Interaction of Filipin III and Amphotericin B with Lecithin–Sterol Vesicles and Cellular Membranes. Spectral and Electron Microscope Studies†

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ABSTRACT: The effects of the polyene antibiotics filipin III and amphotericin B on egg lecithin–sterol vesicles and ciliary membranes from *Tetrahymena pyriformis* W were investigated using ultraviolet absorption, fluorescence, and circular dichroism spectroscopy and negative-staining electron microscopy. Filipin III underwent changes in absorbance and fluorescence excitation peak ratios and enhancements in molecular ellipticity in the presence of lecithin–cholesterol and lecithin–ergosterol vesicles and ergosterol-containing ciliary membranes. Spectral changes were less striking in the presence of tetrahymanol-containing ciliary membranes and vesicles prepared from lecithin alone and from mixtures of lecithin and epicholesterol, thiocholesterol, androstan-3β-ol, or cholestanol. Filipin induced lysis of lecithin–cholesterol and lecithin–ergosterol vesicles, but did not lyse vesicles prepared from lecithin alone and from mixtures of lecithin and epicholesterol, thiocholesterol, androstan-3β-ol, or cholestanol. These spectral and electron microscopic results appear to reflect the strengths of the molecular interactions between lecithin and sterols. A mechanism of formation of "pits" in the vesicles by a process of micelle-mediated fusion of lipid bilayers is discussed. Filipin treatment of ergosterol-containing ciliary membranes produced annuli with mean diameter almost identical with that in filipin-treated lecithin–ergosterol vesicles. The similarities of the effects of filipin on lecithin–ergosterol vesicles and ergosterol-containing ciliary membranes suggest that study of the interaction of filipin with phospholipid bilayers is relevant to the action of the antibiotic on biological membranes and tend to support the validity of using lipid bilayer vesicles as models of cellular membranes. In contrast to results obtained with filipin, spectral changes of amphotericin B are not sensitive to the structure of the sterol incorporated into the vesicle. A homogeneous electron-translucent layer surrounding an inner electron-dense amorphous core was formed when vesicles prepared from lecithin alone and from mixtures of lecithin and cholesterol, ergosterol, epicholesterol, thiocholesterol, androstan-3β-ol, or cholestanol were treated with amphotericin B. Statistically significant swellings of major and minor axes diameters of the prolate-shaped vesicles were observed following amphotericin B treatment of lecithin, lecithin–cholesterol, and lecithin–ergosterol vesicles. Tetrahymanol-containing ciliary membranes were unaffected by filipin III and amphotericin B under the conditions used.

The biological activity of the polyene antibiotics is believed to result from their ability to alter the permeability of membranes of microorganisms containing sterols. Irreversible changes in membrane function and leakage of cellular components result as a consequence of the binding of these antifungal agents to sterols in the membranes of susceptible organisms (Lampen, 1969). Prior studies have shown that the polyene antibiotic filipin interacts reversibly with sterols in aqueous suspension, monolayers, thin lipid membranes, liposomes, and natural membranes such as erythrocytes and cholesterol-containing *Acholeplasma laidlawii* membranes (Lampen *et al.*, 1960; Gottlieb *et al.*, 1961; Kinsky, 1963; Demel *et al.*, 1965, 1968; Kinsky *et al.*, 1967; Sessa and Weissmann, 1968; Bittman and Fischkoff, 1972; Norman *et al.*, 1972a,b; Schroeder *et al.*, 1972). For vesicle-bound sterol, a planar sterol nucleus, 3β-hydroxyl group, and sterol side chain at C-17 are required for maximal binding of filipin to the vesicle (Norman *et al.*, 1972a,b; Bittman and Fischkoff, 1972).

In an attempt to better understand what structural features in the sterol are important in the interaction of polyene antibiotics with sterols in membranes and what alterations in membrane morphology accompany sterol–polyene antibiotic interaction, we have undertaken ultraviolet, fluorescence, and circular dichroism (CD) spectral studies and electron microscopic studies of the interaction of filipin III and amphotericin B with sterols in vesicles and membranes. Dual application of spectral and electron microscopic methods offers the advantage that correlations may be made between molecular interactions and morphological changes which may yield a more comprehensive view of biological action than can be obtained from the separate application of either method.

Lecithin was chosen as the phospholipid from which to prepare phospholipid–sterol vesicles because it is the principal phospholipid in polyene-sensitive organisms. Attention was focused on ergosterol because the binding sites for polyenes on the fungal membrane may be unesterified ergosterol (Lampen *et al.*, 1960, 1962; Zygmunt and Tavormina, 1966). The ciliate *Tetrahymena pyriformis* W is particularly appealing for studies of the relationship of membrane structure to polyene specificity. In lipid-free (unsupplemented) medium, this ciliate synthesizes the pentacyclic triterpenoid alcohol, tetrahymanol. Tetrahymanol is structurally similar to sterols in that it possesses a 3β-hydroxyl group and planar ring structure. However, the presence of 4,4,14-trimethyl groups and cyclization of the side chain may introduce important

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structural differences that could have functional consequences. It has been speculated that the "sterol-like" tetrahymanol may play the sterol's role in *Tetrahymena pyriformis* W (Nes, 1974). Supplementation of the growth medium with ergosterol results in normal cell growth and replacement of tetrahymanol by ergosterol (Conner *et al.*, 1968). Unlike many other sterols which may be assimilated by this ciliate, ergosterol is incorporated without alteration (Conner *et al.*, 1969; 1971). Furthermore, fatty acyl esters of tetrahymanol or ergosterol do not occur in this strain (Mallory and Conner, 1971). The ciliary membrane of *Tetrahymena pyriformis* W is easily isolated; moreover, the molar ratio of lipid phosphorus to alcohol (tetrahymanol or ergosterol) in the ciliary membranes obtained from the two cell types is the same, implying that ergosterol molecules may be inserted into membranes at sites that are normally occupied by tetrahymanol (Conner *et al.*, 1971).

The chemical structures of filipin III, amphotericin B, and tetrahymanol are shown in Figure 1.

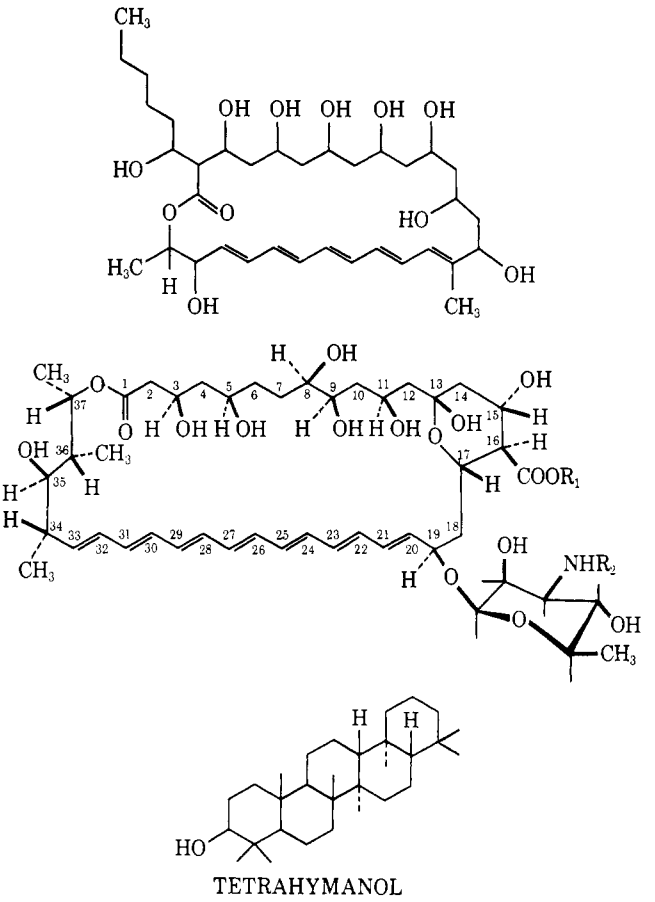

FIGURE 1: Structures of filipin (top), amphotericin (middle, R = H), and tetrahymanol (bottom).

