J. Biochem. 129, 725–732 (2001)

Effect of Aggregation of Amphotericin B on Lysophosphatidylcholine Micelles as Related to Its Complex Formation with Cholesterol or Ergosterol[1]

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Received October 16, 2000; accepted February 9, 2001

The effect of aggregation of amphotericin B (AmB), as well as the complex formation of AmB with cholesterol or ergosterol, was investigated in micelles and vesicles. AmB in lysophosphatidylcholine (LPC) micelles adopted a more favorable monomeric form than that in other drug formulations. At an LPC/AmB ratio of 200, AmB existed only in monomeric form. Such monomeric behavior is likely dependent upon the fluidity and size of the micelles. In LPC micelles composed of 90% monomeric AmB, AmB-ergosterol complex formation occurred with an increase in the sterol concentration, but the complex formation of AmB-cholesterol was slight. On the other hand, in LPC micelles composed of 40% monomeric AmB, the complex formation of AmB-cholesterol as well as AmB-ergosterol was extensive. These results suggest that the complex formation of AmB with both sterols is highly dependent upon the aggregated state of AmB. In addition, using monolayers, mixtures of AmB/LPC/ergosterol were became more stable with rising temperature, while the stability of mixtures of AmB/LPC/cholesterol remained unchanged, implying that complex formation of AmB with cholesterol is different from that of AmB with ergosterol.

Key words: amphotericin B, complex formation, ergosterol, lysophosphatidylcholine, micelle.

Amphotericin B (AmB), a heptaene macrolide produced by *Streptomyces nodosus*, is one of the most potent and effective antibiotics used to combat systemic fungal infections, despite unpleasant toxicity. The increased frequency of organ transplantation and epidemics of acquired immunodeficiency syndrome (AIDS) are the main reasons for the significant increase in fungal infections observed in recent years (*1*, *2*). The most widely accepted model for the anticellular activity of AmB involves the formation of AmB-sterol complexes in cell membranes, which subsequently associate into a transmembrane barrel with a large -OH lined aqueous pore down the middle, although no direct experimental evidence for this is available (*3*). The primary target of AmB is ergosterol, the main sterol in the fungal cell membrane, because AmB-ergosterol complexes are stronger than AmB-cholesterol complexes. However, there are only subtle differences in the synthesis and structure of ergosterol and cholesterol, the major sterol in mammalian cell membranes. The molecular basis for the interaction of AmB with either ergosterol or cholesterol is not known in detail, nor is the relationship between the aggregation states of AmB and its consequences on the better selectivity of AmB toward ergosterol.

Hamilton *et al.* (*4*) have proposed that drug toxicity might be related to the existence of AmB aggregates forming phase-separated domains in the lipid phase, and the aggregates having a tendency to separate into a rigid phase within the membrane. Wójtowicz *et al.* (*5*) have also pointed out that the presence of aggregated AmB in preparations used for pharmacological treatment correlates with drug-selective toxicity. Hence, much effort to reduce the toxicity of AmB has focused on formulating the drug in association with a variety of amphipathic molecules (*6*, *7*). On the other hand, the selectivity of the drug for ergosterol rather than cholesterol has been shown to be maximal when monomeric or, preferably, slightly aggregated AmB is used. In this way, it is generally accepted that there exists a close correlation between the aggregation state of AmB in the preparations used and its toxicity (*8*). Therefore, attention has focused recently on the effect of different self-associated AmB species or monomers against fungal and mammalian cells (*9*). The main strategy consists of varied drug delivery systems (DDS) such as surfactant micelles (*10*, *11*), liposomes (*5*, *12*, *13*), and lipid emulsions (*14*, *15*). Unfortunately, a definitive anti-mycotic DDS without side effects has not yet been developed. Again, the pharmacological usefulness of AmB is based on its higher toxicity to ergos-

[1] This work was supported in part by a grant from the Osaka Prefectural Government through the Special Research Project for Environmental Sciences.

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Abbreviations: AmB, amphotericin B; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)]; DMSO, dimethyl sulfoxide; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; LPC, egg lysophosphatidylcholine; π-*A*, surface pressure-area; TMA-DPH, 1-[4-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene; T_m, phase transition temperature.

terol-containing pathogenic microorganisms than to cholesterol-containing animal host cells.

Lysophosphatidylcholine (LPC) micelles as a new AmB formulation may be effective for improving the therapeutic efficiency of AmB, as described below. LPC is a key intermediate in phospholipid metabolism and occurs as a minor constituent in various cell membranes. In recent years, LPC generated from phospholipase A_2 has been suggested to potentiate the activation of protein kinase C by the second messenger diacylglycerol, and thus may play a crucial role in cell proliferation and differentiation (*16*). LPC itself, with a relatively large hydrophilic moiety, organizes in micellar structures, and its hydrophobic core is markedly larger than that of the drug surfactants, such as ionic deoxycholate (*10*) and nonionic lauroyl sucrose (*11*), used to formulate AmB.

