Dissociation Kinetics and Equilibrium Binding Properties of Polyene Antibiotic Complexes with Phosphatidylcholine/Sterol Vesicles†

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ABSTRACT: The interactions of sonicated vesicles with the polyene antibiotics amphotericin B, candicidin, mediocidin, and a water-soluble, guanidine derivative of amphotericin B were examined by UV–visible spectroscopy at concentrations below which the polyenes become self-associated. The association constants, K_{app}, and the numbers of binding sites per sterol or phospholipid molecule (n) were determined at 30 °C and pH 7.4. A single class of binding sites was found, with no evidence of cooperativity. For the binding of mediocidin, amphotericin B, and the guanidine derivative with phosphatidylcholine (PC), PC/cholesterol, and PC/ergosterol vesicles, K_{app} was in the range of (1.0–3.0) × 10^6 M^{-1}; K_{app} was higher for candicidin–vesicle interaction, reaching 9.0 × 10^6 M^{-1} with PC/ergosterol vesicles. Binding of the guanidine derivative of amphotericin B to PC vesicles lacking sterol was extensive (n = 0.46); since the other polyenes, which have low aqueous solubilities, had $n < 0.05$, positive charges in the mycosamine moiety appear to enhance the extent of polyene antibiotic interaction with the glycerophospholipid head group. Higher values of n (and, therefore, of nK_{app}) were found with sterol-containing than with sterol-free vesicles, suggestive of penetration of the polyenes toward the interior of the bilayer when sterol is present. For binding to PC/sterol vesicles, nK_{app} followed the order of candicidin > guanidine derivative of amphotericin B > amphotericin B ≫ mediocidin. The values of n and nK_{app} were appreciably higher for amphotericin B–ergosterol than for amphotericin B–cholesterol interaction in vesicles. The kinetics of dissociation of polyenes from vesicles were studied by rapid 100-fold dilution of the preformed complexes. Introduction of net negative surface charge had no significant effect on the kinetics of amphotericin B dissociation from PC/ergosterol vesicles but enhanced the rates of dissociation from PC/cholesterol vesicles. Thus, polyene binding and/or localization in vesicles may be sensitive to the structure of the sterol B ring and/or side chain. Factors other than dissociation kinetics apparently influence the overall stability of polyene–vesicle complexes.

The biological properties of polyene antibiotics, which include antifungal, antiprotozoal, antitumor, and antiviral activities, result from the interactions between polyenes and membrane components [reviewed by Hamilton-Miller (1973), Norman et al. (1976), Hammond (1977), Bittman (1978), Holz (1979), and Medoff et al. (1983)]. The most extensively studied of the polyene antibiotics is amphotericin B. Despite the disadvantages of numerous side effects, which include nephrotoxicity, poor stability, and low solubility in water, amphotericin B has proven to be "the cornerstone of antifungal chemotherapy" (Herman & Keys, 1983) of many systemic infections in man (Medoff et al., 1983). The clinical usefulness of amphotericin B is apparently based on its preferential interactions with the sterols of fungal vs. animal cells (Kwan et al., 1972; Archer & Gale, 1975; Archer, 1976). Qualitatively, conditions were demonstrated that give rise to spectral changes in amphotericin B in the presence of ergosterol (a fungal sterol) but not with cholesterol (Gruda et al., 1980; Vertut-Croquin et al., 1983). Quantitatively, the apparent binding constant, K_{app}, of the amphotericin B–ergosterol complex in vesicles from egg PC[1] was about 10-fold higher than that of amphotericin B–cholesterol (Readio & Bittman, 1982). Another widely used polyene antibiotic in membrane research, filipin, has a more pronounced effect on animal cells than on fungal cells (Archer & Gale, 1975; Bittman et al., 1983); K_{app} for the filipin–cholesterol complex in vesicles was about 3-fold higher than that of filipin–ergosterol (Bittman et al., 1974). The overall affinity of polyene antibiotics for membranes can be considered as nK_{app}, where n is the number of binding sites per sterol or phospholipid molecule. Since quantitative measurements of polyene–membrane binding constants and numbers of binding sites have not been reported previously except in very few instances (as those cited above), the question remains to what extent the overall affinities of various polyene antibiotics are correlated with the antifungal activities.

We used a procedure developed by Klotz & Hunston (1971) to measure n, in which the entire range of binding data is considered in evaluating n. The *monomeric* polyene concentration was varied over a wide range to enable a quantitative analysis of n, since data in a narrow range of ligand concentration may give erroneous estimates (Klotz, 1982). In addition to the nonaromatic heptaene amphotericin B [the only polyene macrolide for which an X-ray single crystal analysis is available (Mechlinski et al., 1970)], we studied the binding of the aromatic heptaene candicidin. Candicidin contains a *p*-aminophenacyl group in addition to the mycosamine moiety and carboxyl group found in most polyene antibiotics. Aromatic heptaenes have much higher biological activities against many cells than the nonaromatic heptaenes (Hammond, 1977; Kotler-Brajtburg et al., 1979; Malewicz & Borowski, 1979; Malewicz et al., 1980). Quantitative treatment of their interactions with membranes has not been undertaken, probably because until quite recently they were available only as complex mixtures of macrolides (Mechlinski & Schaffner, 1980) and their chemical structures were not established (Zieliński et al., 1979). We also examined the interaction between vesicles and mediocidin, a hexaene macrolide with an aminoalkyl side chain in addition to the amino sugar and car-