Experimental Section

Materials. Lecithin was isolated and purified from fresh hen egg yolk. The isolation and determination of purity were performed as described previously (Bittman and Blau, 1972). Cholest-5-en-3β-ol (cholesterol), cholest-5-en-3α-ol (epicholesterol), 3β-thiocholest-5-ene (thiocholesterol), 5α-cholestan-3β-ol (cholestanol), androstan-3β-ol, and ergosterol were purchased and purified as reported previously (Bittman and Blau, 1972; Bittman and Fischkoff, 1972). Dicetyl phosphate was obtained from Sigma. Filipin III was supplied by Dr. G. B. Whitfield of the Upjohn Co., Kalamazoo, Mich., and amphotericin B by Squibb, New Brunswick, N. J. The lot numbers of the antibiotics, method of preparation of stock solutions, and extinction coefficients were reported previously (Bittman and Fischkoff, 1972). Perhydrofilipin was prepared by hydrogenation of filipin over Adams catalyst in absolute ethanol at atmospheric pressure and room temperature according to the method of Pandey *et al.* (1972). The experiments involving mixtures of perhydrofilipin and filipin were performed with the "filipin complex." The latter is a mixture of filipin I–IV (Bergy and Eble, 1968). All other experiments involving filipin were performed with filipin III, the principal component of the filipin complex (Bergy and Eble, 1968). For the sake of brevity, amphotericin B is referred to here as amphotericin.

Experiments were performed in 1 mM Tris buffer containing 10 mM NaCl (pH 7.4). The final concentration of dimethylformamide (DMF) was 0.3% (v/v) in all experiments involving filipin.

Preparation of Vesicles and Ciliary Membranes. Vesicles were prepared by transferring aliquots of stock solutions of egg lecithin, sterol, and dicetyl phosphate prepared in chloroform to vials. The chloroform was removed under nitrogen, and the lipids were evaporated to dryness under reduced pressure. The thin, dry lipid film was suspended in the buffer. The suspension was subjected to ultrasonic irradiation under nitrogen for 30 sec at 4° with a 20-kHz Branson Sonifier (Model S-110) fitted with a solid tap horn at power level 4. Vesicles contained 4 mol % of dicetyl phosphate. Ciliary membranes from unsupplemented and ergosterol-supplemented cells of *Tetrahymena pyriformis* W were obtained from Dr. E. Kaneshiro and Dr. R. L. Conner of Bryn Mawr College, Bryn Mawr, Pa. They were isolated as described by Conner *et al.* (1971). Sterol, tetrahymanol, and phosphorus analyses were performed as described previously (Conner *et al.*, 1969, 1971). The molar ratio of lecithin to sterol in vesicles was 7:3. The molar ratio of phospholipid to ergosterol or tetrahymanol in ciliary membranes was 2:1. The total lipid concentrations given in the figure captions are those obtained after mixing with antibiotic.

Spectral Measurements. Absorption measurements were carried out on a Cary 14 spectrophotometer. Fluorescence measurements were performed on a Hitachi-Perkin-Elmer spectrofluorometer, Model MPF-2A, using excitation and emission slit widths as reported previously (Bittman and Fischkoff, 1972). CD measurements were made on a Cary 60 spectropolarimeter equipped with a Model 6001 circular dichroism attachment. All spectra were corrected for light scattering of the vesicles and membranes. In the spectral region investigated (λ >280 nm) the base line of the background was flat. Polarized fluorescence intensities were measured at 15°. All other spectra were recorded at room temperature. Freshly prepared aqueous solutions of the antibiotics were added to the vesicles or ciliary membranes and the suspensions were incubated for a minimum of 2 hr at room temperature in the dark before spectral determinations were made.

Electron Microscopy. Vesicles or membranes were permitted to react with filipin or amphotericin for 20 hr at 4°. Untreated vesicles or membranes were allowed to stand for the same period of time at the same temperature. For examination of vesicles, an equal volume of 2% (w/v) ammonium molybdate solution in 1 mM Tris buffer (pH 7.4) or adjusted to pH 6.5 with NH_4OH was added to each sample as a negative stain. Electron microscopy revealed that under

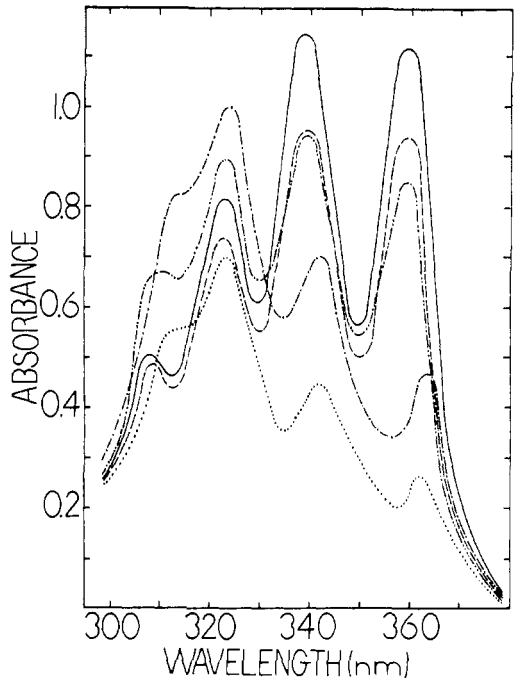

FIGURE 2: Absorption spectra of filipin (—), and of filipin in the presence of lecithin vesicles (---), lecithin–ergosterol vesicles (· · ·), and *Tetrahymena* ciliary membranes containing tetrahymanol (-· · -) and ergosterol (-· -). The concentration of filipin was 21.5 μM. The total lipid concentration in the lecithin vesicles was 0.190 mM. The molar ratio of lecithin to ergosterol in vesicles was 7:3, and the total lipid concentration was 0.38 mM. Tetrahymanol-containing ciliary membranes contained 0.138 mM phospholipid and 0.052 mM tetrahymanol. Ergosterol-containing ciliary membranes contained 0.155 mM phospholipid and 0.069 mM ergosterol.

the experimental conditions used ultrasonic irradiation produced dispersions containing both multilamellar lipid particles and single-lamellar vesicles; since most of the lecithin (about 68% in a typical preparation) was present in the form of single bilayer shells, the lipid dispersions are referred to as vesicles in this paper. Ciliary membrane suspensions were stained with an equal volume of 2% (w/v) phosphotungstic acid, adjusted to pH 6.5 with KOH. Aliquots of the stained vesicle and membrane suspensions were deposited on carbon-reinforced, collodion-covered copper grids. Excess sample suspension was withdrawn by capillary attraction to small pieces of filter paper. The remaining thin film was dried at room temperature. Specimens were examined in a Phillips EM 200 electron microscope with 60-kV accelerating voltage.

Tests were performed to determine the approximate minimum concentrations of filipin and amphotericin that were sufficient to induce vesicle lysis. Lecithin–cholesterol vesicles were prepared at 7:3 molar ratio and total lipid concentration of 1 mM. Aliquots of lecithin–cholesterol vesicles were mixed with an equal volume of filipin solution, giving final filipin concentrations of 4.8, 3.4, 1.9, and 0.96 μM. Similarly, the minimum amphotericin concentration sufficient to induce lecithin–cholesterol vesicle lysis was determined by mixing aliquots of the vesicle suspension with amphotericin solutions to give final amphotericin concentrations of 26.8, 8.9, and 2.7 μM.