It is the purpose of this study to explore the mechanism of the aggregation of AmB in micelles and vesicles as well as the difference in the complex formation of AmB with cholesterol and ergosterol in membranes. We first describe a spectrophotometric study of the conformers of AmB in lysophosphatidylcholine micelles containing various molar ratios of surfactant to AmB. The results are compared with those of AmB in other micelles and liposomes. We then discuss the complex formation between AmB and cholesterol or ergosterol as related to the aggregation state of AmB. Finally, we discuss the mechanism of complex formation of AmB with cholesterol or ergosterol using a monolayer of a ternary mixture of AmB/sterol/LPC.

MATERIALS AND METHODS

Materials—Amphotericin B (AmB) was purchased from Sigma (St. Louis, MO). Cholesterol (99+% pure) and ergosterol (approximately 90% pure) were from Sigma. Ergosterol was recrystallized twice from ethanol. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), and 1,2-dimyristoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (DMPG), all about 99% pure, were purchased from Sigma. 1-[4-(Trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH; approximately 95% pure) was purchased from Sigma. Lysophosphatidylcholine from egg yolk (LPC) and sodium deoxycholate were from Wako Pure Chemicals. The LPC contained 66% palmitic, 24% stearic, 6% oleic, and 1% linolic acid at position 1, with an average molecular weight of 504 (*17*). *N*-Dodecyl-β-D-maltopyranoside (lauroyl maltose) was purchased from Dojin Chemicals. All solvents and other reagents were of the highest purity available, and used without further purification.

Absorption Spectroscopy—Absorption spectroscopy of pure AmB was performed in aqueous solution or organic solvent, with micelles or vesicles. First, a stock solution in which AmB was dissolved in 1-propanol/DMSO (1:1, v/v) at a concentration of 1 mM was prepared. Then, AmB in aqueous solution or organic solvent was diluted by a factor of 100 with water or 1-propanol/DMSO (1:1, v/v) so that the concentration of the AmB was 10 μM. For AmB in micelles such as LPC, sodium deoxycholate, or lauroyl maltose, surfactants were added to the water (0.1 mg/ml), and the mixtures were sonicated in a Bath-type sonifier (Branson Model Sonicator, Yamato) at 50°C. Subsequently, the above-described stock solution was added so that the concentration of AmB was 10 μM. Finally, the mixtures were sonicated at 50°C for 30 min. Incorporation of AmB into DPPC vesicles was performed by the same method as for micelles. In this procedure, the dispersions were shown to consist of small unilamellar vesicles, as described in our previous paper (*18*). In addition, absorption spectroscopy was performed with LPC micelles with varying molar proportions of cholesterol or ergosterol. AmB and sterols at various molar ratios were dissolved in 1-propanol/DMSO (1:1, v/v) so that the concentration of AmB was 1 mM, and samples were prepared by the same method as for micelles. Absorption spectra (between 300 and 450 nm) of AmB in water, 1-propanol/DMSO, LPC micelles, and vesicles were measured at room temperature against water or DPPC vesicles as a blank. Absorption spectra were recorded with a double-beam spectrophotometer (type Ubest V-530, JASCO). All measurements were taken in 1.0 cm pathlength quartz cuvettes.

Surface Pressure Measurements—AmB, LPC, cholesterol, and ergosterol were dissolved separately in 1-propanol/dimethyl sulfoxide (DMSO) (1:1, v/v) so that the concentration of each component was 0.54 mM. For mixed monolayers, ternary mixtures of AmB, LPC, and cholesterol or ergosterol were obtained by mixing equal volumes of the solutions containing each component before application. The apparatus (type HBM-AP, Kyowa Interface Science) used for measuring surface pressure has already been described in detail elsewhere (*18*, *19*). The trough (70 × 14 × 0.5 cm) was coated with Teflon and the entire balance was surrounded by a water jacket, providing temperature control within 1°C. Monolayer spreading was performed by the direct application of numerous small drops (100 μl) of the above-described solutions onto the surface of the water with a microsyringe (Hamilton). Before compression, the surface pressure of the monolayer did not exceed 0.2 $mN \cdot m^{-1}$. Surface pressure-area (π-A) isotherms were obtained using a compression velocity of 0.0567 $m^2 \cdot mg^{-1} \cdot min^{-1}$.

