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EFFECTS OF AMPHOTERICIN B ON MEMBRANE PERMEABILITY—KINETICS OF SPIN PROBE REDUCTION *

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The effect of the polyene antibiotic amphotericin B on the permeability of both unilamellar and multilamellar model membranes is investigated. The method measures the loss of the electron paramagnetic resonance signal of a spin probe, trapped in the aqueous compartment of a lipid dispersion, upon addition of ascorbate ions to the bulk aqueous phase. Amphotericin B causes large increases in the permeability of cholesterol-containing egg phosphatidylcholine membranes, whereas the effects are small in the absence of sterol and do not depend on surface charge. The effect of amphotericin depends upon the antibiotic:sterol mole ratio. The antibiotic appears to be unable to cross the membrane, acting only on the outermost bilayer of a multibilayer dispersion. When a phospholipid in the gel phase is used, amphotericin B causes large increases in permeability, independently of the presence or absence of sterol. It is suggested that the mechanism of action of amphotericin B is different for lipids in the liquid crystalline or gel states.

1. Introduction

It has been shown that the polyene antibiotic amphotericin B can alter the permeability of both biological and model membranes. The effect depends on membrane composition [1–4]. Amphotericin B enhances the permeability of small ions and hydrophilic solutes through uni- and multilamellar vesicles; the effect is more intense when cholesterol is present [5–8]. A model of the cholesterol-amphotericin B active complex has been proposed in which two half-pores are juxtaposed, each formed by 8 cholesterol and 8 amphotericin B molecules. The diameter of the pore (0.8 nm) determines the selectivity towards solute size [9]. If the lipids of a model membrane are in the liquid crystalline state, cholesterol incorporation enhances the effect of amphotericin B, whereas the opposite occurs with gel state lipids [10]. It has been suggested that an ordered state of the phospholipid is required for the interaction with the polyenes, and that the role of cholesterol is to promote the achievement of such a state [11].

We report a study of the effects of amphotericin B on the permeability of multibilayer and single bilayer lipid dispersions of various compositions. The method is based on the measurement of the rate of change of the intensity of the EPR signal displayed by a spin probe, TEMPO choline (see

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Abbreviations: BLM, black lipid membrane; EPC, egg phosphatidylcholine; EPR, electron paramagnetic resonance; DPPC, dipalmitoylphosphatidylcholine; DCP, dicetyl phosphate; CTACl, hexadecyltrimethylammonium chloride; DMSO, dimethyl sulfoxide; TEMPO, 2,2,6,6-tetramethylpiperidine-*N*-oxyl.

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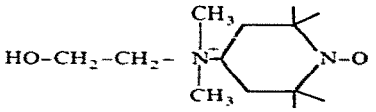
structure below), retained in the internal aqueous



compartments of the membranes, in the presence of ascorbate ions in the bulk aqueous phase [12]. Earlier studies on lipids of different net charge have suggested that the principal permeant species is the ascorbate ion [12].

2. Materials and methods

2.1. Materials

2.1.1. Compounds

Amphotericin B was a gift of Squibb-Indústria Quimica S/A, São Paulo, Brazil. The concentration was corrected according to the potency of each batch. The antibiotic was also obtained from Calbiochem, San Diego, CA, lot 600804, A grade, 851 μg/mg. It was kept at 4°C, protected from light. TEMPO choline chloride was a generous gift of Dr. A.C. Oehlschlager (Simon Fraser University). EPC was purchased from Lipid Products, South Nutfield, U.K., DPPC and dicetyl phosphate (DCP) from Sigma Chemical Co., St. Louis, MO, cholesterol from Steraloids, Inc., Pawling, NY, and CTACl from Eastman Organic Chemicals, Rochester, NY. All other reagents were of analytical grade. The buffer was 300 mosmol NaCl/Na_2HPO_4/KH_2PO_4, pH 7.4.

2.1.2. Equipment

Varian E-4 and E-9 X-band EPR spectrometers were used. Sonication was performed with a model 150 Virsonic Cell Disruptor.

