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INTERACTION OF POLYENE ANTIBIOTICS WITH STEROLS IN PHOSPHATIDYLCHOLINE BILAYER MEMBRANES AS STUDIED BY SPIN PROBES

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Summary

Interaction of filipin and amphotericin B with sterols in phosphatidylcholine membranes has been studied using various spin probes; epiandrosterone, cholestanone, phosphatidylcholine with 12-nitroxide or 5-nitroxide stearate attached to 2 position and also with tempocholine at the head group. Filipin caused increase in the fluidity of cholesterol-containing phosphatidylcholine membranes near the center, while it rather decreased the fluidity near the polar surface. On the other hand, amphotericin B did not apparently affect the fluidity. In the electron spin resonance spectrum of steroid spin probes in the antibiotic-containing membranes, both bound and free signals were observed and the association constant was calculated from the siganal intensity. In the binding of steroids with filipin, both 3 and 17 positions were involved, while the 17 position was less involved in the binding with amphotericin B. Phase change in the host membrane markedly affected the interaction of filipin with epiandrosterone probe. The bound fraction jumped from 0.4 to 0.8 on going to the crystalline state and increased further with decrease in temperature. The overall splitting of the bound signal also increased on lowering the temperature below phase transition. This change was attributed to aggregate formation of filipin-steroid complexes in the crystalline state. On the other hand, effect of phase transition was much smaller on the interaction of amphotericin B with the steroid probe.

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Abbreviations: 12 PC* and 5 PC*, phosphatidylcholine spin probes with a 12-nitroxide and a 5-nitroxide stearic acid, respectively, attached at 2 position; Tempo PC*, Tempo phosphatidylcholine spin probe.

Introduction

Polyene antibiotics interact with sterols in fungal membranes, causing permeability changes, leakage of cellular contents, and ultimately lysis and death of the cells. Because of the crucial role in the antifungal activity, the interaction of polyene antibiotics with sterols in model as well as biological membranes has been studied extensively by various physical means such as spectrophotometry, calorimetry, magnetic resonance, etc. (see [1] for a review). The results have yielded valuable informations on stoichiometry, specificity, and mode of the interactions. On the side of sterols, a 3β-hydroxy group, a planar nucleus, and a hydrophobic side chain at 17 position are involved in the interactions [2—5]. Despite of many common features, filipin had somewhat different mode of action on the sterol-containing membranes. It produces an aggregate of 150—250 Å in diameter (pit) in the membrane [6—9] and causes membrane fragmentation and release of cytoplasmic contents [3]. On the other hand, amphotericin B does not visibly affect the freeze-etch morphology and is thought to form some hydrophilic pores of specific sizes (about 8 Å in diameter) [6].

Using three kinds of phosphatidylcholine spin probes which had their nitroxide moieties at various positions of molecules, effect of the antibiotics on the sterol-containing membranes was studied from the side of phospholipid. Epiandrosterone spin probe and cholestanone spin probe were used to study the interaction of filipin or amphotericin B with steroids in phosphatidylcholine bilayer membranes from the side of steroids. The association constant of polyene antibiotics with the steroid probes and sterols was obtained by directly measuring the bound and unbound steroid probe in the membrane. Effect of phase change in the host membrane on the association was also studied.

The apparent dissociation constant for binding of filipin to sterol-containing phosphatidylcholine liposomes has been measured spectrophotometrically by Bittman et al. [10]. However, the constant is for the equilibrium between the lipid and aqueous phases and not directly related to that in the lipid bilayer membranes. Flick and Gelerinter [11] have published a paper on the interaction of cholestanone spin probe with filipin in egg phosphatidylcholine. These authors observed a rigid component in the ESR spectrum of multibilayers containing large concentrations of filipin and interpreted to be due to binding of the probe to the antibiotic. They were not able to detect the interaction in the lipid vesicles.

Materials and Methods

Polyene antibiotics. Filipin was kindly supplied by Dr. Whitfield, Upjon Co., Kalamazoo, MI. The sample was developed with methylene chloride/methanol (85 : 15) on a type 60 silica gel H thin-layer plate (Merck) containing equimol of KH_2PO_4 and Na_2HPO_4. The chromatogram indicated less than 10% of impurity and the sample was used without further purification. Amphotericin B was a product of Fungizone, E.R. Squibb and Sons, New York, and used without purification. These antibiotics were kept in the dark and dissolved in