Fluorescence Polarization Measurements—Membrane fluidity was followed with a fluorescence probe, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) as a steady-state anisotropy probe. TMA-DPH was dissolved in tetrahydrofuran at a concentration of 5 mM. Aliquots of TMA-DPH solution were added to the micelle or vesicle solutions at a probe-to-lipid ratio of 1:400 for micelles and 1:14 for vesicles, and the mixtures were sonicated in a Bath-type sonifier at 50°C for 30 min, then quickly cooled to room temperature. The final concentration of TMA-DPH was 5 μM. Fluorescence measurements were performed on a fluorescence spectrophotometer (type FP-175, JASCO). The excitation and emission wavelengths were 365 and 450 nm, respectively. The fluorescence measurements were made over the range of 20–50°C. The fluorescence anisotropy, P, was calculated from the following equation:

$$P = (I_{VV} - G \cdot I_{VH})/(I_{VV} + 2G \cdot I_{VH}), \quad (1)$$

where I_{VV} and I_{VH} are the fluorescence intensities measured with parallel and perpendicular polarizers, respectively; G is the instrumental factor, which is derived from $G = I_{HV}/I_{HH}$.

RESULTS

Spectroscopic Characteristics of Amphotericin B in Aqueous Solutions, Organic Solvents, Micelles, and Vesicles—The absorption spectrum of AmB is very sensitive to conformational changes induced by self-aggregation of the polyene or by its association with other compounds. Figure 1 shows absorption spectra of pure AmB in aqueous solution [1% 1-propanol/dimethylsulfoxide (DMSO) (1:1, v/v)], 1-propanol/DMSO, LPC micelles, and DPPC vesicles. The spectra exhibit four bands, at 333 (band 1), 356 (band 2), 386 (band 3), and 413 nm (band 4), except the spectrum in aqueous solution. The spectrum of AmB in aqueous solution is very similar to the others, although it is shifted to shorter wavelength by approximately 4 nm, due to movement in the more polar environment. However, the spectroscopic characteristics are quite different; the magnitude of band 1 is in the order DPPC ≧ aqueous solution > LPC ≫ 1-propanol/DMSO, whereas that of band 4 is in the order 1-propanol ≫ LPC > aqueous solution ≧ DPPC. Band 1 is regarded as characteristic of "aggregates," in which the polyene chromophores are stacked so as to interact electronically (*8*, *18*, *20*, *21*). On the other hand, band 4 is regarded as characteristic of "monomers" (*11*, *12*, *22*, *23*). In this case, the spectrum of AmB in 1-propanol/DMSO completely lacks this characteristic in band 1, consistent with other reports (*12*, *22*). The proportion of monomeric AmB in LPC micelles was found to be markedly larger than in aqueous solution or DPPC vesicles. On the other hand, the absorbance of band 4 in 1-propanol/DMSO showed a linear relationship with AmB concentration (not shown). Hence, the proportion of monomeric AmB can be obtained from the absorbance ratio of band 4 in micelles and/or vesicles to that in 1-propanol/DMSO. From Fig. 1, the proportions of monomeric AmB were found to be 19, 15, and 34% in aqueous solution, micelles, and vesicles, respectively.

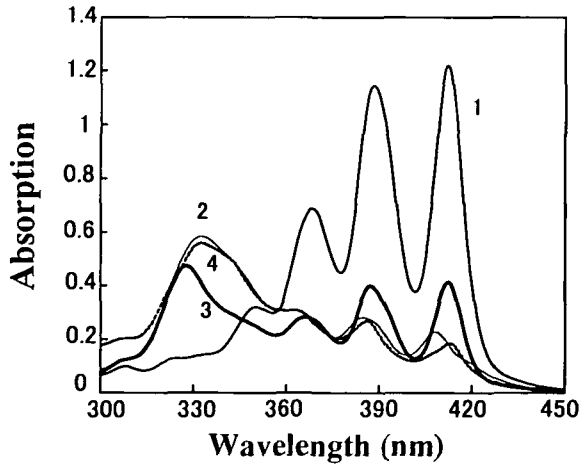

Fig. 1. **Absorption spectra of AmB in 1% 1-propanol/DMSO (1:1, v/v), 1-propanol/DMSO, LPC micelles, and DPPC vesicles.** Absorption spectra were measured as described under "MATERIALS AND METHODS." The concentration of DPPC and LPC was 0.01%. Four representative spectra are displayed with constant AmB concentration (10 μM). Curves 1–4 correspond to 1-propanol/DMSO (100%), 1% 1-propanol/DMSO, LPC micelles, and DPPC vesicles, respectively.