2.2. Methods

2.2.1. Sample preparation

The lipids were dissolved in chloroform and kept in the freezer (−15°C). TEMPO choline chloride was dissolved in the NaCl/phosphate buffer. Solutions of amphotericin B were freshly prepared by dissolving the antibiotic in DMSO. The final concentration of DMSO in the aqueous solutions was 5% (v/v). The membranes had the following compositions (mole%): EPC (100); EPC/cholesterol (75:25); EPC/DCP (92:8); EPC/DCP/cholesterol (67:8:25); EPC/CTACl (85:15); EPC/CTACl/cholesterol (60:15:25); DPPC/DCP (92:8); and DPPC/DCP/cholesterol (67:8:25).

Multilamellar dispersions were prepared by evaporating a chloroform solution of the lipids with a flux of wet nitrogen. Residual solvent was removed by placing the sample under vacuum for at least 2 h. Buffer containing 3.75×10^{-4} M TEMPO choline was added, and the sample shaken in a vortex mixer for 15–20 min. Unilamellar vesicles were obtained by sonication at a power level of 5, duty cycle 50%, for 60–90 min under nitrogen in a water-ice bath, until the sample became translucent. The samples were centrifuged at 20000 *g*, 4°C, for 40 min.

2.3. Permeability studies

Membrane permeability was evaluated via the reduction of the nitroxide radical by the ascorbate ion [12]. The membrane permeability to ascorbate is low (see, however, refs. [12] and [13]) and addition of this agent causes the disappearance of the EPR signal of TEMPO choline in the bulk aqueous phase. The decay of the signal from the remaining trapped probe was followed for 20 min (figs. 1–4), after which the agent under study was added (buffer, DMSO, amphotericin B in DMSO). The low-field spectral line was recorded every minute for 30–40 min. Occasionally, spectra were recorded over periods of 1 or 2 h. The experiments were done at room temperature (22 ± 2°C).

3. Results

Figs. 1–3 show the effect of amphotericin B on the permeability of unilamellar vesicles of various compositions. Note that the initial decay of the EPR signal (first 20 min, before addition of agent) is relatively slow [12].

Amphotericin B has very little effect on the permeability of EPC membranes that do not contain cholesterol (figs. 1a, 2a, and 3a). The effect of

