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Fluorescence Studies of the Binding of the Polyene Antibiotics Filipin III, Amphotericin B, Nystatin, and Lagosin to Cholesterol

(membranes/vesicles/lecithin/probe)

ROBERT BITTMAN AND STEVEN A. FISCHKOFF

Department of Chemistry, Queens College of the City University of New York, Flushing, N.Y. 11367

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ABSTRACT **The interactions of filipin III, amphotericin B, nystatin, and lagosin with sterols in aqueous suspension and in vesicles were followed by fluorescence excitation spectra and by measurement of polarized fluorescence intensities. The equilibrium constants for association of the polyene antibiotics with aqueous suspensions of cholesterol follow the order filipin III > amphotericin B > nystatin > lagosin, in agreement with the order reported for the extent of damage these antibiotics cause in natural and model membranes. Fluorescence polarization measurements show that hydrophobic forces are primarily responsible for the formation of the complexes. Filipin III undergoes a large enhancement in fluorescence polarization on binding to aqueous suspensions of cholesterol and *epi*-cholesterol, and to vesicles of lecithin–cholesterol, lecithin–β-cholestanol, and lecithin–ergosterol. Small increases in polarization occur on interaction of filipin III with vesicles derived from lecithin and *epi*-cholesterol, thiocholesterol, and androstan-3β-ol. Amphotericin B undergoes a relatively constant enhancement in fluorescence polarization on interaction with the various lecithin–sterol vesicles used and does not display the selectivity exhibited by filipin III. It is suggested that filipin III serves as a probe of lecithin–sterol interaction.**

Polyene antibiotics are potent antifungal agents produced by *Streptomycetes*. In addition to their ability to inhibit the sporulation and growth of yeast and other fungi (1), some of the polyene antibiotics possess antiprotozoal activity (2) and, despite their toxicity, they have clinical applicability. For example, polyene antibiotics appear to be capable of controlling serum cholesterol levels (3, 4) and prostate malfunction (5) in dogs; they also exhibit larvicidal and chemosterilant activity in some insects, apparently by blocking the uptake of dietary cholesterol (6, 7). All have a macrolide polyhydroxylic lactone ring of more than 23 atoms, with 4–7 conjugated double bonds. The discovery in 1950 of the first polyene antibiotic (8) generated considerable chemical and biological interest in this group of macrolides. The structure originally assigned to the pentaene antibiotic filipin (9–13) has been revised (14, 15) because the original work was done on a mixture of filipins (now referred to as the filipin complex) consisting of more than four components (16). The related antibiotic lagosin, which appears to be identical to, or a stereoisomer of, fungichromin, and is very similar in structure to the filipins (17), has also been subjected to extensive structural studies (11, 12). Amphotericin B, a conjugated heptaene, and nystatin, whose chromophore is a conjugated tetraene distant by two methylene groups from a diene, are structurally similar. Both have a carboxyl group and an aminoglucoside group linked to the macrocyclic ring, and probably exist in the hemiketal form, at least in the crystalline state (18–20). The chemical structures and absolute configurations of nystatin (20, 21) and amphotericin B (18, 19, 22) have been studied; chemical interest in amphotericin B culminated in the elucidation of the crystal structure of its *N*-iodoacetyl derivative (19).

Because the polyene antibiotics are believed to bind to sterols present in the membranes of polyene-sensitive microorganisms before lethal changes in membrane permeability appear, and because quantitative data concerning the binding constants of polyene antibiotics to cholesterol have not been reported, the fluorescence investigations reported in the present paper were undertaken.

