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INTERACTION OF POLYENE ANTIBIOTICS WITH MODEL AND NATURAL MEMBRANES

by

WINSTON CHUNG HSIN CHEN

A dissertation submitted to the Graduate Faculty in Biochmistry in partial fulfillment of the requirement for the degree of Doctor of Philosophy, The City University of New York

1974

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

May 9, 1974
Date

Robert Bittman
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May 20, 1974
Date

Aaron Lukton
Executive Officer

Supervisory Committee

The City University of New York

This work is in memory of my late wife, Debrah Shan-Yuann, and is lovingly dedicated to my parents, and my wife, Mi-Hong.

ABSTRACT

The polyene antibiotics filipin and amphotericin B have been shown previously to increase the permeability of natural and artificial membranes. In this dissertation the effects of these two polyene antibiotics on egg lecithin-sterol vesicles and ciliary membranes from *Tetrahymena pyriformis* W were investigated using ultraviolet absorption, fluorescence, circular dichroism, and nuclear magnetic resonance spectroscopy, and negative-staining electron microscopy. Equilibrium studies of filipin binding to sterols were made. The aggregation of the polyene antibiotic amphotericin B in aqueous media was investigated. Filipin was used as a fluorescence probe of the transition temperatures (T_c) of dipalmitoyllecithin, dimyristoyllecithin and sphingomyelin in vesicles.

Filipin underwent changes in the absorbance and fluorescence excitation peak ratios and enhancement in molecular ellipticities in the presence of lecithin-cholesterol, lecithin-ergosterol vesicles, ergosterol-containing ciliary membranes, and vesicles derived from ciliary phospholipids containing tetrahymanol and ergosterol. No marked spectral changes were observed in the presence of tetrahymanol-containing ciliary membranes and vesicles prepared from lecithin alone and from mixtures of lecithin and epicholesterol, thiocholesterol, androstan-3α-ol, androstan-3β-ol, androstan-17β-ol, or

cholestanol. The spectra and electron microscopic results suggest that filipin may be used as an indirect probe of the relative strengths of the molecular interactions between lecithin and the sterols.

Formation of "pits" was observed in filipin-treated lecithin-ergosterol vesicles, lecithin-cholesterol vesicles and ergosterol-containing ciliary membranes. 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 in biological membranes and tend to support the validity of using lipid bilayer vesicles as models of cellular membranes.

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 and amphotericin B under the conditions used.

Dissociation constants (K_D) of the binding of filipin to sterols in aqueous dispersion with egg lecithin were obtained by analyzing the absorbance and fluorescence data. The K_D value at 25° for the binding of filipin III to lecithin-sterol vesicles were: epicholesterol, 13 µM; ergosterol, 2.47 µM; and cholesterol, 0.80 µM. Despite the low intrinsic affinity of filipin III for lecithin (K_D=144 µM), at high

lecithin concentrations the polyene binds to vesicles prepared from lecithin alone.

The concentration dependence of the molecular ellipticities of amphotericin B revealed that self-aggregation in aqueous media was pronounced. The aggregates are disrupted by increasing the content of organic solvent, or simply by increasing the temperature. Hydrophobic forces are probably involved in the aggergation of amphotericin B in aqueous media.

The effects of filipin on lecithin-cholesterol vesicles were also investigated by nmr studies. The mobility of the hydrocarbon chain of lecithin was increased in the presence of filipin. The fluorescence polarization of filipin was decreased with increasing temperature in the presence of vesicles prepared from dipalmitoyllecithin, dimyristoyl-lecithin or sphingomyelin.

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INTRODUCTION

Since the discovery of the first polyene antibiotic in 1950 (Hazen and Brown, 1950), approximately 40 polyene antibiotics have been described in the literature. The antibiotics that have been studied most extensively are filipin, nystatin and amphotericin B. These antibiotics have attracted the attention of numerous investigators because of their toxicity to fungi and their lack of effect on bacteria.

Two polyene antibiotics, filipin and amphotericin B, are chosen for study in this dissertation because their specificity toward cholesterol in fungal membranes and their chemical structures have been established.

Filipin was isolated from *Streptomyces filipinensis*, an actinomycete found in a sample of Philippine soil (Ammann *et al*., 1955; Whitfield *et al*., 1955). The isolated antibiotic is a mixture of at least 8 components, all of which possess the typical filipin pentaene chromophore (Bergy and Eble, 1968). A mixture of these components is called the filipin complex. Components II, III, and IV (identified by their differing R_f values on thin-layer chromatography) comprise 96% of the filipin complex. The other five were isolated as a crystalline mixture that

is called filipin I complex. With regard to antifungal activity of these pentaene components, filipins II and III are the most active. The study of interaction of filipin complex and subfractions with lipid monolayers (Kinsky, 1970a) indicated that filipin III increased the surface pressure of cholesterol monolayers to a greater extent than did filipins II and IV. The biological activity of the filipin complex toward Candida albicans abbott is a reflection of the amounts of filipins II (25%) and III (53%) which it contains (Bergy and Eble, 1968). The order of hemolytic activity of filipin components was: filipin II $\cong$ filipin III $\gg$ filipin I $>$ filipin IV, and this order paralleled their biological action (Sessa and Weissmann, 1968).

The structure of filipin is shown in Figure 1. It is characterized by a macrolide ring containing 5 conjugated double bonds. In addition to the pentaene chromophore, filipin contains a lactone group and many hydroxyl groups (Berkoz and Djerassi, 1959; Dhar et al., 1964; Cedar and Ryhage, 1964). Recently, mass spectrometric measurements indicated that filipin I has two hydroxyl groups fewer than filipin III and filipin II has one hydroxyl fewer than filipin III (Pandey and Rinehart, 1970). Filipin IV is apparently stereoisomeric with filipin III (Pandey et al., 1972). Mass spectral analysis of filipins I, II, III and IV indicated that their molecular formulas are $C_{35}H_{58}O_9$, $C_{35}H_{58}O_{10}$, $C_{35}H_{58}O_{11}$ and $C_{35}H_{58}O_{11}$, respectively.

Amphotericin B was isolated from Streptomyces Nodosus (Vandeputte et al., 1956). The chemical structure of this antibiotic (see Figure 1-a) has been investigated since 1957 (Dutcher et al., 1957; Borowski et al., 1963; Cope et al., 1966). The complete structure of the N-iodoacetyl derivative of amphotericin B was determined by X-ray single crystal analysis (Mechlinski et al., 1970; Ganis et al., 1971). Amphotericin B is a macrolide polyene antibiotic with two ionizable groups. One is a carboxyl group, the other is an aminoglucosidic group. Both are linked to the macrolide ring. The 7 conjugated double bonds are in the trans configuration and form a narrow hydrophobic channel (Ganis et al., 1971). The amino sugar moiety, mycosamine, is found in a β-glycoside linkage to the C-19 hydroxyl

Figure 1-a: Structures of filipin (top), and amphotericin B (bottom, R = H).

group, and is present in a pyranoside conformation. The carboxyl group on C-16 and the amino sugar moiety form the most hydrophilic parts of the N-iodoacetylamphotericin B molecule. The other features of the molecule are the polyhydroxyl groups and a ketalic six-membered ring which are included in the macrolactone ring. Most hydroxyl groups are "axial" with respect to the mean plane of the lactone ring. X-Ray analysis also indicated the existence of intermolecular hydrogen bonding in the crystal state. These structural features of the antibiotic are believed to bear an important role in the mode of action of amphotericin B.

It is believed that the biological activity of the polyene antibiotics results from their ability to alter the permeability of membranes of microorganisms containing sterols (Gottlieb et al., 1961; Lampen and Arnow, 1961; Weber and Kinsky, 1965). These polyene antibiotics lyse erythrocytes (Kinsky et al., 1967b) and decrease DNA synthesis and cellular respiration in Ehrlich ascites and Novikoff hepatoma tumor cells (Mondori et al., 1971). Amphotericin B and filipin have medical use for treatment of hypercholesterolemia in animals (Schaffner and Gordon, 1968; Fisher et al., 1969), and for reducing canine prostatic hyperplasia (Gordon and Schaffner, 1968). At low concentrations, filipin had no significant effect on mitochondria isolated from fungi (Gottlieb et al., 1961;

Kinsky et al., 1965), but at higher concentration filipin was bound extensively to mitochondria and inhibited mitochondrial respiration (Balcavage et al., 1968) and ruptured lysozymes (Hakar and Peron, 1972).

Filipin and amphotericin B were found to have the ability to alter the permeability of model membranes (Weissmann and Sessa, 1967; Sessa and Weissmann, 1968; Andreoli et al., 1969; Kinsky, 1970b; Van Zutphen et al., 1971; HsuChen and Feingold, 1973). A report by Bittman and Blau (1972) indicated that filipin III did not alter the kinetics of water permeability of lecithin-cholesterol liposomes at a cholesterol to filipin III molar ratio of 16, but significant alterations were seen at ratios of less than 8 (Bittman, Honig and Majuk, unpublished data). It was reported that both amphotericin B and nystatin, at concentrations of 10^{-5} to 10^{-6} M, alter the glucose permeability of liposomes prepared in the absence of sterol (HsuChen and Feingold, 1973).

