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Interaction of Amphotericin B with Cholesterol in Monolayers, Aqueous Solutions, and Phospholipid Bilayers

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The interaction of amphotericin B (AmB) with cholesterol was investigated in monolayers, aqueous solutions, and phospholipid vesicles. When AmB was mixed with cholesterol, it formed a stable monolayer, implying complex formation in which the stoichiometry was primarily 1:1 AmB:cholesterol. However, the interaction of AmB with cholesterol in aqueous solutions and lipid vesicles was more complex. In aqueous solutions, cholesterol at low concentrations increased the aggregation of AmB. But higher concentrations of cholesterol caused dissociation of the aggregates of AmB due to the formation of AmB-cholesterol complexes. In lipid vesicles, the effect of cholesterol was different from that in aqueous solutions. Both in aqueous solutions and lipid vesicles, the overall dissociation of AmB molecules occurred on interaction with cholesterol. In addition, the interaction of lipid membranes with AmB-cholesterol complexes was investigated by differential scanning calorimetry. The incorporation of AmB into lipid bilayers led to broadening of the lipid transition and a slight decrease in the transition enthalpy, showing that one lipid molecule per AmB molecule was immobilized. However, the number of immobilized lipid molecule per AmB molecule increased in the coexistence of cholesterol, due to the complex formation between AmB and cholesterol.

Key words: amphotericin B, cholesterol, complex formation, monolayer, vesicle.

For a long time, the polyene antibiotic, amphotericin B (AmB), has been one of the most important agents used to combat systemic fungal infections, although it has the side effects (*1*). AmB exhibits potent antifungal activity, while its activity toward sterol-free bacteria cells is very low. This implies that an interaction between sterol and AmB in biomembranes is responsible for this selectivity (*2*). It has been accepted that AmB forms barrel-like pores with cholesterol, thus inducing permeability changes in the membranes (*3*). However, there is evidence that AmB itself interacts with cholesterol-free lipid vesicles to promote and increase the permeability (*4-6*). In this manner, while many studies have been performed to characterize the pore formation by AmB, it has been difficult to obtain a definite answer because of the complicated interaction of the drug with itself, with sterol, and with lipid and aqueous environments. On the other hand, there is evidence that the toxicity of AmB is related to its aggregated state (*7, 8*). These studies have shown that the toxicity of AmB in the monomeric state toward mammalian cells is significantly decreased without impairment of its antifungal activity, whereas AmB in the aggregated state is nonselective and toxic for both kinds of cells. It is therefore very important to have an efficient method for determining the species of AmB present in solutions and lipid vesicles.

During the course of investigation of the complex formation between AmB and cholesterol in membranes, monolayer techniques were used (*9, 10*). However, there is no consistency in the conformational behavior of the monolayers among them. On the other hand, optical spectroscopies appear to useful in the study of the mechanism of action of AmB, since it possesses a conjugated heptaene backbone which exhibits distinctive spectral properties that are remarkably sensitive to change in its local environment (*9, 11*). For example, visible absorbance (*1, 8, 12, 13*), circular dichroism (*1, 14-17*), and fluorescence spectroscopy (*1, 6*) were used to measure the behavior of AmB incorporated into phospholipid vesicles. However, despite the large number of studies devoted to vesicles, no really satisfactory comprehensive explanation of the interaction of AmB with cholesterol has yet been reported.

The change in the structure of a lipid bilayer induced by an AmB-cholesterol complex is also an important factor related to the formation of channels. From this viewpoint, differential scanning calorimetry (DSC) can provide important information. By means of DSC, a model has been proposed for an AmB-lipid complex, in which AmB interacts equally with two lipid acyl chains, forming a 1:1 complex (*18*). Unfortunately, no information on the structure of the lipid bilayer induced by the AmB-cholesterol complex has yet been obtained, except for the observation of the drug-induced phase separation of binary lipid mixtures (*19-21*).

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Abbreviations: AmB, amphotericin B; DSC, differential scanning calorimetry; DMSO, dimethyl sulfoxide; DPPC, 2,3-dipalmitoyl-*sn*-glycero-1-phosphocholine; π-A, surface pressure-area; T_m, temperature at mid-point in gel-to-liquid crystalline phase transition; ΔH, transition enthalpy.

It is the purpose of this study to explore the stoichiometry of the AmB-cholesterol complex related to the aggregated state and the interaction of lipid membranes with the AmB-cholesterol complex. The question which we first address is how AmB interacts with cholesterol in a mixed monolayer. The results are discussed in relation to the molecular structure of the complex of AmB and cholesterol. We then discuss the complex formation between AmB and cholesterol in aqueous solutions as well as the complex formation in lipid vesicles revealed by means of their absorption spectra. Finally, we consider the change in the structure of a lipid bilayer induced by the AmB-cholesterol complex revealed by means of calorimetric measurements.