MATERIALS AND METHODS

Polyene Antibiotics. Filipin III (filipin) was supplied as lot number U-25,639 by Dr. G. B. Whitfield of the Upjohn Co., Kalamazoo, Mich. Each lot was pure by thin-layer chromatography (16). Amphotericin B (amphotericin) and nystatin were obtained as lot numbers 91830 and 73E, respectively, from Squibb, New Brunswick, N.J. Lagosin was provided as lot number H230 from Glaxo Research Ltd., Buckinghamshire, England. Stock solutions of filipin, nystatin, and lagosin were prepared in dimethylformamide (DMF). Experiments were done in 1 mM Tris·HCl–10 mM NaCl (pH 7.4) by transfer of aliquots of the stock solutions to buffer solutions to give the desired concentrations of antibiotic and DMF. Amphotericin was suspended in methanol; it dissolved upon acidification with HCl. In order to prevent formation of amphotericin methyl ester, the methanol was removed promptly and the water-soluble amphotericin hydrochloride thus obtained was dissolved in 1 mM Tris·HCl–10 mM NaCl (pH 7.4). The concentrations of the antibiotics were determined spectrophotometrically on a Cary 14 spectrophotometer. Based on a molecular weight of 670 for filipin and lagosin and 790 for nystatin, the following extinction coefficients were determined in 100% DMF: filipin at 361 nm, 5.06×10^4 M^{-1} cm^{-1}; lagosin at 344 nm, 7.84×10^4 M^{-1} cm^{-1}; nystatin at 307 nm, 3.44×10^4 M^{-1} cm^{-1}. The extinction coefficient at 334 nm of amphotericin in 1 mM Tris·HCl–10 mM NaCl (pH 7.4) immediately after preparation of the solution was 2.65×10^4 M^{-1} cm^{-1}, for an assumed molecular weight of 923.

Lipids and Steroids. Lecithin was isolated and purified from fresh hen egg yolk (23). Purity was analyzed by thin-layer chromatography (24) on silica gel G-coated plates in solvents consisting (by volume) of chloroform–methanol–water 65:25:4 and chloroform–acetone–methanol–acetic acid–water 3:4:1:1:0.5. Steroids were purchased from: cholest-5-en-3β-ol (cholesterol), 5α-cholestan-3β-ol (β-cholestanol), and cholesteryl

Abbreviation: DMF, dimethylformamide.