Results

Absorption Spectra. Previous work showed that the binding of filipin to cholesterol-containing vesicles and membranes is accompanied by an alteration of the peak ratios in the ultraviolet absorption spectrum of the antibiotic (Lampen *et al.*, 1960; Norman *et al.*, 1972a,b). Figure 2 and Table I show that in the presence of the tetrahymanol-containing ciliary membranes of *Tetrahymena pyriformis* W the extinction coefficients of the absorption bands of filipin are diminished somewhat, but their ratios are not altered markedly. Addition of lecithin vesicles or lecithin–epicholesterol vesicles to filipin did not cause significant alteration of the ratios of the absorbance maxima, although the extinction coefficients of filipin were diminished. Interaction of filipin with lecithin–ergosterol vesicles results in a large change in the absorbance peak ratios of the antibiotic, and a strikingly similar spectrum is observed in the presence of ciliary membranes obtained from ergosterol-supplemented cells of *Tetrahymena* (Figure 2). Figure 3 shows that the extinction coefficients of the long wavelength bands of amphotericin undergo small enhancements in the presence of lecithin vesicles, lecithin–cholesterol vesicles, and aqueous suspensions of cholesterol. Similar changes were observed in the absorption spectrum of amphotericin in the presence of tetrahymanol- and ergosterol-containing ciliary membranes.

TABLE I: Effect of Vesicles and Ciliary Membranes on Ultraviolet Absorbance Peak Ratios of Filipin.[a]

Agent Added to Filipin	Absorbance Peak Ratio Differences	
	$(4/1)_b - (4/1)_f$	$(3/1)_b - (3/1)_f$
Buffer	0.00	0.00
Lecithin vesicles	0.05	0.05
Tetrahymanol membranes	0.31	0.32
Ergosterol membranes	0.88	1.32
Lecithin–ergosterol vesicles		
(1)	0.99	1.10
(2)	1.53	1.95

[a] The λ_{max} of filipin (358, 338, 323, 307, and 293 nm) are designated by peak numbers (1, 2, 3, 4, and 5, respectively). The absorbance peak ratio in the presence of the added agent is indicated by "b" (bound); "f" refers to the absorbance peak ratio of free filipin. Absorbances in the presence of vesicles and membranes were measured at λ_{max} of the absorption peaks. The concentrations of filipin and lipids are the same as in Figure 2. In the lecithin–ergosterol vesicles the molar ratio was 7:3 and the total lipid concentrations were 0.15 mM for (1) and 0.38 mM for (2).

Fluorescence Spectra. The effects of sterols incorporated into vesicles and membranes on the fluorescence properties of filipin are shown in Figure 4. In agreement with the absorption spectra, slight enhancements without significant alteration in peak ratios are observed in the presence of lecithin vesicles devoid of sterol and in the presence of lecithin–epicholesterol vesicles. Vesicles and ciliary membranes containing ergosterol and vesicles containing cholesterol caused quenching of the fluorescence intensity of filipin and large changes in the peak ratios (Figure 4). Binding of filipin to aqueous suspensions of cholesterol and epicholesterol resulted in similar changes (Bittman and Fischkoff, 1972). Ciliary membranes containing tetrahymanol caused only slight quenching (spectrum not shown) without alteration of the peak ratios of filipin, as expected from the absorption spectrum of the antibiotic in the presence of membranes isolated

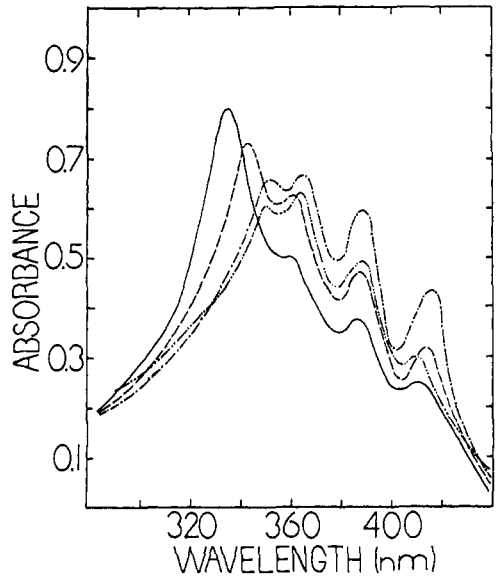

FIGURE 3: Absorption spectra of amphotericin (—), and of amphotericin in the presence of lecithin vesicles (---), lecithin–cholesterol vesicles (- · -), and an aqueous suspension of cholesterol (- · · -). The concentration of amphotericin was 31 μM. The total lipid concentration in the vesicles was 1.4 mM. The concentration of cholesterol in the aqueous suspension was 0.25 mM. The concentration of DMF in the aqueous suspension of cholesterol was 1.0% (v/v).

from unsupplemented cells. At a comparable ratio of lipid to filipin (*ca.* 10), the fluorescence polarization of filipin was 0.33 (for excitation at 325 nm, emission at 480 nm) in the presence of ergosterol-containing membranes and 0.02 in the presence of tetrahymanol-containing membranes. The latter value is equivalent to the fluorescence polarization of filipin in water, indicating that the emitted light from filipin in the presence of tetrahymanol-containing membranes is nearly completely depolarized. In contrast to their effects on the fluorescence properties of filipin (Figure 4), lecithin vesicles and vesicles containing cholesterol and ergosterol produced nearly identical enhancements in the fluorescence excitation and emission spectra of amphotericin (Figure 5). No significant increase in the fluorescence polarization of amphotericin was observed

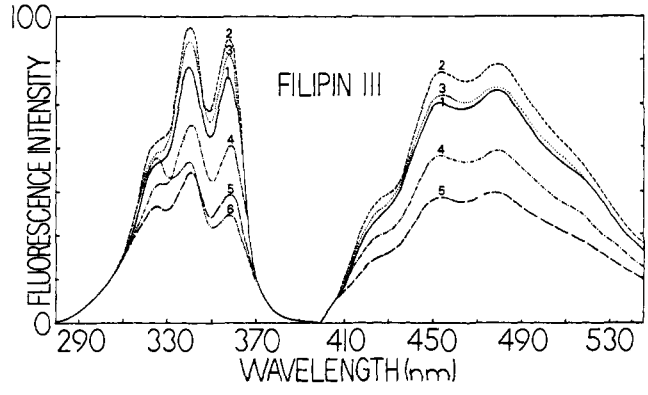

FIGURE 4: Fluorescence excitation (left) and emission (right) spectra of filipin, and of filipin in the presence of lecithin vesicles, lecithin–sterol vesicles at 7:3 molar ratio, and *Tetrahymena* ciliary membranes containing ergosterol. The wavelengths of excitation and emission were 357 and 480 nm, respectively. The concentration of filipin was 3.18 μM. The total lipid concentration in the vesicles was 85 μM. The spectra shown represent filipin in the presence of: **1**, buffer; **2**, lecithin vesicles; **3**, lecithin–epicholesterol vesicles; **4**, lecithin–ergosterol vesicles; **5**, lecithin–cholesterol vesicles; **6**, *Tetrahymena* ciliary membranes containing ergosterol.