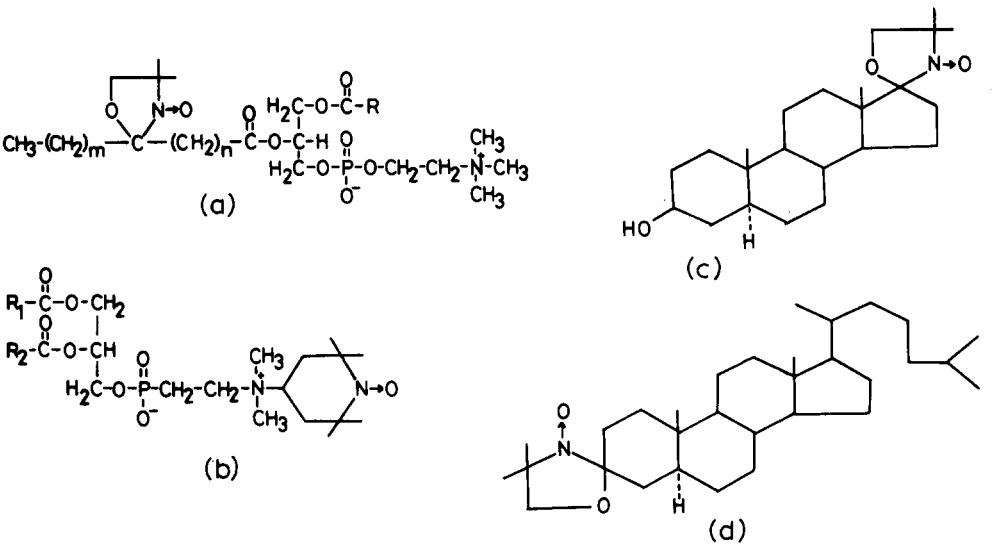

Fig. 1. Phosphatidylcholine spin probes: (a) 5 PC* (m = 12, n = 3), 12 PC* (m = 5, n = 10, and (b) Tempo PC*. Steroid spin probes: (c) epiandrosterone spin probe, and (d) cholestanone spin probe.

distilled dimethylsulfoxide just before use to avoid loss of activity. Sample preparations and ESR measurements were carried out in dim light. No change was observed in the optical spectrum after experiments.

Phospholipids, sterols, and spin probes. Egg yolk phosphatidylcholine was obtained as described by Singleton et al. [12]. Dielaidoyl- and dimyristoylphosphatidylcholines were synthesized by acylation of glycerophosphorylcholine with the corresponding fatty acid (Nakarai Chemicals) by the method of Robles and Van der Berg [13]. Phosphatidylcholine spin probes with a 12-nitroxide (12 PC*) and a 5-nitroxide (5 PC*) stearic acid attached at 2 position, were synthesized according to Hubbel and McConnell [14] (see Fig. 1). Tempo phosphatidylcholine (Tempo PC*) spin probe was synthesized according to Kornberg and McConnell [15] (see Fig. 1). These phospholipids gave a single spot on silica gel G thin-layer plate developed with chloroform/methanol/acetate/water (50 : 30 : 8 : 4, by vol.). And the fatty acid side chains were examined on a gas-liquid chromatography (Shimazu GC-6A), which gave a single peak for dielaidoyl and dimyristoyl phosphatidylcholine.

Two different steroid spin probes were used (see Fig. 1). Epiandrosterone spin probe has a 3β-hydroxy group and the nitroxide moiety at 17 position, while cholestanone spin probe has intact chain at 17 position and the nitroxide moiety at 3 position. These probes were synthesized from the corresponding ketones, 5α-androstan-3β-hydroxy-17-one (Sigma) and 5α-cholestan-3-one (Nakarai) by the general method of Keana et al. [16]. Epiandrosterone spin probe was purified on 100 mesh silicic acid (Mallinckrodt) column chromatography eluted with hexane/ether (50 : 50, v/v). Cholestanone probe was purified by recrystallization in ethanol. These probes were chromatographically pure.

Cholesterol was purchased from Nakarai Chemicals, Kyoto, and recrystallized in ethanol. Ergosterol was kindly provided by Dr. N. Ariga of Gifu

University. These sterols showed a single peak on gas-liquid chromatography.

Preparation of liposomes and vesicles, and treatment with polyene antibiotics. 10 mg of phosphatidylcholine, 1.5 mg of sterol and 1 mol% of spin probe, those in benzene solution were taken into a small test tube, and the solvent was evaporated first by nitrogen stream and then by evacuation overnight. Then, 1 ml of Tris-buffered saline (150 mM NaCl and 50 mM Tris-HCl, pH 7.5) and several small glass beads were added and agitated with Termomixer above 40°C for 30 s. The preparation was called as a liposome. Various concentrations of polyene antibiotics were added to the liposome suspension. The suspension was transferred into a small vial and sonicated at 0°C for 1 min under a nitrogen stream with a 20 kHz sonifier (Kaijo Denki Co., Ltd.) fitted with a titanium tip (7 mm diameter) at a power level of 40. The suspension was used as a vesicle. The amount of dimethylsulfoxide in the suspension was kept smaller than 9%. It was confirmed that addition of that amount of dimethylsulfoxide did not affect the results.

For determination of the association constant of polyene antibiotics with an epiandrosterone spin probe, sonicated vesicles consisting of 10 mg (12 μmol) of phosphatidylcholine, 0.12 μmol of a spin probe and various concentrations of antibiotics in 1 ml of the buffer were prepared. ESR spectrum of the sample was measured and the fraction of the bound and unbound steroid spin probe was obtained from the signal intensity as described in text. These spectra were also used for studies of the effect of phase change on steroid-polyene antibiotics interaction.