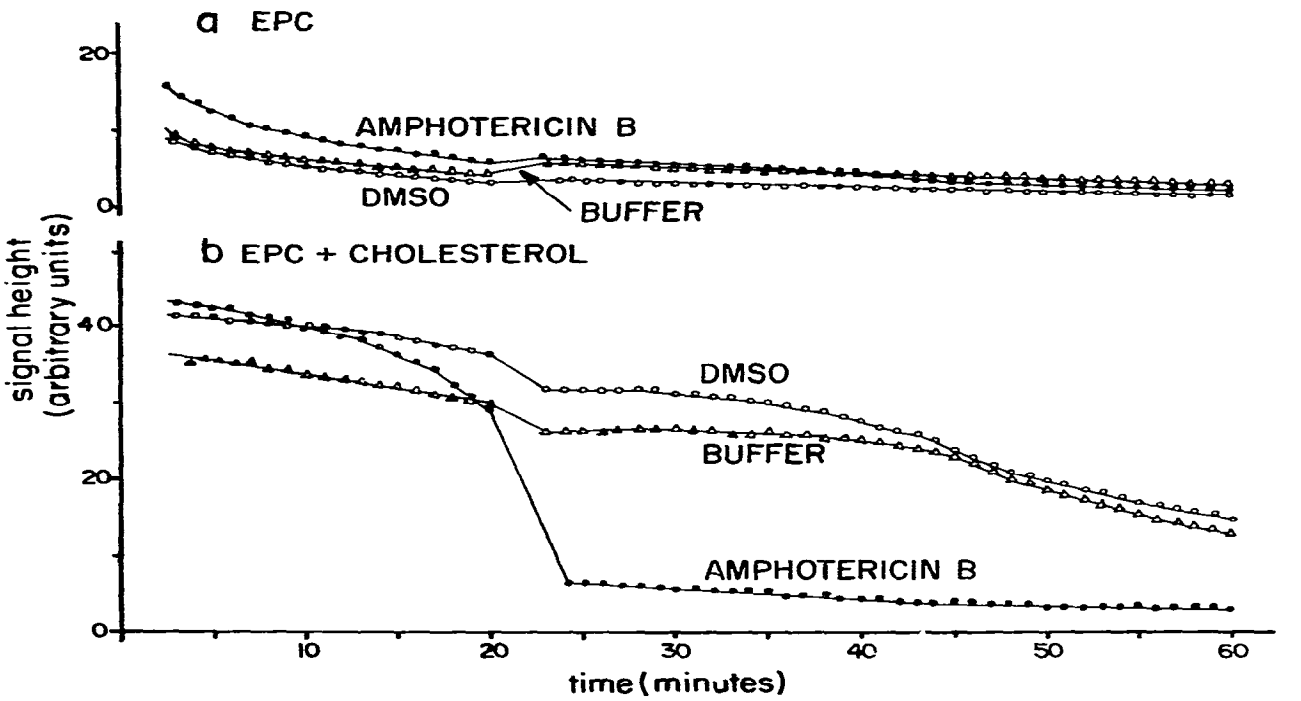


Fig. 1. Loss of EPR signal of TEMPO choline (3.75×10^{-4} M), in arbitrary units, as a function of time after adding ascorbate ions (4×10^{-2} M) to unilamellar vesicles of EPC (a) and EPC/cholesterol (75:25) (b). The signal decay was followed for 20 min and then an agent was added: (△) NaCl/phosphate buffer, 300 mosmol, pH 7.4; (○) DMSO (5%, v/v), and (●) amphotericin B (2.7×10^{-3} M, equimolar to cholesterol).

antibiotic is more pronounced in the cholesterol-containing systems, and is independent of membrane surface charge. Upon addition of amphotericin B in concentrations equimolar to cholesterol, there is an immediate loss of EPR signal and little change takes place after this initial event (figs. 1b, 2b, 3b). Approximately 80% of the EPR signal is lost when the antibiotic is added to these cholesterol-containing samples. Table 1 gives the percent loss of signal for multilamellar vesicles. In this case, amphotericin B causes cholesterol-containing EPC membranes to lose ≈50–60% of their initial TEMPO choline content. The degree of reduction of the EPR signal depends on the concentration of antibiotic (fig. 3). At cholesterol/antibiotic molar ratios of 10:1 and 100:1 (curves A_2 and A_3 in fig. 3b, respectively)

Table 1

Loss (%) of TEMPO choline signal from multilamellar vesicles in the presence of ascorbate

The values are derived from measurements done 3–5 min after adding test agent (buffer, DMSO, or amphotericin B). Experimental details are given in the text and captions to figures. Reproducibility of experiment ±5%

System	Buffer	DMSO	Amphotericin
EPC	16	0	10
EPC/cholesterol	17	8	47
EPC/DCP	0	16	25
EPC/DCP/ cholesterol	9	15	50
EPC/CTACl	30	24	13
EPC/CTACl/ cholesterol	17	17	62

