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EQUILIBRIUM BINDING OF AMPHOTERICIN B AND ITS METHYL ESTER AND BORATE COMPLEX TO STEROLS

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Scatchard analysis of the binding of amphotericin B to sterols in egg phosphatidylcholine/sterol vesicles revealed that amphotericin B was bound to ergosterol by approximately one order of magnitude more tightly than to cholesterol. Amphotericin B methyl ester formed a tighter complex with each of the sterols investigated than did amphotericin B, but the difference in the apparent binding constants toward ergosterol and cholesterol was not as large as with amphotericin B. Amphotericin B borate formed weaker complexes with vesicle-bound sterols than amphotericin B and amphotericin B methyl ester.

Amphotericin B is an important antibiotic in anti-fungal chemotherapy and a useful tool for investigating the role of sterols in biological membranes because it possesses the following characteristics: (a) a specificity for binding to membrane-bound sterols with a 3β-hydroxyl group, planar nucleus, and apolar chain at C-17 [1–3], resulting in disruption of membrane permeability at low polyene concentration; (b) a visible absorption spectrum, reaching to wavelengths where light scattering from dilute membrane suspensions is low; and (c) other spectroscopic properties that can be used to monitor interaction with lipids and membranes [1,4,5]. The selectivity with which the polyene macrolide antibiotics act on membranes of sterol-containing cells varies with the sterol and polyene structures. The small, unionized polyene, filipin, binds more tightly to cholesterol than to ergosterol in vesicles by a factor of three [6]. On the other hand, the implication from the effects of amphotericin B on animal and fungal cells and on model membranes containing cholesterol or ergosterol is that this heptaene macrolide binds more extensively or tightly to ergosterol than to cholesterol [7–9]. Comparisons of the effects of amphotericin B and filipin on cholesterol- and ergosterol-containing membranes have been made by studies of permeability [3,10], electron microscopy [4,11,12], electron spin resonance [13,14], fluorescence polarization [1], and fast kinetics [15,16]. In previous work, we demonstrated that membrane fluidity plays an important role in amphotericin B association with sterols and that amphotericin B-lipid and lipid-lipid interactions appear to be competitive [16].

Although the sterol requirement for polyene antibiotic activity has long been recognized [17], the extent to which the transport of ions and water-soluble solutes is determined by the specificity of binding of these antibiotics with sterols has not yet been established. Qualitative binding data have been reported recently using differential spectroscopy of amphotericin B in aqueous alcohol solutions of ergosterol and cholesterol [18] and

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using the extent of the amphotericin B association with liposomes [10]. These data indicate that the greater sensitivity of ergosterol-containing than cholesterol-containing membranes to amphotericin B may arise from preferential interaction of this polyene with ergosterol. Conventional methods of ligand-drug binding analysis have not been applied to the sterol-amphotericin B system and, to our knowledge, no published data exist concerning intrinsic association constants. (A quantitative treatment involving amphotericin B methyl ester-dehydroergosterol binding [19] was retracted [20] because it was found to be erroneous owing to the large inner-filter effect of emitted light from amphotericin B methyl ester that could not be corrected or ignored. A previous equilibrium study in this laboratory [1] was limited to heterogeneous aqueous suspensions of sterols and did not yield estimates of the number of bound sterol molecules per amphotericin B.) The present spectrophotometric analysis characterizes the amphotericin B-sterol system quantitatively by estimating intrinsic binding constants and stoichiometry.

