One-Sided Action of Amphotericin B on Cholesterol-Containing Membranes Is Determined by Its Self-Association in the Medium†

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ABSTRACT: The inducement of K^+ permeability through membranes by the polyene antibiotic amphotericin B (AmB) has been analyzed as a measure of the antibiotic activity. Dose–response curves have been obtained with cholesterol- and ergosterol-containing egg yolk phosphatidylcholine large unilamellar vesicles (LUVs), human erythrocytes, and *Saccharomyces cerevisiae* cells. Conductance changes induced by AmB in sterol-containing planar bilayer membranes have also been studied. AmB self-association in aqueous buffer was determined by circular dichroism (CD) as a function of the antibiotic concentration. Electronic absorption and CD spectra of AmB were recorded in the presence of LUVs. For given AmB concentrations, the extent of permeability inducement is dependent on the lipid concentration. On the other hand, for cholesterol-containing LUVs or erythrocytes, a critical AmB concentration had to be reached before any permeability is observed. Independent of lipid concentration, this concentration was directly related to antibiotic self-association in the aqueous buffer. The same observation was made for erythrocytes and nystatin. The AmB absorption and CD spectra were totally different for ergosterol- and cholesterol-containing LUVs. Formation of single channels by one-sided addition of AmB could be observed only in ergosterol-containing membranes. These data lead us to propose that the permeability pathways induced by amphotericin B or nystatin, in ergosterol- and in cholesterol-containing membranes, are of different natures. In the latter case the antibiotics are only active, by single-sided addition, in the self-associated form. These findings offer important clues for the design of less toxic derivatives of AmB: they should have a low degree of self-association in water.

The polyene antibiotic amphotericin B (AmB),[1] remains the drug of choice for treating most systemic fungal infections. Unfortunately it has negative side effects, such as nephrotoxicity, which limits its efficacy. Two strategies are possible to improve its therapeutic index. The first one is to modify its distribution among organs by using carrier systems such as liposomes or emulsifiers. This step appears to be very promising [for a review, see Brajtburg et al. (1990b)]. The second possibility is to modify the antibiotic molecule in such a way that, for the derivative, the antifungal activity will be preserved and the toxicity for the host will be remarkably diminished. Many AmB derivatives have been synthesized by substitution at the amino group of mycosamine and/or the carboxyl group. In vitro studies show that some of these derivatives can be designed on a rational basis (Chéron et al., 1988).

The mechanism of antifungal activity as well as of toxicity to the host are not yet completely understood for any of the numerous polyene macrolide antibiotics. The following are generally assumed [see for instance Bolard, (1986) and Brajtburg et al. (1990)]. (i) The cell sensitivity to polyene antibiotics is determined by the presence of sterol in the membrane. For instance, sensitivity increases with the ergosterol content in *Saccharomyces cerevisiae* (Richman-Boytas & Parks, 1989). (ii) The higher sensitivity of fungal cells to AmB action originates from its higher affinity for ergosterol-containing membranes (fungal cells) as compared to cholesterol-containing membranes (mammalian cells). (iii) The mechanism of action on both types of cells is of a similar nature. AmB or AmB–sterol complexes would associate and form transmembrane pores. Through such pores free diffusion of many components essential for cell life occurs. AmB-induced lipid peroxidation also plays an important role (Brajtburg et al., 1990) as does the inhibition of membrane pumps (Surarit & Shepherd, 1987; Vertut-Croquin et al., 1988; Capuozzo et al., 1990). It should be stressed, however, that the disturbances in membrane permeability induced by AmB are not always cytotoxic: At sublethal concentrations, stimulation of cell growth has been shown to occur.

The understanding of the differences in the interaction of AmB with cholesterol and ergosterol seems to be of the first importance for selective toxicity improvement. No significant difference in the sensitivity to lipid peroxidation of ergosterol- and cholesterol-containing membranes has been reported, and therefore this process cannot explain the differences in sensitivity between fungal and mammalian cells. Inducement of permeability, in contrast, is sensitive to the difference between ergosterol and cholesterol.

We have chosen, therefore, to analyze the AmB-induced leakage of K^+ from cells as an indicator of the antibiotic activity, because it is known that inducement of permeability is the first event following AmB addition. Human red blood

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[1] Abbreviations: AmB, amphotericin B; AmE, amphotericin B methyl ester; PC, phosphatidylcholine; GMO, monooleoylglycerol; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; DMSO, dimethyl sulfoxide; Tris, Tris(hydroxymethyl)aminomethane; PBS, phosphate buffer saline; LUV, large unilamellar vesicle; SUV, sonicated unilamellar vesicle; CD, circular dichroism.

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cells and *S. cerevisiae* cells were chosen as representatives of host and fungal cells, respectively. Leakage of K^+ from large lipid unilamellar vesicles (LUVs) was also analyzed since LUVs are good model membranes and allow analysis by spectroscopy. Results with derivatives of AmB were compared as well as results with nystatin. We also tried to demonstrate the existence of single channels in sterol-containing planar lipid bilayers, in the presence of AmB.

Our results indicate that the mechanism by which AmB (or nystatin) induces permeability is different in ergosterol- and in cholesterol-containing membranes. AmB should be added in the self-associated form in order to be active by one-sided action on cholesterol-containing membranes while this is not the case with ergosterol-containing membranes. This finding is important since it may be the clue for the development of less toxic derivatives of AmB.

Materials and Methods

Materials. AmB was a generous gift from Squibb France. Stock solutions were prepared in DMSO and their concentrations determined by UV absorption ($\epsilon_{416} = 1.214 \times 10^5\ M^{-1}\ cm^{-1}$). Egg yolk PC, monooleoylglycerol, dioleoylglycerol, FCCP, DMSO, nystatin, and valinomycin were purchased from Sigma (St. Louis, MO). Cholesterol and ergosterol were purchased from Fluka (Buchs, Switzerland) and were recrystallized in ethanol before use. Pyranine was from Molecular Probes (Eugene, OR). Yeast extract, bactopeptone, and agar were from Difco (Detroit, MI). Polycarbonate filters were from Nuclepore (Pleasanton, CA).

Preparation and Assay of Lipid Vesicle Samples. Large unilamellar liposomes (LUV) were formed by the reverse-phase evaporation method as described by Szoka and Papahadjopoulos (1979). Briefly, 2 mL of the buffer hereafter called "potassium buffer" (100 mM K_2SO_4; 20 mM KH_2PO_4; pH adjusted to 7.1 by KOH) containing 18 mM pyranine was mixed with 6 mL of diethyl ether containing 20 μmol of lipid (egg PC + sterol). The resulting two-phase system was sonicated for 3 min in a sonicator (Branson sonifier B-12) until the mixture became a one-phase dispersion. LUVs were formed after the organic solvent was removed under reduced pressure in a rotatory evaporator. This preparation of liposomes was sequentially filtered through polycarbonate membranes, initially of 1-μm pore size then of 0.4-μm pore size. External pyranine was removed by column dialysis (Sephadex G-25) against the initial pyranine-free buffer. We have reported that such preparations result in liposomes that are fairly homogeneous in size and predominantly unilamellar (Ramos et al., 1989). Lipid phosphorus was determined as reported by Steward (1980), and sterol content was determined by the enzymatic test "C system" provided by Boehringer Mannheim France (Meylan, France).