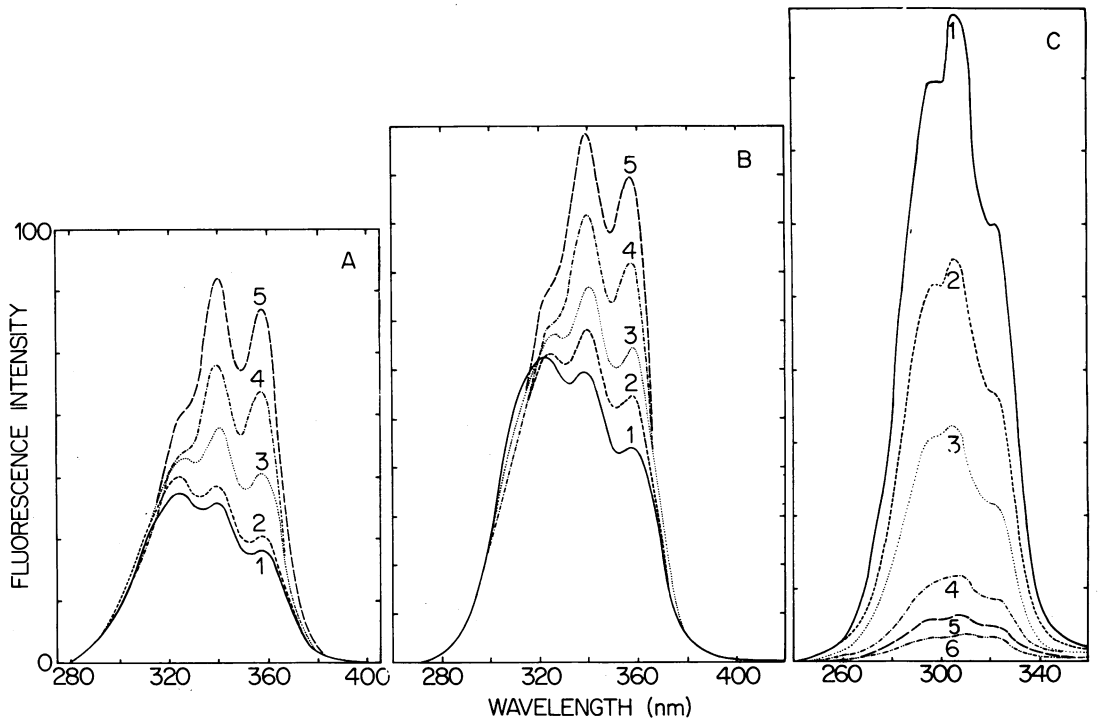

FIG. 1. Fluorescence excitation spectra of filipin, lagosin, and nystatin in the presence of various concentrations of cholesterol and *epi*-cholesterol. The emission was detected at 480 nm for filipin and lagosin and at 400 nm for nystatin. Stock solutions of polyene antibiotic and cholesterol in DMF were diluted with 1 mM Tris·HCl–10 mM NaCl (pH 7.4) so that the final concentration of DMF was 1.0% (v/v). The fluorescene intensities are in arbitrary units. (*A*) The concentration of filipin was 1.08 μM. The concentrations of *epi*-cholesterol were: **1**, 51.7 μM; **2**, 34.5 μM; **3**, 17.2 μM; **4**, 2.46 μM; **5**, 1.01 μM. (*B*) The concentration of lagosin was 0.696 μM. The concentrations of cholesterol were: **1**, 51.7 μM; **2**, 34.5 μM; **3**, 28.7 μM; **4**, 17.2 μM; **5**, 8.62 μM. (*C*) The concentration of nystatin was 0.253 μM. The concentrations of cholesterol were: **1**, 51.7 μM; **2**, 25.9 μM; **3**, 17.2 μM; **4**, 8.62 μM; **5**, 4.70 μM; **6**, 2.46 μM.

ethyl ether from Sigma Chemical Co.; cholest-5-en-3α-ol (*epi*-cholesterol) and ergosterol from Schwarz–Mann; 3β-thiocholest-5-ene(thiocholesterol) from Aldrich Chemical Co.; androstan-3β-ol from Ikapharm, Ramat-Gan, Israel. The sterols were recrystallized several times from acetone. Purity was analyzed by thin-layer chromatography on silica gel G-coated plates in benzene–methanol 92:8 and chloroform–acetone 98:3.5. Dicetyl phosphate was obtained from Sigma.

Preparation of Vesicles. Vesicles were prepared in 1 mM Tris·HCl–10 mM NaCl (pH 7.4). Aliquots of stock solutions of egg lecithin, sterol, and dicetyl phosphate in chloroform were transferred to vials, the chloroform was removed under nitrogen, and the lipids were evaporated to dryness under reduced pressure. The lipid mixtures were dispersed into the buffer solution by ultrasonic irradiation under nitrogen at 4° with a 20-kHz Branson Sonifier (model S-110) fitted with a solid tap horn at power level 4. Vesicles contained 6 mol-% of dicetyl phosphate and were subjected to ultrasonic irradiation for 6 min.

Fluorescence Measurements. Excitation spectra and fluorescence polarization intensities were measured at 15° with a Hitachi-Perkin Elmer model MPF-2A fluorescence spectrophotometer equipped with a polarizer accessory. Excitation and emission slits corresponding to bandpasses of 11 nm were generally used; in some experiments slits of 8, 9, and 10 nm were used. Fluorescence spectra and polarization intensities were corrected for light scattering of the aqueous cholesterol suspensions and vesicles, unless otherwise noted. Polarized fluorescence intensities were corrected for depolarization produced by the emission monochromator grating. Measurements of the fluorescence properties of filipin in the presence of vesicles and membranes were made in buffer containing 0.3% DMF (v/v). When spectra of antibiotics were recorded in the presence of aqueous suspensions of cholesterol, 1% DMF was present. Fluorescence spectra of filipin, lagosin, and nystatin were recorded after a minimum of 2.5 hr of incubation in the dark with the cholesterol suspension or vesicles. Measurements of the fluorescence spectra of amphotericin were made without allowance for an incubation time because the absorbance and fluorescence properties of free amphotericin change markedly with time, probably because of time-dependent formation of aggregates.

RESULTS

Fig. 1 shows the fluorescence excitation spectra of filipin in the presence of aqueous suspensions of *epi*-cholesterol, and of lagosin and nystatin in the presence of aqueous suspensions of cholesterol. The decrease in the intensities of the 340- and 358-nm bands of filipin and lagosin and the increase in the intensity of the band at 323 nm characterize the binding. Similar changes are observed in the absorption spectra. The fluorescence excitation spectrum of nystatin is enhanced in the presence of increased concentrations of cholesterol, without major modification of peak ratios and shape.