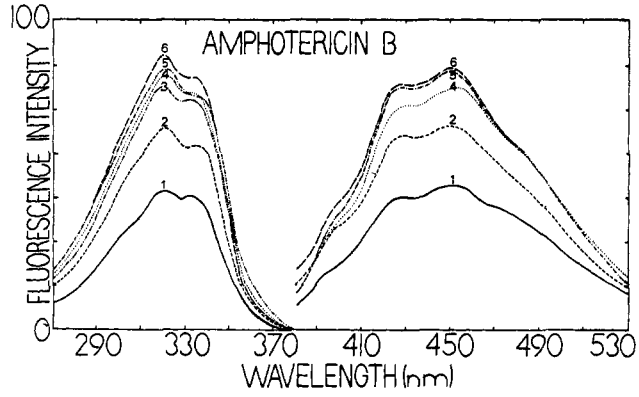

FIGURE 5: Fluorescence excitation (left) and emission (right) spectra of amphotericin, and of amphotericin in the presence of lecithin vesicles, lecithin–sterol vesicles at 7:3 molar ratio, and an aqueous suspension of cholesterol. The wavelengths of excitation and emission were 340 and 480 nm, respectively. The concentration of amphotericin was 8.35 μM (except in curve 3). The total lipid concentration in the vesicles was 0.17 mM. The spectra shown represent amphotericin in the presence of: **1**, buffer; **2**, lecithin—epicholesterol vesicles; **3**, a suspension of 0.25 mM cholesterol in buffer (the amphotericin concentration was 10.8 μM); **4**, lecithin–cholesterol vesicles; **5**, lecithin vesicles; **6**, lecithin–ergosterol vesicles.

in the presence of tetrahymanol- or ergosterol-containing membranes.

Circular Dichroism Spectra. The CD spectrum of filipin resembles the absorption spectrum. Figure 6 shows that when the medium in which the antibiotic is dissolved is changed from water to DMF or dioxane, the molecular ellipticities of the negative dichroic bands are increased dramatically. Figure 6 also shows that filipin undergoes similar increases in molecular ellipticities on binding to an aqueous suspension of cholesterol. Slight shifts toward longer wavelengths are evident.

Filipin appears to be self-associated in aqueous media. In the buffer used, marked deviation from Beer's law was observed in absorption and CD measurements at concentrations

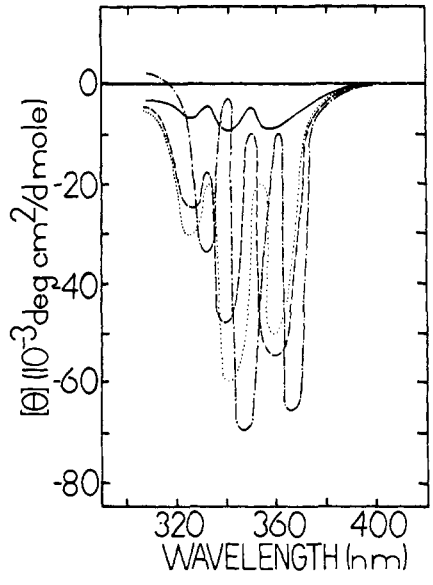

FIGURE 6: CD spectra of filipin in buffer (—), aqueous suspension of cholesterol (- · -), DMF (· · ·), and dioxane (---). The concentration of filipin was 18 μM. The concentration of cholesterol was 0.25 mM. The concentration of DMF in the aqueous suspension was 1.0% (v/v).

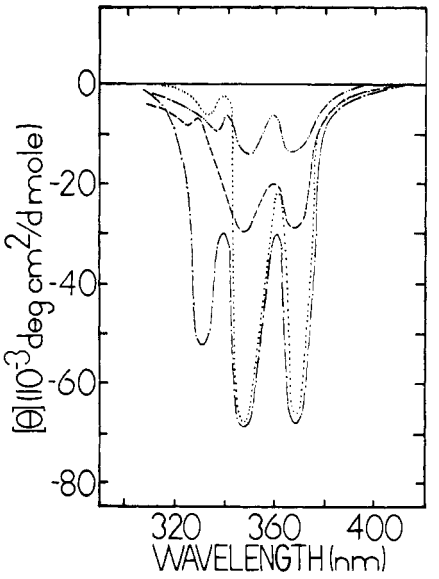

FIGURE 7: CD spectra of filipin in the presence of lecithin–sterol vesicles. The concentration of filipin was 21.9 μM. The molar ratio of lecithin to sterol was 7:3, and the total lipid concentration was 2.5 mM. The sterols used were: (- · ·) thiocholesterol, (---) epicholesterol, (· · ·) ergosterol, and (- · -) cholesterol.

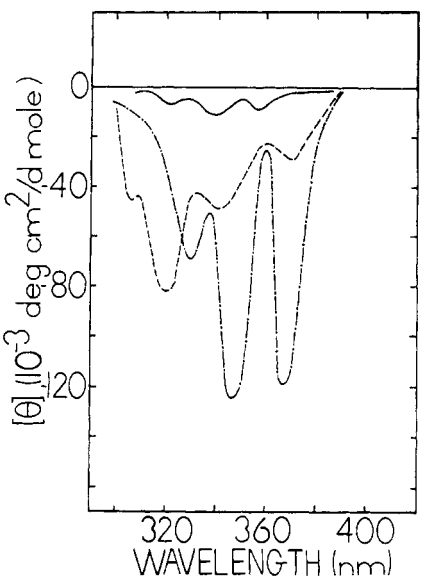

FIGURE 8: CD spectra of filipin (—), and of filipin in the presence of *Tetrahymena* ciliary membranes containing tetrahymanol (---) and ergosterol (- · -). The concentrations were the same as in Figure 1.

higher than about 10–15 μM. The observation that filipin is sedimentable at low speeds in the centrifuge (Lampen *et al.*, 1960) provides additional support for self-association of polyene antibiotics in aqueous media; furthermore, stacked macrolide rings characterize the crystal state of the *N*-iodoacetyl derivative of amphotericin (Ganis *et al.*, 1971). The observed weak rotational strength of filipin in buffer despite the existence of many optically active centers in the vicinity of the chromophore could arise because of (1) interaction between filipin molecules in the aggregate in a manner leading to a partial cancellation of contributions to the CD from chromophores in proximity to each other or (2) a change in the conformation of the macrocyclic ring on exposure to organic solvents such as DMF and dioxane or to aqueous suspensions of cholesterol. These two possibilities could be distinguished experimentally by measuring the molecular ellipticity of filipin in a series of mixed micelles (or aggregates) composed of varying concentrations of filipin and perhydrofilipin. Under the assumption that the conformation of the filipin macrolide ring is the same in the pure filipin micelle as in the mixed filipin–perhydrofilipin micelle, an increase in the molecular ellipticity of filipin upon dilution with perhydrofilipin would suggest that self-association of filipin molecules in water gives rise to the diminished ellipticity shown in Figure 6. On the other hand, if the molecular ellipticity were to remain unchanged upon dilution with perhydrofilipin in the mixed micelle, a medium-induced conformational change could be a plausible explanation for the increase in molecular ellipticity observed in the presence of DMF, dioxane, and aqueous suspensions of cholesterol. Aqueous solutions of filipin with added perhydrofilipin were prepared at ratios of perhydrofilipin to filipin of 4, 2, 0.75, and 0, and the CD spectrum of each solution was recorded. It was found that the molecular ellipticity of filipin was unaffected by dilution with perhydrofilipin (which has no CD spectrum in the region shown in Figure 6), *i.e.*, $[\theta]_{338}$ remained constant. This suggests that cholesterol and organic solvents such as DMF and dioxane induce a conformational change in the macrolide ring.

In the presence of lecithin–cholesterol and lecithin–ergosterol vesicles, the negative dichroic bands of filipin are enhanced dramatically, whereas much smaller enhancements are observed in the presence of lecithin–thiocholesterol and lecithin–epicholesterol vesicles (Figure 7). The CD spectra of filipin in the presence of lecithin vesicles and lecithin–cholestanol vesicles (not shown) resembled the spectrum shown for filipin in the presence of lecithin–epicholesterol vesicles. Figure 8 shows that the CD spectrum of filipin is very strongly enhanced in the presence of ergosterol-containing membranes, whereas smaller enhancement is observed in the presence of tetrahymanol-containing membranes.