MATERIALS AND METHODS

Materials—Amphotericin B (AmB) was purchased from Sigma (St. Louis, MO). 2,3-Dipalmitoyl-*sn*-glycero-1-phosphocholine (DPPC) and cholesterol were from Sigma, the purity being 99+%, respectively. All of the solvents and other reagents were of the highest purity available, and used without further purification.

Surface Pressure Measurement—AmB and cholesterol in various molar ratios were dissolved in 1-propanol/dimethyl sulfoxide (DMSO) (2:1 v/v) so that the concentration of AmB was 0.54 mM. The solutions were stored in a refrigerator and used after about 24 h. The apparatus (type HBM-AP, Kyowa Interface Science) used for measuring the surface pressure has already been described in detail elsewhere (*22, 23*). 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 water with a microsyringe (Hamilton). Thirty minutes was allowed for equilibration of a monolayer before it was compressed. Before compression the surface pressure of the monolayer did not exceed 0.2 $mN \cdot m^{-1}$. The surface pressure-area (π-A) isotherms were obtained using a compression velocity of 0.0567 $m^2 \cdot mg^{-1} \cdot min^{-1}$ at 25°C.

Absorption Spectroscopy—Absorption spectroscopy was performed with aqueous solutions (A) and dilute vesicles (B) with varying the molar proportions of cholesterol, respectively. AmB and cholesterol in various molar ratios were dissolved in 1-propanol/DMSO (1:1 v/v) so that the concentration of AmB was 1 mM. (A) The solutions were then diluted 100 times with water so that the concentration of AmB was 10 μM. (B) After DPPC had been in water (0.1 mg/ml), sonication with a Bath-type sonifier (Branson Model Sonicator, Yamato) was performed at 50°C for 30 min until a transparent solution was obtained. Subsequently, the above-described stock solution was added so that the concentration of AmB was 10 μM. Finally, the mixture was sonicated at 50°C for 30 min. With this procedure, the dispersions were shown to consist of small unilamellar vesicles (*24*). The final concentration of DPPC was 136 μM. Absorption spectra (between 300 and 450 nm) of the aqueous solutions and the vesicle solutions were measured at room temperature against water and DPPC vesicles as blanks, respectively. Absorption spectra were recorded with a double-beam spectrophotometer (type Ubest V-530, JASCO). All measurements were made in 1.0 cm path length quartz cuvettes.

Differential Scanning Calorimetry (DSC)—The samples of vesicles of DPPC and AmB and AmB/cholesterol for DSC measurements were prepared as described by Zhang *et al.* (*25*). After DPPC, and various amounts of AmB and cholesterol had been codissolved in DMSO/chloroform (1:1 v/v), aliquots of the solutions (50 μl) were each transferred to a 70 μl volatile aluminum sample pan, and then the solvent was evaporated off in the dark at 70°C for 5 h to give a thin, homogeneous film. In this sense, complete removal of the solvent was confirmed by measuring the weight of the pan with a thin film. Subsequently, each sample was hydrated, in a pan with 50 μl of water, sealed immediately, then sonicated at the transition temperature of DPPC for 1 h. The final concentration of DPPC was 27.2 mM. DSC measurements were carried out with a DSC 120 (Seiko Electric) equipped with a thermal-analysis data station, calibrated with indium. All of the samples were scanned at the heating rate of $1°C \cdot min^{-1}$. The transition temperature (T_m) was defined as the temperature at the mid-point in the gel-to-liquid crystalline phase transition. Each part of the experiment was repeated at least three times and then averaged.

RESULTS

Conformation of the Amphotericin B/Cholesterol Complex at the Air/Water Interface—Figure 1 shows typical surface pressure-area (π-A) isotherms obtained at 25°C for monolayers of AmB, cholesterol, and a binary mixture of AmB and cholesterol (1:1 mol/mol), on a subphase of water. The AmB monolayer did not show a surface pressure of above 20 $mN \cdot m^{-1}$ even in considerably compressed areas, due to the desorption of AmB from the monolayer (*9*). However, AmB with cholesterol gave a large expansion isotherm compared to cholesterol (limiting area, 0.39 $nm^2 \cdot molecule^{-1}$) even at a high surface pressure above 30 $mN \cdot m^{-1}$, indicating that cholesterol prevented the desorption of