The polyenes differ markedly in the degree of membrane damage which they induce. The degree of membrane alteration may be correlated with the binding affinity of the polyenes to cholesterol (Bittman and Fischkoff, 1972). The effects of polyene antibiotics on lipid monolayers have been reported (Demel et al., 1965, 1968; Norman et al., 1972b). Filipin increased the surface pressure of lecithin monolayers (without cholesterol) at high molar ratios of antibiotic/spread lipid. At low molar ratios of antibiotic/spread

lipid, filipin showed selectivity for monolayers which contained sterol. Only steroids with a 3β-hydroxyl group gave large pressure increases after filipin was added.

The interaction of amphotericin B and nystatin with single bimolecular films containing cholesterol has been reported to increase the conductance and anion permeability of the membranes, possibly because the formation of aqueous pores renders the membrane anion selective (Andreoli and Monahan, 1968; Andreoli _et al_., 1969; Lippe, 1968; Holz and Finkelstein, 1970; Cass _et al_., 1970). Evidence has also been presented that filipin acts as a cation carrier (Van Zutphen _et al_., 1971). The mechanism of ion selectivity is obscure. It has been suggested that the $-NH_3^+$ group is important for the activity of amphotericin B (Cass _et al_., 1970; Schaffner and Mechlinski, 1972), but it is not responsible for selectivity to anions. Cass _et al_. (1970) suggested that the hydroxyl groups of amphotericin B are responsible for anion selectivity by substituting for the outer hydration shell of the anions.

Electron microscopic studies indicated that filipin induces "pit" formation (ca. 80 to 150 Å in diameter) in rat and human erythrocytes (Kinsky _et al_., 1967b). Pit formation in lipid dispersions resembles that in erythrocytes. Pit production by the antibiotic requires the presence of cholesterol in liposomes. No lysis of lecithin-cholesterol dispersions was induced by derivatives of the

antibiotic which have little (perhydrofilipin) or no (ultraviolet-irradiated filipin) hemolytic activity (Kinsky *et al.*, 1968). Recently, freeze-etching electron microscopy indicated that the "pits" do not appear to be holes through the membrane (Tillack and Kinsky 1973; Verlkeij *et al.*, 1973). Unlike filipin, other polyene antibiotics, such as amphotericin B, nystatin and pimaricin, did not form pits in liposomes even in the presence of cholesterol (Verkleij *et al.*, 1973). Although amphotericin B is thought to produce "pores" of 5 Å radius in lipid dispersions, electron microscopy indicated that amphotericin B caused lecithin-cholesterol liposomes to swell (Weissmann and Sessa, 1967). The mechanism of swelling of lecithin-cholesterol liposomes is still unclear.

Fluorescence changes have been used to demonstrate that filipin binds to sterols in aqueous suspensions and membranes (Bittman and Fischkoff, 1972; Schroeder *et al.*, 1972). For a 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.*, 1972b; Bittman and Fischkoff, 1972). Amphotericin B underwent a relatively constant enhancement in fluorescence polarization on interaction with the various lecithin-sterol vesicles used and did not display the selectivity exhibited by filipin III (Bittman and Fischkoff, 1972).

It has been suggested that filipin and other polyenes may exist as micelles or aggregates in aqueous solution (Lampen et al., 1960; Bittman and Fischkoff, 1972; Norman et al., 1972b; Schaffner and Mechlinski, 1972; Schroeder et al., 1973).

In order to understand what structural features in the sterol are important in the interaction of polyene antibiotics with sterols in membranes and what alteration in membrane morphology accompany sterol-polyene antibiotic interaction, ultraviolet, fluorescence, circular dichroism nuclear magnetic resonance spectral studies and electron microscopic studies of the interaction of filipin and amphotericin B with sterols in vesicles and membranes have been undertaken. Both spectral and electron microscopic methods were used in order to correlate the molecular interaction and morphological change. Although the investigation cited above showed that polyenes interact with sterol-containing membranes, binding equilibrium studies of filipin and amphotericin B to membrane-bound sterols have not been carefully studied. The studies described in this dissertation were undertaken in order to obtain more information about the interaction of the polyene antibiotics filipin and amphotericin B with sterols in vesicles and membranes.

Since polyene antibiotics may be self-associated in aqueous solution, the age and temperature of the solution and the percentage of the organic solvent present in solution may affect the binding of polyene antibiotics to sterols in membranes. Therefore, a study of the aggregation of amphotericin B in aqueous solution using spectral methods was undertaken in this dissertation. The binding of polyene antibiotics to sterols in the membranes may provide more information about the molecular interaction between phospholipids and sterols of the membrane (Bittman and Fischkoff, 1972). Therefore, the polyene antibiotics may be used as a probes to investigate the relationship between structure and biological function of membranes .

The principal phospholipid in polyene-sensitive organisms is lecithin. For this reason lecithin was chosen as the phospholipid from which to prepare phospholipid-sterol vesicles. Ergosterol was found to be a binding site for polyenes on the fungal membrane (Lampen et al., 1960, 1962; Zygmunt and Tavormina, 1966). In this dissertation ergosterol was used as one of the major sterols for studying the molecular interactions of polyenes with sterols. Model membranes were prepared by sonication of phospholipid and phospholipid-sterol mixtures in water. Vesicles are formed after prolonged ultrasonic irradiation, and are bounded by bilayer walls enclosing a volume of aqueous solution.

Many investigators have preferred sonicated aqueous egg-yolk lecithin dispersions (vesicles) rather than unsonicated dispersions (liposomes) because vesicles are more homogeneous than liposomes (Chapman et al., 1968; Penkett et al., 1968; Hüang, 1969; Hauser, 1971; Johnson et al., 1971; Finer et al., 1972).

The ciliate Tetrahymena pyriformis W is particularly appealing for investigating the polyene-sterol interaction. In normal unsupplemented medium, the naturally occurring pentacyclic triterpene alcohol, tetrahymanol (Figure 1-b), is synthesized by this ciliate. It has been speculated that

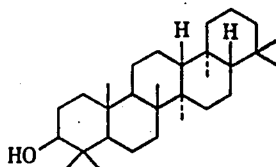


Figure 1-b: Structure of tetrahymanol

the "sterol-like" tetrahymanol may play the sterol's role in Tetrahymena pyriformis W (Nes, 1974). This pentacyclic triterpene alcohol can be replaced by ergosterol as a result of supplementation of the growth medium with that sterol. Ergosterol-grown cell are at least 20 times more sensitive to polyene antibiotics than normal cells (Conner et al., 1971). The molar ratio of lipid phosphorus to alcohol (tetrahymanol or ergosterol) in the ciliary membranes obtained from the

two cell types is the same. This suggests that the ergosterol molecules may occupy the same sites in the ciliary membrane as tetrahymanol. Spectral and electron microscopic studies have been undertaken in this dissertation to investigate the interaction of isolated intact ciliary membranes and vesicles derived from ciliary lipids with the polyene antibiotics filipin and amphotericin B.

MATERIALS AND METHODS

A. Lipids and Steroids. Egg lecithin was isolated and purified from hen egg yolk by the method of Singleton *et al.* (1965). The purity was determined by thin-layer chromatography on silica gel plates using a solvent system consisting (by volume) of chloroform : acetone : methanol : acetic acid : water (3 : 4 : 1 : 1 : 0.5). Synthetic lecithins were purchased from the following sources: β,γ-dimyristoyllecithin from Calbiochem; phosphatidylserine from Schwarz-Mann Bioresearch. Spinach digalactosyl diglyceride was obtained from the Hormel Institute.

Steroids were purchased from the following sources: cholest-5-en-3β-ol (cholesterol) and 5α-cholestan-3β-ol (cholestanol) from Sigma Chemical Co.; cholest-5-en-3α-ol (epicholesterol) and ergosterol from Schwarz-Mann Bioresearch; 3β-thiocholest-5-ene (thiocholesterol) from Aldrich Chemical Co.; androstan-3α-ol, androstan-3β-ol, and androstan-17β-ol from Ikapharm, Ramat-Gan, Israel. The sterols were recrystallized several times from acetone. The purity was analyzed by thin-layer chromatography on silica-gel G-coated plates consisting (by volume) of benzene-methanol (92 : 8) and chloroform-acetone (98 :3.5). Dicetyl phosphate was obtained from Sigma Chemical Co.

B. Antibiotics. The polyene antibiotic filipin III was supplied as lot number U-25,639, reference number 8395-MEB-116 D, by Dr. G. B. Whitfield of the Upjohn Co., Kalamazoo,

Mich. Each lot was found to be pure by thin-layer chromatography using the method of Bergy and Eble (1968). The experiment involving mixtures of perhydrofilipin and filipin were performed with the "filipin complex." 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). Perhydrofilipin exhibited no absorption and circular dichoism spectra between 300-400 nm. Stock solutions of filipin were prepared in N, N-dimethylformamide (DMF) and stored at -20° for not more than one week. Aliquots of the stock solution of filipn were added to water or 0.001 M Tris buffer containing 0.01 M NaCl, pH 7.4, to give the desired concentration of antibiotics and DMF (0.3%). Based on a molecular weight of 670, the molar extinction coefficient of filipin in aqueous solution (immediately after preparation of the solution) was 5.2×10^{4} $M^{-1}cm^{-1}$ at 338 nm.