Isolation of Human Red Blood Cells. Human venous blood collected in tubes containing EDTA (1 mg/mL EDTA in blood) was centrifuged at 1500*g* for 10 min, and the plasma and buffy coat were removed. Erythrocytes were then washed with phosphate-buffered saline (PBS) (155 mM NaCl, 7 mM Na_2HPO_4, and 3 mM KH_2PO_4 adjusted to pH 7.4), dispersed in equal volumes of PBS, and used on the following day.

Isolation of Yeast Cells. S. cerevisiae ATCC 9763 was maintained on agar slants composed of 0.3% yeast extract, 0.5% bactopeptone, 1% glucose, and 2% agar. An inoculum from the culture was transferred to liquid medium composed of yeast extract, bactopeptone, and glucose in the concentrations indicated for agar slants. After 18–20 h of growing at 28 °C with shaking, cells were harvested by centrifugation. The pellet was rinsed twice with distilled water and finally suspended in PBS. The concentration of cells was adjusted according to hematocymeter counts.

Measurements of Antibiotic-Induced Permeability to K^+ in LUVs. We used the method that we have already described (Hervé et al., 1989) in which ^{31}P NMR has been applied to monitor intravesicular pH changes. In the experiments described in the present paper, the intensity of fluorescence of pyranine enclosed in LUVs has been used as an intravesicular pH indicator since this intensity is linearly related to pH between 7.8 and 6.4 (Kano & Fendler, 1978; Deamer & Gutknecht, 1986). This method was therefore similar to that used by Whyte et al. (1989). Its advantages over the use of NMR are easy access, rapidity, and the ability to analyze a large range of vesicle concentrations. The emission fluorescence intensity of pyranine was measured at 510 nm with excitation at 460 nm and recorded as a function of time.

LUVs containing pyranine were diluted in the potassium buffer, the same as in the interior, to obtain the desired concentration. A 2 mL aliquot of the LUV suspension was placed in the spectrofluorometer cuvette. A pH gradient of 0.44 pH units was created by addition of 10 μL of 1 N H_2SO_4. Permeability to protons was increased by addition of 5 μL of 0.2 μM FCCP in DMSO. Finally, various concentrations of 10^{-4} M AmB in DMSO were added.

Under these conditions, changes in fluorescence intensity induced by addition of AmB (AmB concentration dependent) were due to the obligatory H^+/K^+ exchange and are an indirect measure of AmB-induced permeability to K^+. It was ascertained that AmB induces no pyranine leakage; without FCCP no fluorescence changes were observed.

Since changes in fluorescence are linearly related to the changes in pH and the pH gradient is 0.44, the internal pH is 7.1. Assuming that the volume of the LUVs is negligible compared to that of the external medium, then

$$\Delta[H^+] = \Delta[K^+] = 0.78 \times 10^{-7} \times 10^{0.0044x}$$

where x is the percentage of fluorescence change observed after addition of AmB as compared to that obtained after addition of valinomycin at a concentration sufficient to prevent limitation of the pH gradient decay by K^+ flux. In these conditions, the percentage P of K^+ leakage induced by AmB as compared to the maximum leakage observed in the presence of valinomycin could be written

$$P = 56(10^{0.0044x} - 1)$$

Measurement of Antibiotic-Induced K^+ Leakage from Erythrocytes. Serial dilutions of a stock solution of 10^{-3} M AmB in DMSO (5, 2.5, 1.25, 0.62, and 0.31 μM) were prepared in PBS. The solutions were then brought to the desired temperature by a 5-min incubation. Erythrocytes were dispersed in these solutions, and incubations were performed for 1 h at the chosen temperature with occasional shaking. Erythrocytes were then harvested by centrifugation, rinsed once with PBS, and lysed. Erythrocytes incubated in PBS alone plus 1% or 5% DMSO did not lose K^+ during the experiments. The concentration of K^+ was measured in a flame photometer (model PHF90, Lisabio, France). Experiments were done at 37 °C (unless specified) and in triplicate.

Measurement of Antibiotic-Induced K^+ Leakage from Yeast Cells. Two batches of cell suspension, containing 3×10^7 and 3×10^8 cells/mL were prepared for the determination of the effect of cell concentration. These two preparations were distributed in 2-mL aliquots and incubated with the desired concentration of AmB at 30 °C (the temperature effect was also analyzed at 20 and 37 °C with 3×10^7 cells/mL). The stock solution of AmB was 10^{-3} M in DMSO. AmB was

injected directly in microliter amounts to cell suspensions. After a 1-h incubation, the suspensions were centrifuged. Pelleted cells were suspended in 2 mL of bidistilled water and boiled for 15 min. Samples were centrifuged. The concentration of K^+ in the supernatants was determined with a flame photometer (model PHF 90, Lisabio, France). 100% of the K^+ release was obtained from boiled cell suspensions without antibiotic.

Spectroscopic Measurements. CD spectra were recorded with a Jobin-Yvon Mark V dichrograph. Spectral wavelengths are given ±0.5 nm. The dichrograph was calibrated with a low-pressure Hg lamp, and a constant bandwidth of 5 nm was used. Each spectrum was corrected for light scattered by vesicles. $\Delta\epsilon$ is the differential molar dichroic absorption coefficient (10^3 cm^2 mol^{-1}). Electronic absorption spectra were recorded with a Cary 219 (Varian) spectrophotometer. Fluorescence intensities were measured with a Jobin-Yvon JY3D spectrophotometer.

Single-Channel Experiments. Single-channel experiments were performed on lipid bilayers formed from a 1% solution of monooleoylglycerol (GMO) (or dioleoyl-PC) and sterol (at a 9:1 molar ratio) in decane by using Teflon cells filled with 1 M KCl. Ag/AgCl electrodes were used for current detection. The membrane areas were 0.1–0.2 mm^2. A current-to-voltage convertor (Keithley model 427) was used as current amplifier.

Results

Circular Dichroism Spectra of AmB, Some of Its Derivatives, and Nystatin in Buffer. Solutions of AmB were obtained, as usual, by dilution of a stock solution in DMSO in the chosen buffer. The CD spectra as a function of concentration of AmB in Tris-HCl buffer at pH 7 and 20 °C have already been described (Bolard et al., 1980). Their general characteristics are similar to those observed with the buffers used in the present study: At low concentrations, that is below 5×10^{-8} M, small positive bands are observed at 409, 385, 365, and 347 nm. When the concentration of AmB is increased, a critical value is obtained resulting in an intense doublet centered around 345 nm (see Figure 4b) and with an increasing $\Delta\epsilon$.