Electron Microscopy. Electron micrographs of amphotericin-treated vesicles, filipin-treated vesicles, and untreated vesicles were examined to determine whether the polyenes cause structural changes that depend on the type of sterol present. The effects of filipin treatment on vesicle structure are described first and then changes in vesicle structure and size produced by amphotericin treatment are presented.

Filipin-treated vesicles prepared from lecithin alone or from a mixture of lecithin and cholestanol, thiocholesterol, androstan-3β-ol, or epicholesterol looked essentially like the untreated vesicles (Figure 9), *i.e.*, no filipin-induced effect was observed. Vesicles prepared from lecithin and cholesterol or ergosterol were lysed by filipin treatment. Figure 10 shows lecithin–ergosterol vesicles lysed by filipin. The treated vesicles exhibited various stages of disruption characterized by a budding outward of vesicle boundary layers and a collapse of the vesicles to form a thin film perforated with "pits" containing an electron-dense deposit of aqueous negative stain. The fine organization of the lipid film and the electron-dense "pits" are shown in Figure 10. The electron-dense center of each pit is surrounded by an electron-translucent annulus. The mean inner diameter of the annulus is 200 ± 15 Å and the mean outer diameter is 340 ± 17 Å.

It is not possible to directly observe the lytic events with the electron microscope since the specimen is fixed or "frozen" within a glass-like coating of negative stain when observed. However, by examining a succession of images obtained for vesicles in various stages of lysis, one can deduce a probable sequence of events. It appears (Figure 10) that filipin-

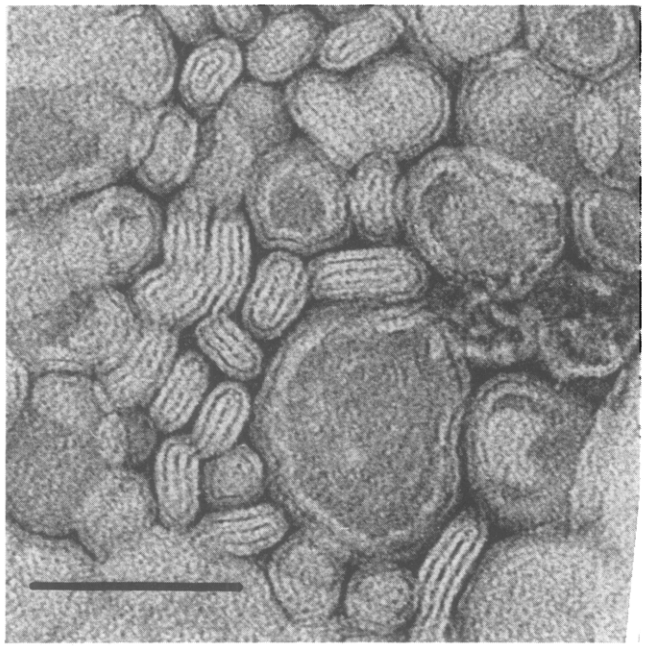

FIGURE 9: Untreated lecithin–ergosterol vesicles. Total lipid conte it in the vesicles was 1 mM. Lipid bilayers appear as white bar ls surrounded by black-stained aqueous spaces. This preparation ε id those shown in Figures 10–12 were stained with 2% (w/v) ε n-monium molybdate, pH 6.5. Marker = 100 nm.

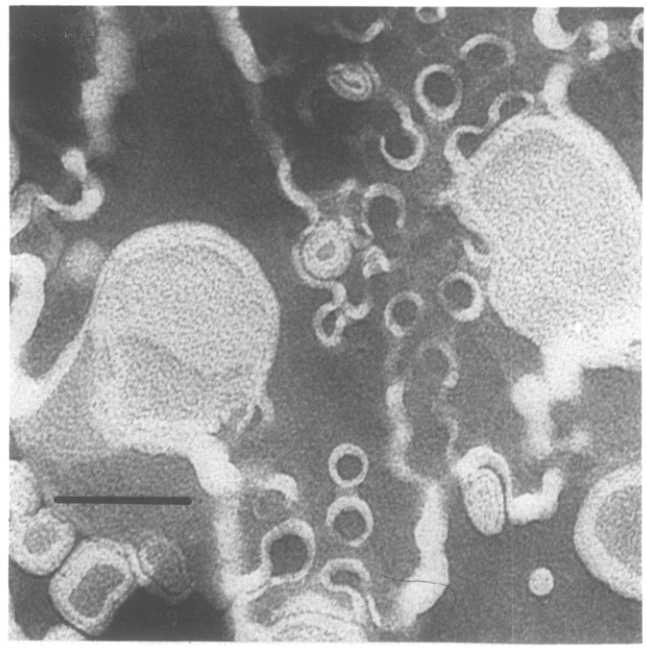

FIGURE 10: Lecithin–ergosterol vesicles treated with filipin at a final concentration of 12.7 μM. The total lipid content in the vesicles was 1 mM. The orderly array of lipid bilayers as presented in Figure 9 has been destroyed and annular hydrophobic bodies, "pits," appear within the thin film produced by lysis of the vesicle. Each annulus contains an electron dense core. Marker = 100 nm.

induced lysis first causes the outer lipid bilayer at the peripl ery of the vesicles to become inflated, resulting in bud-like ex rusions into the surrounding medium. This process continu s as the concentric boundary lipid bilayers begin to dissolve ir o a homogeneous electron-dense film that spreads outward om the lysing vesicles. We propose that the pit annulus is fo med by a loop-like protrusion from the vesicle bilayer, wh h is gradually constricted at the point of origin from the v sicle. The adjacent bilayer segments in the constricted region f the loop fuse through a process of micelle-mediated coale ence to produce a closed ring suspended within the lipid r sidue thus forming a lysed vesicle. Lucy (1969) has propose l that surface-active agents facilitate fusion of adjacent mer brane lipid bilayers through the production of micelles in he bilayers. These micellar regions in apposed bilayer se gments interdigitate, thereby producing a coalesced membra ie segment. The micellar regions shown in Figure 11 lo k convincingly like the regions of fused membrane segme its presented in Lucy's diagram. Our studies give further su port to the possibility that surface-active agents induce f sion of lipid bilayer regions of natural membranes by a micelle-mediated process.

Amphotericin-treated vesicles do not exhibit lys s as observed with filipin treatment. We have observed two effects of amphotericin treatment on vesicle structure: (1) th e peripheral concentric lipid bilayers of all of the treate l vesicles are disorganized to form a homogeneous electron-t anslucent layer surrounding an inner electron-dense amorp ous core, and (2) vesicle swelling was observed when vesicle prepared from lecithin alone, or lecithin and cholesterol or ergosterol were treated with amphotericin. Each of these eff cts is discussed below.

The homogeneous lipid boundary layer at the su face of the amphotericin-treated vesicles prepared from le ithin–ergosterol is shown in Figure 12. The observed change in lecithin–cholesterol vesicles induced by amphotericin tr atment are almost identical with those obtained with lecithin–ergosterol vesicles.