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[1] Abbreviations: DAPEG-AB, *N*-[*N*-[3-(dimethylamino)propyl]-*N'*-ethylguanyl]amphotericin B, a water-soluble guanidine-type derivative of amphotericin B in which the 3-amino group of mycosamine is replaced by —NHC(=$N^+HC_2H_5$)NH$(CH_2)_3N^+H(CH_3)_2$; PC, phosphatidylcholine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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boxylic acid groups. Mediocidin has been suggested to be useful for preventing and eliminating fungal contamination in tissue cultures (Fisher et al., 1978) because it has high antifungal activity but does not affect growth of mammalian cell lines at efficacious antifungal concentrations (1–5 μg/mL) (Utahara et al., 1954; Bonner et al., 1972). Finally, a water-soluble derivative of amphotericin B with a net positive charge at neutral pH, DAPEG-AB, was studied. The antifungal activity of DAPEG-AB is comparable to that of the parent compound (Witzke, 1981), but the partition coefficient between the lipid and aqueous phases differs from the other polyenes we investigated.

We found that the nK_{app} values for the interaction of these polyene antibiotics with vesicles are not correlated with their antifungal activities. Furthermore, we monitored the increase in absorbance of the polyene antibiotics on dissociation of polyene–membrane complexes. We observed that the dissociation rates are also not correlated with the order of their cell toxicities, although it may be predicted that recovery of cells exposed to polyenes would depend on the dissociation of the complexes formed. These studies suggest that the sensitivity of membranes to polyene antibiotics depends on many factors. In addition to the importance of the polyene to sterol ratio, phospholipid and sterol structure, and time of exposure to polyene, other determinants of the sensitivity of cells to polyenes may be the localization of the polyene in the membrane and the number, size, and/or lifetimes of the pores or interfacial membrane defects they induce in cells of sterol-containing organisms.

Experimental Procedures

Materials. Amphotericin B was obtained from E. R. Squibb, New Brunswick, NJ. Candicidin and mediocidin were kindly provided by Dr. C. P. Schaffner, Waksman Institute of Microbiology, Piscataway, NJ. DAPEG-AB was prepared as described previously (Witzke, 1981). The characterization of these antibiotics with respect to the components present was established by high-pressure liquid chromatography. Three major components were present in candicidin (Hansen & Thomsen, 1976; Mechlinski & Schaffner, 1980). Amphotericin B and DAPEG-AB were essentially homogeneous, containing only trace amounts of two minor components (Mechlinski & Schaffner, 1974; Witzke, 1981). Mediocidin consisted of one major and two minor components (Patel, 1980). Stock solutions of the polyenes were prepared in dimethyl sulfoxide except for DAPEG-AB, which was dissolved in water containing 20 ppm of *n*-propyl gallate (Aldrich Chemical Co.). The stock solutions were stored under argon at −20 °C.

Cholesterol and ergosterol were obtained from Sigma Chemical Co., St. Louis, MO, and were recrystallized from methanol. Egg PC was purchased from Makor Chemicals Ltd., Jerusalem, Israel. The sterols and PC were pure, on the basis of their migration as single spots on silica gel G plates in *n*-hexane–diethyl ether–acetic acid (70:30:1 v/v/v) and chloroform–methanol–water (65:25:4 v/v/v), respectively. Dicetyl phosphate was from Sigma, and *n*-octadecylamine was from K & K Laboratories.

The buffer used in all of the experiments consisted of 2.5% glucose and 5 mM HEPES, pH 7.4. The buffer was deoxygenated before use by boiling for 2 min; after it was cooled under a stream of argon, *n*-propyl gallate was added to a concentration of 2–20 ppm.

Preparation of Vesicles. Appropriate amounts of egg PC or egg PC and sterol (and, where indicated, dicetyl phosphate or *n*-octadecylamine) in chloroform were mixed, and the solvent was removed under a stream of nitrogen. Residual chloroform was evaporated under vacuum. Vesicles were prepared from PC or PC and sterol (3:1 molar ratio) in 5 mM HEPES containing 2.5% glucose, pH 7.4, by sonication under a nitrogen atmosphere. A Heat Systems Ultrasonics W375A sonicator equipped with a 0.5-in. tipped horn was used, and the dispersions were centrifuged at 12000*g* for 30 min at 4 °C to remove undispersed lipids and titanium fragments.