Amphotericin B was obtained from E.R. Squibb and Sons, Inc., New Brunswick, NJ and from Sigma Chemical Co., St. Louis, MO (Type A). Amphotericin B methyl ester was kindly provided by Dr. C.P. Schaffner, and partricin and partricin methyl ester were from Dr. R. Muir (G.D. Searle, lot numbers SN-644 and SN-654). A borate complex of amphotericin B (amphotericin B borate), which is reported to have a molecular weight of approx. 100000 and high solubility in aqueous solution [21], was a generous gift from Dr. G. Strauss; the lipid bilayer/water distribution coefficient is approximately 100-times lower for the borate complex than for amphotericin B [22]. The polyene antibiotics were dissolved in dimethylformamide and stored in the dark at −5°C for no longer than 3 days. The concentrations of stock solutions of the polyenes in dimethylformamide were determined using the following extinction coefficients: amphotericin B, $\epsilon = 12.3 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ (409 nm); amphotericin B methyl ester, $\epsilon = 5.7 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ (409 nm); amphotericin B borate, $\epsilon = 4.0 \cdot 10^6\ M^{-1} \cdot cm^{-1}$ (409 nm). The amphotericins were used without additional purification. Unpurified partricin (free acid) contained several major impurities when analyzed by silica gel thin-layer chromatography using methanol/chloroform/borax (3:2:1, v/v) as the eluting solvent. Partricin was purified by dissolving the solid in the minimum volume of methanol, and filtration of the insolubles. The filtrate was evaporated and washed with ether. The procedure was repeated twice, yielding material ($\epsilon = 7.2 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ at 402 nm) that gave one spot on thin-layer chromatography. Partricin methyl ester was purified by filtering a suspension of the methyl ester in acetonitrile and then dissolving the insoluble portion in methanol; after filtration and evaporation of the solvent, a yellow powder was obtained, which was washed with ether, chloroform, and dried ($\epsilon = 5.5 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ at 402 nm). The structure of partricin was reported recently by Golik et al. [23].

Sterols were obtained from the following sources: cholesterol was from Sigma; β-sitosterol, stigmasterol, ergosterol, and desmosterol were from Steraloids, Inc., Wilton, NH; lanosterol was from Dr. S. Rottem. All sterols were recrystallized at least twice from ethanol. The purity was checked by thin-layer chromatography on silica gel in ether/petroleum ether (9:3, v/v). Egg phosphatidylcholine (PC) was purchased from Makor Chemicals Ltd., Jerusalem, Israel and from Sigma. Dicetylphosphoric acid was from Sigma.

Vesicles from PC and sterol (or from PC alone) were prepared as described previously [15]. The PC:sterol mole ratio was 3:1 and vesicles contained 1 mol % of dicetylphosphoric acid. The lipids were suspended in 2.5% aqueous dextrose at a concentration of 10–20 μmol of total lipid per ml. The suspensions were sonicated at 5°C under nitrogen using a W-375 (Heat Systems-Ultrasonics, Inc.) sonifier for about 30 min at 50% duty cycle until the turbidity was constant ($A_{600} = 0.15$–0.2). The lipid dispersions were centrifuged at $18000 \times g$ for 15 min in a Beckman J-21B centrifuge.

Solutions of polyene antibiotics in dimethylformamide were added in microliter quantities to 10-cm pathlength cuvettes containing 2.5% aqueous dextrose, giving polyene and dimethylformamide concentrations of $< 1\ \mu M$ and 3% (by volume), respectively. Vesicles were added to both the sample and reference cuvettes in order to avoid errors from light scattering of vesicles. To minimize errors due to dilution, aliquots of relatively