Figure 2 shows the absorption spectra of AmB in LPC and deoxycholate micelles containing different molar ratios of surfactant to AmB. The spectrum of AmB in LPC micelles changed gradually with increasing amounts of LPC at relatively low molar ratios of LPC to AmB, and approached that characteristic of AmB in 1-propanol/DMSO. On the other hand, the spectrum in deoxycholate micelles changed little even at high molar ratios of surfactant to

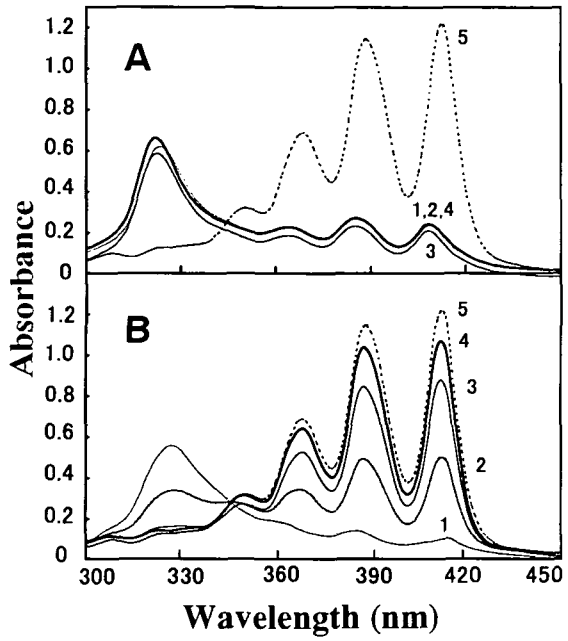

Fig. 2. **Absorption spectra of AmB in micelles with different molar ratios of surfactant to AmB.** (A) deoxycholate micelles. (B) LPC micelles. The surfactant concentrations in (A) and (B) correspond to 20 μM, 200 μM, 400 μM, and 2 mM for curves 1, 2, 3, and 4, respectively. Curve 5 in (A) and (B) corresponds to the spectrum of monomeric AmB in 1-propanol/DMSO. All spectra are displayed at a constant AmB concentration (10 μM).

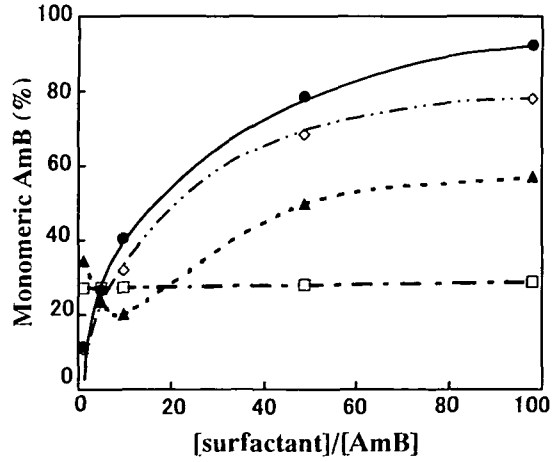

Fig. 3. **Plots of the proportion of monomeric AmB *versus* the molar ratio of surfactant to AmB.** Curves ●, ▲, □, and ◇ correspond to LPC, lauroyl maltose, deoxycholate micelles, and DMPC/DMPG (7:3, mol/mol) vesicles, respectively. The proportion of monomeric AmB was derived from the absorbance ratio of band 4 in the micelles and/or vesicles in Fig. 2 to that in 1-propanol/DMSO.

AmB. Figure 3 shows plots of the increase in monomeric AmB *versus* the molar ratios of surfactant to AmB in various micelles, including LPC, lauroyl maltose, and sodium deoxycholate (commercial form, Fungizone), and vesicles, including DMPC, DPPC, and DMPC/DMPG (7:3) (commercial form, AmBisome). When the concentration of LPC was increased, the aggregated state of AmB in micelles changed to a molecularly dispersed state, with more than 90% of the AmB in the monomeric form at an LPC to AmB ratio of 200. With lauroyl maltose micelles, AmB dissociated into the monomeric form as well, but the AmB in the monomeric form was less than 60% even at a ratio of 200. In contrast, AmB in deoxycholate micelles existed mainly in aggregates, even when the surfactant was further increased. In DMPC/DMPG (7:3) vesicles, the behavior of AmB was similar to that in LPC micelles, but less than 80% was in the monomeric form at ratio of 200. On the other hand, the behavior of AmB in DMPC or DPPC vesicles was markedly different from that in DMPC/DMPG (7:3) vesicles, an increase in the monomeric form being scarcely found over a wide range of ratios (data not shown), consistent with a report by Fujii *et al.* (*20*).

Complex Formation of Amphotericin B with Cholesterol and Ergosterol in LPC Micelles—It is probable that there is a greater affinity of AmB for ergosterol than cholesterol. It is also considered that ergosterol has a much higher affinity for aggregated AmB as compared with monomeric AmB (*24*), and does not react with monomeric AmB (*25*). Nevertheless, no direct evidence for this has been obtained so far. Absorption spectra of AmB in 1-propanol/DMSO, where AmB exists only in the monomeric form, were first measured when cholesterol or ergosterol was added in various molar proportions. The resulting spectra for sterol-containing AmB were the same as the spectrum of pure AmB, showing no complex formation by monomeric AmB with either sterol (data not shown). Figure 4 shows absorption spectra of AmB when cholesterol or ergosterol was added in various molar proportions to LPC micelles. The spectrum changed progressively with increasing amounts of cholesterol or ergosterol, accompanying a decrease in band 4. The decrease in band 4 is attributable to complex formation between AmB and sterols. This characteristic was striking in the ergosterol-containing system.