Dissociation Kinetics. The kinetics of dissociation of polyene antibiotics from vesicles was monitored by the absorbance change at one of the maxima of the polyene spectrum (408 nm for amphotericin B, 402.5 nm for candicidin, and 381 nm for mediocidin). The complexes were formed (prior to dissociation experiments) as follows. To 1 mL of vesicle suspension (2 mM total lipid) was added 10 μL of 0.2% *n*-propyl gallate. Then, 10 μL of a polyene stock solution (5 mM) was injected into the solution, and the mixture was incubated under argon at 5 °C overnight in the dark. For dissociation experiments at a 100-fold dilution, 30-μL aliquots of vesicle-bound polyene were mixed rapidly with 3 mL of buffer at 30 °C in a septum-capped cuvette (1-cm path length) under argon. The increase in absorbance accompanying dissociation was followed on a Perkin-Elmer/Hitachi Model 320 spectrophotometer. Semilogarithmic plots of the absorbance change, $A_\infty - A_t$, vs. time were curved in all cases (although with candicidin in the presence of PC vesicles, the faster rate process could not be measured with high precision because the amplitude was small). The plots were resolved into two first-order rate processes by extrapolation of the slow phase toward zero time to give a corrected base line for the faster process. Rate constant k_2 was calculated from the slope of the slow phase of the reaction, and rate constant k_1 was determined from a first-order plot of its reaction amplitude vs. time (Figure 1). The two first-order plots were fitted by least-squares linear regression analysis with the aid of a TI-58 calculator to the equation $\ln(\Delta A) = -k_{1,2}t + \ln(\Delta A_{1,2})$, where k_1 and k_2 are the rate constants of the fast and slow components, t is the time in minutes, and $\ln(\Delta A_{1,2})$ is the y intercept from which ΔA_1 and ΔA_2 were calculated. The overall dissociation rate constant, k_{av}, is the weighted average of k_1 and k_2, which was calculated as the ratio $(\Delta A_1 k_1 + \Delta A_2 k_2)/(\Delta A_1 + \Delta A_2)$.

Equilibrium Studies. Microliter aliquots of stock solutions of polyenes were injected into a septum-capped 10-cm path-length cell containing 28 mL of buffer under argon, giving a final concentration of 0.6–0.9 μM polyene and <0.01% dimethyl sulfoxide. After the absorbance of the free polyene solution had stabilized (30–60 min), the solution was titrated with aliquots of sonicated vesicle suspensions (1–50 mM total lipid). These aliquots were added by microliter syringe. To avoid errors from light scattering, equal amounts of vesicles were added to the reference cuvette as well. The time required for the absorbance of the polyene to attain equilibrium after addition of vesicle aliquots varied between 2 and 5 min, except for amphotericin B in the presence of ergosterol- and cholesterol-containing vesicles (which required up to 30 min). For binding of DAPEG-AB to vesicles, the titrations were conducted as described above, except we used an aqueous stock solution of DAPEG-AB and 1-cm path-length cells containing 3 mL of buffer. The final DAPEG-AB concentration was 2.0–2.4 μM.

Absorption measurements were conducted at 30 °C. The total polyene concentrations were determined spectrophotometrically with the following wavelengths and aqueous extinction coefficients (M^{-1} cm^{-1}): amphotericin B, 408 nm, 10.6

Table I: Apparent Rate Constants and Absorbance Changes for Dissociation of Polyene Antibiotics from Vesicles[a]

polyene	vesicles[b]	$10k_1$ (min^{-1})	$10k_2$ (min^{-1})	$\% \Delta A_1 : \% \Delta A_2$	k_{av} rel[c]
amphotericin B	PC	17.81 ± 0.23	0.38 ± 0.05	48:52	0.76
	PC/cholesterol	5.59 ± 0.29	0.60 ± 0.08	37:63	0.21
	PC/ergosterol	6.83 ± 0.23	0.49 ± 0.01	56:44	0.35
candicidin	PC	10.73 ± 3.69	0.68 ± 0.05	57:43	0.55
	PC/cholesterol	7.72 ± 0.67	0.27 ± 0.04	46:54	0.32
	PC/ergosterol	7.07 ± 0.81	1.14 ± 0.08	47:53	0.34
mediocidin	PC	21.97 ± 1.63	2.31 ± 0.55	75:25	0.91
	PC/cholesterol	22.50 ± 3.07	3.86 ± 0.34	60:40	0.80
	PC/ergosterol	21.80 ± 3.11	3.10 ± 0.23	66:34	0.83

[a] Dissociation of polyene–vesicle complexes and polyene aggregates was achieved by rapid dilution with 100 volumes of buffer at 30 °C. The absorbance increase was fitted to a two-exponential decay as described under Experimental Procedures, where k_1 and k_2 are the first-order rate constants of the fast and slow components, respectively, and ΔA_1 and ΔA_2 are the ratios of the absorbance change relative to the total absorbance change, times 100. The rate constants of the fast and slow components are weighted by the percentages of absorbance change in determining the overall decay, k_{av}. The errors represent the SEM of triplicate measurements made with each sample. The linear regression correlation coefficients ranged from 0.97 to 0.9998. [b] Sterol, when present, was incorporated at 25 mol %. [c] k_{av} rel is the average rate constant for dissociation from vesicles relative to dissociation of polyene oligomers or aggregates. In the absence of vesicles, two exponential decay curves were recorded following a 100-fold dilution with buffer (to 0.5 μM final concentration of polyene). The following values of k_{av} (numbers in parentheses indicate $\% \Delta A_1 : \% \Delta A_2$) were obtained: amphotericin B, 1.15 min^{-1} (68:32); candicidin, 1.17 min^{-1} (59:41); mediocidin, 1.88 min^{-1} (86:14).

× 10^4; DAPEG-AB, 409 nm, 10.9 × 10^4; candicidin, 381 nm, 9.3 × 10^4; mediocidin, 381 nm, 9.0 × 10^4. The values for the absorbance decreases, ΔA, were corrected for the dilution during the titration, under the assumption that Beer's law applies at the low polyene concentrations we used.