To obtain information about the type of forces responsible for the binding of filipin to cholesterol, the effect of methanol concentration on the excitation spectrum of the filipin–choles-

terol complex was studied. Fig. 2*A* shows that addition of methanol leads to reversal of the spectral changes that occur on binding. On binding of filipin to cholesterol, the fluorescence polarization at 323 nm increases from 0.017 to 0.366. Fig. 2*B* shows the decrease in fluorescence polarization and percent of bound antibiotic with increased methanol concentration.

The fluorescence polarization spectrum of filipin in the presence of an aqueous suspension of *epi*-cholesterol resembles the absorption and excitation spectra. Fig. 3*A* demonstrates the correlation between the electronic transitions of the bound polyene and the polarization values. Filipin bound to cholesterol exhibits a similar polarization spectrum, but the polarization intensities are slightly higher than those obtained with *epi*-cholesterol (Fig. 3*B*). The polarized fluorescence intensities of filipin, lagosin, and amphotericin increase on binding to cholesterol (Fig. 3*B, C*). The fluorescence polarization of nystatin did not increase markedly on binding to cholesterol. In the absence of cholesterol, nystatin has a fluorescence polarization of 0.341 in Tris·HCl (at λ_{ex} 310 nm, λ_{em} 400 nm). Since the polarization of nystatin in methanol is 0.091, the higher polarization value in water probably results from the formation of nystatin aggregates or micelles.

Under the conditions used in the fluorescence polarization titrations, the total cholesterol concentration exceeds the concentration of bound antibiotic by molar ratios between at least 10 and 100, and the free cholesterol concentration varies between at least 90 and 99% of the total cholesterol concentration if a 1:1 stoichiometry is assumed. The antibiotic–cholesterol equilibria were analyzed with Eqs. 1 and 2 (see ref. 25):

$$\frac{1}{p} = \frac{1}{K_s p_{max,s}} \frac{1}{[\text{cholesterol}]} + \frac{1}{p_{max,s}} \quad [1]$$

$$\frac{1}{p} = \frac{1}{K_w p_{max,w}} \frac{1}{[\text{cholesterol}]} + \frac{1}{p_{max,w}} \quad [2]$$

where s and w represent the strong and weak classes of binding sites, K is the association equilibrium constant, and p is the fluorescence polarization of the antibiotic at a given cholesterol concentration. K_s and K_w were calculated from the quotient of $-(y\text{-intercept/slope})$ for each class of lines in the double-reciprocal plot. The two lines were chosen such that the standard deviation for both lines was minimized by the method of least squares. The equilibrium constants are reported in Table 1.

Fig. 4 shows the fluorescence polarizations of filipin and amphotericin resulting from the interaction of these polyenes with lecithin–sterol vesicles. The polarization of amphotericin does not vary markedly with the sterol, whereas filipin undergoes marked changes in polarization on binding to vesicles containing different sterols.