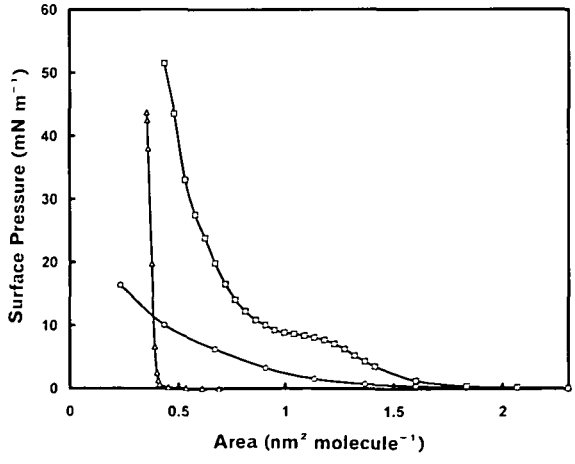

Fig. 1. **Typical surface pressure-area (π-A) isotherms for monolayers of AmB, cholesterol, and a binary mixture of AmB and cholesterol, on a subphase of water at 25°C.** Surface pressures were measured as described under "MATERIALS AND METHODS." Open circles, open triangles, and open squares show AmB, cholesterol, and the binary mixture of AmB and cholesterol (1:1 mol/mol), respectively.

AmB due to the interaction of AmB with it. Then the extent of the interaction of AmB with cholesterol was studied using mixtures of AmB and cholesterol in various molar ratios. Figure 2 shows plots of the increases in area from that of a pure cholesterol monolayer at 20 and 30 mN·m^{-1} as a function of the molar ratio of cholesterol to AmB in the binary mixture. Such extreme surface pressure as 30 mN·m^{-1} was selected for two reasons: (A) The change in pressure *versus* area is great enough to make measurement errors insignificant. (B) From the relationship between the internal pressure of a bilayer and the surface pressure of a monolayer, the packing of lipid in a biological membrane is comparable to that in a monolayer with a surface pressure of $30 < \pi < 35$ mN·m^{-1} (*26*). The curve at 20 mN·m^{-1} was increased by increasing the amount of cholesterol, the maximal value being attained at the AmB/cholesterol molar ratio of approximately 0.5, whereas the curve at 30 mN·m^{-1} exhibited a maximal value at the ratio of approximately 1. This result implies that the complex formation between AmB and cholesterol differs with the magnitude of the surface pressure.

On the other hand, the interfacial behavior of AmB and DPPC was investigated. Figure 3 shows the π-A isotherms for monolayers of AmB, DPPC, and a binary mixture of AmB and DPPC (1:1 mol/mol) spread on water. When AmB was mixed with DPPC, slight expansion from the curve of ideal mixing occurred at low surface pressures below 10 mN·m^{-1}, due to the interaction of AmB with DPPC. However, at high surface pressures above 30 mN·m^{-1}, the π-A curve of a mixed monolayer became close to that of a DPPC monolayer. Thus the AmB molecules were easily squeezed out from the mixed monolayer beyond the state of closed-packing of lipid.

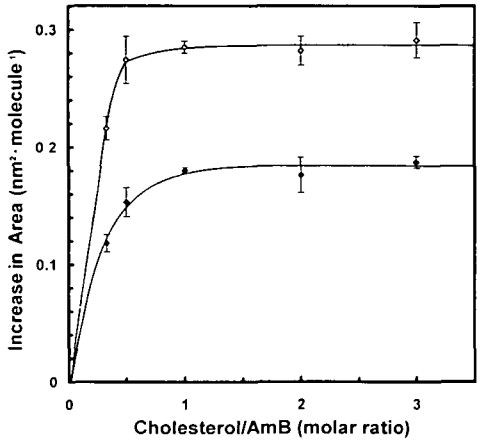

Fig. 2. **Plots of the increase in area of cholesterol monolayer as a function of the molar ratio of cholesterol to AmB in the binary mixture.** Open diamonds (upper) and closed diamonds (lower) show the increases in area at film pressures of 20 and 30 mN·m^{-1}, respectively. The concentration of AmB in each spreading solution was 0.54 mM. The values are means±SE for three experiments.

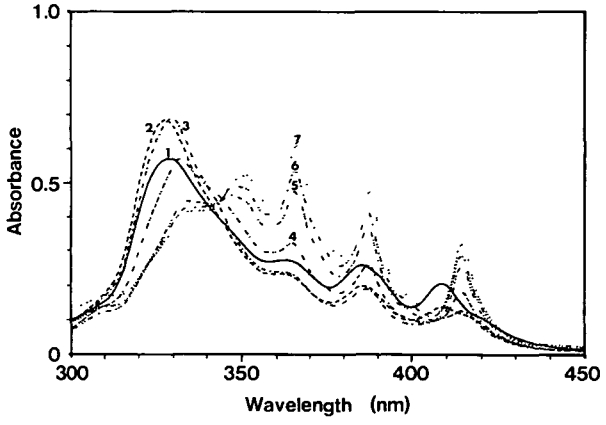

Fig. 4. **Absorption spectra of AmB with different molar ratios of cholesterol to AmB in 1% 1-propanol/DMSO (1:1 v/v).** Absorption spectra were measured as described under "MATERIALS AND METHODS." Seven representative spectra are displayed with a constant AmB concentration (10 μM) but with the following different relative cholesterol/AmB ratios. Curves 1-7 correspond ratios of 0, 0.25, 0.5, 1, 2, 4, and 8, respectively.