Amphotericin B was obtained from Squibb, New Brunswick, N. J., as lot number 91830. It was suspended in methanol and dissolved upon acidification with hydrochloric acid. In order to prevent formation of amphotericin B methyl ester, the methanol was removed promptly and the water-soluble amphotericin B hydrochloride thus obtained was dissolved in 0.001 M Tris-0.01 M NaCl solution. The extinction coefficient at 334 nm of amphotericin B in 0.001 M NaCl solution (pH 7.4), immediately after preparation of the

solution, was found to be $2.65 \times 10^4\ M^{-1}cm^{-1}$, assuming a molecular weight of 923.

C. Preparation of Vesicles. Vesicles were prepared in 0.001 M Tris-0.01 M NaCl, pH 7.4, or H_2O. Aliquots of stock solution of 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 vacuum. After a glass bead (1-mm diameter) was added, the thin film of lipid mixtures was dispersed into the buffer solution by mixing the suspension on a Vortex mixer for 60 sec. The dispersion was then subjected to ultrasonic irradiation under nitrogen at 4^oC with a 20-kHz Branson sonifier (Model S-110) fitted with a solid tap horn at power level 4. Vesicles contained 4 mole-percent dicetyl phosphate and were subjected to ultrasonic irradiation for 1 min except for those vesicles containing ergosterol. Ergosterol-containing vesicles were sonicated for 2 min.

D. Membranes. Ciliary membranes from unsupplemented and ergosterol-supplemented cells of *Tetrahymena pyriformis* W were obtained from Drs. R. L. Conner and E. Kaneshiro of Bryn Mawr College, Bryn Mawr, Pa. These membranes were isolated as described by Conner *et al*. (1971). Sterol, tetrahymanol and phosphorus analyses were performed as described by Conner *et al*. (1969, 1971). The molar ratio of phospholipid to ergosterol or tetrahymanol in the ciliary membranes was 2 : 1.

E. Absorption Spectra Measurements. Absorption measurements were carried out on a Cary Model 14 spectrophotometer. Equal volumes of antibiotic in buffer solution or H_2O were added to the vesicles or membrane preparation, then were incubated in the dark for 2 hr at room temperature. The regions of wavelength recorded were 280-380 nm for filipin and 280-420 nm for amphotericin B. The time and temperature of incubation are important because aggregation of antibiotics in aqueous solution may affect the ability of antibiotics to interact with sterols (Schroeder *et al.*, 1973).

F. Fluorescence Measurements. Excitation spectra, emission spectra and fluorescence polarization intensities were measured at 20° with a Hitachi-Perkin Elmer model MPF-2A fluorescence spectrophotometer equipped with a polarizer accessory. Excitation and emission slits corresponding to bandpasses of 10 nm were used in general. Fluorescence spectra and polarization intensities were corrected for light scattering of the vesicles and membranes, unless otherwised 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 by volume. Spectra were recorded after a minimum of 2 hr of incubation of filipin with the

vesicles or membranes. Measurements of the fluorescence spectra of amphotericin B 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.

G. Circular Dichroism Spectra. CD measurements were made on a Cary Model 60 spectropolarimeter equipped with a model 6001 circular dichroism attachment. All spectra were corrected for light scattering of the vesicles and membranes. The wavelength regions between 290-380 nm for filipin and 290-420 nm for amphotericin B were chosen because of the corresponding absorption spectra. All measurements were taken at room temperature.

H. Nuclear Magnetic Resonance Spectra. The nuclear magnetic resonance spectra of lecithin vesicles prepared by sonication in the presence of D_2O containing an internal standard, 0.13% silanor-D_2O-TSP (sodium-3-trimethylsilyl propionate-2, 2, 3, 3-d_4, obtained from Merck, Sharp and Dohme Co.), were scanned repetitively on a Varian 100-MHz instrument connected to a time-average computer.

I. Electron Microscopy. Vesicles or membranes were permitted to react with filipin or amphotericin B for 20 hr at 4^oC. 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 0.001 M Tris buffer

(pH 7.4) or adjusted to pH 6.5 with NH_4OH was added to each sample as a negative stain. 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 vesicles 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 by Dr. O. R. Anderson of Lamont-Doherty Geological Observatory of Columbia University, Palisades, N.Y., in a Philips EM 200 electron microscope with 60 kV accelerating voltage.

Tests were performed to determine the approximate minimum concentrations of filipin and amphotericin B 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 B concentration sufficient to induce lecithin-cholesterol vesicle lysis was determined by mixing aliqouts of the vesicle suspension with amphotericin B solution to give final amphotericin B concentrations of 26.8, 8.9, and 2.7 µM.

RESULTS

I. Spectral Studies for Interaction of Filipin III and Amphotericin B with Lecithin-Cholesterol Vesicles and Cellular Membranes.

A. Absorption Spectral Studies

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., 1972 a, b). Figure 2 and Table I show that in the presence of 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. Interaction of filipin with lecithin-ergosterol vesicles results in a large change in the absorbance peak ratios of the antibiotic and a closely similar spectrum is observed in the presence of ciliary membranes obtained from ergosterol-supplemented cells of Tetrahymena. The extinction coefficients of the absorption bands of filipin were diminished in the presence of lecithin vesicles, lecithin-epicholesterol vesicles, lecithin-cholestanol vesicles, lecithin-androstan-3α-ol vesicles and lecithin-androstan-17β-ol vesicles (Table II).

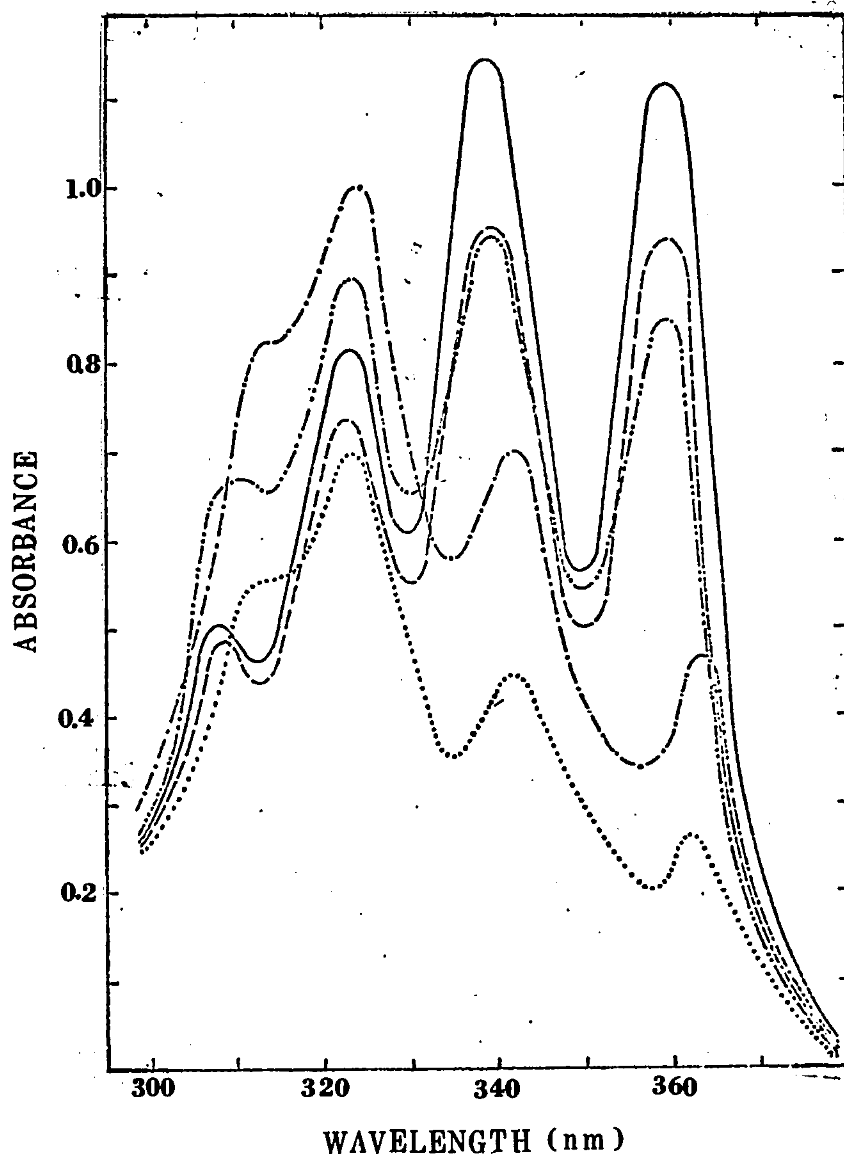


Figure 2. Absorption spectra of filipin (—), and of filipin in the presence of lecithin vesicles (-----), lecithin-ergosterol vesicles (•••••), and Tetrahymena ciliary membranes containing tetrahymanol (—••—) and ergosterol (—•—). The concentration of filipin was 21.5 μM. The total lipid concentration 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.