In order to analyze the AmB inducement of permeability of K^+ from LUVs, erythrocytes, and *S. cerevisiae* cells, we have recorded the differential molar dichroic absorption coefficient ($\Delta\epsilon$) of this doublet under the different experimental conditions used. Indeed, it had appeared that $\Delta\epsilon$ depends on several factors: temperature, time elapsed after dilution, and the nature of the buffer. Therefore spectra were recorded 5 min after dilution of the stock solution in potassium buffer, pH 6.6 (at 20 and 37 °C), and in PBS buffer, pH 7.4 (at 37 °C), starting from 10^{-4} and 10^{-3} M AmB stock solutions in DMSO, respectively. The critical concentration at which the dichroic doublet begins to appear (see Figure 1) was strongly dependent on the temperature, shifting in potassium buffer from approximately 8×10^{-8} M at 20 °C to 3×10^{-7} M at 37 °C (3×10^{-7} M at 37 °C in PBS). It depended also on the concentration of the stock solution (data not shown).

CD spectra of AmE and *N*-acetyl AmB were similar to those of AmB. The variation of the intensity of their dichroic doublet ($\Delta\epsilon$) with concentration is shown in Figure 7a.

The nystatin CD spectra in aqueous media and their concentration dependence have not been reported. At a concentration below 10^{-5} M, in PBS and at 37 °C, three weak positive bands were observed at 320, 303, and 290 nm ($\Delta\epsilon$ around 3). Above this concentration, new spectra were observed with negative bands at 324, 308, and 297 nm and with $\Delta\epsilon$ increasing with concentration (see Figure 7a).

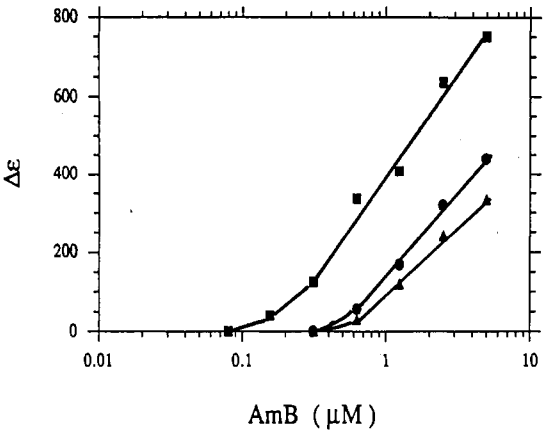

FIGURE 1: Concentration dependence of the AmB 345-nm centered dichroic doublet under different experimental conditions. The amplitude of the doublet with positive and negative peaks at 335 and 355 nm, respectively, is expressed as the differential molar dichroic absorption coefficient $\Delta\epsilon$ (10^3 cm^2 mol^{-1}). Spectra were recorded in potassium buffer at pH 6.6 or 7.1 at 20 °C (■) and 37 °C (●) and in PBS buffer, pH 7.4, at 37 °C (▲) (10^{-4} M AmB stock solution in DMSO), 5 min after dilution of the stock solution in the buffer.

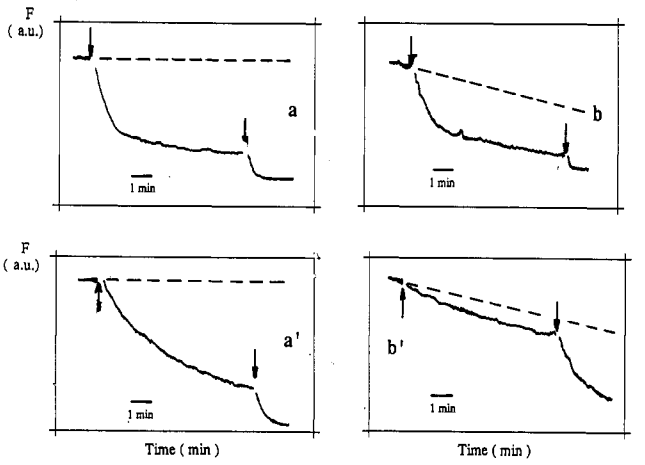

FIGURE 2: AmB-induced fluorescence changes of pyranine encapsulated in sterol-containing egg yolk PC LUVs, in the presence of a pH gradient and of the protonophore FCCP, as a function of time. The influence of the sterol nature (ergosterol or cholesterol) and lipid concentration (variation from 1 to 10) were assessed. (Traces a and b) 10 mol % ergosterol egg yolk PC LUVs at a phospholipid concentration of 1.8 mM and 0.18 mM, respectively, in the presence of AmB at a concentration of 0.75 μM and 0.075 μM, respectively. (Traces a′ and b′) 20 mol % cholesterol egg yolk PC LUVs at a phospholipid concentration of 3.48 mM and 0.348 mM, respectively, in the presence of AmB at a concentration of 0.75 μM and 0.075 μM, respectively. The internal pH of the LUVs was 7.1, and the external pH was 6.66. The dotted lines show the fluorescence changes in the absence of AmB. On each recording the first arrow indicates the addition of AmB and the second one the addition of valinomycin.

AmB-Induced K^+ Leakage from Sterol-Containing LUVs. The fluorescence of pyranine encapsulated in LUVs was quite stable even after addition of 5 μL of 1 N H_2SO_4 per milliliter of buffer. After addition of FCCP, a progressive slight decrease was observed, in particular for vesicles at low concentration. This time-dependent effect was taken into account in the subsequent analysis of the AmB-induced decrease of fluorescence. Figure 2 shows the variations obtained for two lipid concentrations of sterol-containing LUVs at two antibiotic concentrations and reveals the striking similarity of the curves for similar AmB to lipid ratios in ergosterol-containing LUVs, in contrast with the changes in cholesterol-containing LUVs. After 10 min, valinomycin was added at a ratio of 1 mole per