The association of polyene antibiotics with cholesterol and ergosterol was examined in dielaidoylphosphatidylcholine vesicles containing 7 mol% of sterol through the competition to epiandrosterone spin probe (see text).

The phosphorus content was assayed according to Bartlett [17]. The concentration of cholesterol in vesicles untreated with polyene antibiotics was determined by use of 'Cholesterol-test Wako (Wako Pure Chemicals)', a modified method of Zak [18]. To obtain the concentration of polyene antibiotics, 50 ml of 50% ethanol was added to 50 μl of the sample and the optical absorption spectrum was measured with a commercial spectrophotometer (RA 112, Union Giken). Molecular extinction coefficients 32 400 $M^{-1} \cdot cm^{-1}$ at 337 nm and 84 000 $M^{-1} \cdot cm^{-1}$ at 385 nm were used for filipin and amphotericin B, respectively. The concentration of the antibiotics in the membranes was estimated by subtracting the concentration in the suspending medium from the total concentration in the dispersion. The former was 0.68 mM for filipin and 0.01 mM for amphotericin B in the medium containing 9% of dimethylsulfoxide.

ESR spectroscopy. ESR spectra were measured with JEOL ME-1X and FE-2X ESR spectrometers equipped with variable temperature control. The rate of temperature decrease was not greater than 0.5°C/min. Integration of the spectra was done by JEOL 980A computer using a commercial program (CWEC 02-741212 CW ESR ACCUM, JEOL).

Results

Interaction of polyene antibiotics with sterol in phosphatidylcholine membranes detected by phosphatidylcholine spin probes

The effect of polyene antibiotics on vesicles was investigated using the three kinds of phosphatidylcholine spin probes. The Pauling-Corey-Koltun molecular model shows that 12 PC* has its nitroxide moiety at the position of about 26 Å from the top of choline moiety and 5 PC* at about 17 Å. Tempo PC* has its nitroxide moiety attached to the choline moiety. The order parameter of the phospholipid acyl chain was increased, by addition of 25 mol% cholesterol to egg yolk phosphatidylcholine membranes, to 0.38 for 12 PC* and from 0.748 to 0.796 for 5 PC* at 20°C. When Tempo PC* was used, the peak height ratio of the low magnetic field line to the central line was decreased from 1.08 to 1.00. When filipin was added to the cholesterol-containing membranes, the order parameter of 12 PC* was decreased and approached that for pure phosphatidylcholine membranes. However, the order parameter of 5 PC* was rather increased by addition of filipin (Fig. 2A). The peak height ratio of Tempo PC* was also affected, decreasing remarkably at low molar ratio and showing further decrease at higher ratio (Fig. 2A). On the other hand, the addition of

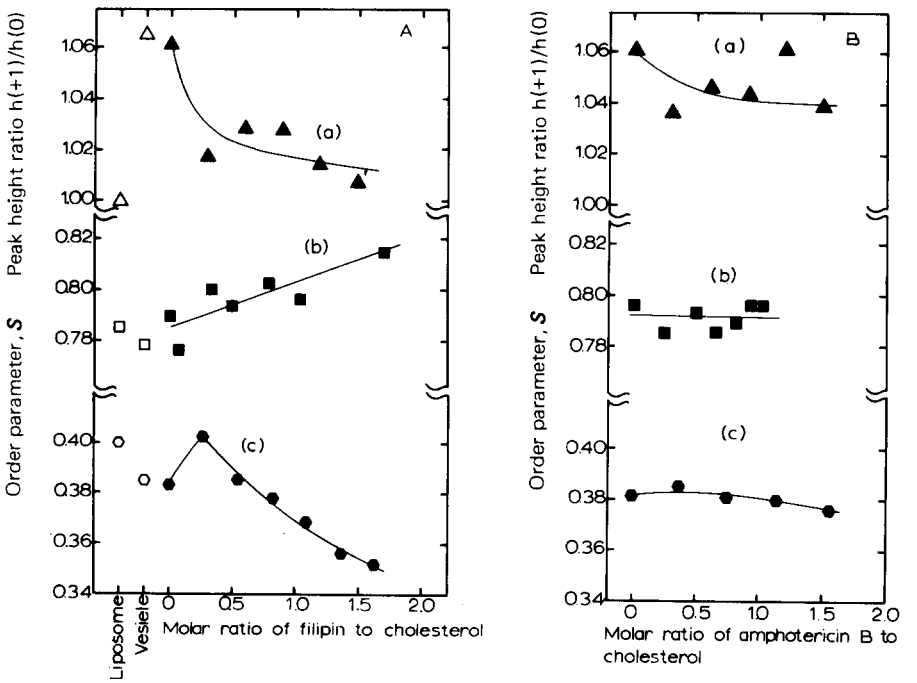

Fig. 2. Effect of filipin (A) or amphotericin B (B) on the order parameter of phosphatidylcholine spin probe ((a) 12 PC*, and (b) 5 PC*) and on the peak height ratio of low field to central lines of Tempo PC* (c) in cholesterol-containing phosphatidylcholine vesicles. Egg yolk phosphatidylcholine containing 1 mol% of spin probe and 27 mol% of cholesterol were mixed with various concentrations of filipin, and then sonicated for 1 min at 0°C under N_2 stream. ESR spectrum was measured at 20°C.

amphotericin B did not change the order parameters of 12 PC* and 5 PC* and also the peak height ratio of Tempo PC* (Fig. 2B).