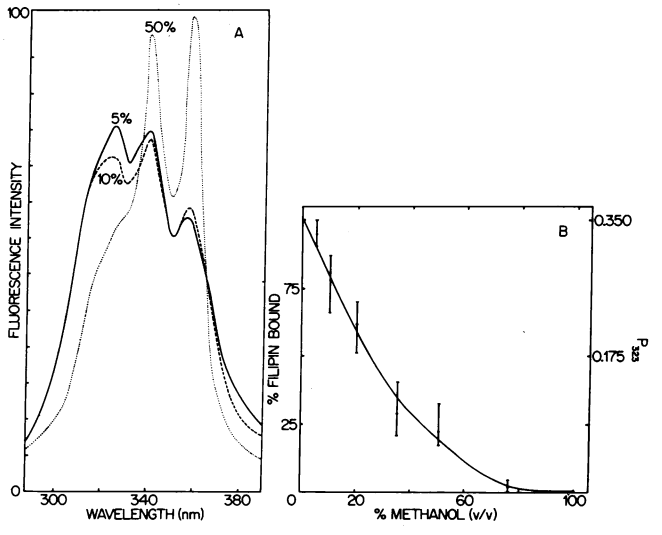

FIG. 2. Effect of methanol concentration on the binding of filipin to cholesterol. (*A*) Excitation spectra were recorded for emission at 480 nm. The concentration of filipin was 0.515 μM. The concentration of cholesterol was 51.7 μM. DMF was present at a concentration of 0.5% (v/v) in all suspensions, and the concentration of methanol was varied as shown. Spectra are not corrected for light scattering by cholesterol alone. The curve labeled 5% methanol was identical to those obtained in 0, 0.5, 1.0, and 2.0% methanol (v/v). (*B*) Polarized fluorescence intensities were measured at 323 nm as a function of methanol concentration. The emission was detected at 480 nm. The % of bound filipin is the product of the fraction bound and the total concentration of filipin (0.515 μM) $\times$ 100. The fraction of bound filipin is $(p - p_{free})/(p_{max} - p_{free})$, where p is the polarization at a given concentration of methanol and, at the wavelengths used in these measurements, p_{max} and p_{free} are 0.366 and 0.017, respectively. The ordinate on the *right* represents the fluorescence polarization at 323 nm, corrected for the contribution of 0.017 made by filipin in the absence of cholesterol.

DISCUSSION

Spectral changes characteristic of the filipin–cholesterol interaction occur in the excitation spectrum of filipin in the pres-

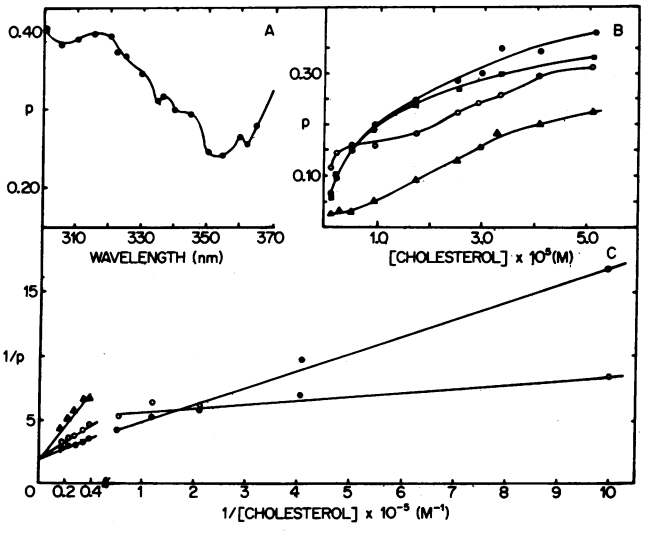

FIG. 3. Effect of cholesterol concentration on the fluorescence polarization of filipin, lagosin, and amphotericin. The emission wavelength was 480 nm. (*A*) The fluorescence polarization spectrum of filipin (1.08 μM) was recorded in the presence of 51.7 μM *epi*-cholesterol. (*B*). The polarized fluorescence intensities of filipin (1.08 μM), lagosin (0.696 μM), and amphotericin (0.923 μM) were recorded in aqueous suspensions containing different concentrations of cholesterol. The excitation wavelength was 340 nm for lagosin and amphotericin, and 325 nm for filipin: ●, filipin in the presence of cholesterol; ■, filipin in the presence of *epi*-cholesterol; ○, amphotericin and ▲, lagosin in the presence of cholesterol. (*C*) Double-reciprocal plots of fluorescence polarization against cholesterol concentration were constructed from the data shown in *B*.