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 peak. 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).

TABLE II. Effect of Lecithin-Sterol Vesicles on the Absorbance of Filipin at Peak 2. [a]

Agent Added to Filipin	% Decrease in Absorbance at 338 nm $\frac{A_f - A_b}{A_f} \times 100\%$
Buffer	0
Lecithin vesicles	26
Lecithin-androstan-3α-ol vesicles	52
Lecithin-androstan-17β-ol vesicles	61
Lecithin-ergosterol vesicles	65
Lecithin-cholestanol vesicles	66
Lecithin-epicholesterol vesicles	70
Lecithin-cholesterol vesicles	81

[a] The percent decrease in the absorbance of filipin in the presence of vesicles was measured at the λ_{max} of the absorption peak near 338 nm (The λ_{max} is shifted toward longer wavelength by approximately 3 nm in the presence of cholesterol-containing vesicles.) The concentration of filipin was 21.5 µM. The total lipid concentration in the lecithin vesicles, lecithin-epicholesterol vesicles, lecithin-cholestanol vesicles and lecithin-cholesterol vesicles was 0.19 mM. The concentrations of total lipid in the lecithin-ergosterol vesicles, lecithin-androstan-3α ol vesicles, and lecithin-androstan-17β-ol vesicles were 0.38, 0.25, and 0.25 mM, respectively. The molar ratio of lecithin to sterols was 7:3. The absorbance in presence of the added agent in indicated by "b" (bound); "f" refers to the absorbance of free filipin.

Figure 3 indicates that the degree of interaction of filipin and vesicles depends on the relative concentrations of lipid and antibiotic. The effects of vesicles which were prepared from phosphatidylserine and cholesterol or from digalactosyl diglyceride and cholesterol on the ultraviolet absorption spectrum of filipin are shown in Table III.

The effects of vesicles derived from phosphatidylserine, digalactosyl diglyceride, or ciliary phospholipids on the ultraviolet absorbance peak ratios of filipin are shown in Table III. The phospholipids isolated from the cilia of ergosterol-supplemented (PLE) and unsupplemented (PLT) cells of <u>Tetrahymena</u> <u>pyriformis</u> W were used to prepare vesicles. Table III shows that the absorbance at 338 nm of filipin is reduced by approximately 60% in the presence of vesicles containing tetrahymanol or ergosterol. Only slight decreases in absorbance were observed in the presence of vesicles derived from the cilia phospholipids alone.

Figure 4 shows that the extinction coefficients of the long wavelength bands of amphotericin B undergo small enhancements in the presence of lecithin vesicles, lecithin-cholesterol vesilces and aqueous suspensions of cholesterol. Similar changes were observed in the absorption spectrum of amphotericin B in the presence of tetrahymanol- and ergosterol-containing ciliary membranes. However, from the differences of absorbance peak ratios of bound and unbound

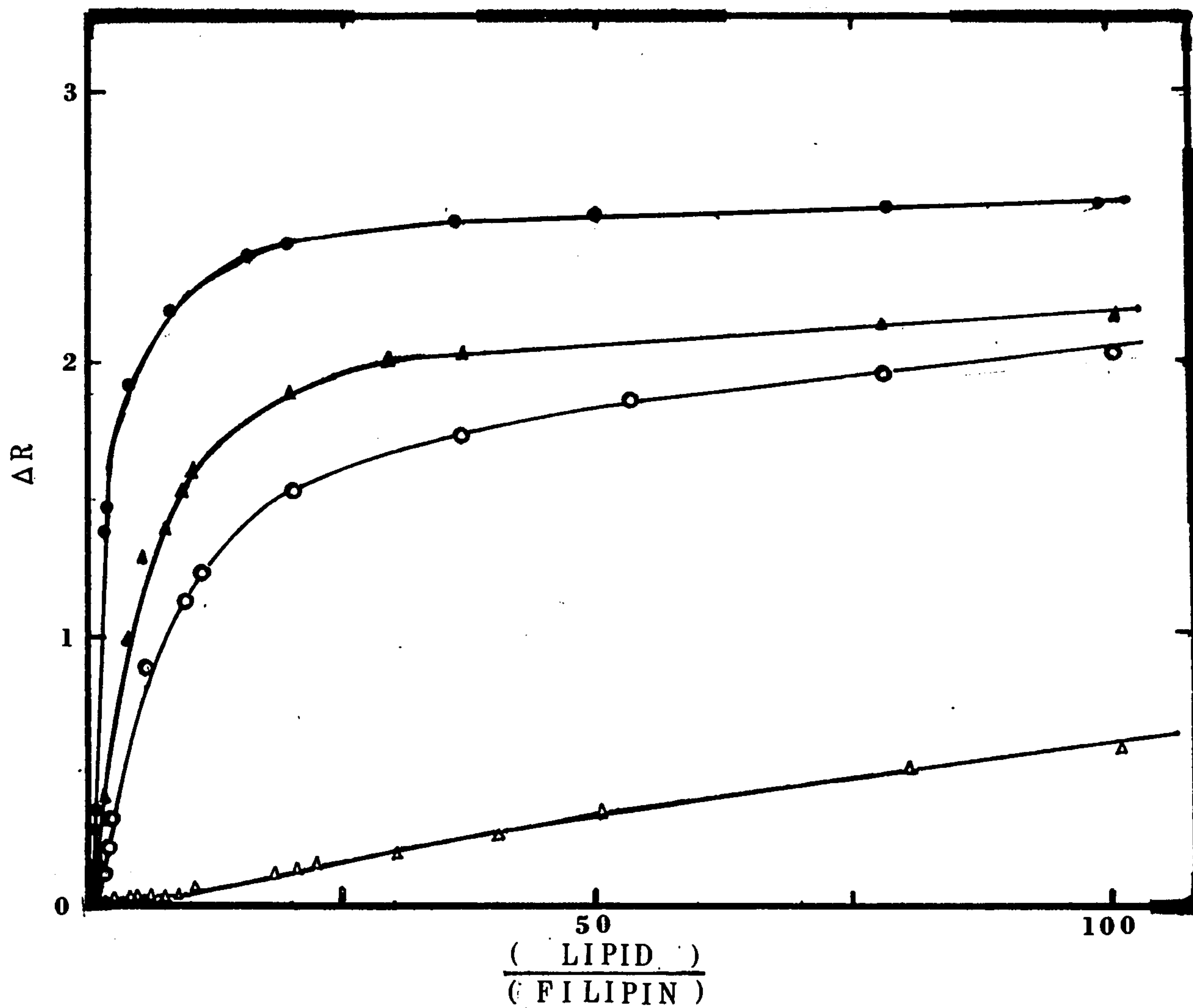


Figure 3. Effect of lipid/filipin ratio on the ultraviolet absorbance peak ratio difference, Δ R, of filipin. Vesicles were prepared from lecithin-cholesterol (7:3) (—●—), lecithin-cholesterol (7:1) (—▲—), lecithin-epicholesterol (7:3) (–o–), and lecithin (—△—). The concentration of filipin was 10.5 µM. The absorbance peak ratio, Δ R, is $(4/1)_b - (4/1)_f$, where "b" indicates the absorbance of filipin in the presence of vesicles and "f" refers to the absorbance peak ratio of free filipin. The absorbance peaks 1 and 4 are defined in Table I, p.20.

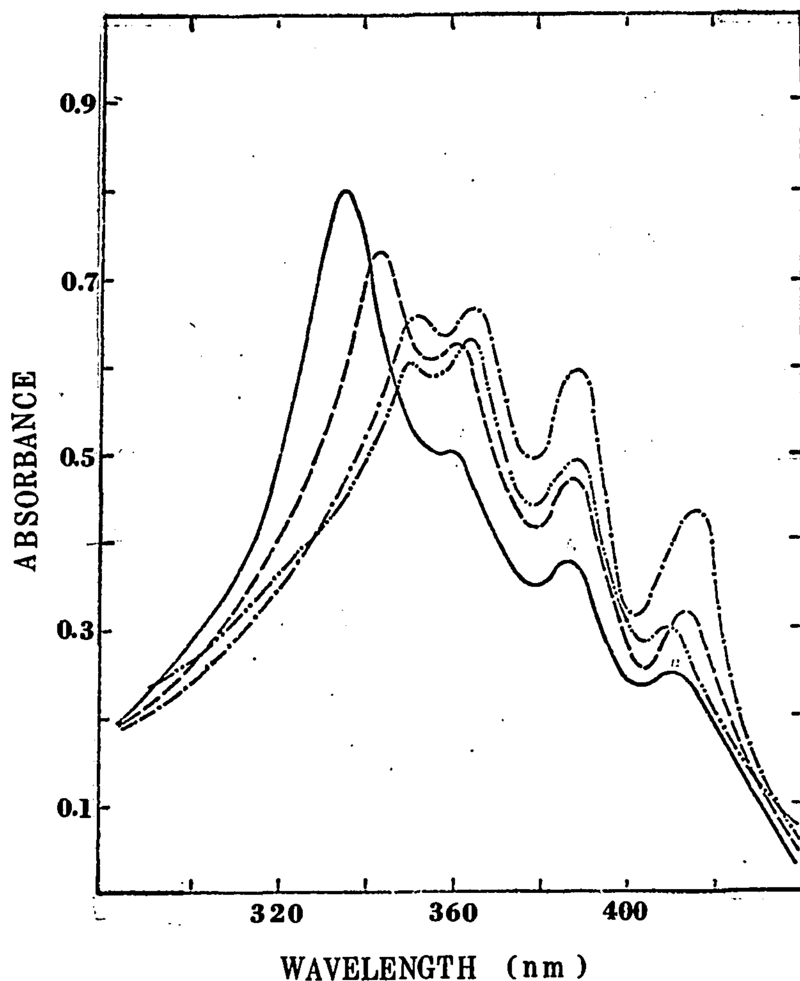


Figure 4. Absorption spectra of amphotericin B (—), and of amphotericin B in the presence of lecithin vesicles (---), lecithin-cholesterol vesicles (—•—), and an aqueous suspension of cholesterol (—••—). The concentration of amphotericin B was 31 μM. The total lipid concentration in the vesicles was 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).