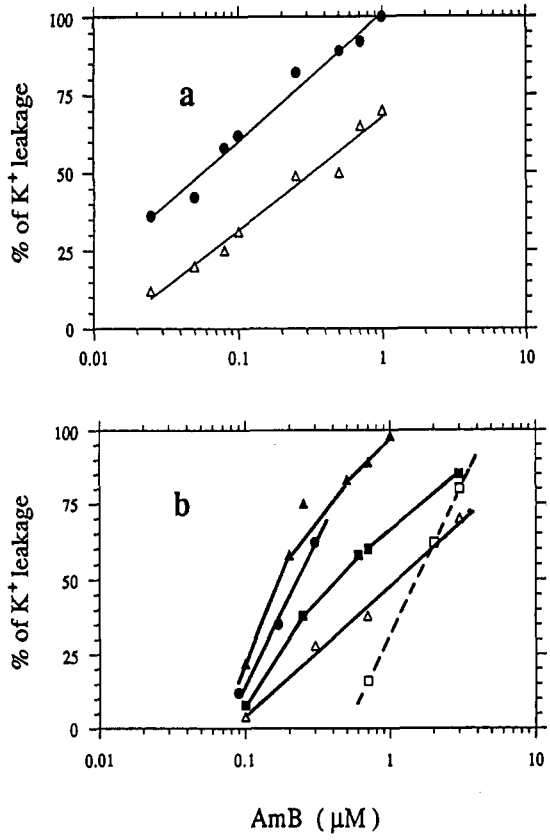

FIGURE 3: AmB-induced leakage of K^+ through sterol-containing LUVs as a function of AmB and vesicle concentrations. K^+ movement was followed by the internal pH changes resulting from the obligatory K^+/H^+ exchange occurring in the presence of FCCP and the antibiotic (see Materials and Methods). Measurements were done 10 min after the addition of AmB. The maximum K^+ efflux was that obtained by final addition of valinomycin. The temperature was 23 °C. (a) 10 mol % ergosterol egg yolk PC LUVs. The concentration of phospholipids was (△) 1.8 mM and (●) 0.18 mM. (b) 10 mol % cholesterol egg yolk PC LUVs. The concentration of phospholipids was (△) 2.2 mM and (●) 0.11 mM; 20 mol % cholesterol egg yolk PC LUVs. The concentration of phospholipids was (■) 2.2 mM and (▲, □) 0.22 mM, where one experiment (□) was done at 37 °C.

10^3 mole of lipid (Grzesiek & Dencher, 1986) to determine the maximum observable fluorescence change. Figure 3 shows the percentages of the total observable fluorescence changes obtained for various AmB concentrations in ergosterol-containing membranes (Figure 3a) and in cholesterol-containing membranes (Figure 3b).

Our results confirm the documented higher sensitivity of ergosterol-containing membranes to AmB action, at least for low K^+ leaks. Increased cholesterol content, from 10–20 mol %, was directly related to increased sensitivity to K^+ permeability. Most of the studies were done at 23 °C. One experiment was done at 37 °C with 20 mol % cholesterol egg PC LUVs. In this case, the dose inducing 50% leakage of K^+ was shifted to 1.5 μM (see Figure 3b).

The most striking result of this analysis is that at concentrations of AmB below 8×10^{-8} M (5×10^{-7} M at 37 °C) no effect is observed in cholesterol-containing LUVs whatever the lipid concentration or sterol content. Conversely, just above this concentration of antibiotic, K^+ permeability is induced. On the contrary, for ergosterol-containing LUVs, the AmB effect occurs at lower concentrations (8×10^{-9} M), and no critical concentration could be detected.

Binding of AmB to LUVs As Determined by Electronic Absorption and CD. Ergosterol-containing LUVs induce modifications of the AmB spectra that are different from those

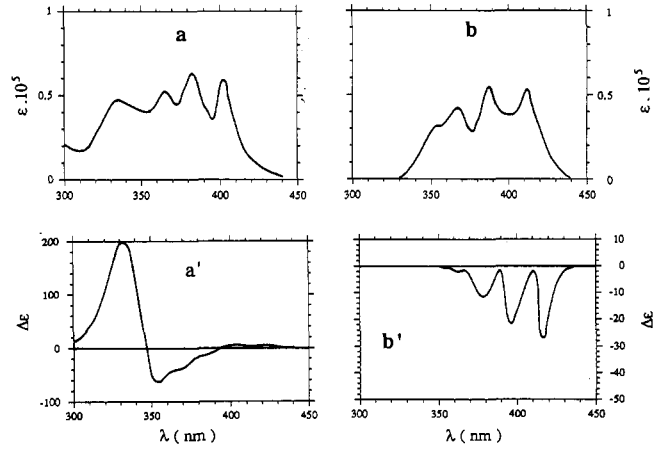

FIGURE 4: Spectra of the interaction of 0.5 μM AmB with sterol-containing egg yolk PC LUVs at 23 °C (phospholipids concentration of 240 μM). Absorption and circular dichroism spectra of (a, a') free AmB or AmB in the presence of 20 mol % cholesterol-containing LUVs. (b, b') AmB in the presence of 10 mol % ergosterol-containing LUVs.

reported for cholesterol-containing LUVs. We have described the general features of these changes in CD spectra (Bolard et al., 1984; Milhaud et al., 1989). To better characterize these differences, we recorded the spectra of AmB at concentrations between 2×10^{-7} and 2×10^{-4} M in the presence of LUVs (2.4×10^{-4} M in phospholipids) containing either 10% ergosterol or 20% cholesterol, that is, at AmB to sterol ratios varying over a range of 1000, wider than that already covered (Milhaud et al., 1989) and in the experimental conditions used for permeability measurements. As an example, Figure 4 shows the spectra of 2×10^{-7} M AmB recorded in the presence of LUVs after a 3-min incubation. For these AmB to lipid ratios, the extent of K^+ leakage is the same with both types of LUVs. In the presence of ergosterol-containing LUVs, CD spectra are totally changed as compared to those observed in buffer. In particular, the dichroic doublet, characteristic of self-associated unbound AmB, has totally disappeared. Narrow negative bands are observed at 420, 395, and 375 nm. By absorption spectra, the band at 409 nm characteristic of monomeric free AmB is seen to be strongly diminished, and new bands have appeared at 415, 390, and 368 nm. In striking contrast, no changes could be observed in absorption and CD spectra of AmB after addition of cholesterol-containing LUVs.

When increasing the ratio of AmB to ergosterol above 0.5, the spectra of unbound aggregated AmB progressively reappeared; while for cholesterol-containing LUVs, when AmB concentration was increased beyond 2×10^{-7} M, the dichroic doublet of free aggregated AmB decreased but beyond 3×10^{-6} M no further decrease occurred. From both studies, we inferred the amount of bound AmB as a function of the total AmB concentration, using the method already described (Jullien et al., 1989). The amplitude of the dichroic doublet was a measure of the amount of AmB remaining free in the presence of LUVs. In Figure 5 the variation of the ratio of bound AmB to sterol as a function of the ratio of total AmB to sterol is represented. (Note the logarithmic scale.) As already noted, binding of AmB to cholesterol-containing LUVs was saturated for a ratio of bound AmB to sterol of 0.025. In striking contrast, the binding of AmB to ergosterol-containing LUVs was saturated for a ratio of 1.