We chose to evaluate binding constants and site stoichiometries from plots of C_f/r vs. C_f. Here C_f represents the concentration of free polyene, and r represents C_{bound}/total lipid concentration. For a single class of binding sites, or multiple classes of independent, noninteracting sites with identical binding constants, this plot gives a straight line with slope of $1/n$, y intercept of $1/(nK_{app})$, and x intercept of $-1/K_{app}$ (Klotz & Hunston, 1971). In the present study, r is the ratio of bound polyene to total sterol (or to PC in the sterol-free vesicles), n is the number of binding sites per sterol (or PC) molecule, and K_{app} is the apparent binding constant. To determine the fraction of polyene bound ($\Delta A/\Delta A_{max}$), an initial value of $1/\Delta A_{max}$ was estimated from a double-reciprocal plot of $1/\Delta A$ vs. 1/total lipid concentration. The best value of $1/\Delta A_{max}$ was then determined by iteration with a program on a TI-58 calculator or TI-99/4A computer. The best linear fit of C_f/r vs. C_f was obtained, from which n, nK_{app}, and K_{app} were calculated.

Results

Dissociation Kinetics. The kinetics of the dissociation of polyenes from PC or PC/sterol vesicles have until now not been reported. Dissociation by dilution rather than by chemical disruption was chosen, reducing the risk of misinterpretation of the results because of competing processes. A 100-fold dilution was found to give complete dissociation of amphotericin B– and mediocidin–vesicle complexes, since the absorption spectra at equilibrium were identical with those of the free polyenes in dilute aqueous solution. In the case of candicidin–PC/sterol vesicle complexes, the spectra obtained after equilibration on 100-fold dilution showed a red-shift of ~1 nm, and dissociation was thus incomplete. By comparison with results from dilutions of more than 100-fold, it was estimated that candicidin–PC/sterol vesicle complexes were approximately two-thirds dissociated on 100-fold dilution.

In order to minimize interference from dissociation of aggregated forms (oligomers or micelles) of the polyenes, we used a molar ratio of 40:1 of total lipid (PC plus sterol) to polyene in forming the complexes. Another advantage of using a high lipid to polyene molar ratio is that disruption of vesicles is minimized (Gent & Prestegard, 1976). Figure 1 shows a

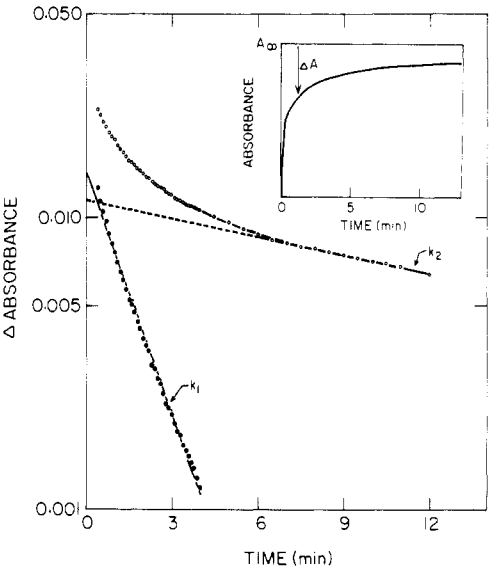

FIGURE 1: Typical kinetic analysis of the rate processes observed in amphotericin B dissociation from PC/ergosterol (molar ratio 3:1) vesicles. Preformed complexes of amphotericin B (0.5 μM final concentration) and vesicles were prepared as described under Experimental Procedures. The absorbance change at 408 nm, $A_\infty - A_t$, is plotted on a semilogarithmic scale vs. time, and k_2 is obtained from the slope of the slow phase. Extrapolation to zero time gives the change in absorbance associated with this phase and a base line for calculating the absorbance change associated with the fast phase. Rate constant k_1 is obtained from the slope of the semilogarithmic plot of the fast phase (●) vs. time, and the y intercept gives the absorbance change of this process. (Inset) The increase in absorbance at 408 nm on 100-fold dilution of amphotericin B–PC/ergosterol vesicles. A_∞ is the absorbance after equilibration. Absorbance changes during the initial 0.4 min following dilution are obscured by the response time of the spectrophotometer.

semilogarithmic plot for the dissociation of amphotericin B from PC/ergosterol vesicles. The plot of the absorbance change against time is curved, and the curve was analyzed by assuming that the absorbance change is the resultant of two superimposed rate processes. Table I summarizes the rate constants and relative absorbance changes of the two reactions, which were derived as described under Experimental Procedures. Incorporation of sterol into the vesicles caused k_1 to decrease significantly for amphotericin B and candicidin, whereas k_1 was independent of the type of vesicles with mediocidin. The overall rate constant, k_{av}, in which the fast and slow rate constants are weighted by the relative absorbance