concentrated PC/sterol suspensions (10–20 mM total lipid) or of sterol in organic solvent (0.12–0.17 mM) were added by means of microliter syringe to the cuvettes. The maximum volume of added vesicles or sterol was 0.4 ml, i.e., the maximum dilution error was 1.5%. Values of ΔA were corrected for the small dilution factor, and also for any small decrease in solubility of the polyene antibiotic in 2.5% aqueous dextrose during the time course of a blank titration in which dextrose solution was added without vesicles. This correction was considerably greater for amphotericin B methyl ester than for amphotericin B and the borate complex. Absorption measurements were conducted at 409 nm for amphotericin and 402 nm for partricin using a Varian Superscan 3 spectrophotometer. The temperature was 30°C, and the time interval between the addition of vesicle aliquots was about 1 min (about 1 h per titration). In the titrations of amphotericin B and its methyl ester and borate complex with vesicles, small decreases in the absorbance at 409 nm were observed over the course of several minutes upon the addition of the first few aliquots of vesicles (the amount of bound sterol per polyene, $r = 0.1$–0.2). Values at low r values often deviated from the Scatchard plot curve and have been omitted. However, when subsequent aliquots of vesicles were added, the ΔA_{409} values were not changed appreciably after about 1 min, suggesting that equilibrium in this system was essentially attained. The initial time-dependent absorbance changes may reflect binding and partitioning into the membrane phase and interconversions involving bound forms of amphotericin or its aggregates. Time-dependent changes have been detected at much higher amphotericin concentrations by absorbance [16], circular dichroism [24], and electron spin resonance spectroscopy [25]. In the present study mea-