AmB-, AmB Derivative-, and Nystatin-Induced K^+ Leakage from Red Blood Cells. AmB induces K^+ leakage from erythrocytes as previously demonstrated [see Bolard (1986); Chêron et al. (1988); and Vertut-Croquin et al. (1989)]. The

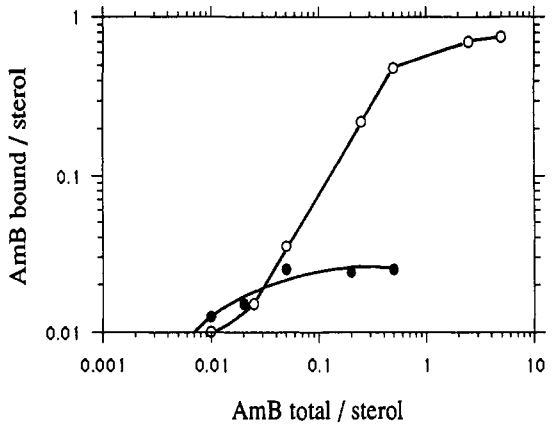

FIGURE 5: Binding of AmB to sterol-containing LUVs. The amount of AmB bound per sterol as a function of total AmB added per sterol is shown. The free AmB concentration was determined from the amplitude of the 345-nm centered dichroic doublet. The phospholipid concentration was 240 μM. Symbols: (●) 20 mol % cholesterol LUVs [redrawn from Milhaud et al. (1989)]. (○) 10 mol % ergosterol LUVs. Note the logarithmic scale.

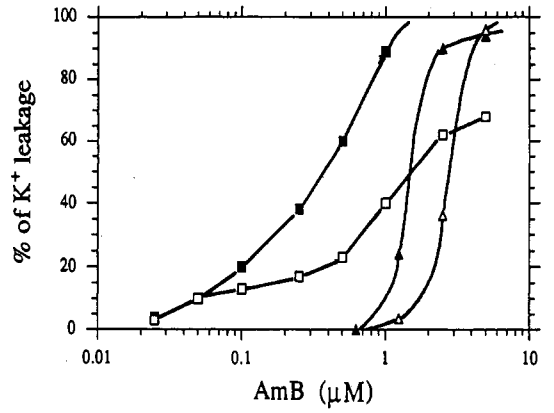

FIGURE 6: AmB-induced leakage of K^+ from *S. cerevisiae* cells and human erythrocytes. The dependence on AmB and cell concentrations is shown. The concentrations of K^+ ions leaked from the cells were measured and expressed in percentages of control values found in AmB-untreated cells. Yeast cells at 30 °C: (■) 3×10^7 cells/mL, (□) 3×10^8 cells/mL. Erythrocytes at 37 °C: (▲) 3×10^7 cells/mL, (△) 3×10^8 cells/mL.

antibiotic was more active at 20 °C than at 37 °C; the dose inducing 50% K^+ release was generally 2-fold smaller at 20 °C (data not shown). The dose–response curves depended on the erythrocyte concentration; 50% K^+ release at 37 °C occurred at 1.7 μM with 3×10^7 cells/mL and 3 μM with 3×10^8 cells/mL (Figure 6). In both cases, the inducement of permeability started around 0.7 μM. If the absolute values of K^+ release were plotted as a function of AmB concentration, the initial curves at both cell concentrations were superimposed but separated when all the K^+ ions of the 3×10^7 cells had been released. This characteristic was different from that observed with yeast cells (see below).

Measurements were also performed with AmB methyl ester and *N*-acetyl-AmB at 37 °C with 3×10^7 cells/mL (Figure 7b). While AmE activity was similar to that of AmB, *N*-acetyl-AmB was much less active (50% K^+ release at 10^{-5} M). Nystatin also appeared to be much less active (50% K^+ release at 4×10^{-5}M).

AmB-Induced Leakage from Yeast. The effect of cell concentration on the activity of AmB at 30 °C, measured by K^+ release from *S. cerevisiae* cells, is shown in Figure 6. The activity of AmB increases both with the concentration of antibiotic and with temperature: a 50% release was observed with 1.1, 0.28, and 0.075 μM AmB at 20, 30, and 37 °C,

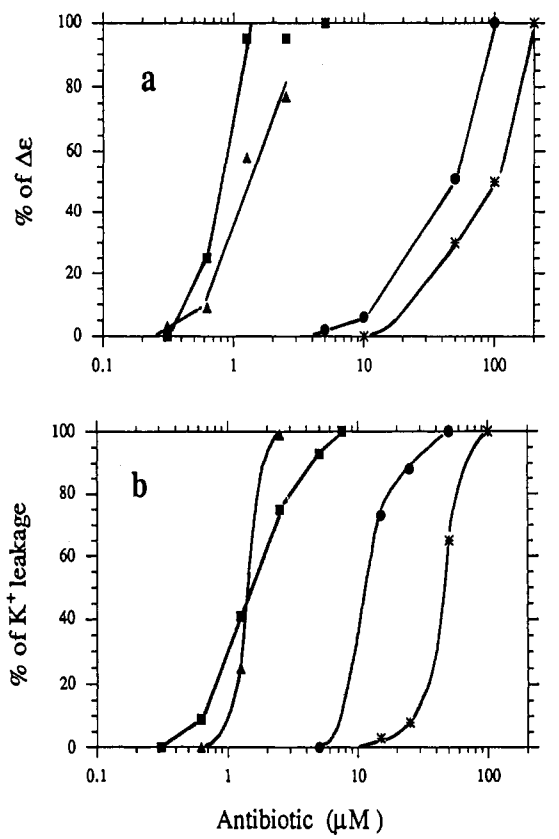

FIGURE 7: Comparisons of the polyene antibiotic-induced K^+ leakage from erythrocytes with the intensity of the dichroic doublet, as a function of the antibiotic concentration. The erythrocyte concentration was 3×10^7 cells/mL. The temperature 37 °C. Symbols: (▲) AmB, (■) AmE, (●) *N*-acetyl-AmB, and (*) nystatin. For the measurement of K^+ leakage and $\Delta\epsilon$, see Figures 1 and 6. (a) The percentage of the maximum $\Delta\epsilon$ observed (for nystatin, $\Delta\epsilon$ was measured at 297 nm). (b) The percentage of the maximum K^+ leakage observed.

respectively. Therefore, an elevation of temperature by 17 °C (20–37 °C) corresponds to almost a 15-fold increase in the efficacy of AmB. Dose–response curves obtained for these three temperatures are shifted in parallel (data not shown).

The optimal growth temperature for yeast cells is below 30 °C, and at 37 °C the spontaneous release of K^+ from cells in the absence of antibiotic is much higher than at 30 °C. To avoid spontaneous K^+ leakage, the effect of cell concentration was therefore studied at 30 °C (Figure 6). The dose–response curves were dependent on the cell concentration, 50% release occurring at 3.5×10^{-7} M with 3×10^7 cells/mL and 1.5×10^{-6} M with 3×10^8 cells/mL. For both cell concentrations (3×10^7 cells/mL and 3×10^8 cells/mL), the threshold antibiotic concentration necessary to induce potassium release was similar (5×10^{-8}M). However, the shape of the curves was more complex than with erythrocytes: it appeared biphasic, as if two mechanisms were in competition. The first one, observed below 5×10^{-7} M, would start to be active above 5×10^{-8} M and would not be dependent on cell concentration; for both cell concentrations, the same percentage of K^+ was released (approximately 10%). In the case of erythrocytes, it was the absolute value of K^+ release that was identical for concentrations near the threshold of activity. The second mechanism, which corresponds to the largest activity, would be observed in the region where the dose–response curves obtained at different cell concentrations are parallel.