Table II: Effect of Surface Charge on Dissociation Kinetics of Amphotericin B from Vesicles

vesicles	surface charge[a]	$10k_1$ (min^{-1})	$10k_2$ (min^{-1})	% ΔA_1:% ΔA_2	k_{av} (min^{-1})	relative ΔA_{total} of dissociation[b]
PC	none	17.81 ± 0.23	0.38 ± 0.05	48:52	0.87	1.00
	positive	9.77 ± 0.23	0.36 ± 0.01	64:36	0.64	1.09
	negative	17.20 ± 1.02	1.28 ± 0.19	68:32	1.21	0.85
PC/cholesterol	none	5.59 ± 0.29	0.60 ± 0.08	37:63	0.25	1.00
	negative	18.01 ± 1.67	1.09 ± 0.09	51:49	0.97	0.27
PC/ergosterol	none	6.83 ± 0.23	0.49 ± 0.01	56:44	0.41	1.00
	negative	8.10 ± 0.28	0.68 ± 0.10	51:49	0.45	1.00

[a] Dicetyl phosphate or *n*-octadecylamine was incorporated at 1 mol % to confer negative or positive membrane surface charge, respectively. [b] The relative increases in absorbance on dissociation were estimated as the ratio of $(\Delta A_1 + \Delta A_2)_{charged}/(\Delta A_1 + \Delta A_2)_{uncharged}$.

changes, was dependent on vesicle composition for dissociation of amphotericin B and candicidin in the order: free polyene aggregates > PC > PC/sterol. The dissociation rate constants were higher with mediocidin than with amphotericin B and candicidin, and k_{av} varied only slightly with vesicle composition.

The effect of membrane surface charge on the dissociation kinetics of amphotericin B from vesicles was examined by incorporation of dicetyl phosphate and *n*-octadecylamine into the bilayers. Table II shows that the overall rate of dissociation from PC vesicles is faster in the order of negatively charged > net zero charge > positively charged bilayers. Since amphotericin B has a net partial negative charge at neutral pH, it is not surprising that the introduction of negative charge increased k_{av} and reduced the extent of binding to PC vesicles. The enhancement in the rate of amphotericin B dissociation by a negative surface charge was much more pronounced in vesicles containing cholesterol than in the corresponding sterol-free and ergosterol-containing vesicles. However, incorporation of dicetyl phosphate into PC/ergosterol bilayers had a negligible effect on amphotericin B dissociation. The dependence of dissociation kinetics on membrane surface charge in PC/cholesterol but not in PC/ergosterol vesicles suggests that the polyene is localized differently in these two systems. Deeper penetration of the polyene into the hydrophobic core of the membrane apparently takes place when ergosterol is present. In contrast to our results with PC and PC/cholesterol vesicles, amphotericin-induced permeation of ascorbate into vesicles was found to be independent of surface charge (Aracava et al., 1981a).

Equilibrium Studies. The approach of analyzing binding data in the form of the well-known Scatchard (1949) plot was criticized recently by Klotz (1982). He pointed out that many investigators have derived the number of sites per mole of receptor (n) by extrapolating to the x intercept with data covering a narrow range of concentrations. Klotz (1982) emphasized the importance of plots of the type r vs. log C_f, which indicate the range of the experimental data in the binding isotherm. Such a plot is expected to yield an S-shaped curve, approaching n as log C_f approaches infinity and with an inflection point at $n_{1/2}$. Figure 2 shows that the spectrophotometric titration data we obtained for amphotericin B and candicidin binding to PC/ergosterol vesicles describe only points to about $n_{1/2}$. For these polyenes, data above $n_{1/2}$ exceed their critical micelle concentrations (0.6–0.8 μM). DAPEG-AB and mediocidin, however, have much higher critical micelle concentrations (Witzke, 1981; Schaffner & Mechlinski, 1972), and the experimental data include part of the curve above the inflection point for each of these polyenes.

In a plot of C_f/r vs. C_f (Figure 3), the slope is $1/n$, the y intercept is $1/nK_{app}$, and the x intercept is $-1/K_{app}$ (Klotz & Hunston, 1971). In this plot, n (the number of binding sites per mole of sterol or PC) is obtained from the slope, whereas

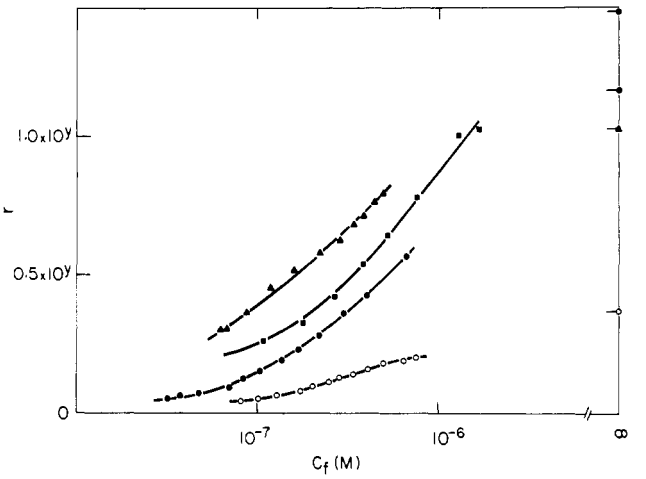

FIGURE 2: Plot of r vs. log C_f for binding of polyene antibiotics to PC/ergosterol vesicles. C_f is the molar concentration of free polyene; r is the molar ratio of bound polyene to total ergosterol. The horizontal lines and symbols on the right-hand ordinate indicate the values of n (the number of sites for polyene per mole of ergosterol); n values were estimated from the slopes of the lines in Figure 4. The polyenes are (▲) candicidin, (■) DAPEG-AB, (●) amphotericin B, and (○) mediocidin. For candicidin, amphotericin B, and DAPEG-AB, y = 0; for mediocidin, $y = -1$. The following concentrations of total polyene were used: candicidin, 0.75 μM; amphotericin B, 0.79 μM; mediocidin, 0.92 μM; DAPEG-AB, 2.40 μM.