The effect of polyene antibiotics on ergosterol-containing membranes was also studied with 12 PC* spin probe. The two polyene antibiotics caused almost no change in the order parameter up to the molar ratio of 1.0. Above the molar ratio 1.0, filipin slightly increased and amphotericin B slightly decreased the order parameter.

Control experiments showed that these polyene antibiotics did not affect the parameters when added to pure phosphatidylcholine membranes.

The order parameters and peak height ratio were different between vesicles and liposomes as shown in Fig. 2A. However, after the vesicles were treated with polyene antibiotics, they became sedimentable by a low speed centrifugation ($2000 \times g$, 10 min) and the ESR parameters were almost the same as those of liposomes.

ESR spectrum of steroid spin probe in phospholipid vesicles and liposomes, and effect of polyene antibiotics

ESR spectra of epiandrosterone spin probe in dielaidoylphosphatidylcholine liposomes at temperatures higher and lower than the phase transition temperature (13°C) are shown in Fig. 3. In the liquid crystalline phase, a sharp three line spectrum was obtained, indicating a rapid rotational motion of the spin probe in the membrane. The spectrum for sonicated vesicles was indistinguishable from that for liposomes. The rotational correlation time was calculated using Kivelson's equation [19] based on rapid isotropic rotation. The value obtained from the peak height ratio of the high field to the central lines was 19.5 ns, while the value from the ratio of the low field to the central lines was 4 ns. The difference suggests an anisotropic rotation of the steroid spin probe in the membrane.

The spectral features for epiandrosterone spin probe were different from those for cholestanone spin probe (see Fig. 4c). This is due to different orientation of the spin probe principal axis with respect to the long axis of the steroid molecule. The Pauling-Corey-Koltun molecular model indicated that the π-orbital direction was almost perpendicular to the long axis for cholestanone probe, while it inclined and made an angle of 60° for epiandrosterone spin probe.

On lowering the temperature below the phase transition, the ESR spectrum became broadened but only to small extent (Fig. 3). The spectrum for sonicated vesicles (dotted line) was different from that for liposomes (full line) in this temperature region, the former being much narrower. The difference may be explained by the generally observed broadening phenomena of the phase transition on sonication of phospholipid bilayer membrane [20]. Cholesterol fluidizes the crystalline phase and rigidifies the liquid crystalline phase of membranes [21]. Since the epiandrosterone probe is an analogue to cholesterol, it may disturb the local environments in the crystalline phase.

Effect of polyene antibiotics on the steroid-containing phosphatidylcholine vesicles was investigated using steroid spin probes. Fig. 4 shows the effects of filipin on epiandrosterone spin probe and on cholestanone probe, and the effect of amphotericin B on epiandrosterone probe in dielaidoylphosphatidyl-

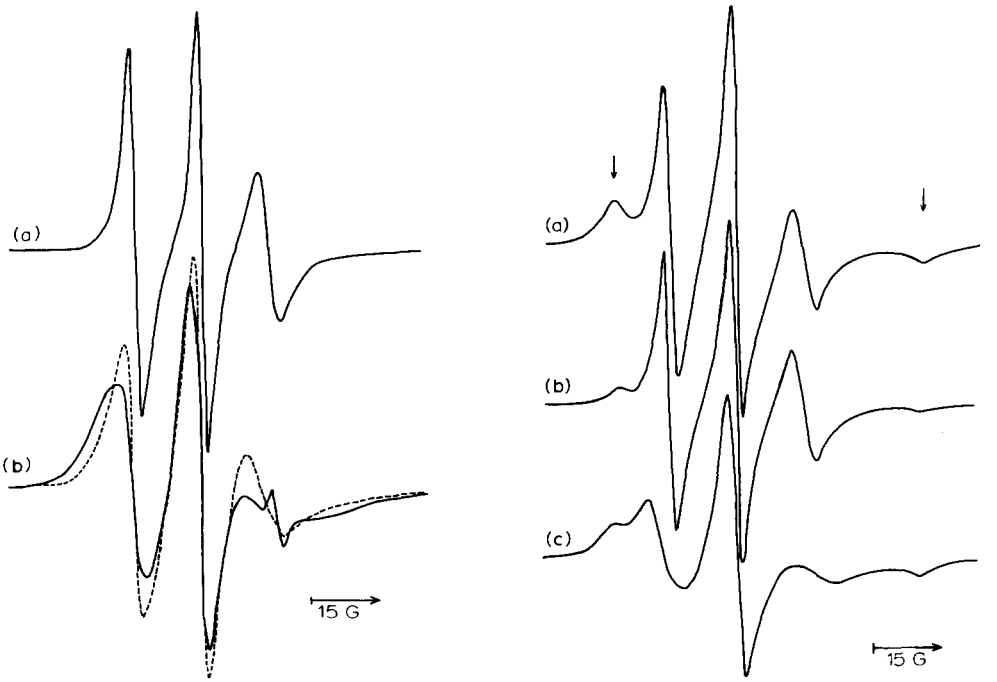

Fig. 3. ESR spectrum of epiandrosterone spin probe in dielaidoylphosphatidylcholine liposomes at (a) 20°C and (b) 5°C. . . . . ., the spectrum for sonicated vesicles at 5°C. 10 mg of dielaidoylphosphatidylcholine containing 1 mol% of spin probe was agitated with glass beads in 1 ml of Tris-buffered saline, pH 7.5, and then sonicated.