TABLE III. Effect of Vesicles Derived from Phosphatidylserine, Digalactosyl Diglyceride, or Ciliary Phospholipid on Ultraviolet Absorbance Peak Ratios of Filipin. [a]

Agent Added to Filipin	Absorbance Peak Ratio Difference $(4/1)_b - (4/1)_f$	% Decrease in Absorbance $\frac{A_f - A_b}{A_f} \times 100\%$ 338nm
Buffer	0.00	0
Phosphatidylserine vesicles	0.18	28
" -epicholesterol vesicles	1.30	56
" -cholesterol vesicles	2.35	61
Digalactosyl diglyceride vesicles	0.15	25
" -epicholesterol vesicles	0.60	56
" -cholesterol vesicles	2.92	80
PLT vesicles [b]	0.03	10
PLT-tetrahymanol vesicles	1.41	65
PLT-ergosterol vesicles	1.43	60
PLE vesicles [c]	0.05	21
PLE-tetrahymanol vesicles	1.46	60
PLE-ergosterol vesicles	1.56	59

(TABLE III)

[a] The filipin concentration was 12 μM. The molar ratio of phospholipid to alcohol was 7:3. The total lipid concentration in the vesicles derived from cilia lipids was 0.25 mM, and that in the phosphatidylserine or phosphatidylserine-sterol vesicles was 0.3 mM. The total lipid concentration in the digalactosyl diglyceride and digalactosyl diglyceride-sterol vesicles was 0.15 mM.

[b] PLT stands for ciliary phospholipids obtained from unsupplemented cells.

[c] PLE stands for ciliary phospholipids obtained from ergosterol-supplemented cells.

TABLE IV. Effect of Vesicles on Ultraviolet Absorbance Peak Ratio of Amphotericin B. [a]

Agent Added to Amphotericin B	Absorbance Peak Ratio Differences $(2/4)_b - (2/4)_f$
Buffer	0.00
Lecithin vesicles	0.16
Lecithin-cholesterol vesicles	0.44
Cholesterol aqueous suspension	0.36

[a] The λ_{max} of amphotericin B (405, 385, 360, and 335 nm) are designated by peak numbers (1, 2, 3, and 4, respectively). The concentration of amphotericin B was 31 μM. The total lipid concentration was 1.4 mM. The molar ratio of phospholipid to sterol was 7:3. The concentration of cholesterol and DMF in aqueous suspension of sterol were 0.25 mM and 0.3% (v/v), respectively.

amphotericin B indicated that the preference on the binding of amphotericin B to the vesicles containing cholesterol are shown in Table IV. These results are consistent with the report by Weissmann and Sessa (1967), in which the amount of marker released was approximately doubled by prior incorporation of cholesterol or ergosterol into the liposomes.

B. Circular Dichroism Studies

The CD spectrum of filipin resembles the absorption spectrum. Figure 5 shows that when the dielectric constant of the medium in which the antibiotic is dissolved is decreased, *e.g.*, from water to DMF and dioxane, the molecular ellipticities of the negative dichroic bands are increased dramatically. Filipin undergoes similar increase in molecular ellipticities on binding to an aqueous suspension of cholesterol (Figure 5). Slight shifts toward longer wavelengths are evident. 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 the molecular ellipticity of filipin at 340 nm was unaffected by dilution with perhydrofilipin. Perhydrofilipin has no CD spectrum in the region 300-400 nm.

The effects of lecithin-cholesterol vesicles and other vesicles containing different sterols on the circular dichroic bands of filipin are shown in Figure 6. In the presence of lecithin-cholesterol and lecithin-

Figure 5. 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).

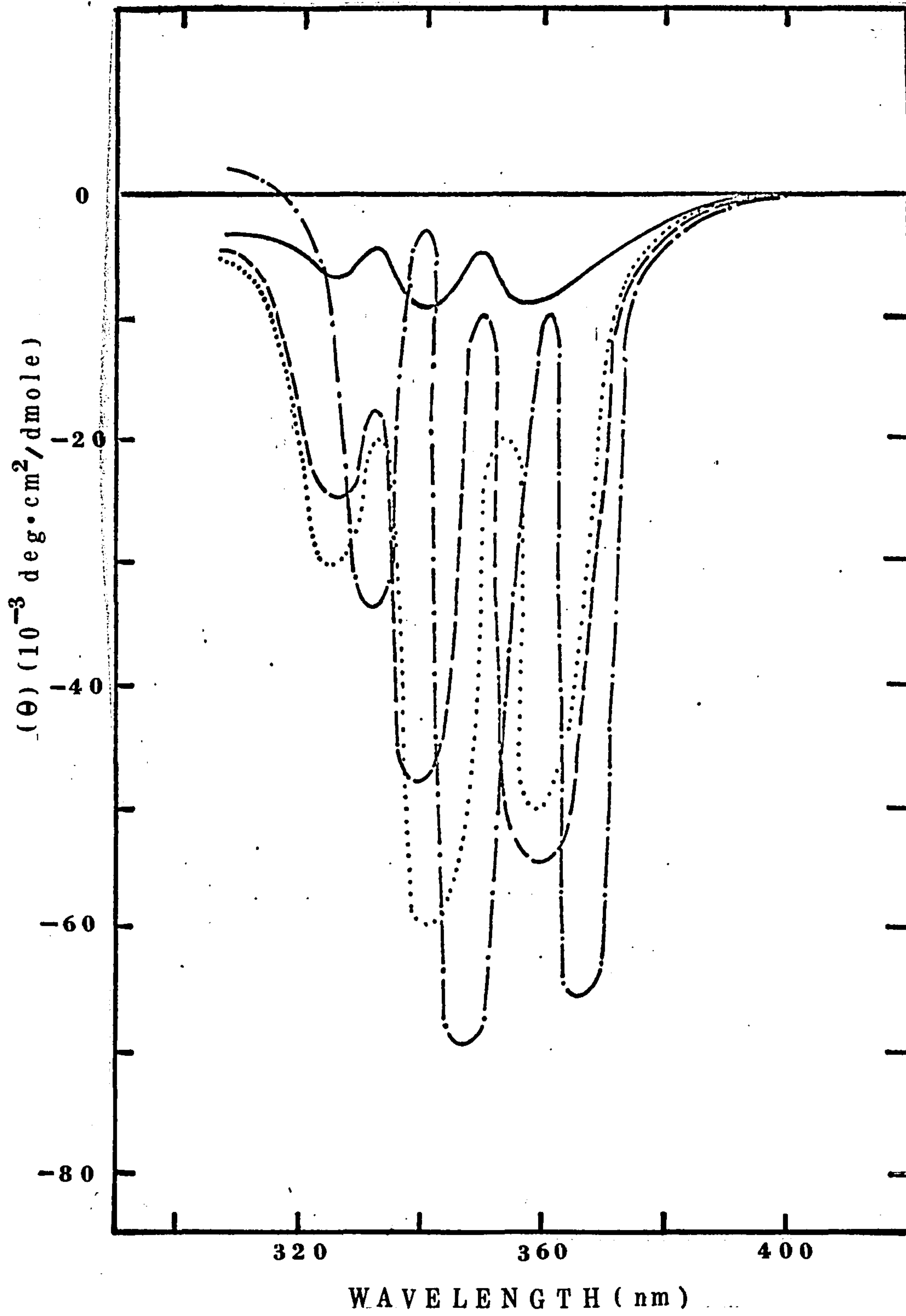


Figure 5.

Figure 6. 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.