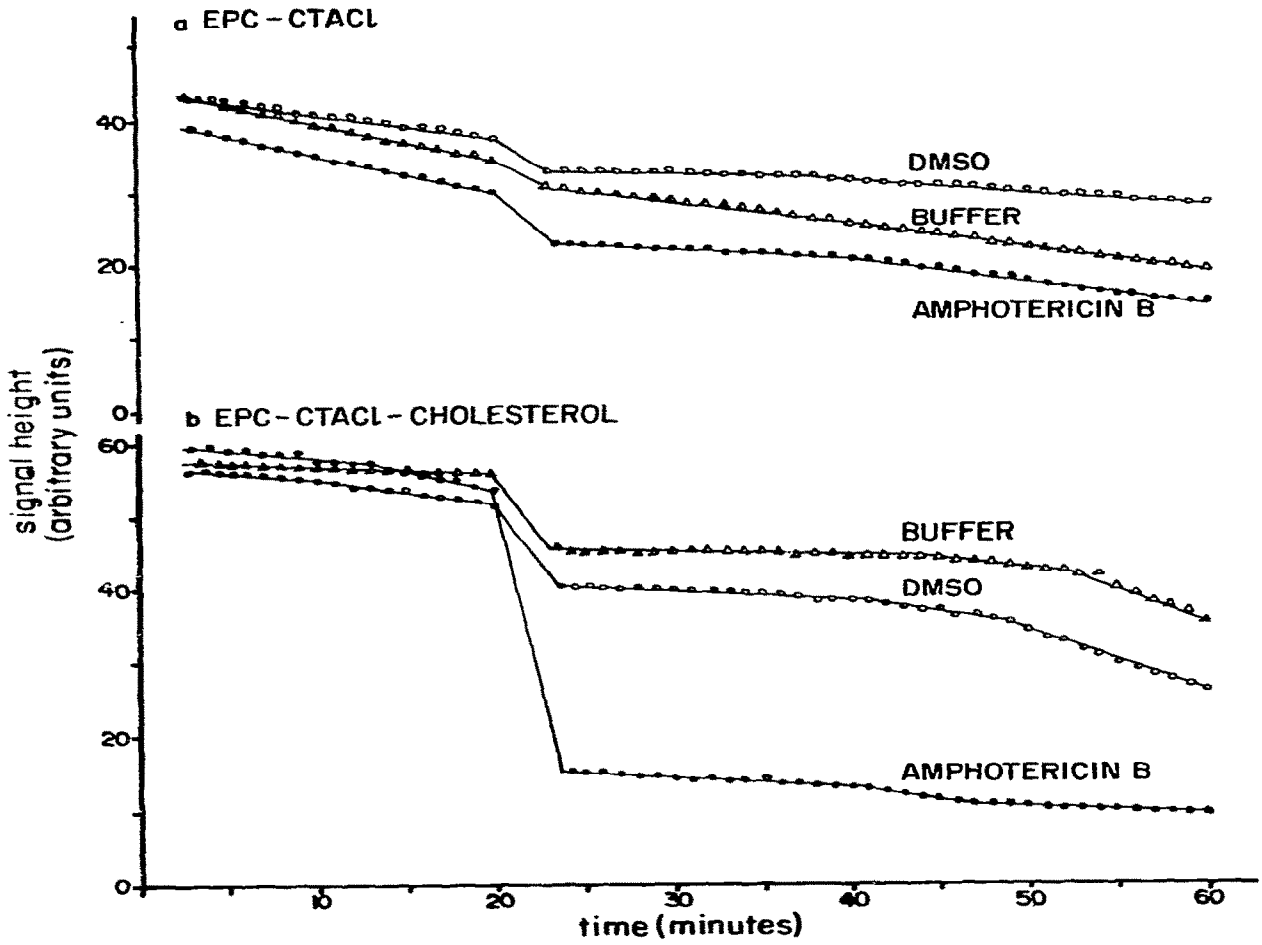


Fig. 2. As in fig. 1, unilamellar vesicles of EPC/CTACl (85:15) (a) and EPC/CTACl/cholesterol (60:15:25) (b).

the pattern of the kinetic behavior is qualitatively the same as for equimolar ratios (curve A_1); there is a large initial loss of TEMPO choline paramagnetism, and a slow decay of the remaining signal. Curve A_3 shows the smallest initial loss of signal after adding antibiotic. The subsequent signal loss follows a pattern similar to that of the control sample containing DMSO (fig. 3b). DMSO has been shown to increase the permeability of model membranes [12].

The results obtained for DPPC/DCP and DPPC/DCP/cholesterol unilamellar vesicles are given in fig. 4. The effect of amphotericin B on both systems was immediate and very pronounced. In vesicles without cholesterol, the loss of EPR signal was almost complete after 15 min, whereas in those containing sterol, the remaining paramagnetic signal was significant even after 60 min ($\approx$ 30%). While the kinetic behavior of the cholesterol-containing DPPC/DCP system is similar to that of sterol-containing EPC membranes, the cholesterol-free systems differ significantly;