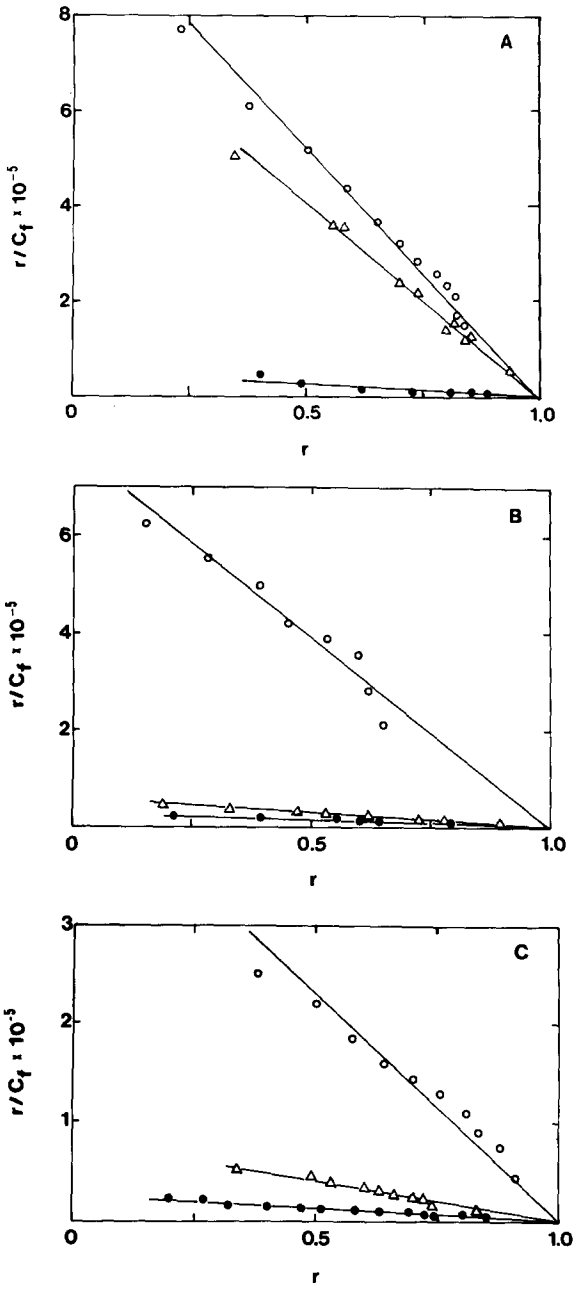

Fig. 1. Scatchard plots for amphotericin binding to sterols in egg phosphatidylcholine/sterol vesicles at 30°C. Interaction of (○) amphotericin B methyl ester, (△) amphotericin B, and (●) amphotericin B borate with (A) ergosterol, (B) cholesterol, and (C) β-sitosterol. The molar ratio of phosphatidylcholine/sterol was 3:1. C_f = free sterol concentration, r = bound sterol per polyene, calculated from $\Delta A/\Delta A_{max}$ as described in the text. The concentrations of the sterols in the vesicle suspensions were varied as follows: For titrations with amphotericin B methyl ester, in (A) from 0.45 to 7.6 μM, in (B) from 0.45 to 7.1 μM, and in (C) from 1.8 to 12.5 μM. For titrations with amphotericin B, in (A) from 0.67 to 10.7 μM, in (B) from 1.8 to 107 μM, and in (C) from 1.8 to 125 μM. For titrations with amphotericin B borate, in (A) from 6.7 to 98.0 μM, in (B) from 7.7 to 74.7 μM, and in (C) from 6.7 to 156 μM. The concentrations of the amphotericins (calculated from their extinction coefficients) were as follows: amphotericin B methyl ester, 0.74 μM; amphotericin B, 0.38 μM; and amphotericin B borate, 0.015 μM.

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g constants, which were determined by least-squares linear regression analysis of data points from Scatchard plots of amphotericin binding to sterols in PC
s, are the mean values of at least three measurements. Error limits are reported as ± S.E. For the six sterol-containing vesicles reported here, the range of n va
ined from the average of $-y$-intercept/slope, was 0.95–1.15.

	Cholesterol	Ergosterol	β-Sitosterol	Stigmasterol	Desmosterol	Lanosterol
otericin B	$(5.2 \pm 1.4) \cdot 10^4$	$(6.9 \pm 1.1) \cdot 10^5$	$(4.6 \pm 1.3) \cdot 10^4$	$(2.4 \pm 0.7) \cdot 10^4$	$(1.3 \pm 0.5) \cdot 10^4$	$(5.4 \pm 0.8) \cdot 10^3$
otericin B hyl ester	$(6.4 \pm 1.8) \cdot 10^5$	$(9.8 \pm 0.4) \cdot 10^5$	$(4.4 \pm 0.8) \cdot 10^5$	$(3.7 \pm 1.4) \cdot 10^5$	$(3.0 \pm 0.8) \cdot 10^5$	$(2.9 \pm 1.0) \cdot 10^5$
otericin B borate	$(4.0 \pm 1.0) \cdot 10^4$	$(3.5 \pm 1.2) \cdot 10^4$	$(2.3 \pm 0.4) \cdot 10^4$	$(1.5 \pm 0.3) \cdot 10^4$	$(1.9 \pm 0.2) \cdot 10^4$	–

surements with amphotericin concentrations less than 1 μM were made possible by use of 10-cm pathlength cuvettes. Scatchard plots were fitted statistically to the equation $r/C_{free} = K_{app}(n-r)$ with the aid of a TI58 programmable calculator. The fraction of sites occupied was calculated from $\Delta A/\Delta A_{max}$, and ΔA_{max} was estimated from the intercept upon extrapolation of a plot of $1/\Delta A$ vs. $1/C_{free}$. A least-squares linear regression analysis of the data points (TI Programmable 58/59 Owner's Manual) gave the equilibrium constant K_{app} as the negative of the slope and the number of binding sites n from the y intercept (nK_{app}).

The traditional approach to analyzing binding affinity quantitatively is to measure the amount of bound complex as a function of free ligand concentration, and to plot the data in the form of a Scatchard [26] or similar binding isotherm. Fig. 1A shows the Scatchard plots of the binding of amphotericin B and the methyl ester and borate complex to ergosterol in egg PC vesicles. Similar plots are presented in Figs. 1B and C for binding to cholesterol and β-sitosterol. The slopes and intercepts of these plots provide estimates of the apparent equilibrium constants, K_{app}, and the saturation limit of r (the amount of bound ligand (sterol) per total polyene). Table I shows that amphotericin B methyl ester is bound more tightly at equilibrium than amphotericin B to cholesterol, β-sitosterol, stigmasterol, and desmosterol in