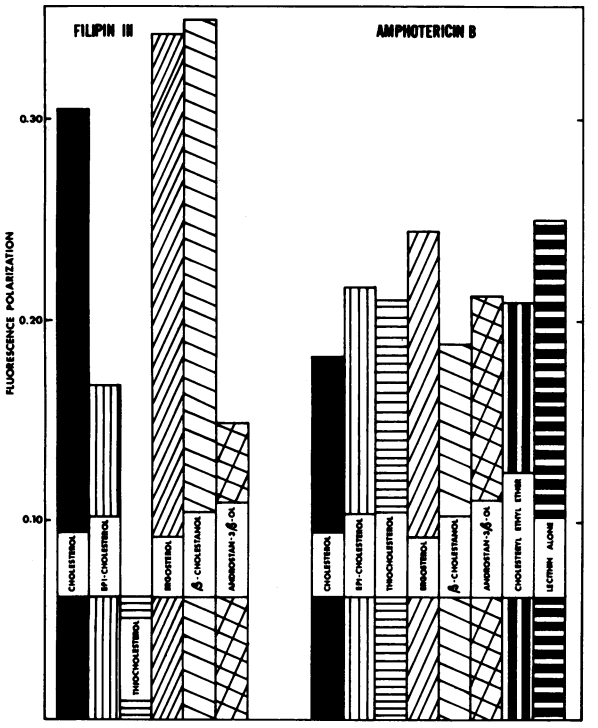

FIG. 4. Effect of lecithin–sterol vesicles on the fluorescence polarization of filipin and amphotericin. The wavelengths of excitation and emission were 325 and 480 nm, respectively. The concentrations of filipin and amphotericin were 3.85 μM and 7.42 μM, respectively. The lecithin and sterol were present at 1:1 molar ratio, and the total lipid concentration in the vesicles was 0.71 mM.

ence of aqueous cholesterol suspensions and lecithin–cholesterol vesicles (Fig. 1*A*). The fluorescence polarization of free filipin is 0.017 at the excitation and emission wavelengths used, indicating that the emitted light is nearly completely depolarized. Therefore, rapid rotational Brownian motion occurs during the lifetime of the excited state of filipin, a process that results in randomization of the direction of the emission oscillator. On being bound to cholesterol, the polarization of filipin increases to a maximal value of 0.366. Since the degree of quenching accompanying binding is relatively low, the excited lifetime of filipin probably is not shortened appreciably on binding. The increase in polarization, therefore, probably results from slower Brownian motion in bound filipin than in free filipin.

The magnitude of the enhancement in fluorescence polarization of filipin depends on the type of steroid incorporated into the vesicles (Fig. 4). *Epi*-cholesterol and androstanol exert only slight effects on the phase transitions of lecithin (26) and on the permeability properties of liposomes (27), indicating that these sterols do not reduce the motional freedom of the hydrocarbon chains of lecithin. Fig. 4 shows that filipin undergoes large enhancements in fluorescence polarization when bound to vesicles in which the lecithin–sterol interaction is strong, presumably because the antibiotic is firmly oriented during the lifetime of the excited state. Thus, the fluorescence polarization of filipin appears to reflect the properties of the molecular interactions in the lecithin–sterol complex. For example, although *epi*-cholesterol in aqueous suspensions interacts with filipin almost as strongly as cholesterol (Fig. 3), interaction of lecithin–*epi*-cholesterol vesicles with filipin results in only a small enhancement of the fluorescence polarization of the antibiotic (Fig. 4). This result is in marked contrast to the effect of lecithin–cholesterol vesicles on filipin; on binding they cause quenching of the fluorescence intensity, alteration of the peak ratios in the excitation spectrum, and a large enhancement in polarization. These findings agree with those of Van Deenen and coworkers (28), who observed that monolayers of *epi*-cholesterol alone gave significant increases in surface pressure after addition of filipin, but mixed lecithin–*epi*-cholesterol monolayers gave only small pressure increases. In contrast, mixed lecithin–cholesterol monolayers gave significant pressure increases, as did monolayers of cholesterol alone. Furthermore, we observed (unpublished data) large enhancements in the circular dichroism bands of filipin on binding to aqueous suspensions of cholesterol and *epi*-cholesterol, and to lecithin–cholesterol vesicles; however, only small enhancements occur on interaction with lecithin–*epi*-cholesterol vesicles. The results reported here are consistent with recent studies showing that the stereochemical orientation of the hydroxyl group of cholesterol at the lecithin polar headgroup plays an important role in lecithin–cholesterol interaction (26–30). Furthermore, the small increase in fluorescence polarization of filipin caused by lecithin–thiocholesterol vesicles is consistent with the small enhancement these vesicles produce in the circular dichroism spectrum of filipin, with the inability of filipin to cause lysis of these vesicles at concentrations that lyse lecithin–cholesterol vesicles (manuscript in preparation), and with the diminished effect of thiocholesterol on the initial rates of osmotic shrinking of liposomes (30). These results support the involvement of hydrogen bonding between lecithin and cholesterol, and suggest that the association of lecithin and thiocholesterol may be weaker, or the lifetime of the complex may be shorter, than that formed between lecithin and cholesterol itself. The small enhancement in polarization caused by lecithin–androstanol vesicles indicates that the side chain on the steroid nucleus of cholesterol is involved in the interaction of lecithin with cholesterol. This result is in agreement with permeability and differential scanning calorimetry studies (26, 27). A sterol with a different side chain structure, ergosterol, does enhance the polarization of filipin when it is present in vesicles (Fig. 4). Vesicles containing β-cholestanol give rise to a large increase in polarization, indicating that the double bond at the 5 position of cholesterol may not play a crucial role in lecithin–cholesterol interaction. This suggestion also is consistent with the strong interac-