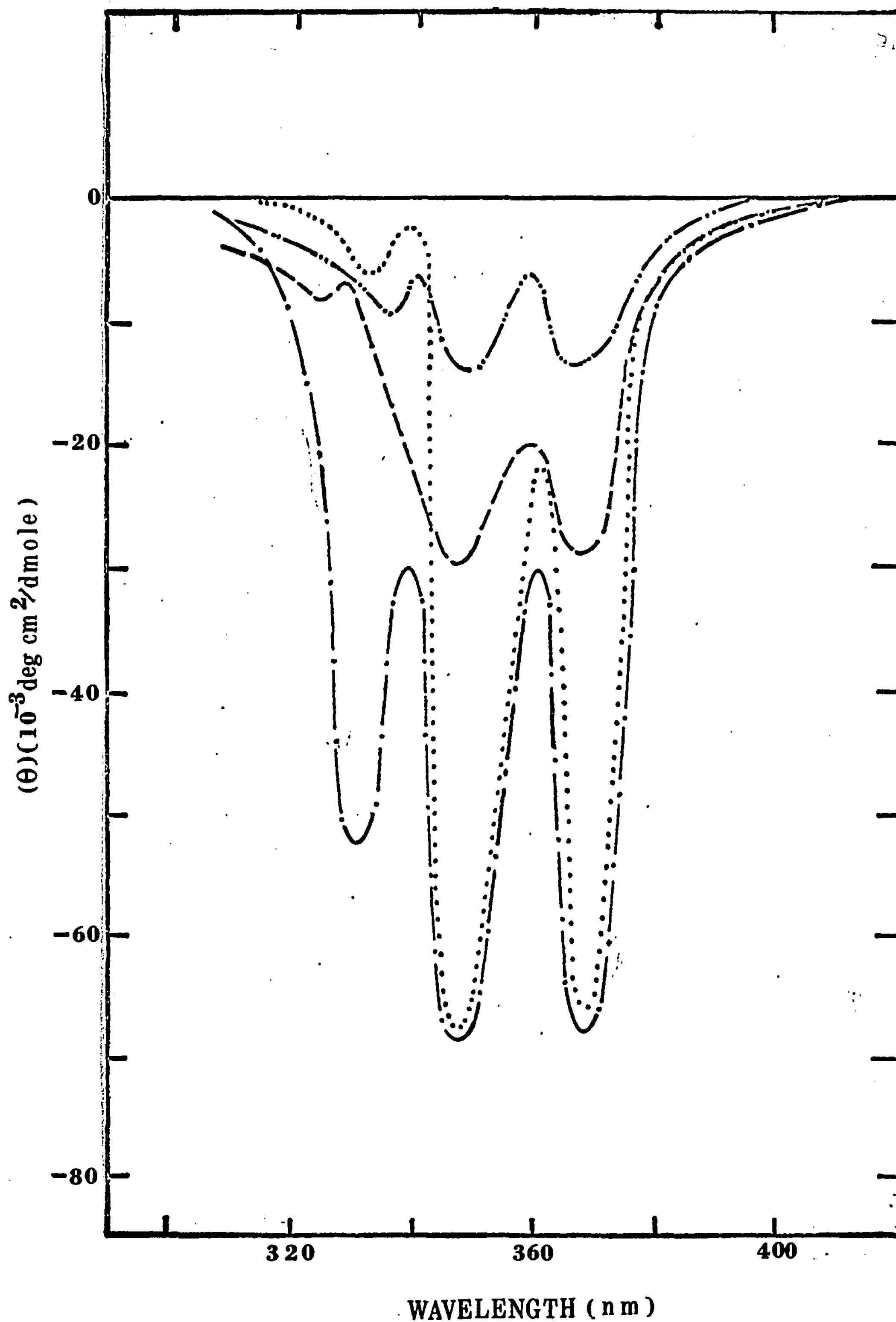


Figure 6.

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. The CD spectrum of filipin in the presence of lecithin vesicles resembled the spectrum shown for filipin in the presence of lecithin-epicholesterol vesicles.

Figure 7 shows that the CD spectrum of filipin is strongly enhanced in the presence of ergosterol-containing ciliary membranes, whereas smaller enhancements of the long wavelength bands are observed in the presence of tetrahymanol-containing ciliary membranes. Small enhancements of the negative dichroic bands of filipin in the presence of vesicles derived from the lipids isolated from the cilia of Tetrahymena (Figure 8) were also observed. Ergosterol-containing vesicles caused stronger enhancements of the CD bands of filipin than vesicles containing tetrahymanol. The CD spectra of filipin and digalactosyl diglyceride-cholesterol vesicles are shown in Figure 9. A strong enhancement of the negative dichroic bands of filipin is observed in cholesterol-containing vesicles, and smaller effects are found in vesicles containing epicholesterol and in vesicles lacking sterol.

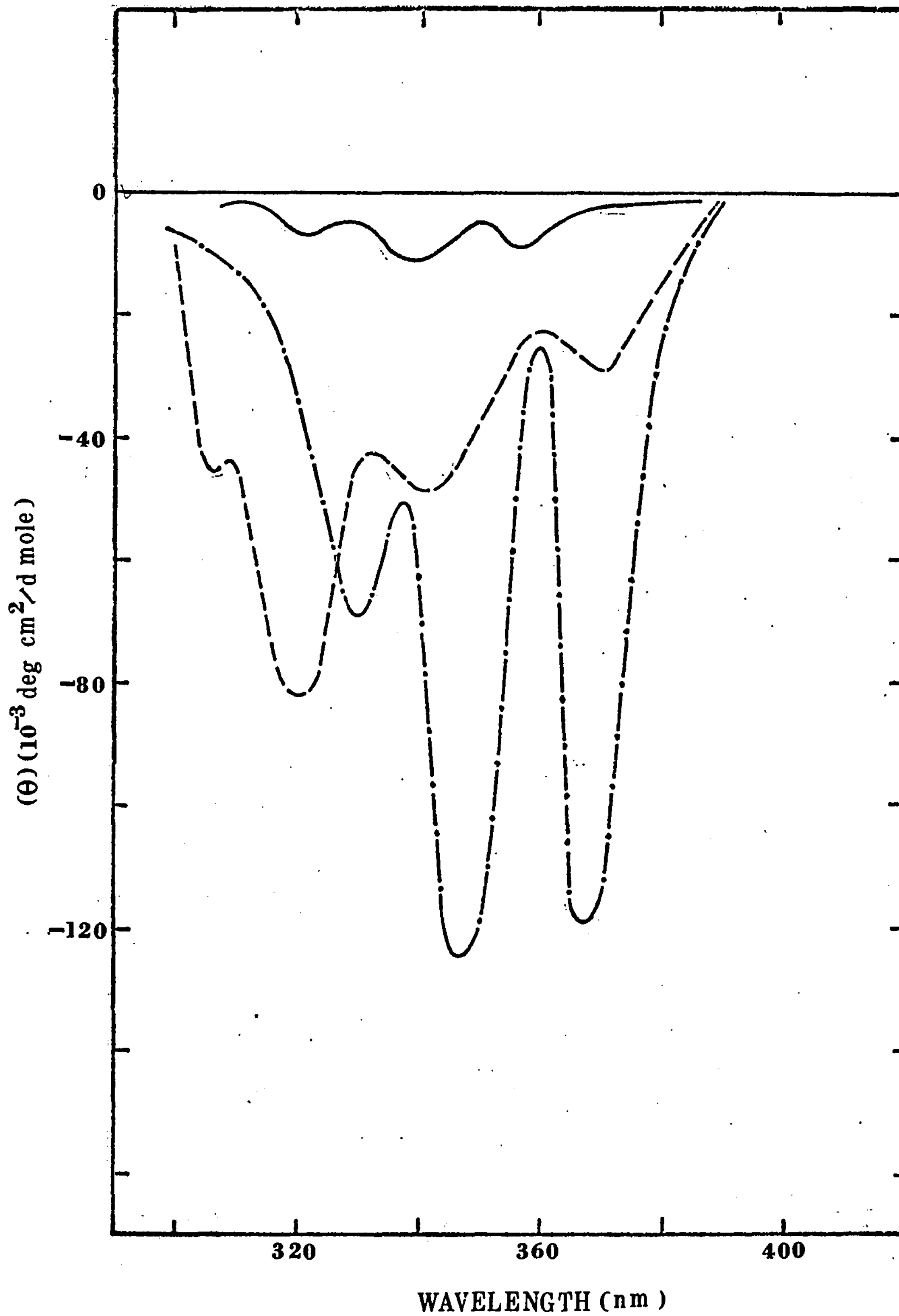


Figure 7. CD spectra of filipin (—), and of filipin in the presence of Tetrahymena ciliary membranes containing tetrahymanol (---) and ergosterol (-•-). The concentrations were the same as in Figure 2.

Figure 8. CD spectra of filipin (---), and of filipin in the presence of vesicles derived from ciliary phospholipids.
A. Vesicles were prepared from PLT (–▲–), PLT-tetrahymanol (–o–), and PLT-ergosterol (–•–).
B. Vesicles were prepared from PLE (– –), PLE-tetrahymanol (–o–), and PLE-ergosterol (–•–).
The concentration of filipin was 16 μM. The phospholipid and alcohol were present in vesicles at 7:3 molar ratio, and the total lipid concentration in the vesicles was 250 μM.