According to Mazerski *et al.* (*26*), the absorption spectrum of free AmB in the monomeric form differs markedly from that of the AmB-sterol complex, *i.e.*, free AmB in the monomeric form exhibits a very high molar extinction coefficient ($\varepsilon = 1.7 \times 10^5$ $M^{-1}\cdot cm^{-1}$), while complexes exhibit a low value ($\varepsilon = 2 \times 10^4$ $M^{-1}\cdot cm^{-1}$) with an acceptable level of error (± 5%). On the other hand, it has been shown that AmB in the aggregated form has no band 4 (*11*, *27*, *28*). Therefore, the mole fraction of AmB-sterol complex in sterol-containing AmB/LPC micelles can be obtained from the following equation:

$$\frac{A_{II}}{A_I} = \frac{1.7 \times 10^5 (1 - X)C + 2 \times 10^4 XC}{1.7 \times 10^5 C} \qquad (2)$$

where A_I and A_{II} are the respective absorbances of free AmB and sterol-containing AmB in band 4, C the concentration of free AmB in the monomeric form, and X the mole

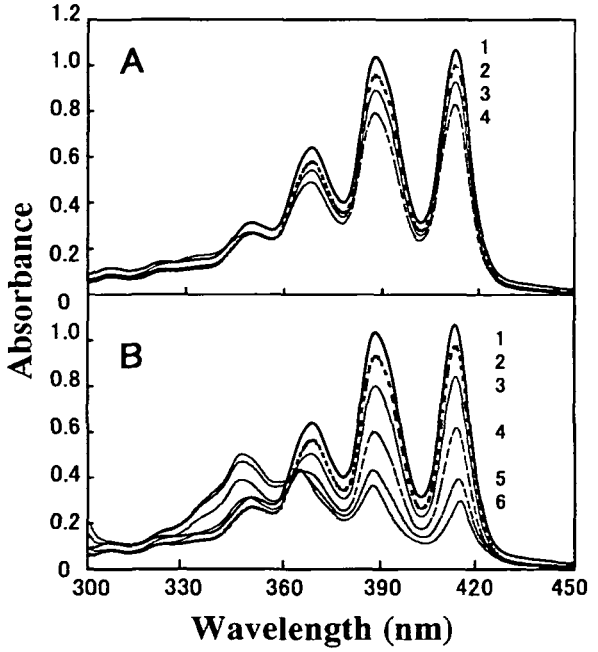

Fig. 4. **Absorption spectra of AmB with various molar ratios of sterol to AmB in LPC micelles.** (A) cholesterol. (B) ergosterol. In (A), curves 1–4 correspond to molar ratios of 0, 0.5, 4, 8, respectively. In (B), curves 1–6 correspond to molar ratios of 0, 0.25, 1, 2, 4, 8, respectively. All spectra are displayed with constant AmB (10 μM) and LPC (2 mM) concentrations.

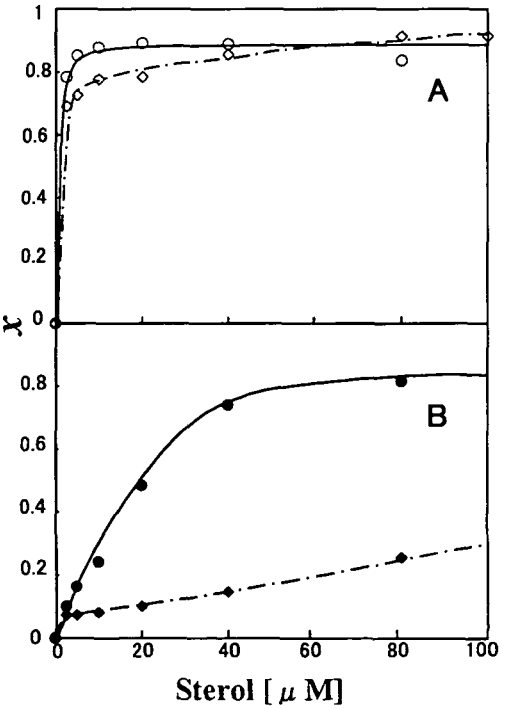

Fig. 5. **Plots of the mole fraction of AmB-sterol complex (*X*) with AmB (A) in 40% monomeric form and (B) in 90% monomeric form as a function of the concentration of sterol.** Curves ○, ◇ in (A) and ●, ◆ in (B) correspond to ergosterol and cholesterol, respectively. The values of X were derived from Eq. 3 using the absorbances of band 4 in Fig. 4. The concentrations of AmB and LPC were 10 μM and 2 mM, respectively. The values are the averages of three experiments.