TABLE 1. *Equilibrium constants K for complexes of polyene antibiotics and cholesterol in aqueous suspensions**

Antibiotic	K_s (M^{-1})	K_w (M^{-1})
Filipin	7.6×10^6	4.5×10^5
Amphotericin	2.0×10^6	8.6×10^4
Nystatin	7.0×10^5	1.2×10^4
Lagosin	2.4×10^4	8.8×10^3

* Association constants were determined at 15° in the presence of 1% DMF by volume from double-reciprocal plots of fluorescence polarization (except for nystatin) against cholesterol concentration, as described in the text and in Fig. 3. For nystatin, the equilibrium constants were determined from double-reciprocal plots of fluorescence intensity against cholesterol concentration. The concentrations of filipin, amphotericin, and lagosin are given in Fig. 3. The concentration of nystatin was 0.253 μM.

tion of β-cholestanol and lecithin observed from the condensing effect of this sterol on lecithin monolayers (29).

The ability of methanol to disrupt the filipin–cholesterol complex suggests that hydrophobic forces are involved in maintaining the complex, and shows that covalent bond formation does not occur. We have reached the same conclusion from circular dichroism studies. These results are consistent with those obtained by others (28, 31, 32).

Different polyene antibiotics display a spectrum of effects with regard to their relative potencies in natural and model membranes. In view of the hypothesis that sterols are a prerequisite for sensitivity of organisms to polyene antibiotics, different degrees of physical damage in membranes may arise because of differing affinities among the polyene antibiotics for cholesterol. Table 1 shows that the order of affinities is filipin > amphotericin > nystatin > lagosin. This agrees with the order of potency filipin > amphotericin $\gtrsim$ nystatin that has been observed in several model and natural membranes. (Data to allow inclusion of lagosin in the following comparison of potency have not been published.) The severity of damage to single bimolecular films of lecithin and cholesterol, as measured by the decrease in membrane resistance, followed the order filipin > amphotericin > nystatin (33, 34), as did the extent of hemolysis of rat erythrocytes (35). Filipin caused greater lysis of *Neurospora* protoplasts than nystatin (36), and the same trend was found for the inhibition of yeast glycolysis and the extent of leakage of K^+ and inorganic phosphate from yeast cell membranes (37). However, quantitative comparisons between the relative affinities of the polyene antibiotics toward cholesterol and the extent of damage they induced in membranes are not possible because one must consider discrepancies in the concentrations of the antibiotics, difficulties in their solubilization, and differences in potency and specificity for cholesterol among components of the filipin complex (38).

Attempts to construct binding isotherms by the method of Scatchard (39) failed. Quantitative analysis of the binding equilibria are complicated by the tendency of free polyene antibiotics to aggregate, even in the low concentration range used in the present study, as evidenced by the departure from Beer's law (unpublished results) and by the high fluorescence polarization observed for aqueous solutions of nystatin. In addition, cholesterol has a very low solubility in water. Dispersions prepared by mixture of ethanolic solutions of the steroid with an excess of water have been observed by electron microscopy of negatively stained preparations to consist of "microcrystals" of various shapes and sizes (40, 41). Thus, free cholesterol in aqueous suspensions probably exists as aggregates or micelles of different degrees of self-association, perhaps explaining why more than one class of binding sites is observed.

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