Single-Channel Experiments. As it was shown that the activity (leakage) depends on the nature of the sterol associated with the antibiotic (see above), it was tempting to correlate these results with the possible formation of transmembrane

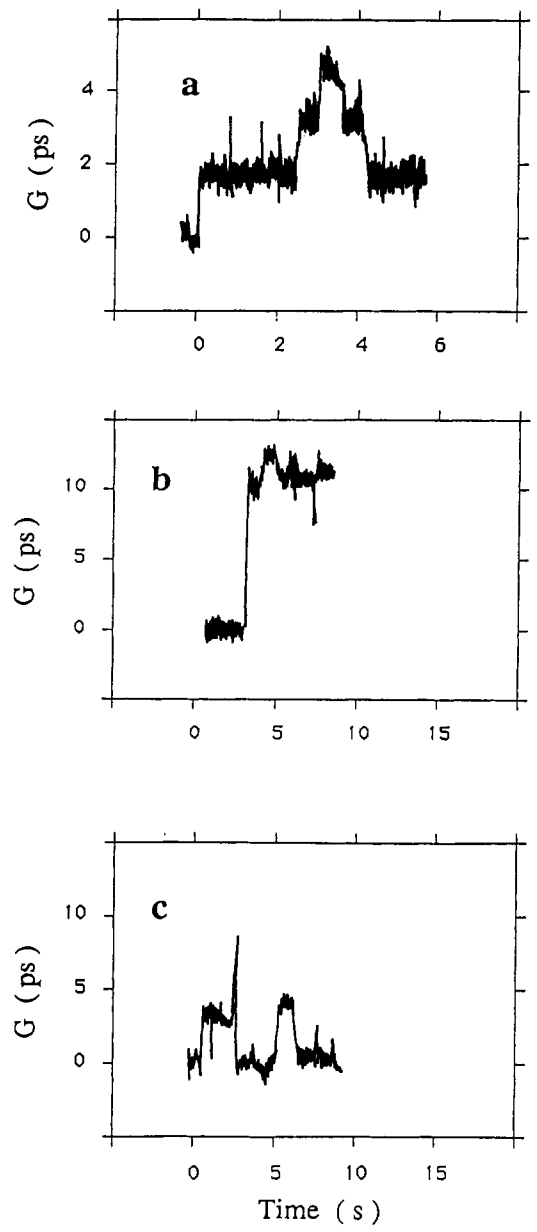

FIGURE 8: Current records of one-sided AmB-induced single channels through 10 mol % ergosterol monooleoylglycerol bilayers. The nominal AmB concentrations were (a) 10^{-8} M, (b) 4×10^{-11} M, and (c) 10^{-10} M. The applied voltage was 100 mV.

pores. From the experimental point of view, when incorporated into GMP/ergosterol (10:1) membranes, AmB is shown to be able to induce the formation of transmembrane channels that are characterized by discrete fluctuations of the transmembrane current (Figure 8). It must be noticed that the amplitude of the single events can vary from one membrane to another (compare Figure 8, panels a, b, and c) but with well-defined conductances (2–20 pS) and that within the same membranes all three types of events shown in Figure 8 can be detected. Furthermore, all of these defined conductances could be observed in a reproducible manner on a large number (≈50) of membranes formed to carry out these experiments. The finding of various conductances is very probably due to the formation of channels of different sizes that may have their origin in the procedure used to introduce AmB in the membrane: AmB is dissolved in DMSO, and aliquots of this solution are added to the electrolyte in which the antibiotic is poorly soluble, leading very probably to transient aggregates of various sizes that then incorporate in the bilayer.

When the same experiments as these described just above were carried out on membranes where ergosterol was replaced by cholesterol no single channel events could be observed. Only some rare nonreproducible and therefore nonsignificant events could be observed, the origin of which probably lies in a partial destabilization of the membrane by DMSO.

It must also be mentioned here that a similar behavior, with lower fluctuation intensities however, also occurred when the lipid was DOPC, a lipid that is closer to natural ones than GMO. This indicates that the experiments described here are relevant for comparison with the other experiments described in this article.

Discussion

AmB-Induced K^+ Leakage from LUV and Self-Association of the Antibiotic. A dichroic doublet centered at 345 nm is observed in the spectra of AmB dispersed in aqueous media above a critical concentration. The doublet is indicative of the self-association of the antibiotic (Mazerski et al., 1982). Below this critical concentration, AmB is in a monomeric form. As long as the total concentration is below 10^{-6} M, AmB is water soluble (Mazerski et al., 1990). In the present study, we observed that the concentration threshold at which the dichroic doublet begins to appear is temperature dependent. It depends also on the nature of the buffer, being different when AmB is dispersed in PBS or in "potassium buffer".

Concerning the inducement of K^+ leakage, our results are in excellent agreement with published reports (Gary-Bobo & Cybulska, 1982; Capuozzo et al., 1989; Cohen, 1986).

The lipid concentration dependences of the dose–response curves with ergosterol- and cholesterol-containing LUVs are essentially different. In the case of cholesterol-containing LUVs, AmB concentration should reach a critical level (5×10^{-8}M at 23 °C) for the drug to induce K^+ release. In contrast, in the case of ergosterol, the extent of leakage depends only on the [AmB]:[lipid] molar ratio, which indicates that no limiting lower concentration for activity development exists in this case.

The critical concentration at which permeability is induced in cholesterol-containing LUVs correlates with the appearance of the doublet in the CD spectrum of AmB in aqueous medium and thus with the beginning of AmB self-association. In the buffer used ("K^+ buffer"), this concentration is in both cases around 8×10^{-8} M at 23 °C. At 37 °C, inducement of permeability as well as self-association shifted to higher concentrations. Therefore, the species responsible for the inducement of K^+ leakage from cholesterol-containing LUVs is the self-associated species. In contrast, monomeric AmB is active on ergosterol-containing LUVs since activity is observed well below the threshold of self-association.

It is the first time that the effect of lipid concentration on the dose–response curve has been addressed. Neither the absolute antibiotic concentration alone nor the AmB:lipid ratio can be assumed to be sufficient to determine the extent of induced leakage, without prior careful analysis. It is important to note that erroneous conclusions can be drawn about cholesterol/ergosterol selectivity if the dose–response curves for cation leakage do not cover a large range of concentrations.