Measurements of vesicle diameter were taken to determine if the amphotericin treatment caused vesicle enlargement. The control vesicles are sometimes prolate in shape and contain a peripheral boundary of several concentric bilayers surrounding an internal space. We therefore decided to measure the major axis diameter and minor axis diameter of each

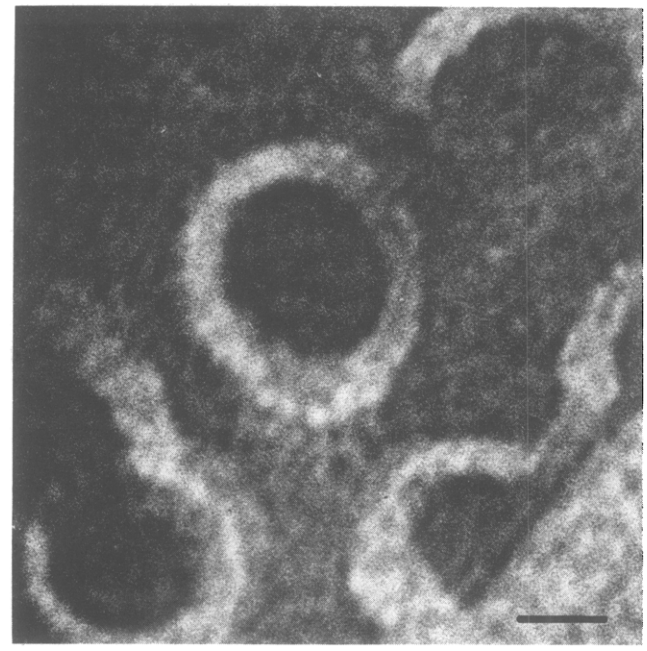

FIGURE 11: An enlarged view of an annulus from a filipin-treated lecithin–ergosterol vesicle. Spherical micelle-like bodies occur within the hydrophobic ring of the annulus at one side which appears to be a point where apposed lipid bilayers fused to form the closed annulus. Marker = 10 nm.

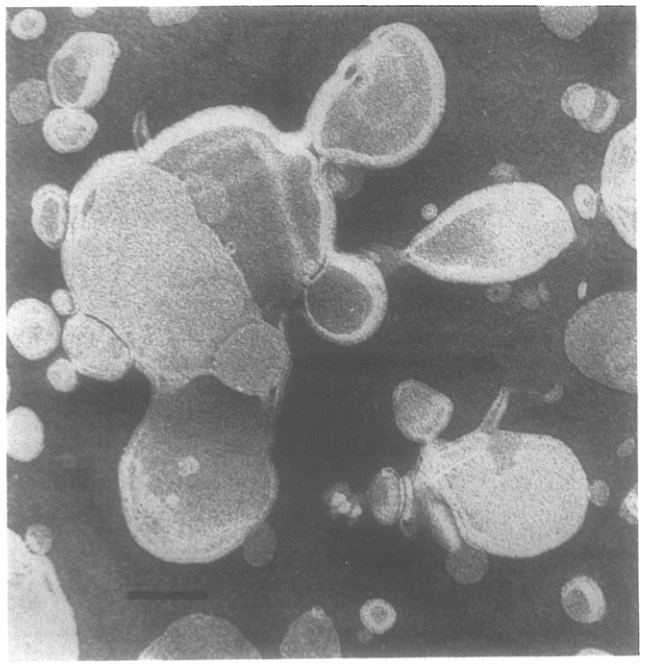

FIGURE 12: Lecithin–ergosterol vesicles treated with amphotericin at a final concentration of 17.7 μM. The total lipid content in the vesicles was 1 mM. The vesicles are swollen and contain an internal amorphous space. The orderly arrangement of the stratified vesicle boundary bilayers has been disrupted to form a nonstratified lipoidal envelope. Marker = 100 nm.

vesicle. The outer diameter and the inner core diameter along each axis was also measured. A t test was performed to determine if statistically significant differences in mean diameters existed when amphotericin-treated vesicles were compared to their controls. A summary of changes in vesicle diameters induced by amphotericin treatment is presented in Table II. Statistically significant increases ($p < 0.05$) in mean outside and inside diameters were found when vesicles prepared from lecithin alone and from a mixture of lecithin and cholesterol or ergosterol were treated with amphotericin. No statistically significant change in mean outer or inner diameters was observed when vesicles prepared from a mixture of lecithin and epicholesterol were treated with amphotericin. Amphotericin-treated lecithin–thiocholesterol vesicles showed no statistically significant increase in inner or outer mean major diameters. However, there was a statistically significant increase in mean outer and inner minor diameters. The vesicles in the control preparation were prolate. The increase in mean minor diameters is apparently a result of swelling induced by amphotericin treatment yielding a more spherical body. Although there was no change in major diameter, the spherical swelling results in an increase in minor diameter.

A test of minimum filipin concentration sufficient to induce lysis of lecithin–cholesterol vesicles showed that filipin concentrations of 3.4 μM or greater produced extensive lysis resulting in formation of thin films containing electron-dense, pit-like regions. At filipin concentrations of 1.9 μM, most vesicles were swollen, but few showed signs of complete disruption. Flilipin concentrations below 1.9 μM produced little or no effect on vesicle morphology and gave vesicles that appeared essentially like those of the untreated sample. Although a thorough examination of minimum amphotericin concentration sufficient to induce swelling in lecithin–cholesterol vesicles was not performed, we found that concentrations as low as 26.8 μM induced swelling. The range of amphotericin concentrations used in this study was 26.8–268 μM.

A preparation of ergosterol-containing ciliary membranes that is negatively stained with phosphotungstic acid is shown in Figure 13. Following filipin treatment, membrane lysis and formation of electron-dense pits containing aqueous negative stain resulted (Figure 14). The pits are very similar to those observed in lecithin–ergosterol vesicles treated with filipin (Figure 10). The mean diameter of the pits in the lysed vesicles is almost identical with that in the *Tetrahymena* ciliary membrane. The mean inner diameter of pits in the ergosterol-containing ciliary membranes was 177 ± 32 Å and the mean outer diameter was 293 ± 32 Å. Ciliary membranes containing tetrahymanol were not affected by amphotericin or filipin. The treated membranes looked essentially like the control membranes. We could not detect differences in structure between untreated tetrahymanol-containing membrane fragments and those containing ergosterol. The tetrahymanol-containing membranes looked like the ergosterol-containing membranes as presented in Figure 13.

Discussion

Changes in the ratios of extinction coefficients (Figure 2), fluorescence intensity (Figure 3), and molecular ellipticity

TABLE II: Mean Diameters of Amphotericin-Treated and Untreated Vesicles.[a]

	Lecithin Alone		Lecithin–Cholesterol		Lecithin–Ergosterol		Lecithin–Thiocholesterol		Lecithin–Epicholesterol	
Major axis o.d.	75	91*	105	180*	81	137*	124	122	95	100
Major axis i.d.	62	71*	86	156*	68	111*	88	95	85	74
Minor axis o.d.	48	67*	73	148*	52	97*	79	104*	66	73
Minor axis i.d.	38	49*	56	129*	41	78*	45	73*	59	49

[a] Diameters are reported in nanometers. The first number in each pair is for the untreated vesicles and the second number is for the amphotericin-treated vesicles. An asterisk indicates a statistically significant mean diameter increase ($p < 0.05$). For each entry the data represent measurements obtained from at least two separate vesicle preparations. The molar ratio of total lipid to amphotericin was varied from 60 to 90. Preparation of vesicles and time of incubation with polyene were as described in the Experimental Section. Although there are apparent differences in mean diameters of vesicles among the untreated vesicles it is not advisable to assume that these differences are due to the composition of the vesicles. Since the vesicles were prepared at different times, differences in size could be attributed to differences in preparation technique such as sonication. A detailed study of lipid composition effects on vesicle size should be undertaken before conclusions concerning the differences in diameters of untreated vesicles are reached.

