. Even 30% cholesterol is very likely to be in excess of the amount required for pore formation. Free cholesterol has been shown to enhance the order of the bilayer by somehow facilitating interaction between the phospholipids (very likely through head group interactions). If the pores remove cholesterol from association with the lecithin, then we would expect the order to decrease as observed. It is also not surprising that the 30% cholesterol samples are more sensitive to this effect than the 55% samples. Removal of the same amount of cholesterol from associated with the lecithin is more critical at lower initial concentrations (since less free cholesterol is then available). Thus the epr spectra lend support to the theory that cholesterol is involved in pore formation.

It is interesting to contrast the nystatin and amphotericin results with a similar study of the related polyene filipin.[13] A current model of filipin action involves aggregation of many filipin-cholesterol complexes that rupture the membrane.[1] This model requires that cholesterol occupy a position that is perpendicular to its usual position in the membrane. The cholestane probe is structurally similar to cholesterol and has been shown by an ultraviolet study to be capable of complexing with filipin *in vitro* like

cholesterol. We dissolved the cholestane probe in lipid multibilayers as described above in the presence of filipin. We have seen spectral features from the cholesterol-free systems that strongly suggest that the probe takes on just such a position as required by the aggregate model, i.e., "frozen" with its long molecular axis perpendicular to the bilayer normal. The dose dependence of the intensity of the "frozen" spectral component and the absence of such lines when the stearic acid probe was used supported this interpretation. The cholesterol-free samples involving nystatin and amphotericin B showed no indication of the "frozen" component so prominent in the analogous filipin systems. This strengthens our interpretation of the filipin data for two reasons. First, complexation of nystatin and amphotericin in the manner suggested for filipin is unlikely for steric reasons. Presence of "frozen" components in the nystatin–amphotericin spectra would cast serious doubt on our claim that such features in the filipin spectra are due to complexed probe. Secondly, it may be argued that the "frozen" components in the filipin data merely reflected hindered probe motion outside of the postulated aggregate, due to the presence of bulky molecules within the bilayer. If this is correct, then probe motion should be similarly hindered by nystatin and amphotericin B. However, we see no indication of such an effect.

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