Fig. 4. ESR spectrum of (a) epiandrosterone spin probe-filipin; (b) epiandrosterone spin probe-amphotericin B, and (c) cholestanone spin probe-filipin in dielaidoylphosphatidylcholine vesicles at 20°C. 10 μl of (a) 50 mM filipin, and (b) 50 mM amphotericin B in dimethylsulfoxide was added to 0.1 ml of sonicated vesicles of dielaidoylphosphatidylcholine (12.6 mM) containing epiandrosterone spin probe (0.21 mM) and incubated at 30°C for 30 min. In (c), 30 μl of 50 mM filipin was added to 0.1 ml of sonicated vesicles of dielaidoylphosphatidylcholine (12.6 mM) containing cholestanone spin probe (0.37 mM) and incubated. The bound component is indicated by arrows.

choline vesicles. In all systems, a new component with a large overall splitting (≈65 G) appeared in the ESR spectrum (indicated by arrows in Fig. 4). The component increased with the amount of antibiotics added, e.g. 14%, 17% and 23% of bound component was observed for 1.3 mM, 1.9 mM and 2.4 mM of filipin, respectively, in egg yolk phosphatidylcholine vesicles at 20°C. The large splitting value indicates strong immobilization of the steroid probe and suggests its binding to polyene antibiotics in the membrane. The splitting value for epiandrosterone probe-filipin (62.5 G) was the same as the for cholestanone probe-filipin (63.5 G). This suggests an equivalent involvement of both 3 and 17 positions of steroid molecule in the binding to filipin. The splitting value for amphotericin B-epiandrosterone probe (64.2 G) was also quite similar to those values. However, the immobilized component was not detected in the system of amphotericin B-cholestanone spin probe. The 3β-hydroxy group is more essential in the binding to amphotericin B than to filipin.

The specific requirement of the 3β-hydroxy group was much stronger in the interaction with a saponin, digitonin. The epiandrosterone spin probe gave the

bound signal predominantly (overall splitting: 65.2 G) in egg yolk phosphatidylcholine membrane, while neither cholestanone probe nor androstan-17β-ol-3-one derivative probe, having 17β-hydroxy group, showed bound signal (data not shown).

Association constant of steroid-polyene antibiotic in phosphatidylcholine membranes

Egg yolk phosphatidylcholine vesicles containing polyene antibiotic and epiandrosterone spin probe were prepared as described in Materials and Methods and the ESR spectrum was measured at 20°C. The fractions of the unbound and bound components were obtained from the spectrum after integration. The association constant was defined as

$$K_a = \frac{[A\text{-}E]}{[A][E]}$$

where [A], [E] and [A-E] are the concentrations of polyene antibiotic, unbound and bound epiandrosterone spin probe in the vesicle membrane, respectively. The 1 : 1 stoichiometry was assumed for the complex formation. The equation was rewritten as

$$\frac{[E]_{total}}{[E]_{bound}} = \frac{1}{K_a}\frac{1}{[A]_{free}} + 1$$

where [A-E] was put equal to $[E]_{bound}$. $[E]_{total}$ was measured from the ESR spectrum compared with that of standard of spin probe. $[E]_{bound} = [E]_{total} \times$ [fraction of the bound component]. $[A]_{free}$ was calculated as described in Materials and Methods. Fig. 5. shows a plot of $([E]_{total})/([E]_{bound})$ vs. $(1)/([A]_{free})$. The linear relationship supports the assumption of 1 : 1 stoichiometry and $1/K_a$ was obtained from the slope. The association constant was 0.28 mM^{-1} for filipin-epiandrosterone spin probe and 0.13 mM^{-1} for amphotericin B-epiandrosterone probe in egg yolk phosphatidylcholine membrane at 20°C.

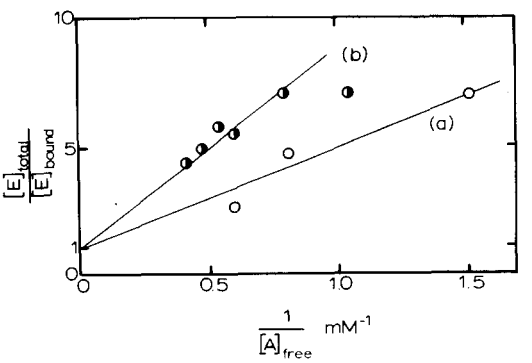

Fig. 5. Determination of the association constant of polyene antibiotics ((a) filipin, and (b) amphotericin B) with epiandrosterone spin probe. Egg yolk phosphatidylcholine vesicles containing 1 mol% of epiandrosterone spin probe and various concentration of polyene antibiotics were prepared and the ESR spectrum was measured at 20°C. The concentrations of $[E]_{total}$, $[E]_{bound}$, and $[A]_{free}$ were obtained as described in text.