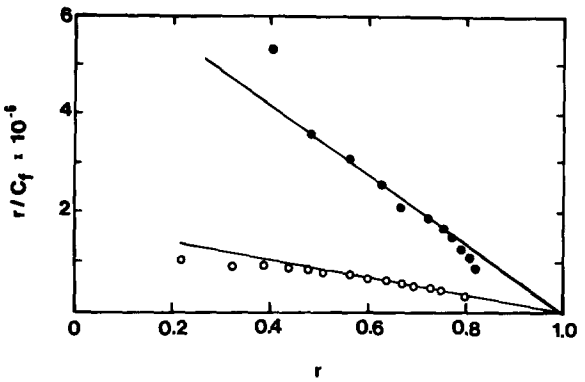

Fig. 2. Scatchard plots for amphotericin B binding to dispersions of cholesterol (●) and ergosterol (○) in 5% aqueous acetonitrile at 30°C. The concentration of amphotericin B was 0.47 μM. The cholesterol and ergosterol concentrations were varied from 8.9 to 133 μM and from 12.0 to 275 μM, respectively.

vesicles by approximately one order of magnitude. The affinity of amphotericin B (and its methyl ester) toward ergosterol is indeed greater than that toward cholesterol in vesicles prepared from PC, confirming qualitative binding data in liposomes [10]. Comparisons of the binding constants also reveal that the selectivity of amphotericin B for ergosterol vs. cholesterol ($K_a = 6.9 \cdot 10^5$ M^{-1} vs. $5.2 \cdot 10^4$ M^{-1}) is greater than that of amphotericin B methyl ester ($K_a = 9.8 \cdot 10^5$ M^{-1} vs. $6.4 \cdot 10^5$ M^{-1}) (Table I). Amphotericin B borate binds more weakly to cholesterol, ergosterol, and β-sitosterol than amphotericin B and the methyl ester, and does not display appreciable binding selectivity (Table I). Amphotericin B methyl ester binds to PC vesicles devoid of sterol considerably more tightly than amphotericin B and the borate complex. Each of the three amphotericin B molecules forms a complex with the sterols investigated in which the stoichiometry is approx. 1 bound amphotericin per sterol molecule (Fig. 1 and Table I).

In contrast to the suggestion that amphotericin B binds more tightly to ergosterol than cholesterol in aqueous alcohol solutions [18], we found tighter binding of amphotericin B to cholesterol ($K_a = 7.6 \cdot 10^6$ M^{-1}, $n = 0.97$) than to ergosterol ($K_a = 1.5 \cdot 10^6$ M^{-1}, $n = 1.1$) in 5% aqueous acetonitrile solution (Fig. 2). The binding of amphotericin B to cholesterol in 15% and 25% aqueous methanol is slightly stronger than that to ergosterol ($K_a = 1.7 \cdot 10^6$ for cholesterol in 25% methanol vs. $1.0 \cdot 10^6$ M^{-1} for ergosterol, $n = 0.97$–1.05). It has been reported previously that steroid structural requirements for interaction with polyenes differ when steroid dispersions or steroids incorporated into phospholipid bilayers are used [1,5].

Scatchard plots for the interactions of the heptaene macrolide partricin and its methyl ester with cholesterol in vesicles with PC indicate that partricin methyl ester is bound more tightly ($K_a = (2.6 \pm 0.1) \cdot 10^6$ M^{-1}, $n = 0.93$) than is partricin free acid ($K_a = (8.3 \pm 1.7) \cdot 10^5$ M^{-1}, $n = 0.93 \pm 0.05$).

The significant difference in K_a for amphotericin B-ergosterol interaction compared with amphotericin B-cholesterol interaction may explain why lower amphotericin concentrations were sufficient to induce K^+ leakage from ergosterol-

containing liposomes than from cholesterol-containing liposomes [10] and why ergosterol-containing cells were found to be more sensitive to amphotericin B than cholesterol-containing cells [8,9].

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