fraction of the AmB-sterol complex. Equation 2 becomes approximately

$$X = 1.13(1 - A_{II}/A_I) \quad (3)$$

The values for the mole fraction of AmB-sterol complex (X), were derived from Eq. 3 using the absorbances of band 4 in Fig. 4. Figure 5 shows plots of X as a function of the concentration of sterol when 90 and 40% of the AmB is in the monomeric form, respectively. With LPC micelles composed of 90% monomeric AmB, formation of AmB-ergosterol complexes occurred slowly with increasing concentration of sterol, but only a small proportion of AmB formed AmB-cholesterol complexes. In contrast, with LPC micelles composed of 40% monomeric AmB, the complex formation of both AmB-ergosterol and AmB-cholesterol complexes occurred more markedly than when 90% of the AmB was in the monomeric form. These results indicate that the formation of complexes of AmB with both sterols is highly dependent upon the aggregation state of AmB. In addition, the AmB-ergosterol curve increases steeply with increasing amounts of sterol, with a maximum value at a concentration of 10 μM (corresponding to an AmB/ergosterol molar ratio of 1).

Effect of Temperature on Monolayers of Ternary Mixtures of Amphotericin B/Sterols/LPC at the Air/Water Interface—By means of surface pressure measurements, the interactions of AmB with cholesterol or ergosterol in LPC monolayers were investigated. Figure 6 shows π-A isotherms for ternary mixtures of AmB/cholesterol/LPC (1:1:1 molar ratio) and AmB/LPC/ergosterol (1:1:1 molar ratio) on a subphase of water as a function of temperature. For cholesterol and ergosterol, the limiting area was almost same (0.4 $nm^2 \cdot molecule^{-1}$) and the spreading isotherm of sterols was independent of temperature in the range of 20–35°C (data not shown). This is consistent with a report by Baglioni *et al.* (*29*). The monolayer of AmB did not show surface pressure above 20 $mN \cdot m^{-1}$, and its spreading isotherm showed a small decremental shift (data not shown). As shown in Fig. 6A, the isotherm of AmB/LPC/cholesterol showed both a small decremental shift and a decrease in collapse pressure with increasing temperatures up to 30°C. In contrast, the isotherm of AmB/LPC/ergosterol showed both a large incremental shift and a large increase in collapse pressure with increasing temperatures up to 35°C (Fig. 6B). These results indicate that the formation of complexes of AmB and cholesterol differs from that of complexes of AmB and ergosterol.

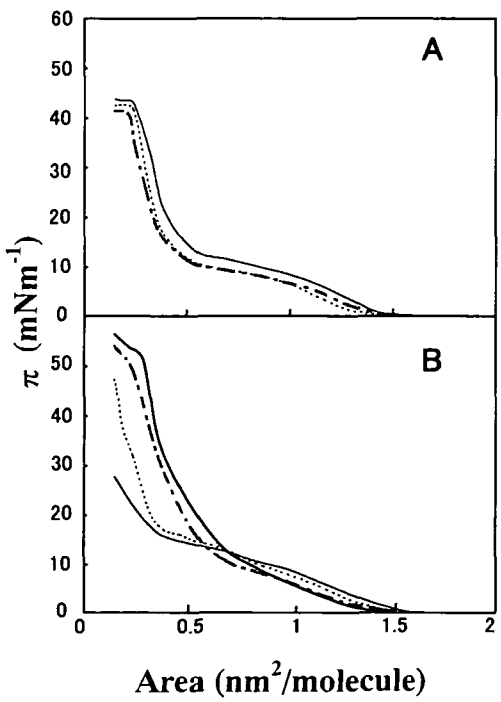

Fig. 6. **Surface pressure-area (π-A) for ternary mixtures of AmB/LPC/sterol on a subphase of water as a function of temperature.** (A) AmB/LPC/cholesterol (1:1:1, mol/mol/mol). (B) AmB/LPC/ergosterol (1:1:1, mol/mol/mol). π-A isotherms were measured as described under "MATERIALS AND METHODS." Curves ——, ------, – – –, —— in (A) and (B) correspond to 20, 25, 30, and 35°C.

DISCUSSION

AmB should be in a self-associated form in order to induce K^+ permeation in cholesterol-containing liposomes, and any form of the antibiotic (monomeric or self associated) induces K^+ leakage from ergosterol-containing membrane models (*21*, *30*, *31*). In a monomeric form, AmB shows pharmacologically less acute and long-term toxicity in terms of mouse survival (*10*). Similarly, in mixed micellar systems, where AmB exists almost in a monomeric form, the hemolytic effects on erythrocytes are minimal with full retention of antifungal activity (*32*). In this way, the aggregation state of AmB is a determinant in toxicity. Therefore, we intended to improve the toxicodynamic profile of AmB through the development of a novel micellar formulation.