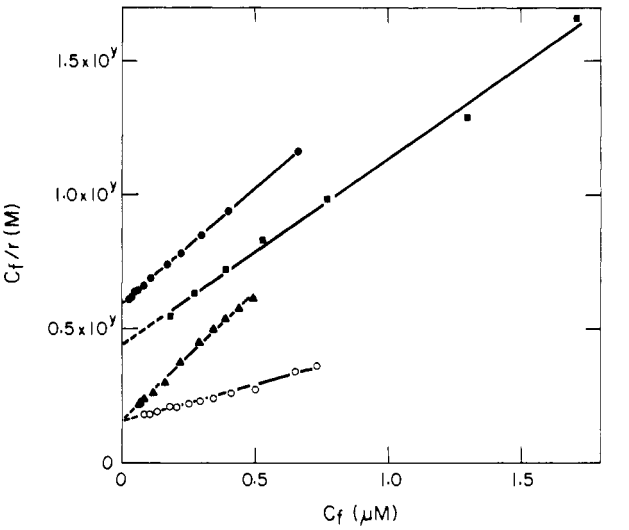

FIGURE 3: Plot of C_f/r vs. C_f for binding of polyene antibiotics to PC/ergosterol vesicles. For mediocidin (○), $y = -4$; for amphotericin B (●), DAPEG-AB (■), and candicidin (▲), $y = -6$.

in Scatchard plots the x intercept determines n; alternatively, n can be estimated from the y intercept (nK_{app}). Figure 4 shows Scatchard plots of the same binding data used in Figure 3, indicating the extent of extrapolation necessary to the x and

Table III: Association Constants and Numbers of Binding Sites for Polyene Antibiotics Interacting with Vesicles[a]

polyene	vesicles	K_{app} ($\times 10^{-6}$ M^{-1})	n	nK_{app} (M^{-1})	relative nK_{app}[b]
amphotericin B	PC	3.31 ± 0.51	0.00175 ± 0.00004	(5.84 ± 1.01) × 10^5	1
	PC/cholesterol	0.97 ± 0.17	0.219 ± 0.022	(2.05 ± 0.16) × 10^5	35.1
	PC/ergosterol	1.52 ± 0.04	1.094 ± 0.044	(1.66 ± 0.06) × 10^6	284.2
DAPEG-AB	PC	2.11 ± 0.21	0.458 ± 0.036	(0.95 ± 0.02) × 10^6	1
	PC/cholesterol	3.17 ± 0.78	0.888 ± 0.068	(2.66 ± 0.55) × 10^6	2.8
	PC/ergosterol	1.42 ± 0.07	1.468 ± 0.060	(2.08 ± 0.11) × 10^6	2.2
candicidin	PC	4.49 ± 0.50	0.054 ± 0.004	(2.51 ± 0.11) × 10^5	1
	PC/cholesterol	4.91 ± 0.93	1.272 ± 0.016	(6.56 ± 0.37) × 10^6	26.1
	PC/ergosterol	8.89 ± 0.90	0.978 ± 0.039	(8.77 ± 1.09) × 10^6	34.9
mediocidin	PC	1.01 ± 0.12	0.015 ± 0.001	(1.53 ± 0.27) × 10^4	1
	PC/cholesterol	2.50 ± 0.49	0.032 ± 0.005	(7.65 ± 0.54) × 10^4	5.0
	PC/ergosterol	2.17 ± 0.31	0.035 ± 0.001	(7.64 ± 0.94) × 10^4	5.0

[a] The apparent binding constant, K_{app}, and the apparent number of binding sites, n (polyene per lipid), were determined as described under Experimental Procedures, and nK_{app} was obtained from the reciprocal of the y intercept of plots of C_f/r vs. C_f. The correlation coefficients varied from 0.933 to 0.9997. Each K_{app} and n is the average ± SEM of three experiments. [b] Values of nK_{app} with each polyene are relative to the nK_{app} obtained with vesicles prepared from PC without sterol.

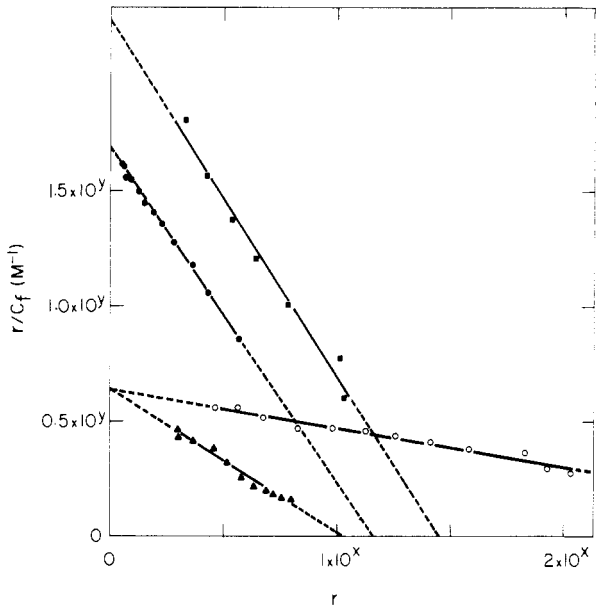

FIGURE 4: Scatchard plot of binding of polyene antibiotics to PC/ergosterol vesicles. The values of x and y are as follows: DAPEG-AB (■), $x = 0$ and $y = 6$; amphotericin B (●), $x = 0$ and $y = 6$; mediocidin (○), $x = -2$ and $y = 5$; candicidin (▲), $x = 0$ and $y = 7$.