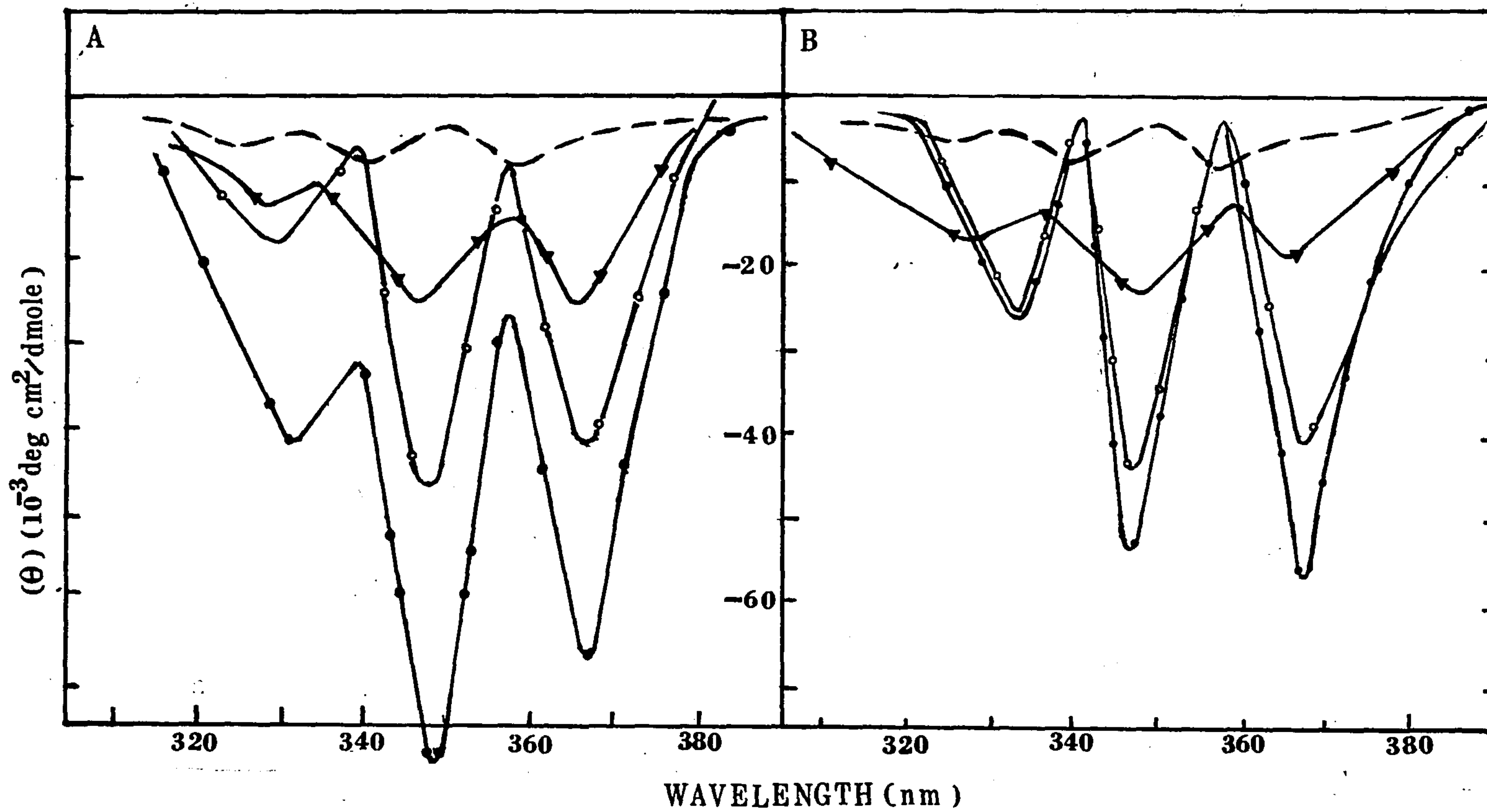


Figure 8.

Figure 9. CD spectra of filipin in the presence of vesicles derived from digalactosyl diglyceride and phosphatidylserine.
A. Vesicles were prepared from phosphatidylserine (---), phosphatidylserine-epicholesterol (—o—), and phosphatidylserine-cholesterol (—•—).
B. Vesicles were prepared from digalactosyl diglyceride (---), digalactosyl diglyceride-epicholesterol (—o—), and digalactosyl diglyceride-cholesterol (—•—).
The molar ratio of lipid to sterol was 7:3. The total lipid concentrations were 0.3 mM and 0.15 mM for the vesicles derived from phosphatidylserine and digalactosyl diglyceride, respectively. The concentration of filipin was 11 μM.

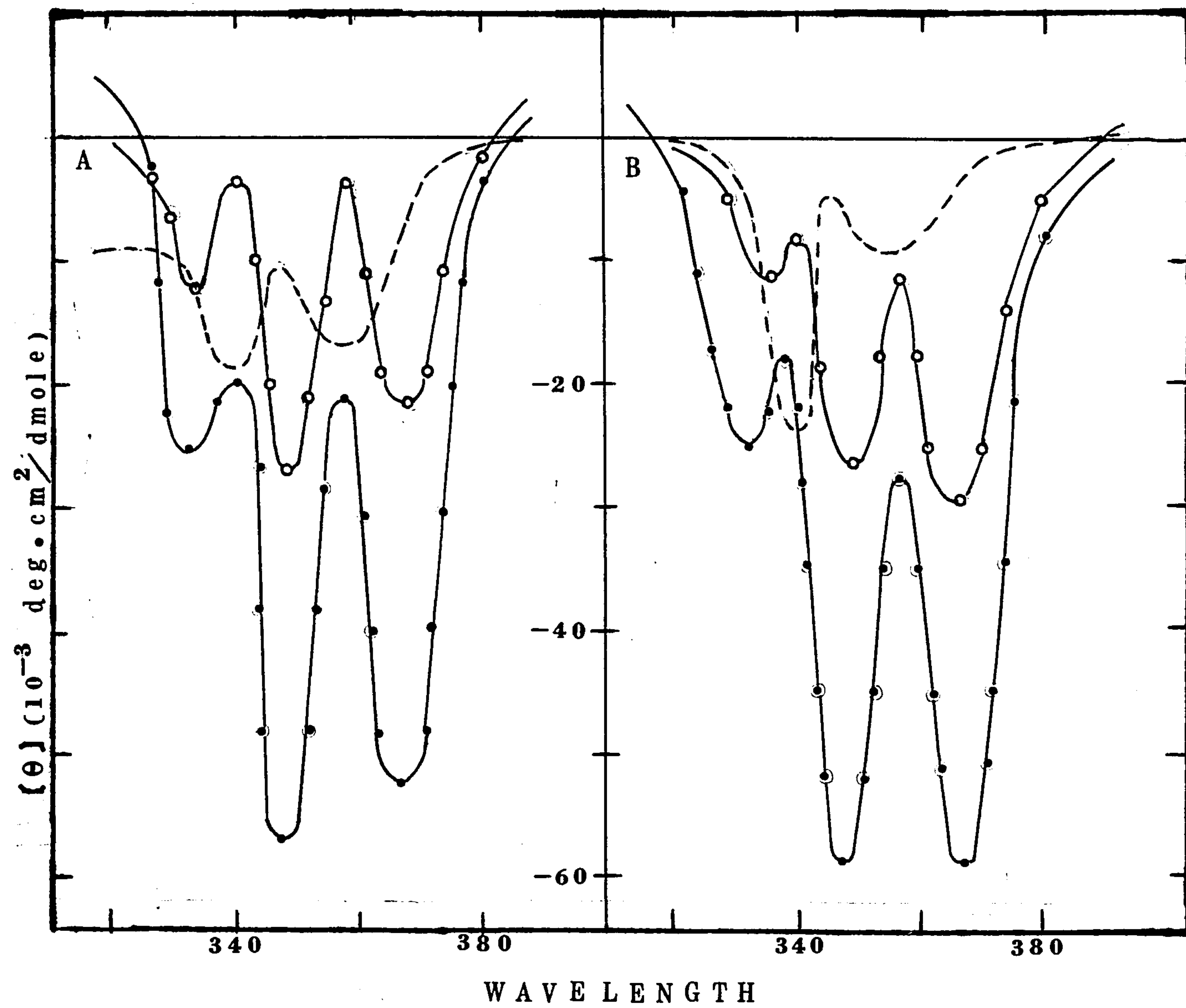


Figure 9.

The optical activity of amphotericin B in aqueous solution is complicated. The CD spectrum of amphotericin B in aqueous solution shows strong negative and positive ellipticity bands at 347 and 335 nm, respectively. The effect of lecithin and lecithin-cholesterol vesicles on the CD bands of amphotericin B are shown in Figure 10. These spectra were taken at times varying from 0-20 min after mixing the antibiotic solution with the vesicles. The diminution of positive and negative CD bands were found to be accompanied by a shift of the positive band toward longer wavelength. The spectra measured at different times cross at a common point called the isoellipticity point. No significant shift in the wavelength of isoellipticity point of amphotericin B was found in the presence of lecithin vesicles and lecithin-cholesterol vesicles.