Optical spectroscopy appears to useful for studying the mechanism of the action of AmB, since AmB possesses a conjugated heptaene backbone that exhibits distinctive spectral properties that are remarkably sensitive to changes in the local environment. AmB also has very characteristic spectral properties when solubilized in some organic solvents in which AmB exists in a monomeric form. When AmB is present in lipid membranes, however, it is difficult to obtain spectra comparable to the spectra in organic solvent because of the overlapping of the spectra of the membrane-bound forms of AmB and the self-aggregation of AmB (*30*, *33*). When AmB was incorporated into LPC micelles, the changes in the absorbance spectra due to the aggregation of AmB and its formation of complexes with sterols such as cholesterol and ergosterol could be monitored reliably, because most of the AmB in a monomeric form could be incorporated into LPC micelles, as described below. When AmB in LPC micelles is mostly in a monomeric form, its absorbance spectrum is the same as that of AmB in 1-propanol/DMSO (1:1), where the drug is dissociated completely into monomers, with maximal peak at 412 nm. This suggests that the ε value at 412 nm is unaltered, even if AmB forms a complex with LPC. On the other hand, the absorbance at 412 nm (band 4) of AmB in 1-propanol/DMSO is proportional to the concentration of AmB. In addition, band 4 disappears completely when most of the AmB is in aggregated forms. Thus, the proportion of monomeric AmB in LPC micelles can be obtained from the absorbance ratio of AmB in membranes to AmB in 1-propanol/DMSO.

In LPC micelles, 90% or more of the AmB is in a mono-

meric form when the LPC to AmB ratio approaches 200, but other drug formulations have less than 60% of the AmB in a monomeric form. Even in DMPC/DMPG (7:3) liposomes, regarded as one of the best drug systems (*34*), less than 80% of the AmB is in a monomeric form (Fig. 3). Fujii *et al.* (*20*) have reported that the transition from the "monomeric" to the "aggregated" state of AmB begins at a critical concentration of 1 AmB per 1,000 lipids in the membrane of soy phosphatidylcholine vesicles. Such a lipid/AmB ratio corresponds to a fivefold concentration of lipid in LPC micelles, implying that this formulation of LPC is very effective for the monomerization of AmB.

As pointed out by Hamilton *et al.* (*4*), the aggregated state of AmB in micelles and vesicles is likely to be correlated with the membrane fluidity of the lipids. Therefore, we measured the membrane fluidity of micelles and vesicles by fluorescence polarization of 1-[4-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH) (*35*) as fluorescent probe. As shown in Fig. 7, the value of *P*, which refers to membrane fluidity, changes around the phase transition temperatures, T_m, of both DMPC (T_m = 24°C) and DPPC (T_m = 42°C) vesicles, indicating that the membrane fluidity of DMPC and DPPC vesicles declines steeply above T_m. On the other hand, the *P* values for micelles are markedly lower than those of vesicles below T_m, indicating the higher membrane fluidity of micelles. These behaviors suggest that micelles are effective for incorporating AmB in the monomeric form. Unfortunately, the size of micelles other than LPC is very small, generally 2.6–5.2 nm in diameter (*36*). LPC micelles are significantly larger than other micelles, about 8 nm in diameter (*37*), resulting in enhanced incorporation of the monomeric form of AmB.

It is generally accepted that AmB exerts toxic effects on biological membranes by adhering to sterols within cellular membranes. It binds to ergosterol in the fungal cell membrane and to cholesterol in mammalian cells *via* hydrogen bonds and van der Waals forces. This causes the formation of hydrophilic pores, which results in lethal permeability changes (*38*). It is mainly admitted that hydrophobic interactions occur between the steroid skeleton and alkyl tail of sterols and the rigid polyenic part of the macrocyclic ring of AmB (*39*). Based on mechanical calculations of the intra- and inter-molecular energies of AmB-sterol complexes, Langlet *et al.* (*40*) have proposed that van der Waals forces slightly favor the AmB-ergosterol complex. The presence of the double bond at the C-22 position in the terminal hydrocarbon chain of ergosterol makes it more rigid than cholesterol. Such rigidity may be the reason why the contraction is greater in the former system than in the latter. Besides the hydrophobic interactions, it is also believed that the intermolecular complexes between AmB and cholesterol or ergosterol proceed from H-bonding forces *via* water molecules (*39*). The formation of a H-bond is established between the protonable amino group of AmB, as a hydrogen donor, and the 3β OH of the sterol, as an acceptor. On the other hand, Mazerski *et al.* (*26*) have shown that at pH 10, where the amino group is no longer protonated, only about 10% of AmB binds to cholesterol, but about 80% still binds ergosterol. Thus, the van der Waals interaction between the side chain of ergosterol and lipophilic part of AmB could be stronger.