y intercepts. At low r values (high vesicle concentrations), we often found large deviations in Scatchard plots with PC and PC/cholesterol vesicles (data not shown). These deviations, which may arise because of the difficulty in measuring small absorbance changes accurately in turbid samples, can lead to errors in estimation of nK_{app} from the y intercept. Plots of C_f/r vs. C_f showed only negligible deviations at low values of r and C_f.

A single class of binding sites was found for the interaction of each of the four polyene antibiotics with PC/ergosterol vesicles (Figures 3 and 4). Using vesicles prepared from PC or PC/cholesterol, we also observed a single class of binding sites with these polyenes when the data were plotted on C_f/r vs. C_f or Scatchard graphs (plots not shown). Table III summarizes our measurements of polyene binding to vesicles from plots of C_f/r vs. C_f. Each polyene was bound to vesicles prepared from PC and from mixtures of PC and sterols, with little variation in apparent affinity on vesicle type or polyene structure. Candicidin gave slightly higher values of K_{app} than the other three polyenes. Major differences among the polyenes were noted in the values of n and, therefore, in the overall vesicle affinity, nK_{app}. The values of n and nK_{app} are higher for polyene binding to sterol-containing vesicles compared with those for sterol-free vesicles. The overall affinity of polyenes for sterol-containing vesicles followed the order of candicidin > DAPEG-AB ≥ amphotericin B >> mediocidin. Mediocidin has an nK_{app} value approximately 100-fold lower than that of candicidin. Amphotericin B showed the highest selectivity toward ergosterol relative to cholesterol (a factor of 284.2/35.1, or 8.1). However, candicidin, mediocidin, and DAPEG-AB showed no significant difference for interaction with cholesterol compared with ergosterol. The nK_{app} values for mediocidin and DAPEG-AB interaction with PC/sterol vesicles exceeded the nK_{app} values for interaction with PC vesicles by factors of only 5 and about 2.5, respectively. In contrast, candicidin and amphotericin B showed considerably higher nK_{app} values with sterol-containing vesicles compared with sterol-free vesicles.

Discussion

n Values. In a recent Scatchard analysis of amphotericin B binding to sterols, we treated r as the number of moles of bound sterol per mole of total polyene (Readio & Bittman, 1982). In the present paper, we consider r as the number of moles of bound polyene per total sterol (or PC in sterol-free vesicles). The overall affinity (nK_{app}) of amphotericin B for ergosterol in vesicles exceeds that for cholesterol by about 1 order of magnitude. The previous method of analysis, however, could not detect differences between the number of binding sites (n) because the fraction of bound sterol was equated with the fraction of bound polyene. We have now estimated the numbers of receptor sites for polyene antibiotics by using experimental data covering a wide range of free polyene concentration. The binding data obtained with each of the polyenes (Figure 2) ranged at least to the inflection point ($n_{1/2}$), which corresponds to half-saturation for a receptor with n identical binding sites. Measurements of monomeric polyene binding to vesicles could not be made above about $n_{1/2}$ for amphotericin B and candicidin because of self-association above about 10^{-6} M (Rinnert et al., 1977; Witzke, 1981; Mazerski et al., 1982; Strauss, 1982). The use of 10-cm path-length cuvettes allowed measurements to be made at very low polyene concentrations, where absorbance changes are small. The n values obtained from the slopes of C_f/r vs. C_f plots are very low for the binding of all of the polyenes to PC vesicles except DAPEG-AB (Table III). The difference in the n values of amphotericin B and DAPEG-AB is particularly striking. The rate of association of this water-soluble derivative of amphotericin B with vesicles is more rapid than that of amphotericin B at the same concentration (N. M. Witzke and R. Bittman, unpublished results), suggesting that the positive

charges in the mycosamine moiety of DAPEG-AB enhance the interaction with the glycerophospholipid head group. Spin-label studies of amphotericin B in PC bilayers have also concluded that this polyene is located at the bilayer surface at low ratios of polyene to sterol (Oehlschlager & Laks, 1980) and that interactions in the aqueous phase may be established slowly (Aracava et al., 1981b).