Relative magnitude of the association constant of cholesterol or ergosterol with polyene antibiotics was estimated from ESR spectrum of dielaidoylphosphatidylcholine vesicles containing epiandrosterone probe, sterol and antibiotic at 20°C. In this three component system, a simultaneous equilibrium will hold and the association constant K_s will be assumed as follows

$$A + E \leftrightarrow A\text{-}E$$

$$A + S \leftrightarrow A\text{-}S$$

$$K_s = \frac{[A\text{-}S]}{[A][S]}$$

where [S] and [A-S] are the concentration of the unbound and bound sterols in the membrane, respectively. The association constant can be obtained since $[S] = [S]_0 - [A\text{-}S]$ and $[A\text{-}S] = [A]_0 - [A] - [A\text{-}E]$, and [E] and [A-E] can be measured from the ESR spectrum. The subscripts 0 stands for the total concentration. The K_s value was obtained as a mean of three experiments. The association constant of filipin with cholesterol, ergosterol, epiandrosterone probe and cholestanone probe was in a ratio of 1 : 0.05 : 0.02 : 0.003. That of amphotericin B with cholesterol, ergosterol and epiandrosterone probe was in a ratio of 1 : 0.64 : 0.60.

Effect of phase change on steroid-polyene antibiocitic interaction

Phase change in the phospholipid medium greatly affected the complex formation between filipin and epiandrosterone spin probe. Fig. 6 shows change in the ESR spectrum of epiandrosterone-filipin in dielaidoylphosphatidylcholine vesicles when the temperature was lowered from 20°C to 10°C. Fraction of the bound component was estimated and plotted in Fig. 7. A jump in the bound fraction on going from the liquid crystalline to crystalline state is clearly demonstrated. In the estimation of unbound and bound component from the spectrum such as shown in Fig. 6, enough attention was paid to broadening of the spectrum. This correction is necessary not only because of change in the temperature but also because of increase in the vesicular size. The ESR spectrum of vesicles and liposomes were different in the crystalline state as described previously (compare, for example, the two spectra in Fig. 3). In addition to the jump in the bound fraction, the overall splitting of the bound spectrum started to increase below the phase transition (Fig. 8).

On the other hand, when egg yolk phosphatidylcholine was used as the host medium, the spectrum of epiandrosterone probe-filipin complex changed only slightly on lowering the temperature from 40°C to 5°C. Neither the bound fraction nor the overall splitting (Fig. 8) changed markedly. The large changes observed for dielaidoylphosphatidylcholine are therefore due to the phase transition and not to mere change in the temperature. This was further confirmed by experiments using dimyristoylphosphatidylcholine vesicles above and below the transition temperature (22°C).

A discontinuity in the temperature dependence of the bound fraction was also observed for amphotericin B-epiandrosterone probe in dielaidoylphosphatidylcholine vesicles (Fig. 7). However, the jump at the phase transition was