AmB-, AmB Derivatives-, and Nystatin-Induced K^+ Leakage from Red Blood Cells and Yeasts: Correlation with Self-Association of the Antibiotic (Figure 7). The response of red blood cells to the action of AmB appeared to depend on several parameters such as temperature, pH of the buffer, cell density, and concentration of AmB in the stock solution (data not shown). Consequently, depending on the exact experimental conditions, the dose of antibiotic inducing a 50%

release, commonly used as a measure of AmB activity, can vary between 0.8 and 3 μM This explains the variability of the results found in the literature. Interestingly, for a given pH and temperature, the dose at which K^+ release begins to be observed is the same whatever the cell concentration. This is similar to what we observed with cholesterol-containing LUVs.

Strikingly, the self-association of AmB, as determined by CD, appeared to depend on the same parameters. Furthermore, the different concentrations at which AmB started to induce K^+ release under different experimental conditions corresponded to the different concentrations at which self-association started to occur. This result also is similar to that observed with cholesterol-containing LUV.

The activity of the AmB derivatives studied (AmE and *N*-acetyl-AmB) was also in close relationship with the appearance of the dichroic doublet. As for nystatin, the appearance above 10^{-5} M of a new CD spectrum with higher $\Delta\epsilon$, assumed to reflect self-association, could also be related to induction of K^+ release from erythrocytes.

With *S. cerevisiae* the situation is more complex. The important result is that K^+ leakage is already observed at 3×10^{-8} M, far below the critical concentration at which AmB starts to self-associate. At these concentrations, AmB is totally in the monomeric form.

Different Nature of the Permeability Pathways Induced by AmB in Ergosterol- and in Cholesterol-Containing LUVs. This difference is strongly suggested by the converging set of data we obtained using different approaches. (1) The dose–response curves for the inducement of K^+ leakage in cholesterol- and ergosterol-containing LUVs have different characteristics, as described above. (2) Single channels are induced by AmB in ergosterol-containing planar bilayers and not in cholesterol-containing bilayers. (3) The amount of AmB bound to cholesterol-containing LUVs, as determined from CD spectra, saturates at a ratio of AmB to cholesterol of 0.025, in agreement with what we observed with hepatocytes (Binet & Bolard, 1988) or thymocytes (Henry-Toulmé et al., 1989). In these examples, the maximum amount of bound AmB was approximately 1 AmB for 100 lipids. In contrast, the amount of AmB bound to ergosterol-containing LUVs saturates at a ratio of AmB to ergosterol of 1. (4) There is an opposite temperature dependence of inducement of K^+ leakage by AmB from erythrocytes and from *S. cerevisiae* cells.

Additionally, data from the literature on the one-sided action of polyene antibiotics also confirm this assumption: (1) Ergosterol greatly potentiates the one-sided action of nystatin, which is supposed to act similarly to AmB, on decholesterolized brain lipids but cholesterol did not (Marty & Finkelstein, 1975). Furthermore, the difference in sensitivity to the one-sided and two-sided action of nystatin is much more pronounced in cholesterol-containing membranes formed with asolectin or *cis*-monounsaturated monoglycerides than in ergosterol-containing membranes (Kleinberg & Finkelstein, 1984). AmB added on one side of a membrane constituted of ox brain phospholipids and cholesterol does not increase conductance (Kasumov et al., 1979). (2) AmB induces Ca^{2+} leakage from ergosterol-containing EPC LUV (Ramos et al., 1989) or from leishmania cells (Cohen et al., 1990). In contrast, the AmB activity is much weaker with cholesterol-containing EPC LUV or nonexistent in lymphocytes (Henry-Toulmé et al., 1989). Furthermore, the characteristics of AmB-induced Ca^{2+} leakage from sterol-containing EPC LUVs (Ramos et al., 1989) also presented the differences observed for K^+ leakage, that is, a critical AmB concentration for cholesterol-containing membranes and not for ergosterol-containing membranes. (3) AmB derivatives such as AmE have been claimed to act as a transporter through cholesterol-containing membranes and as a channel former through ergosterol-containing membranes (Hervé et al., 1989).

AmB (or Nystatin) Self-Association and One-Sided Action on Cholesterol-Containing Membranes. We have seen that AmB self-association in "potassium buffer" begins around 0.8 μM at 37 °C and around 0.08 μM at 20 °C. In our experiments with LUVs and erythrocytes, the beginning of K^+ inducement of permeability is always correlated with these values. Examination of the data previously given in literature indicates that the one-sided addition of AmB to mammalian cells at 37 °C results in activity only for concentrations higher than 0.8 μM, that is, above the threshold of self-association. This is the case for mammalian cells such as L, HeLa, 3T3, BHK, HL 60 [for references, see Bolard, (1986)], and hepatocytes (Binet & Bolard, 1988) although the exact values vary from one experiment to another, due to differences in the incubation media, temperature, and times of incubation.

We therefore assume that, when added on one side of a cholesterol-containing membrane, AmB or nystatin should be in the self-associated form to induce K^+ permeability. At the concentration that begins to induce permeability, self-associated AmB is soluble and oligomeric (Mazerski et al., 1990). Soluble oligomers may therefore be considered as the active species. Once bound to the membrane, oligomeric AmB may, as demonstrated with nystatin (O'Neill et al., 1986), form micelles that induce permeability (Wietzerbin et al., 1990). It is interesting to note that we reach the same conclusion as Gruda and Dussault (1987), who hypothesized that the toxicity of antibiotics administered in the monomeric form with only traces of the dimer would be lower. This proposal was obtained from studies in aqueous solutions of propanol, mimicking membranes and related therefore to the mechanism of action of AmB already embedded in the membrane.

Of course parameters other than self-association may influence the activity of AmB or AmB derivatives: for instance, the affinity of the self-associated species for membranes, the stability of the pores formed, and the rate of exchange of the permeabilizing species between membranes of different cells [see Szponarski et al. (1988)]. However the beginning of self-association represents a determinant lower concentration, below which no inducement of permeability can be expected to occur.

Our data lead to a refinement of the model proposed by Finkelstein's group (Marty & Finkelstein, 1975; Kleinberg & Finkelstein, 1984) and Hoogevest et al. (1978) for the one-sided action of AmB, by introducing a difference between ergosterol- and cholesterol-containing membranes. The former model applies to ergosterol-containing membranes (Figure 9). In this latter case AmB complexes with ergosterol via hydrogen bonding between the mycosamine NH_2 group and the sterol OH group and a van der Waals interaction between the steroid nucleus, its side chain, and the lactone group of the antibiotic (Hervé et al., 1989; Baginski et al., 1990). The 1:1 complex may aggregate in the membrane and form a transmembrane pore in the hydrophobic interior of the membrane up to a thickness of 24 Å with cis monosaturated monoglycerides (Kleinberg & Finkelstein, 1984). AmB remains localized solely on the external leaflet of the membrane as seen by EPR studies of a paramagnetic derivative of AmB (Urbina et al., 1986).