Our data show that extensive interaction can take place in vesicles from PC alone. At saturation, there are about 4.7 amphotericin B molecules per vesicle, 135 candicidin molecules, and 40 mediocidin molecules, with the assumption that there are about 2700 PC molecules per vesicle. Several studies have demonstrated binding of polyene antibiotics to PC vesicles [see references in Chen & Bittman (1977) and Medoff et al. (1983)], but the stoichiometry has not been previously reported. The large increases in the n values of amphotericin B, DAPEG-AB, and candicidin on the introduction of sterols into vesicles suggest that the polyenes penetrate toward the interior of the bilayer, explaining the sterol-dependent increases in permeability of model membrane systems induced by polyene antibiotics. The stoichiometry of 0.22 amphotericin B molecule per cholesterol (Table III) is in good agreement with previous estimates in vesicles and *Acholeplasma laidlawii* membranes (Norman et al., 1972; deKruijff et al., 1974a). However, the stoichiometry does not agree with a value of 0.7 cholesterol molecule per amphotericin B (deKruijff et al., 1974b). For amphotericin B–ergosterol complexation, we found $n = 1.09 \pm 0.04$.

Influence of Sterol Structure on Vesicle–Amphotericin B Interaction. A possible explanation for the higher n value with ergosterol-containing vesicles is that the interaction of amphotericin B with ergosterol differs from that with cholesterol; the lack of effect of surface charge on amphotericin B–ergosterol dissociation kinetics, in contrast with the marked dependence on surface charge in cholesterol-containing vesicles, suggests a difference in polyene localization in these two systems. Moreover, the circular dichroism spectra of amphotericin B complexes with ergosterol and cholesterol in PC vesicles are different at various sterol concentrations (Vertut-Croquin et al., 1983). The structural differences between the two sterols are in the side chain and B ring, with ergosterol containing a Δ-22 bond, a 24β-methyl group, and a Δ-7 bond. The amphotericin-induced increase in membrane permeability is sensitive to sterol side-chain length (Nakamura et al., 1980); moreover, the effects of amphotericin B, candicidin, and nystatin on the conductance of lipid bilayers followed the order of ergosterol and cholesta-5,7,22-trien-3β-ol (which differ from cholesterol in both the B ring and the side chain) > cholesterol and 24β-methyl-cholesta-5,22-dien-3β-ol (which have only one double bond in the B ring) > 5α-cholestan-3β-ol (which has no double bond in the B ring) (Feigin et al., 1978). It therefore would be of interest to study other sterols to gain a better understanding of the structural requirements in the aliphatic side chain at C-17 and in the B ring for interaction with amphotericin B.

nK_{app} Values. The equilibrium binding data (Table III) do not show a correlation with the order of antifungal activity. The order of nK_{app} for PC/sterol vesicles is candicidin > DAPEG-AB and amphotericin B >> mediocidin, whereas the order of in vitro activity against *Saccharomyces cerevisiae* and *Candida albicans* is candicidin and mediocidin > amphotericin B ≥ DAPEG-AB (Bonner et al., 1972; Mechlinski, 1973; Witzke, 1981). These results suggest that caution should be exerted in making inferences about the mode of action of polyene antibiotics with PC/sterol vesicles only.

There are several indications that attention should be given to phospholipid structure as well as to sterol structure when analyzing polyene–membrane interactions. Bilayer thickness was demonstrated to be an important parameter in the extent of K^+ leakage from vesicles induced by amphotericin B (Van Hoogevest & deKruijff, 1978). The sensitivity of *S. cerevisiae* to nystatin was dependent on membrane phospholipid content (Jirku et al., 1981), and mutants of *C. tropicals* that were resistant to nystatin differed in lipid classes other than sterols (Danilenko & Stepanyuk, 1982). Furthermore, the abilities of amphotericin B and candicidin to promote proton permeability in PC/sterol vesicles were not related to their relative activities against sterol-containing organisms (Cybulska et al., 1981).

Dissociation Kinetics. Since the toxic side effects of polyene antibiotics probably result from interaction with cell membranes, dissociation of polyene–membrane complexes may be expected to play a role in the resistance to and recovery from polyene-induced membrane damage. However, our dissociation and equilibrium binding data are not consistent with the order of acute intraperitoneal toxicity (mediocidin ~ candicidin >> amphotericin B) found in mice (Bonner et al., 1972). The dissociation data presented in Table I indicate that mediocidin is displaced from vesicles more rapidly than candicidin and amphotericin B. The dissociation of amphotericin B from PC/ergosterol vesicles proceeds slightly faster than that from PC/cholesterol vesicles. Since nK_{app} for interaction of amphotericin B with ergosterol is about 8-fold higher than nK_{app} for interaction with cholesterol, we conclude that high nK_{app} values do not result from slow rates of dissociation. Therefore, factors other than dissociation kinetics apparently influence the overall stability of polyene–membrane complexes. In a previous study of the association kinetics of polyene association with sterols, we found that the extent of competition between lipid–lipid and polyene–lipid interactions is a factor to consider (Chen & Bittman, 1977). The permeability increases induced by polyene antibiotics in sterol-containing organisms appear to involve formation of pores or phase boundaries in the cell membrane [reviewed by Medoff et al. (1983) and Aracava et al. (1981b)]. The properties of the ionic channels formed in phospholipid–cholesterol bilayers were reported to vary with polyene antibiotic structure (Kasumov et al., 1979). It is therefore likely that the number, size, and/or lifetimes of the pores or phase boundary defects are other factors that play important roles in determining the sensitivity of cells to polyene antibiotics.

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Registry No. DAPEG-AB, 87263-93-2; amphotericin B, 1397-89-3; candicidin, 1403-17-4; mediocidin, 1403-95-8; cholesterol, 57-88-5; ergosterol, 57-87-4.

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