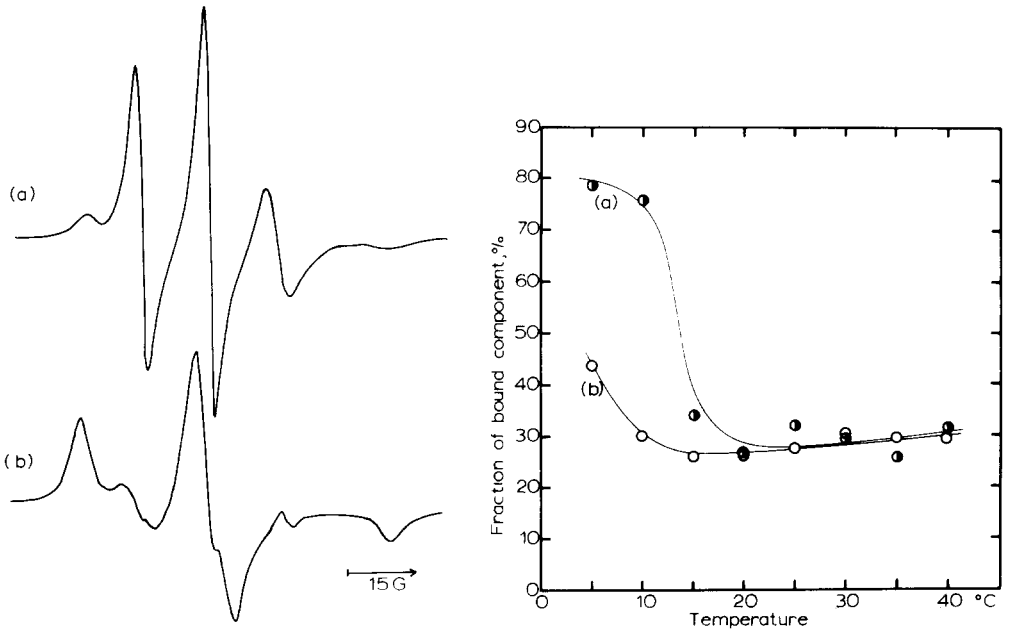

Fig. 6. ESR spectrum of epiandrosterone spin probe-filipin in dielaidoylphosphatidylcholine vesicles at (a) 20° C, and (b) 10° C. Dielaidoyl phosphatidylcholine liposomes containing 1 mol% of epiandrosterone spin probe were mixed filipin and sonicated for 1 min at 0° C under N_2 stream. The final concentration was 9.2 mM, 0.1 mM and 1.2 mM for dielaidoyl phosphatidylcholine, epiandrosterone probe and filipin, respectively.

Fig. 7. Effect of phase transition on the bound fraction of epiandrosterone spin probe to filipin (a) and to amphotericin B (b) in dielaidoylphosphatidylcholine vesicles. (a) Dielaidoyl phosphatidylcholine 9.2 mM, epiandrosterone probe 0.1 mM, filipin 1.2 mM, and (b) dielaidoyl phosphatidylcholine 9.1 mM, epiandrosterone probe 0.1 mM and amphotericin B 0.7 mM. The bound fraction was obtained from the integrated ESR spectrum measured at various temperatures as described in the text.

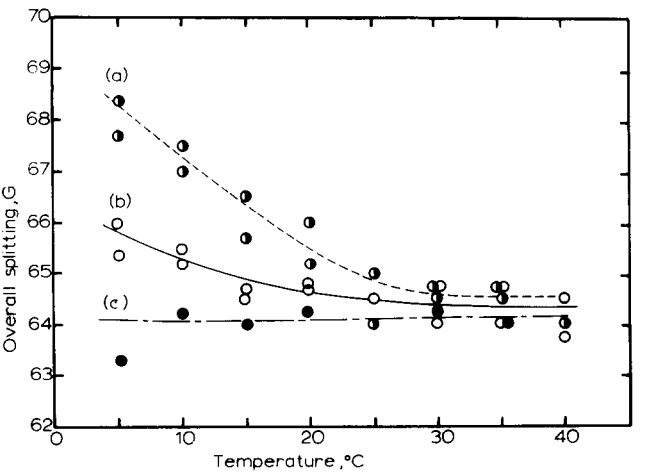

Fig. 8. The overall splitting of the bound spectrum for epiandrosterone spin probe to filipin (a) and to amphotericin B (b) in dielaidoylphosphatidylcholine vesicles. (c) The overall splitting value for epiandrosterone spin probe to filipin in egg yolk phosphatidylcholine vesicles. The sample preparations were similar to those in Fig. 7.

much smaller than that for filipin-epiandrosterone probe. The change in the overall splitting was also slight (Fig. 8). In the liquid crystalline phase, both types of complexes gave the same splitting value.

In egg yolk phosphatidylcholine vesicles, the temperature dependence of the spectrum of amphotericin B-epiandrosterone probe was so small that the bound fraction and the overall splitting value were nearly constant in the temperature rage from 40 to 5° C (Fig. 8).

Discussion

The present spin label study has yielded some insight into the interaction of polyene antibiotics with steroids in phosphatidylcholine bilayer membranes. Several characteristic differences were found in the interactions of filipin and amphotericin B with steroids. Firstly, filipin increased the fluidity in the hydrophobic core of the cholesterol-containing bilayer membranes and decreased the fluidity near carbonyl group and near the polar surface, while amphotericin B did not apparently affect the fluidity in any regions. The effect of filipin can be interpreted to be due to sweeping of cholesterol from the middle of bilayer membranes by the formation of cholesterol-filipin complexes in the region near carbonyl group. The fluidity in the middle region, which had been decreased by cholesterol, now became close to its own value. Flick and Gelerinter [11] have shown that cholestanone spin probe altered the direction of the molecular long axis from perpendicular to parallel to the lipid bilayer when filipin was added. The result for amphotericin B indicates that the complex still interacts with the surrounding phospholipid to maintain the decreased fluidity. The marked difference in the effect on fluidity does suggest a substantial difference in the complex structure between the two antibiotics.

The fluidity change as detected by the spin probe appears to be consistent with some calorimetric studies. Norman et al. [22] have observed a complete reappearance of the phase transition on addition of filipin to cholesterol-phosphatidylcholine dispersion. As for the effect of amphotericin B, De Kruijff et al. [23] obtained only partial recovery of the phase transition. Feinstein et al. [24] have observed using fluorescence polarization technique the amphotericin B did not decrease but even slightly increased the microviscosity of cholesterol-phosphatidylcholine liposomes.