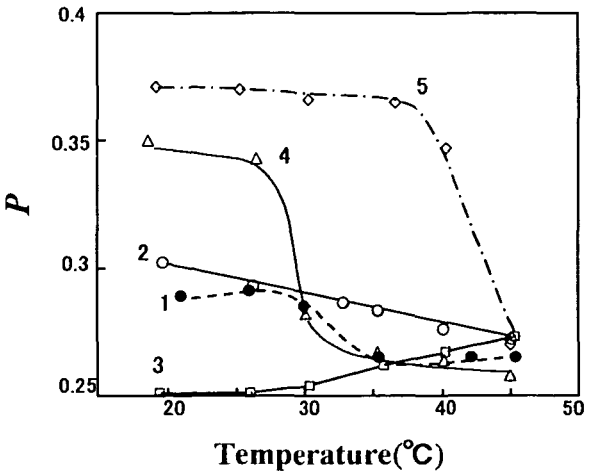

Fig. 7. **Change in the fluorescence anisotropy (*P*) as a function of temperature for various micelles and vesicles.** Fluorescence anisotropy was measured as described under "MATERIALS AND METHODS." Representative curves are shown with a constant TMA-DPH concentration (5 μM). Curves ●, ○, □, △, and ◇ correspond to LPC, lauroyl maltose, deoxycholate, DMPC, and DPPC, respectively.

Gruda and Dussault (*25*) have proposed that ergosterol does not react (or reacts extremely slowly) with monomeric AmB, but dimeric AmB allows complexation with ergosterol. When traces of dimeric AmB are present in the medium (the remainder being monomeric AmB), the complex formed is likely to have the structure AmB-ergosterol-AmB (complex 1). In this process, the kinetics may be the very slow stage known as the rate-determining stage. Subsequently, complex 1 reacts with ergosterol where the stoichiometry is 1:1 AmB:ergosterol (complex 2). In the second process, the kinetics may be fast. On the other hand, it is considered that cholesterol does not react with monomeric or dimeric AmB, but reacts with AmB in a self-associated form. In addition, Scatchard analysis of the binding of AmB to sterols in phospholipid vesicles has revealed that AmB is bound to ergosterol approximately one order of magnitude more tightly than to cholesterol (*41*).

However, to confirm the above-described behavior, it is necessary to obtain an AmB-membrane system containing various proportions of monomeric AmB. This could be elucidated directly by incorporating AmB into LPC micelles. As shown in Fig. 5, with LPC micelles composed of a small aggregated AmB (90% monomeric form), the formation of AmB-ergosterol complexes occurs progressively with increasing ergosterol, but the formation of AmB-cholesterol complexes is slight. In contrast, with LPC micelles composed of large amounts of aggregated AmB (40% monomeric form), the formation of AmB-cholesterol as well as AmB-ergosterol complexes occurs rapidly by adding sterols. These results indicate that the selectivity of AmB appears when the monomeric or, preferably, slightly aggregated drug is used. This is the first observation of the difference between cholesterol and ergosterol in complex form-ation with AmB in membranes.

Using the mixed monolayers, Seoane *et al.* (*42*) have reported that the condensing effect of ergosterol on AmB is larger than that of cholesterol, implying that the interaction is stronger with ergosterol than with cholesterol. As shown in Fig. 6, a similar condensing effect was obtained in

this study. However, it is more important that the π-*A* isotherms between cholesterol- and ergosterol-containing monolayers are quite different in temperature-dependence (Fig. 6); *i.e.*, ternary mixtures of AmB/ergosterol/LPC become more stable as the temperature rises from 20 to 35°C, while ternary mixtures of AmB/cholesterol/LPC remain almost unaltered. This is the first observation of the difference between the two sterols in AmB-containing lipid monolayer systems. It is considered in general that the electrostatic interaction between the molecules is weakened as the temperature rises, but that the hydrophobic interactions increase in strength as the temperature rises in limited ranges (*43*). Thus, the results obtained imply that AmB interacts more tightly with ergosterol than with cholesterol due to hydrophobic interactions between the side chain of ergosterol and the lipophilic part of the AmB molecule. In this connection, Bolard *et al.* (*21*) have shown that the ability of AmB to induce K^+ leakage from cholesterol-containing human erythrocyte cells decreases with increasing temperatures, while, in contrast, K^+ release from ergosterol-containing *Saccharomyces cerevisiae* cells increases 15-fold when the temperature rises from 20 to 37°C. Such behavior may be correlated with an increase in temperature accelerating the formation of AmB ergosterol complexes in monolayers.

The authors thank Dr. Y. Matsumura (Kyoto University, Japan) for use of the fluorescence spectrometer.

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