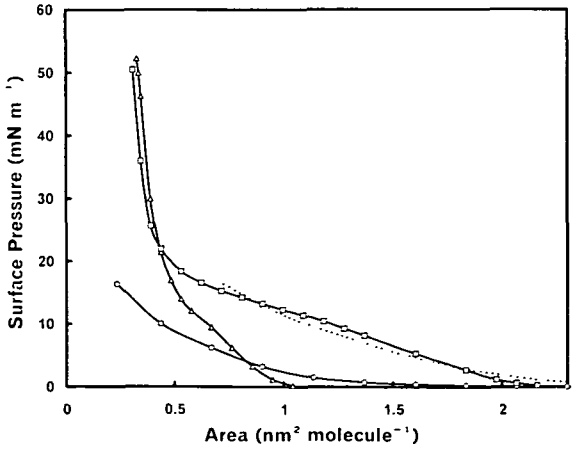

Fig. 3. **π-A isotherms for monolayers of AmB, DPPC, and the binary mixture of AmB and DPPC, on a subphase of water at 25°C.** Open circles, open triangles, and open squares show AmB, DPPC, and the binary mixture of AmB and DPPC (1:1 mol/mol), respectively. The curve (·····) represents the behavior found when the additivity rule was obeyed by the two components.

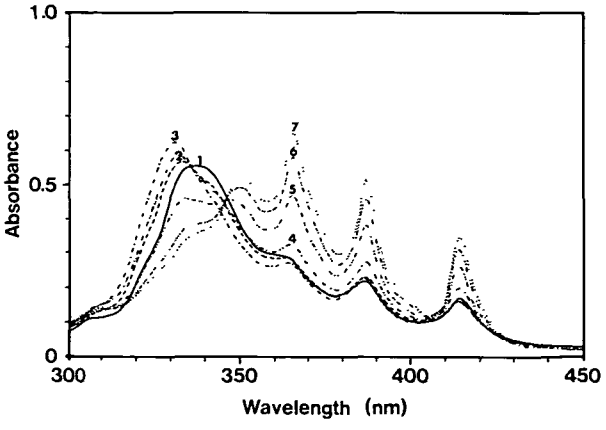

Fig. 5. **Absorption spectra of AmB with different molar ratios of cholesterol to AmB in DPPC vesicles.** The concentration of DPPC in each sample was 1.86 μM. Seven representative spectra are displayed with a constant AmB concentration (10 μM) but with the following different relative cholesterol/AmB ratios. Curves 1-7 correspond ratios of 0, 0.25, 0.5, 1, 2, 4, and 8, respectively.

Spectroscopic Characteristics of Amphotericin B in an Aqueous Phase and DPPC Vesicles—Figure 4 shows typical absorption spectra of AmB in an aqueous solution when cholesterol was added in various molar proportions. The pure AmB solution exhibited a spectrum with four bands at 329 (band 1), 362 (band 2), 385 (band 3), and 408 nm (band 4). The spectrum changed gradually with increasing amounts of cholesterol at relatively low molar proportions, *i.e.*, below a cholesterol to AmB ratio of 0.5, accompanying an increase in band 1, and decreases in bands 2, 3, and 4. Such a spectral change is attributable to an increase in the aggregates of AmB due to cholesterol, since band 1 can be regarded as characteristic of "aggregates" in which the polyene chromophores are stacked so as to interact electronically (*1, 8, 27, 28*). However, on further increases in the amount of cholesterol beyond the cholesterol/AmB ratio of 1, bands 2, 3, and 4 became more pronounced with a simultaneous decrease in band 1. It is noteworthy that on increasing the amount of cholesterol to greater than the cholesterol/AmB ratio of 1, the band at 408 nm shifted to 413 nm (band 4′), and a new band (band 5) appeared around 350 nm. Gruda and Dussault (*29*) have proposed that such a spectral modification is attributable to complex formation between AmB and sterol. Therefore, we attributed the above-described spectral change to complex formation between AmB and cholesterol.

The interaction of AmB with cholesterol was further examined when AmB was incorporated into a lipid environment. Figure 5 shows several representative absorption spectra of AmB in DPPC vesicles when cholesterol was added in various molar proportions. The pure AmB exhibited a spectrum with four bands at 333 (band 1′), 362 (band 2), 386 (band 3), and 414 (band 4′). Thus, band 4′, due to the complex formation between AmB and DPPC, as described under "DISCUSSION," appeared, whereas the band at 408 nm, specific for free monomeric AmB in an aqueous solution, disappeared. The spectrum also showed a strong absorption band (band 1′) which was not observed for the aqueous solutions. When the amount of cholesterol was increased up to the cholesterol/AmB ratio of 0.5, band 1′ alone showed a blue-shift. However, on further increases in the amount of cholesterol beyond the cholesterol/AmB ratio of 1, band 1′ was seen to strongly decrease, and a new intense but flattened band appeared around 350 nm (band 5), accompanying progressive increases in bands 2, 3, and 4. These results indicate that AmB in lipid vesicles changes from an aggregated state into a dissociated state through interaction with cholesterol.