Secondly, the phase change in the host phosphatidylcholine membranes affected the filipin-steroid interaction dramatically, while it affected the amphotericin B-steroid interaction only to small extent. In the liquid crystalline state, the association constant decreased slightly as the temperature was lowered. The overall splitting of the bound signal was also slightly dependent on the temperature. However, the association constant for filipin jumped at the phase transition (Fig. 7). The overall splitting value also largely increased with the decrease in temperature (Fig. 8), indicating further immobilization of the bound steroid probe. These changes in filipin-steroid interaction on going to the crystalline state may be attributed to aggregate formation of smaller complexes. Following the increase in quantity of complexes, the crystallization of the host medium may sequester smaller complexes to form larger aggregates. In the aggregates, the steroid probe may be bound to adjacent complexes addi-

tionally and immobilized to more extent. Moreover, the rotational mobility of the complex as a whole will be much reduced by aggregate formation.

De Kruiff et al. [23] have studied temperature dependence of polyene antibiotic-sterol interactions in *Acholeplasma laidlawii* cells grown on elaidic acid and observed changes in permeability and ATPase activity dependent on the phase. This observation may be relevant to our results.

De Kruijff and Demel [25] constructed the molecular model for the amphotericin B-cholesterol complex as a ciricular arrangement of eight amphotericin B molecules interdigitated by eight cholesterol molecules. The model for filipin was that filipin and cholesterol make a larger aggregate of 150—250 Å in diameter located in the hydrophobic core of the membrane. Our results suggest that filipin-cholesterol aggregates locate near the carbonyl group rather than in the core of lipid bilayer membranes. Similar result was also obtained for biological membranes. We have observed using 5-nitroxide stearic acid probe that the fluidity of human erythrocyte membrane was decreased by filipin. Majuk et al. [26] have observed that the fluidity of vesicular stomatis virion was reduced by filipin treatment using the same probe.

References

1 Norman, A.W., Spielvogel, A.M. and Wong, R.G. (1976) Adv. Lipid Res. 14, 127—170
2 Norman, A.W., Demel, R.A., De Kruyff, B. and Van Deenen, L.L.M. (1972) J. Biol. Chem. 247, 1918—1929
3 De Kruijff, B., Gerritsen, W.J., Oerlemans, A., Demel, R.A. and Van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 339, 30—43
4 Norman, A.W., Demel, R.A., De Kruyff, B., Guerts Van Kessel, W.S.M. and Van Deenen, L.L.M. (1972) Biochim. Biophys. Acta 290, 1—14
5 Bittman, R. and Fischkoff, S.A. (1972) Proc. Natl. Acad. Sci. U.S. 69, 3795—3799
6 Verkleij, A.J., De Kruijff, B., Gerritsen, W.F., Demel, R.A., Van Deenen, L.L.M. and Ververgaert, P.H.J. (1973) Biochim. Biophys. Acta 291, 577—581
7 Tillack, T.W. and Kinsky, S.C. (1973) Biochim. Biophys. Acta 323, 43—54
8 Nozawa, Y., Kitajima, Y., Sekiya, T. and Ito, Y. (1974) Biochim. Biophys. Acta 367, 32—38
9 Kitajima, Y., Sekiya, T. and Nozawa, Y. (1976) Biochim. Biophys. Acta 445, 452—465
10 Bittman, R., Chen, W.C. and Blau, L. (1974) Biochemistry 7, 653—659
11 Flick, C. and Gelerinter, E. (1977) Chem. Phys. Lipids 18, 62—72
12 Singleton, W.S., Gray, M.S., Brown, W.L. and White, J.L. (1965) J. Am. Oil Chem. Soc. 42, 53—56
13 Robles, E.C. and Van Den Berg, D. (1969) Biochim. Biophys. Acta 187, 520—526
14 Hubbel, W.L. and McConnell, H.M. (1971) J. Am. Chem. Soc. 93, 314—326
15 Kornberg, R.D. and McConnell, H.M. (1971) Biochemistry 10, 1111—1120
16 Keana, J.F.W., Keana, S.B. and Beetham, D. (1967) J. Am. Chem. Soc. 89, 3055—3056
17 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466—468
18 Zak, B. (1957) Am. J. Clin. Pathol. 27, 583—588
19 Kivelson, D. (1960) J. Chem. Phys. 33, 1094—1106
20 Marsh, D., Watts, A. and Knowles, P.F. (1977) Biochim. Biophys. Acta 457, 109—132
21 Demel, R.A. and De Kruyff, B. (1976) Biochim. Biophys. Acta 457, 109—132
22 Norman, A.W., Demel, R.A., De Kruyff, B. and Van Deenen, L.L.M. (1972) J. Biol. Chem. 247, 1918—1929
23 De Kruijff, B., Gerritsen, W.J., Oerlemans, A., Van Dijck, P.W.M., Demel, R.A. and Van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 339, 44—56
24 Feinstein, M.B., Fernandez, S.M. and Sha'afi, R.I. (1975) Biochim. Biophys. Acta 413, 354—370
25 De Kruijff, B. and Demel, R.A. (1974) Biochim. Biophys. Acta 339, 57—70
26 Majuk, Z., Bittman, R., Landsberger, F.R. and Compans, R.W. (1977) J. Virol. 24, 883—892