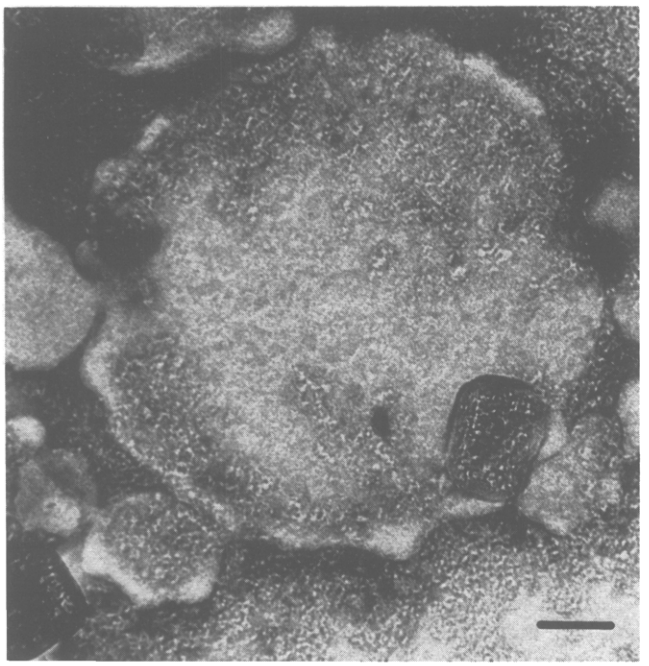

FIGURE 13: *Tetrahymena* ciliary membrane fragment containing ergosterol. This preparation and the one shown in Figure 14 were stained with 2% (w/v) aqueous potassium phosphotungstate, pH 6.5. Marker = 100 nm.

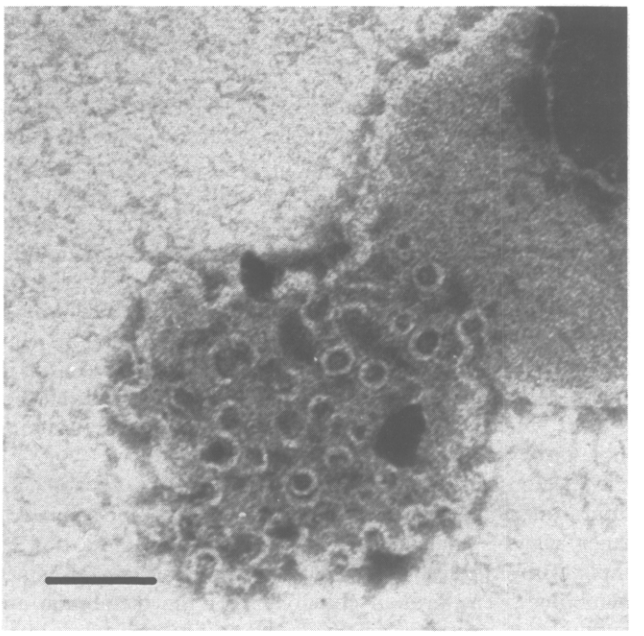

FIGURE 14: *Tetrahymena* ciliary membrane fragment containing ergosterol treated with filipin at a final concentration of 14.9 μM. The annuli formed in the disrupted membrane fragment look remarkably like those observed in filipin-treated lecithin–ergosterol vesicles (Figure 10). Marker = 100 nm.

(Figure 7) of filipin on binding to lecithin–sterol vesicles and ciliary membranes depend on the type of sterol incorporated into the vesicles and membranes. The same dependence was observed in electron microscope studies of the appearance of morphological changes induced in the sterol-containing vesicles on filipin treatment. These results are consistent with fluorescence polarization studies that showed that filipin did not interact significantly with lecithin vesicles containing epicholesterol, androstan-3β-ol, or thiocholesterol (Bittman and Fischkoff, 1972), and with ultraviolet absorption studies that showed that filipin did not interact with intact cell suspensions of *A. laidlawii* containing membrane-bound epicholesterol (Norman *et al.*, 1972b). These findings are also consistent with the observations that filipin increased the surface pressure of lecithin–cholesterol and lecithin–stigmasterol monolayers, but not of lecithin–epicholesterol monolayers (Norman *et al.*, 1972b). Under the same conditions of filipin treatment, lecithin–ergosterol monolayers exhibited only a significant pressure increase at an initial surface pressure of 14 dyn/cm. Mixed lipid monolayers were not prepared containing androstan-3β-ol. However, pure monolayers of androstan-3β-ol showed little increase in surface pressure when treated with filipin. This was particularly true at initial surface pressures of 15–25 dyn/cm. There is a clear correlation between the findings of (a) the monolayer and fluorescence polarization experiments and (b) the results of the vesicle studies reported in this study. Those sterols found to give increases in monolayer surface pressure with filipin treatment and enhancements in the polarized fluorescence intensity of the antibiotic are also those that make lecithin–sterol vesicles susceptible to filipin disruption. On the other hand, those sterols that do not interact with filipin to produce increases in surface pressure of monolayers or marked fluorescence polarization enhancement do not produce filipin sensitivity when incorporated in lecithin–sterol vesicles.

Many studies have shown that lecithin–sterol interactions are strong when the sterol has a planar nuclear ring structure, a cholestane-type side chain at C-17, and a 3β-hydroxyl group (Butler *et al.*, 1970; Demel *et al.*, 1972a,b; Bittman and Blau, 1972). The first two features presumably lead to maximal hydrophobic bonding with the paraffin chains of the phospholipids, and the presence of an equatorially oriented 3-hydroxyl group apparently permits formation of stable hydrogen bonding or dipole–dipole interactions with acceptor groups such as the negatively charged lecithin phosphate oxygen atom. Since significant morphological changes in vesicle structure and spectral changes in filipin were observed following interaction of the antibiotic with lecithin–ergosterol and lecithin–cholesterol vesicles, but not with vesicles prepared from lecithin, lecithin–epicholesterol, lecithin–thiocholesterol, and lecithin–androstan-3β-ol, it appears that filipin binding reflects the relative strengths of lecithin–sterol interactions. The results obtained with lecithin–cholestanol vesicles are inconsistent, however, with reports suggesting that cholestanol does participate in strong phospholipid–sterol interactions (Clayton and Bloch, 1963; Butler *et al.*, 1970; Bittman and Fischkoff, 1972; Demel *et al.*, 1972a,b; De Kruyff *et al.*, 1973). We found that filipin did not cause lysis of lecithin–cholestanol vesicles at polyene concentrations that caused lysis of cholesterol- and ergosterol-containing vesicles. Furthermore, the molecular ellipticities of filipin did not increase markedly in the presence of lecithin–cholestanol vesicles.

The observations from ultraviolet absorption (Figure 3) and fluorescence spectra (Figure 5) and from electron microscopy (Table II) that interaction of amphotericin with lecithin and lecithin–sterol vesicles lacks the selectivity exhibited by filipin agree with the findings that (a) amphotericin undergoes a relatively constant enhancement in fluorescence polarization in the presence of various lecithin–sterol vesicles (Bittman and Fischkoff, 1972), and (b) at low concentrations of amphotericin the polyene undergoes alteration in extinction coefficients in the presence of membranes of *A. laidlawii* lacking cholesterol, as well as with those containing cholesterol (Norman *et al.*, 1972a). Other reports, however, have indicated that

amphotericin affects only cholesterol-containing membranes (Lippe, 1968; Andreoli and Monahan, 1968; Finkelstein and Cass, 1968; Dennis *et al.*, 1970; Cass *et al.*, 1970; Rottem *et al.*, 1971). It should be noted, however, that at the concentration of amphotericin used for the studies reported in Table II, the swelling of lecithin vesicles is less than that of lecithin–cholesterol or lecithin–ergosterol vesicles. A distinction between the capacity of amphotericin to affect lecithin vesicles compared to its capacity to affect lecithin–cholesterol and lecithin–ergosterol vesicles may become even more apparent with the use of lower antibiotic concentrations or higher lipid concentrations. In fact, at higher lipid concentrations (but comparable amphotericin concentration) than those used in the present study, Weissmann and Sessa (1967) reported that lecithin vesicles did not exhibit swelling, whereas lecithin–cholesterol vesicles did.