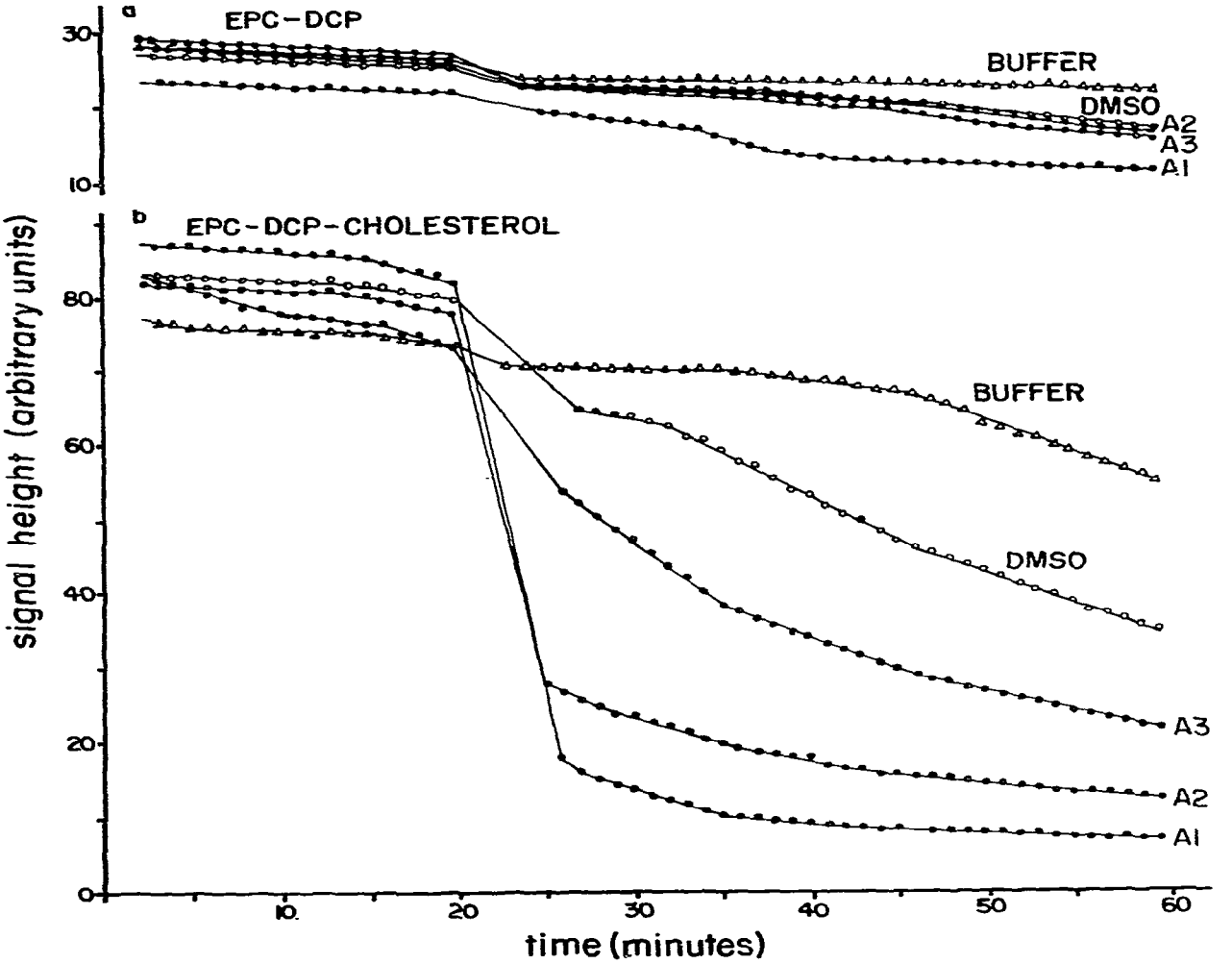


Fig. 3. As in fig. 1, unilamellar vesicles of EPC/DCP (92:8) (a) and EPC/DCP/cholesterol (67:8:25) (b). Amphotericin B concentrations were: A_1, 2.7×10^{-3} M (equimolar to cholesterol); A_2, 2.7×10^{-4} M; A_3, 2.7×10^{-5} M. (Data from ref. [12].)

there is a steeper signal decrease in the DPPC/DCP (fig. 4a) than in the EPC/DCP system (fig. 3a).