Calorimetric Behavior of DPPC Vesicles Containing Amphotericin B, Cholesterol, and a Binary Mixture of Amphotericin B and Cholesterol—Figure 6 shows DSC scans of DPPC vesicles containing various molar ratios of AmB, cholesterol, and a binary mixture (1:1 mol/mol) to DPPC, respectively. With increasing concentrations of AmB, the transition enthalpy gradually decreased so that at the AmB/DPPC ratio of 0.4, there was only a very weak remnant of a transition and at the AmB/DPPC ratio of 0.8, no transition was visible. Such behavior was consistent with that reported by other researchers (*19, 20*). Figure 7 shows plots of T_m of DPPC *versus* molar ratios of AmB, cholesterol, and the binary mixture (1:1 mol/mol) to DPPC, derived from Fig. 6. With increasing concentrations of AmB, T_m of DPPC increased somewhat. In contrast to AmB, cholesterol progressively lowered T_m within 2°C. On the other hand, the binary mixture lowered T_m up to the

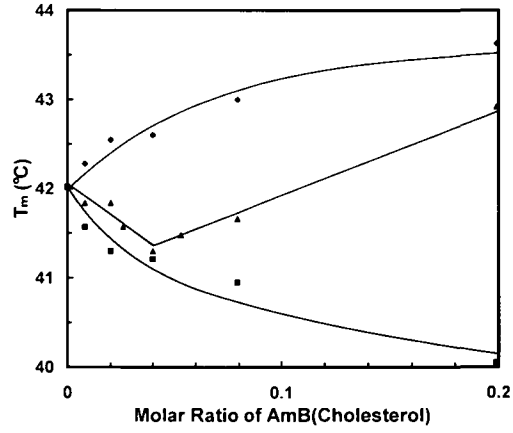

Fig. 7. **Plots of T_m of DPPC *versus* the molar ratios of AmB, cholesterol, and the binary mixture (1:1 mol/mol) to DPPC.** T_m was defined as the temperature at the mid-point in the gel-to-liquid crystalline phase transition of DPPC. Closed diamonds, closed squares, and closed triangles show AmB, cholesterol, and the binary mixture of AmB and cholesterol (1:1 mol/mol), respectively.

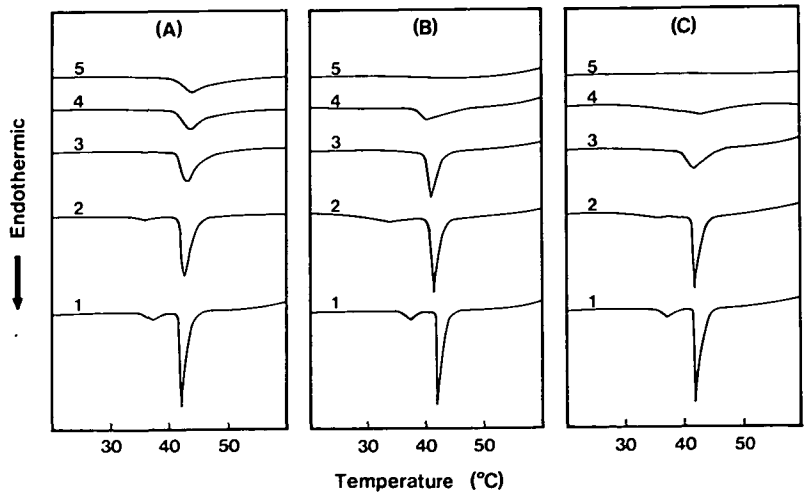

Fig. 6. **DSC scans of various molar ratios of AmB (A), cholesterol (B), and the binary mixture (1:1 mol/mol) (C) to DPPC in lipid vesicles.** Each lot of sample vesicles was prepared as described under "MATERIALS AND METHODS." All samples were scanned at the heating rate of 1°C·min^{-1}. Representative curves are displayed with a constant DPPC concentration (27.2 mM) but with the following different relative molar ratios of AmB, cholesterol, and AmB/cholesterol (1:1 mol/mol) to DPPC. Curves 1-5 correspond to AmB/DPPC molar ratios of 0, 0.02, 0.08, 0.2, and 0.4, respectively.