The good correlation between the behavior of filipin in natural membranes and lipid bilayer vesicles as reported in this paper suggests that the matrix provided for interaction of filipin with ergosterol may be similar in vesicles and ciliary membranes. The protein component of the membrane apparently does not interfere with the ability of ergosterol to interact with filipin. The results reported here thus lend further credence to the validity of using phospholipid bilayer vesicles as models of the phospholipid bilayer region of cellular membranes.

The failure to observe appreciable interaction of filipin with tetrahymanol-containing membranes by both spectral and electron microscopic techniques is consistent with the report that unsupplemented *Tetrahymena* cells are 20 times more resistant to lysis by filipin than ergosterol-containing cells within 2–3 min of exposure to a given concentration of the antibiotic (Conner *et al.*, 1971). Two possible explanations can be advanced for the lack of appreciable interaction of filipin with tetrahymanol-containing membranes under the conditions used. First, filipin may be incapable of interacting with tetrahymanol because of structural dissimilarities between sterols (the "normal" receptor molecules for polyene antibiotics) and the triterpenoid alcohol. Second, the arrangement of tetrahymanol in the membrane may preclude filipin–tetrahymanol interaction. Evidence has been gathered which supports the second explanation. Spectral data obtained in this laboratory show that filipin interacts with vesicles prepared from a mixture of tetrahymanol and the total phospholipids isolated from the cilia of unsupplemented cells. Since no interaction was observed in tetrahymanol-containing membranes, we infer that the packing of tetrahymanol with phospholipid molecules in the vesicle is different from that in the ciliary membrane. The reasons for this are not understood. Since filipin does not interact appreciably with tetrahymanol-containing ciliary membranes but does interact with ergosterol-containing membranes, it would be interesting to know whether the arrangement of the two alcohols in their respective membranes could be sufficiently different to account for the observed difference in filipin-binding capacity. Corey–Pauling–Koltun molecular models indicate that tetrahymanol and ergosterol have a generally similar rod-like shape, but tetrahymanol is about 2 Å thicker than ergosterol at the C/D ring juncture and about 4.5 Å shorter. The *gem*-dimethyl groups at C-4 of tetrahymanol may inhibit the hydrogen-bonding capacity or modify the dipole–dipole bonding properties of tetrahymanol relative to ergosterol. The methyl groups at the 4 and 14 positions and cyclization of the side chain to form the fifth ring may result in important structural differences. Such differences may cause the interaction of tetrahymanol with other membrane components in the *Tetrahymena* ciliary membrane to be different from that of ergosterol. It is therefore not unreasonable to assume that the accessibility of the two alcohols to filipin may be different in the membranes obtained from the two cell types. Moreover, ergosterol supplementation leads to alterations in phospholipid fatty acid composition (Ferguson *et al.*, 1971) which may also contribute to possible differences in the arrangement of the alcohols in their membranes. Therefore, the existence of differences in filipin-binding capacity in membranes from two different cell types of *Tetrahymena pyriformis* W as revealed by spectral and electron microscopic studies suggests that filipin may serve as an indirect probe of phospholipid–sterol interactions in ciliary membranes.

Our electron micrographs of filipin-induced pit formation in ergosterol-containing lecithin vesicles are very similar to those obtained for cholesterol-containing lecithin vesicles (Kinsky *et al.*, 1967). Moreover, the morphology of pits observed in ergosterol-containing *Tetrahymena* ciliary membranes is remarkably similar if not identical with those observed in human erythrocytes. It should be acknowledged, however, that Kinsky *et al.* (1967) found considerable variation in pit size produced by filipin treatment depending on the source of the erythrocytes.

The molecular organization of pits in filipin-lysed lecithin vesicles containing cholesterol or ergosterol remains unsolved. Verkleij *et al.* (1973) used freeze-etching techniques to examine natural membranes and lipid vesicles treated with filipin. They concluded from their observations that the pits observed with negative-stained preparations are clumps of lipid within the hydrophobic layer of the membrane and that no evidence for a continuous pore through the "pit" was observed. Thus filipin-induced membrane lysis may be due largely to disruption of the lipid layer and production of the micellar aggregates ("pits") within the hydrophobic region rather than formation of pores through the membrane. Although further research is needed to clarify the molecular organization of filipin-induced pits in membranes, their presence has been clearly observed by several researchers using cholesterol-containing membranes and in this study using ergosterol-containing membranes.

The sequential events during pit formation in lipid vesicles cannot be determined precisely using negative staining methods with the electron microscope. However, we have varied the concentration of filipin applied to the vesicles and observed preparations in various stages of lysis as a means of constructing an explanation for the sequence of events during vesicle lysis. An apparently early event and one observed with low concentrations of filipin (1.9–3.4 μM) is swelling of the vesicle followed by a budding outward of the peripheral bilayer of the vesicle. The process of "budding" could be explained by an increase in surface pressure caused by penetration of filipin into the lipid bilayer. Demel *et al.* (1965, 1968) have shown that lecithin–cholesterol monolayers exhibit an increase in surface pressure when filipin is injected beneath them; moreover, filipin caused cholesterol monolayers held at constant surface pressure to expand in area. Since it is not possible to determine the molecular composition of the bud-like protrusion by electron microscopy, we do not know whether it contains a ternary mixture of lecithin, sterol, and filipin or represents a filipin–sterol complex in which the sterol has been removed from its interaction with lecithin as suggested by Kinsky *et al.* (1967), Norman *et al.* (1972b), and Verkleij *et al.* (1973).

The outward budding of the lipid vesicle peripheral layers continues until all or much of the vesicle is lysed, producing a thin lipid film containing internal annular pits. The origin of the pits remains undetermined. The formation of micellar-like lipoidal aggregates may represent the mechanism whereby fusion of the adjacent bilayers in the bud-like protrusions is accomplished at the point of separation from the vesicle. Repeated formation of these annular elements could account for the scattered pits observed within the lysed remains of the vesicle.

Amphotericin-treated vesicles were not lysed, but exhibited varying degrees of swelling and distortion. When vesicles prepared from lecithin alone, lecithin and cholesterol, lecithin and ergosterol, or lecithin and thiocholesterol were treated with amphotericin, a statistically significant increase in diameter compared to their untreated controls was observed. In confirmation of the spectral results (Figures 3 and 5), electron microscopy shows that amphotericin is not as specific as filipin in its interaction with vesicle lipid components. Whereas filipin-induced vesicle lysis occurred only with ergosterol- or cholesterol-containing vesicles among the various kinds we studied, amphotericin induced statistically significant changes in vesicle swelling in four of the five vesicle types as reported in Table II. The mechanism of amphotericin-induced swelling of vesicles and the change in bilayer organization at the vesicle periphery has not been determined. From our electron microscope examination, it appears that several of the bilayers at the periphery are disorganized to form a nonstratified envelope surrounding the swollen vesicle. The large central amorphous space observed in our electron micrographs of the swollen vesicles resulting from amphotericin treatment looks very much like that reported for amphotericin-treated vesicles of lecithin and cholesterol reported by Weissmann and Sessa (1967).

Acknowledgment

We thank Dr. Edna Kaneshiro and Professor Robert L. Conner for providing preparations of ciliary membranes from *Tetrahymena pyriformis* W.

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