The relative rates of signal loss for the control systems, calculated according to Aracava et al. [12], are 1.71 and 5.31 min^{-1} for the EPC systems, while the values are 0.07 min^{-1} for DPPC/DCP and 0.06 min^{-1} for DPPC/DCP/cholesterol. This large difference in permeability between a phospholipid in the liquid crystalline state (EPC) and in the gel state (DPPC) parallels the difference in rotational mobility of a spin probe intercalated in bilayers of those lipids (13). The rate of rotational motion for the spin probe along its long molecular

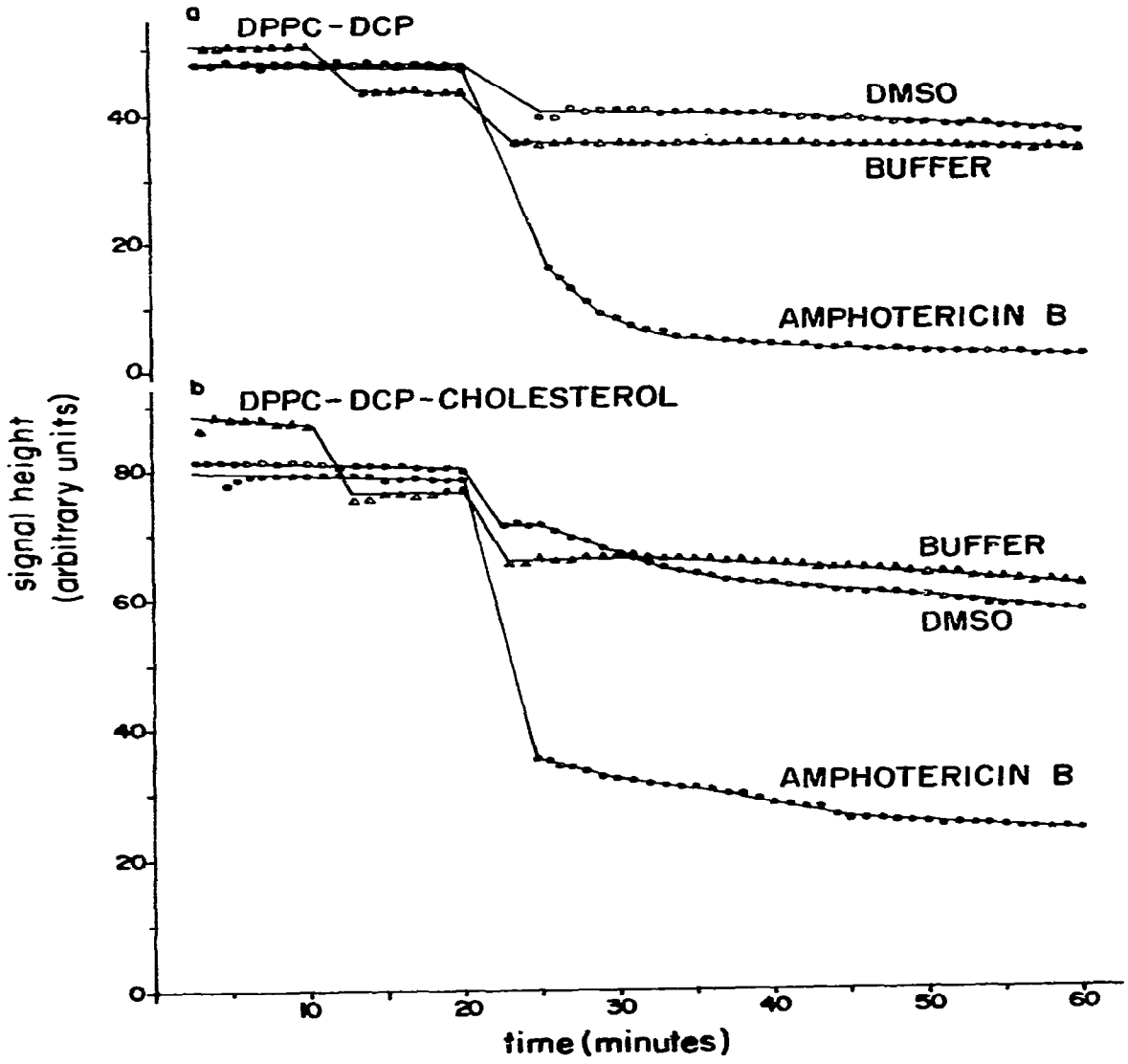


Fig. 4. As in fig. 1, unilamellar vesicles of DPPC/DCP (29:8:5) (a) and DPPC/DCP/cholesterol (67:8:25) (b).

axis is largely decreased in DPPC bilayers (with or without cholesterol) when compared to analogous EPC systems.