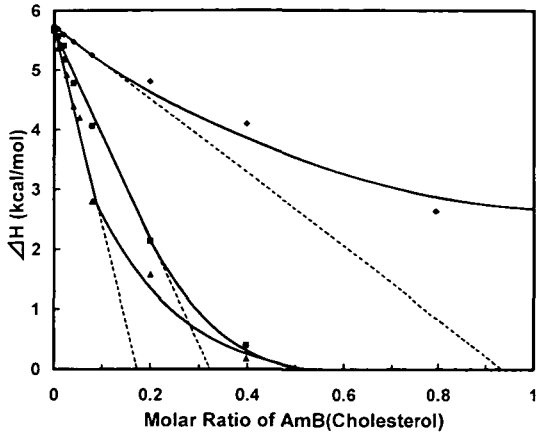

Fig. 8. **Plots of ΔH of the DPPC phase transition as a function of the respective molar ratios of AmB, cholesterol, and the binary mixture (1:1 mol/mol) to DPPC.** The three intersections of the curves with the abscissa (·····) represent the numbers of immobilized DPPC molecules.

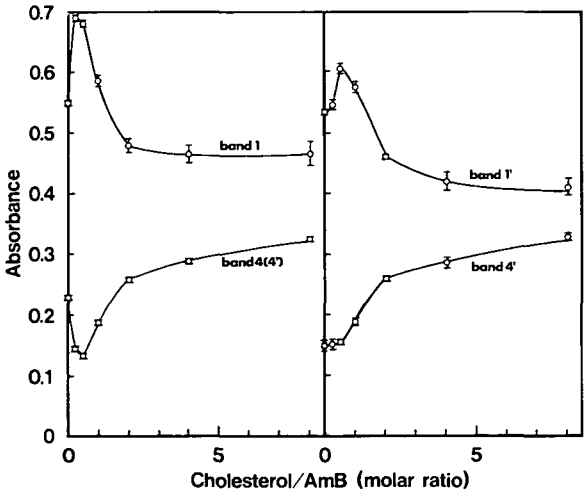

Fig. 9. **Plots of the intensities of the absorbance of AmB/cholesterol of band 1 (band 1') and band 4 (band 4') in 1% 1-propanol/DMSO (1:1 v/v) (left) and DPPC vesicles (right) as a function of the molar ratio of cholesterol to AmB.** The data were derived from the absorption spectra in Figs. 4 and 5. The values are means±SE for three experiments.

binary mixture/DPPC ratio of 0.04, but raised T_m with additional increases.

Assuming that lipid adjacent to such hydrophobic proteins or ionophores does not participate in the transition and that the transition can only be broken down into contributions from free lipid components, the following equation is obtained (*23, 30*).

$$\Delta H = \Delta H^{\circ}\ (1 - N\frac{I}{L}) \qquad (1)$$

where ΔH is the measured enthalpy change of the gel to liquid-crystalline phase transition, ΔH° the value for the pure lipid system, I/L the molar ionophore-to-lipid ratio, and N the number of lipid molecules withdrawn per ionophore, regarded as boundary lipid, which is motionally restricted by the interaction with the ionophore. Figure 8 shows plots of ΔH of the DPPC phase transition as a function of the molar ratios of AmB, cholesterol, and the binary mixture (1:1 mol/mol) to DPPC. From the intersection with the abscissa the number of immobilized DPPC molecules was found to be about one per AmB molecule and about three per cholesterol molecule, respectively. On the other hand, the number of immobilized DPPC molecule per one molecule of the AmB-cholesterol complex was found to be a markedly larger (six) than in the case of AmB and cholesterol separately.

DISCUSSION

De Kruijff and Demel (*31*) have proposed a model based on the complex formation of a circular arrangement of 8 AmB molecules interdigitated by 8 cholesterol molecules. From the results of Scatchard analysis (*32*) and DSC (*18*), it has also been proposed that AmB molecules form a complex with cholesterol molecules in which the stoichiometry is approximately one AmB molecule per cholesterol molecule. In this way, the fact that the stoichiometry is 1:1 AmB:cholesterol in general may be attributable to the following. AmB is characterized by the presence of an amino group of the amino sugar moiety and a carboxyl group at the C-18 position in the macrolide ring. On the other hand, cholesterol has 3β OH in its molecule. Such a molecular feature stimulates the formation of a H-bond, which is established between the protonable amino group of AmB, as a hydrogen donor, and the 3β OH of cholesterol, as an acceptor. Consequently, it may be reasonably assumed to form a complex in which the stoichiometry is 1:1 AmB : cholesterol. However, it has been reported that an AmB-cholesterol complex with 2:1 stoichiometry is formed (*10*). Furthermore, as shown by the circular dichroism results, the conformers in the complexes were too numerous, depending upon the AmB to cholesterol molar ratio (*15, 33*). On the contrary, it is supposed that the binding between cholesterol and AmB is weak or nonexistent in sterol-containing membranes (*27*). In this sense, the stoichiometry of the AmB-cholesterol complex remains unresolved.