In cholesterol-containing membranes, the binding between the sterol and AmB is weak or nonexistent in the conditions

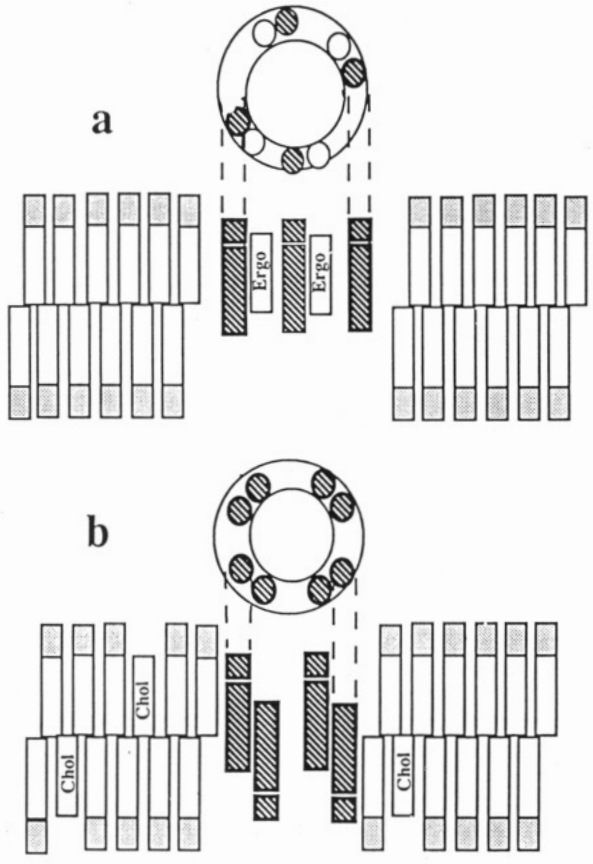

FIGURE 9: Schematic representation of the AmB (or nystatin) pores inducing K^+ permeability by one-sided action through ergosterol- and cholesterol-containing membranes. With ergosterol (a), the model is that of van Hoogevest and de Kruijff (1978) or Kleinberg and Finkelstein (1984). The pore is constituted of AmB–ergosterol complexes, and ergosterol is removed from the phospholipid phase. With cholesterol (b), the pore is formed of AmB dimers or groups of dimers. In the dimers the AmB molecules are associated in a head-to-tail configuration. The number of AmB molecules forming the pore was arbitrarily chosen. Cholesterol does not participate in this structure and remains in the phospholipid phase. The thickness of the bilayer represented is that of pure di-18:1 PC small unilamellar vesicles, that is, 43 Å (Caffrey & Feigenson, 1981); that of the polar region is 7 Å (Kleinfeld, 1987). The lactone ring of AmB is 22 Å long, and the polar head is 6 Å long (Kleinberg & Finkelstein, 1984). Amphotericin B is represented by black rectangles.

of our experiments, that is, at AmB/phospholipid ratios below 10^{-2} M; it has never been demonstrated in membranes [see Bolard (1986)], and the ratio at saturation of cholesterol to AmB bound is approximately 30 (Binet & Bolard, 1988; Henry-Toulmé et al., 1989; Milhaud et al., 1989). The difference between ergosterol and cholesterol could be due to conformational changes of the side chain that lead, in the latter case, to smaller van der Waals interactions with AmB. Cholesterol would interfere only in rigidifying the phospholipids and thereby allow a better incorporation of the antibiotic as already proposed by Hsu Chen and Feingold (1973) and Finkelstein's group (Marty & Finkelstein, 1975; Kleinberg & Finkelstein, 1984). Monomers of AmB when they bind to cholesterol-containing membranes, are too short to form transmembrane channels, even if they aggregate subsequently. In contrast, a head-to-tail arrangement of the oligomers increases the length of the assembly and enables the formation of transmembrane channels. The existence of an excitonic dichroic doublet in the self-associated species indicates that the polyenes are in close proximity and possibly in a helical structure (Hemenger et al., 1983). This assembly may be only slightly longer than monomeric AmB. However, this small increase in length may be sufficient because it has been shown that the one-sided action of AmB displays a sharp chain-length dependence (Kleinberg & Finkelstein, 1984; Whyte et al., 1989). Anyway, the assembly is certainly shorter than the thickness of the membrane of cholesterol-containing $22{:}1_c$/$22{:}1_c$ PC liposomes since in this case even 10 μM AmB is not active by single-sided action (van Hoogevest et al., 1978). Our model can explain the greater toxicity of aromatic polyene antibiotics such as vacidin A (partricin B) that can induce K^+ leakage from erythrocytes at a concentration of 10^{-8} M (Cybulska et al., 1989), a concentration at which the antibiotic is not self-associated. The greater length of vacidin A, due to the presence of the aromatic group, enables the antibiotic to form a transmembrane channel, even in monomeric form.

The model that we propose has limitations: it is valid for membranes in the liquid-crystalline state and with a large radius of curvature, which is actually the case with cell membranes. It cannot be extended to small unilamellar vesicles without further examinations [for a discussion on the differences of activity of AmB on LUVs and SUVs, See Milhaud et al. (1989) and Hartsel et al. (1991)]. As far as nystatin is concerned, supplementary studies are needed to establish whether the mechanism proposed here can be extended to it. However, the fact that the antibiotic self-associates at a much higher concentration than AmB can offer a clue for the observation that AmB is active on sterol-free phosphatidylcholine small unilamellar vesicles while nystatin is not, at least up to a ratio of nystatin to lipid of 0.5×10^{-2} (Whyte et al., 1990). Finally, our model is proposed for membranes in the fluid state and may not be valid for membranes in the gel state. In particular, erythrocytes present a phase transition around 20 °C, and it may well be that the mechanism of formation of AmB channels is different below this temperature (Szponarski & Bolard, 1987).

Conclusion. Whatever the exact mechanism of cell killing by heptaene antibiotics, the inducement of transmembrane permeability and ion fluxes is an essential step that always precedes cell death. For instance, without AmB-induced Na^+ entry into erythrocytes, hemolysis does not occur (Cybulska et al., 1984; Szponarski & Bolard, 1987). Certainly permeability inducement is responsible for the selective action of AmB against fungi and host cells. Therefore our results afford clues for the improvement of AmB therapeutic index by chemical modifications. The derivatives should have a free mycosamine NH_2 group to form the hydrogen bond with ergosterol, which is necessary for polyene antifungal activity (Chéron et al., 1988). On the other hand, the modification brought should decrease self-association of the antibiotic molecules in water, which is very easily determined by measuring the intensity of the dichroic doublet. We are currently investigating this characteristic in the numerous derivatives already described in the literature and take it into account in the design of new derivatives.

Registry No. AmB, 1397-89-3; cholesterol, 57-88-5; ergosterol, 57-87-4.

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