4. Discussion

The role of surface charge and cholesterol on the entrapped aqueous volume, as well as the effect of DMSO on membrane permeability, have

been discussed earlier [12].

EPC vesicles containing cholesterol are highly sensitive to amphotericin B; the first measurement after addition of antibiotic indicated an almost complete loss of the TEMPO choline EPR signal (figs. 1–3). The lack of total signal reduction in multilamellar vesicles (table 1) is most likely due to the fact that the antibiotic is incapable of transversing the lipid bilayer. Only the contents of the first interlamellar space are liberated. This observation is in agreement with the results of de Kruijff et al. [6], Singer [7] and van Hoogevest and de Kruijff [8].

It has been suggested that disruption of unilamellar vesicles occurs at high amphotericin B:cholesterol ratios (1:1 or 2:1) [14]. We believe that if this were the case in our permeability studies the antibiotic should have been able to reach all bilayers of liposomes and release all internal contents, unless disruption of the vesicles were incomplete.

When unilamellar vesicles are used, the percentage of signal decay for cholesterol-containing EPC systems is $\approx 80\%$. It is possible that the remaining TEMPO choline is retained in a small population of multilamellar vesicles.

The above experiments were performed with equimolar concentrations of antibiotic and sterol. The results depend on antibiotic concentration (fig. 3). 3–5 min after adding amphotericin B to A_1 78% of the signal is lost, close to the maximum (88%) observed. For A_3 the decay at long times is apparently due to the action of DMSO alone. Fig. 3 suggests that the action of amphotericin B is immediate, and that the antibiotic acts only on a fraction of the vesicle population when the mole ratio polyene:sterol is low. These data do not agree with the work by van Hoogevest and de Kruijff [8], who have reported that all trapped K^+ is released in 15 min in a system that contains 3 molecules of antibiotic per vesicle; in system A_3 this ratio is approximately 6.

The results obtained with DPPC in the presence of cholesterol (fig. 4) are analogous to those found with EPC. However, in contrast with EPC, DPPC (which is in the gel state at 22°C) seems to interact with amphotericin B even in the absence of cholesterol, with changes in permeability of the same magnitude as those observed in the presence of sterol. These results agree with those of Hsu Chen and Feingold [10] who found a pronounced effect of amphotericin B upon the permeability of glucose in DPPC liposomes at temperatures below that of the gel-liquid crystal transition. The incorporation of increasing cholesterol concentrations caused a decrease in the effect of the antibiotic. This is consistent with the reduced loss of EPR signal in our cholesterol-containing systems. Alternatively, the remaining 30% of TEMPO choline could be trapped in a population of multilamellar vesicles.

The work by Hsu Chen and Feingold [10] has been used to argue against a sterol requirement for antibiotic activity; the important factor would be the degree of packing in the membrane; more tightly packed systems would be more capable of interacting with amphotericin B. Another possible explanation for the difference between DPPC and EPC could reside in different mechanisms of action of the antibiotic in the two systems [15,16].

In conclusion, we have shown that at a 1:1 antibiotic:sterol mole ratio there is an immediate loss of 80% of the signal in unilamellar vesicles and 50–60% in multilamellar vesicles of cholesterol-containing EPC systems. The effect is much smaller in the absence of sterol and does not depend on surface charge. These results are in agreement with the proposed requirement of sterol for antibiotic action and also suggest that the antibiotic is incapable of crossing the membrane, acting only on the outermost bilayer of multilamellar systems.

The studies using variable antibiotic concentrations suggest that at low amphotericin B:cholesterol ratios the polyene is incapable of interacting with all vesicles present in the medium. This is in agreement with the suggestion that the (re)equilibration of antibiotic between water and membrane is slow [13].

Cholesterol is not required for the interaction between amphotericin B and gel phase phospholipid, the total loss of the entrapped spin probe being observed after addition of the antibiotic. The different kinetics of signal decay suggest that the mechanism of action of amphotericin B differs in cholesterol-containing systems and in gel phase systems.

No membrane structural changes, as monitored by incorporated lipid spin probes [13], were observed under the conditions of any of the above permeability studies, presumably due to the low population density of active amphotericin complexes.

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