In order to elucidate the stoichiometry of the AmB-cholesterol complex, we first attempted to elucidate the interaction of AmB with cholesterol in a monolayer. The results imply that the presence of cholesterol prevents the desorption of AmB because of the interaction of AmB with cholesterol, and that AmB interacts with cholesterol (Fig. 2). The stoichiometry at 20 mN·m^{-1} in this work agrees with a value of two AmB molecule per cholesterol molecule which was obtained in the monolayer study by Saint-Pierre-Chazalet *et al.* (*10*), whereas the stoichiometry at 30 mN·m^{-1} does not agree. A possible explanation for such a discrepancy is that there is a difference between the surface pressures chosen for the interaction of AmB with cholesterol. Saint-Pierre-Chazalet *et al.* (*10*) selected two pressures, 5 and 20 mN·m^{-1}, *i.e.*, lower than the 30 mN·m^{-1} in this work. According to Readio and Bittman (*32*), it was implied that AmB forms both tighter and weaker complexes with cholesterol. From the above-mentioned fact, we deduce that the weaker complex formation at surface pressures below 20 mN·m^{-1} occurred through the association due to the hydrophobic interaction between AmB and cholesterol, whereas the tighter complex formation at surface pressures above 30 mN·m^{-1} occurred

through the formation of an electrostatic H-bond between AmB and cholesterol, with which AmB forms a 1-to-1 complex with cholesterol.

When AmB was mixed with DPPC, slight expansion from the curve of ideal mixing at low surface pressures was observed (Fig. 3). This suggests that AmB weakly interacts with DPPC molecules. Hartsel *et al.* (*5*) proposed that the insertion of AmB toward the lipid bilayer brings the polar polyol region in direct contact with the lipid hydrocarbon, and thereby induces some of the local lipid head groups to "fold around" so as to interact with the polyol. Such an unfavorable juxtaposition between AmB and phospholipid appears to occur in monolayers, resulting in the occurrence of a defect in the monolayers. Hence, at low surface pressures the expansion of a monolayer could be observed. On the other hand, at high surface pressures the interaction between AmB and phospholipid was not observed, since AmB was completely desorbed from the monolayer. Our finding is supported by an earlier monolayer study (*10*).

As judged on absorption spectroscopy, the interaction between AmB and cholesterol in aqueous solutions and lipid vesicles was more complex than that in monolayers. This is mainly attributable to both the monomer and aggregates of AmB being almost always present in both the aqueous solutions and the vesicles, their spectra being intrinsically overlapped (*7, 8*). In the past, complex formation was monitored as the absorption ratios of 408/348 nm (*8*) and 408/363 nm (*34*). Recently, Chapados *et al.* (*7*) revealed, by means of factor analysis to separate the spectra of individual species from the experimental absorption spectra, that both the monomer and aggregates of AmB exhibit absorption bands at 348 (band 5), 363 (band 2), and 385 nm (band 3). So, it is not appropriate to monitor the complex formation by means of earlier methods. On the other hand, it is obvious that the band at 408 nm (band 4) is seen only for the monomer of free AmB in aqueous solutions (*7, 12, 32*). Furthermore, it is implied that band 4 is red-shifted when AmB interacts with sterol in aqueous solutions (*29*). Hence, in Fig. 4, the red-shift from 408 nm to 414 nm is assigned to the complex formation of AmB with cholesterol in aqueous solutions. Similarly, Wietzerbin *et al.* (*35*) first found that the absorption band for AmB at 409 nm, characteristic of the monomeric form in an aqueous medium, shifted to 416 nm when incorporated into red cell ghosts, revealing that an absorption band at 416 nm was characteristic of AmB molecules which had penetrated into the hydrophobic membrane interior, since the positions of the absorption band were dependent on the solvent polarity, expressed as the relative dielectric constant. As shown in Fig. 5, not band 4 at 408 nm but band 4′ at 414 nm was found for all vesicle preparations including cholesterol-free vesicles. This implies that all AmB molecules are incorporated into the lipid bilayer. In addition, with small increases in the amount of cholesterol in the lipid bilayer, band 1′ at 333 nm showed a blue-shift. Based on the concept presented by Wietzerbin *et al.* (*35*), we suppose that the aggregates are transferred from the hydrophobic membrane interior to the outer membrane on the addition of cholesterol.

Figure 9 shows plots of the intensities of the absorption of AmB/cholesterol of band 1 (band 1′) and band 4 (band 4′) in aqueous solutions and DPPC vesicles as a function of the molar ratio of cholesterol to AmB, derived from the curves in Figs. 4 and 5. In aqueous solutions, the complex formation between AmB and cholesterol would not occur at low concentrations below the cholesterol/AmB ratio of 0.5, but accompanying an increase in the aggregates of AmB. Additional increases in the amount of cholesterol obviously decreased the aggregates of AmB. In lipid vesicles, however, an increase in the aggregates was rarely found even at low concentrations below the cholesterol/AmB ratio of 0.5. Additional increases in the amount of cholesterol decreased the aggregates as well as the behavior in aqueous solutions. These results constitute the first spectroscopic observation of the AmB-cholesterol interaction in aqueous solutions and lipid vesicles. Anyhow, the above-mentioned results suggest that the aggregates of AmB dissociate in lipid membranes on interacting with cholesterol.

In addition, with further increases in the amount of cholesterol beyond the cholesterol/AmB ratio of 2, the intensities of both bands 1 and 4 were little affected by the addition of cholesterol. From this result, a stoichiometry of roughly 1:2 AmB:cholesterol could be estimated. In this sense, the stoichiometry obtained on spectroscopy differed from that obtained in the present monolayer study. The reason why alternate stoichiometries exist is as follows. A particular ordered state such as a monolayer at 30 $mN \cdot m^{-1}$ may be considerably favorable for an electrostatic interaction between AmB and cholesterol. Consequently, the most probable binding site of the complex may be one at which the H-bond between the amino group of AmB and the 3β OH of cholesterol is formed, but the complex formation may become not so specific with high ratios of cholesterol to AmB, since the complex formation due to the hydrophobic interaction between the steroid nucleus and the alkyl tail of the lipid in the membrane environment is superimposed (*36*), leading to the formation of numerous conformers (*15*). We consider that such a hypothesis explains why saturable binding stoichiometry cannot be observed on absorption spectroscopy.

Thus far, refinement of the interaction between a lipid bilayer and an AmB-cholesterol complex has not been performed by means of calorimetric measurement, although there have been several investigations on the interaction between a lipid bilayer and AmB (*17-20*). Hence, we investigated the structure of a lipid bilayer induced by a 1:1 AmB-cholesterol complex. The incorporation of both AmB and cholesterol into DPPC vesicles at low concentrations lowered T_m but additional incorporation of the mixture raised T_m (Fig. 7). Assuming that there is no interaction between AmB and cholesterol in bilayers, the mixture should show the mid-point of the T_m of AmB and cholesterol at every concentration. However, the observed curve markedly deviated from the corresponding curve in which there was no interaction between AmB and cholesterol. From our finding, it is obvious that a lipid bilayer is influenced by the complex formation between AmB and cholesterol.

From the intersection with the abscissa, the number of immobilized DPPC molecules was found to be about one per AmB molecule (Fig. 8). Our finding is supported by earlier NMR studies (*14, 37*). However, the incorporation of larger amounts of AmB beyond the AmB/DPPC ratio of 0.08 resulted in only a relatively small further decrease in ΔH. The present work provided the first calorimetric finding. It is apparent that when gramicidin A, a typical

"channel-forming" ionophore, associates in a lipid bilayer, deviation from the linear portion of the enthalpy curve occurs because the number of lipid molecules around the ionophore molecule decreases, as reported in our previous paper (*23*). Hence, we deduce that when AmB molecules are incorporated into a lipid bilayer, AmB markedly tends to form aggregates, as shown by the present spectroscopic observation for lipid vesicles without cholesterol. Plots of ΔH *versus* pure cholesterol revealed an approximately linear relationship in the range measured, and showed that the number of immobilized lipid molecules was approximately three per cholesterol molecule. Our result is supported by an earlier calorimetric study (*38*). On the other hand, plots of ΔH *versus* the binary mixture (1:1 mol/mol) revealed an approximately linear relationship in a rather wide range. Such behavior suggests that the aggregates of AmB dissociate in a lipid bilayer through the formation of the AmB-cholesterol complex. In addition, the number of immobilized lipid molecules per AmB and cholesterol mixture was found to be 1.5-fold as great as with AmB and cholesterol separately. This was the first calorimetric observation of the interaction of an AmB-cholesterol complex with a lipid bilayer. The above-mentioned result implies that the action of AmB toward a lipid bilayer becomes progressively greater as cholesterol is added, contributing to the stabilization of the barrel-like pores formed by AmB and cholesterol in a lipid bilayer.

In conclusion, the mechanism underlying the complex formation between AmB and cholesterol was revealed in monolayers, aqueous solutions and lipid vesicles. From the surface pressure and spectroscopic properties, it is clear that the stoichiometry of 1:1 AmB-cholesterol is the most probable but weaker complexes are formed in the presence of a larger amount of cholesterol. From the spectroscopic and calorimetric characteristics, it is obvious that AmB in the aggregated state dissociates in a lipid bilayer on the complex formation with cholesterol.

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