Figure 10. CD spectra of amphotericin B (---), and of amphotericin B in the presence of lecithin (LEC) and lecithin-cholesterol (LEC-CHOL) vesicles. The spectra were taken at intervals of 2.5 min from 0 to 20 min after mixing of the amphotericin B solution with the vesicles. The isoellipticity point is indicated by the arrow. The total lipid concentration was 1.5 mM. The molar ratio of lecithin to cholesterol was 7:3, The concentration of amphotericin B in aqueous solution was 32 µM.

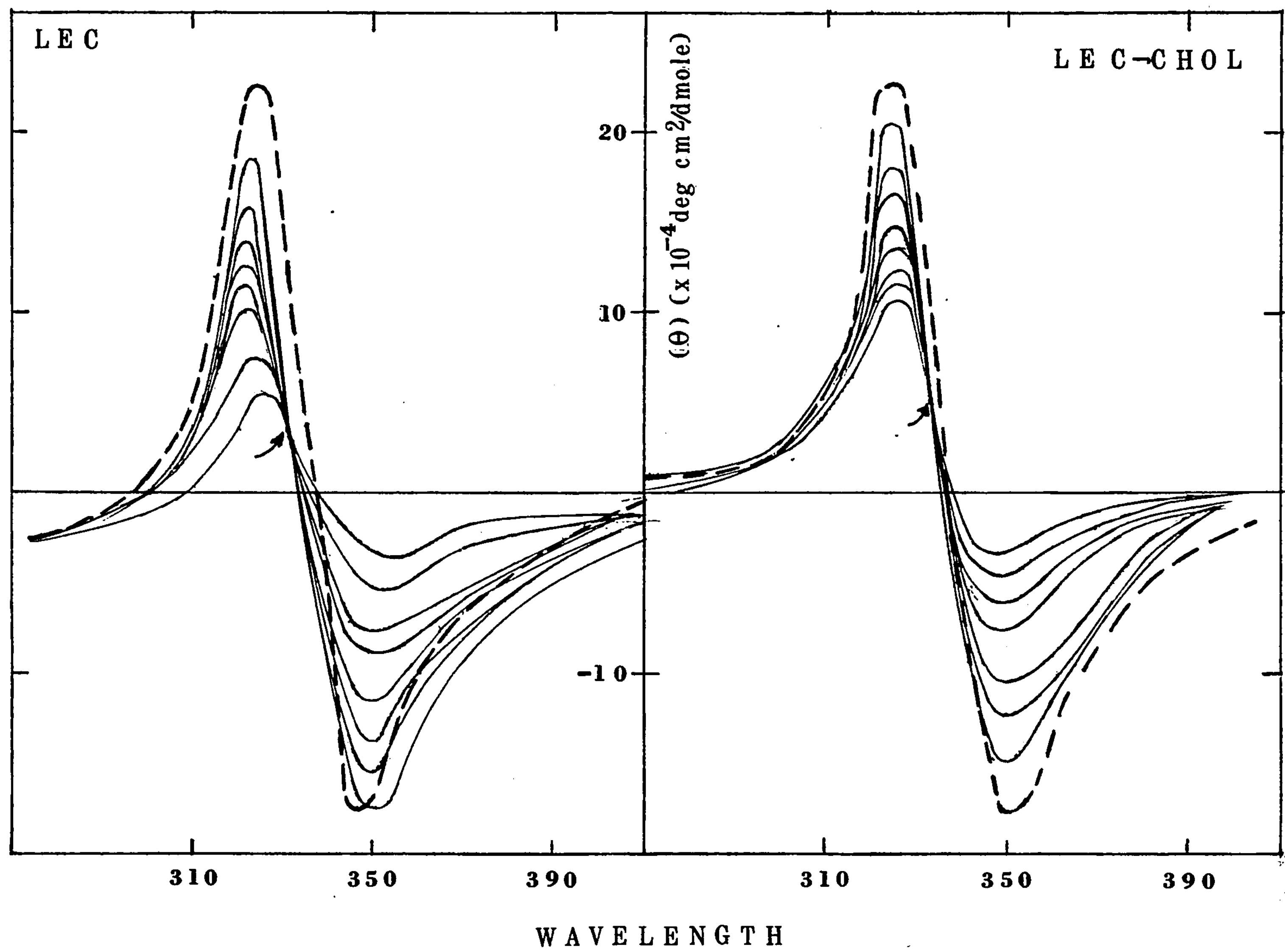


Figure 10.

C. Fluorescence Studies

Figure 11 shows the effects of sterols incorporated into vesicles and membranes on the fluorescence properties of filipin. 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 of the excitation spectra (Figure 11). Although vesicles derived from PLT and tetrahymanol, PLE and ergosterol, PLE and tetrahymanol, and PLT and ergosterol caused quenching of the fluorescence intensity and large changes in the peak ratios (Figure 12), ciliary membranes containing tetrahymanol caused only slight quenching without alteration of the peak ratios of filipin. Vesicles derived from the phospholipids of the cilia of both cell types caused only slight enhancement without alteration of the peak ratios (Figure 12). Fluorescence polarization measurements of filipin in the presence of vesicles and intact ciliary membranes are shown in Table V.

The effects of aqueous suspensions of sterols and of vesicles derived from lecithin, phosphatidylserine and digalactosyl diglyceride, with and without sterol,

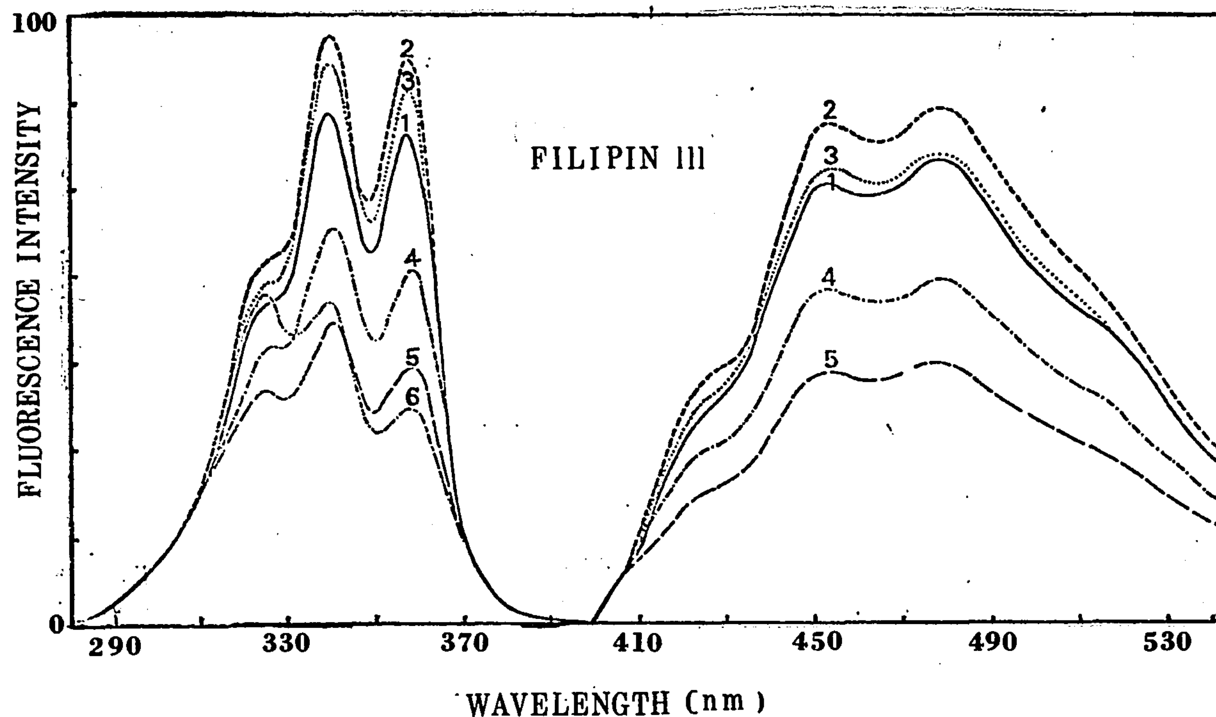


Figure 11. Fluorescence excitation (left) and emission (right) spectra of filipin, and of filipin in the presence of lecithin vesicles, lecithin-sterol vesicles at 7:3 molar ratio, and Tetrahymena ciliary membranes containing ergosterol. The wavelengths of excitation and emission were 357 and 480 nm, 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 12. Fluorescence excitation spectra of filipin (---), and of filipin in the presence of vesicles derived from ciliary phospholipids.

A. Vesicles were prepared from PLE (—•—), PLE-ergosterol (—o—), and PLE-tetrahymanol (—▵—).

B. Vesicles were prepared from PLT (—•—), PLT-ergosterol (—o—), and PLT-tetrahymanol (—▵—).

The concentration of filipin was 3 μM. The phospholipid and alcohol were present in vesicles at 7:3 molar ratio, and the total lipid concentration in the vesicles was 0.25 mM.

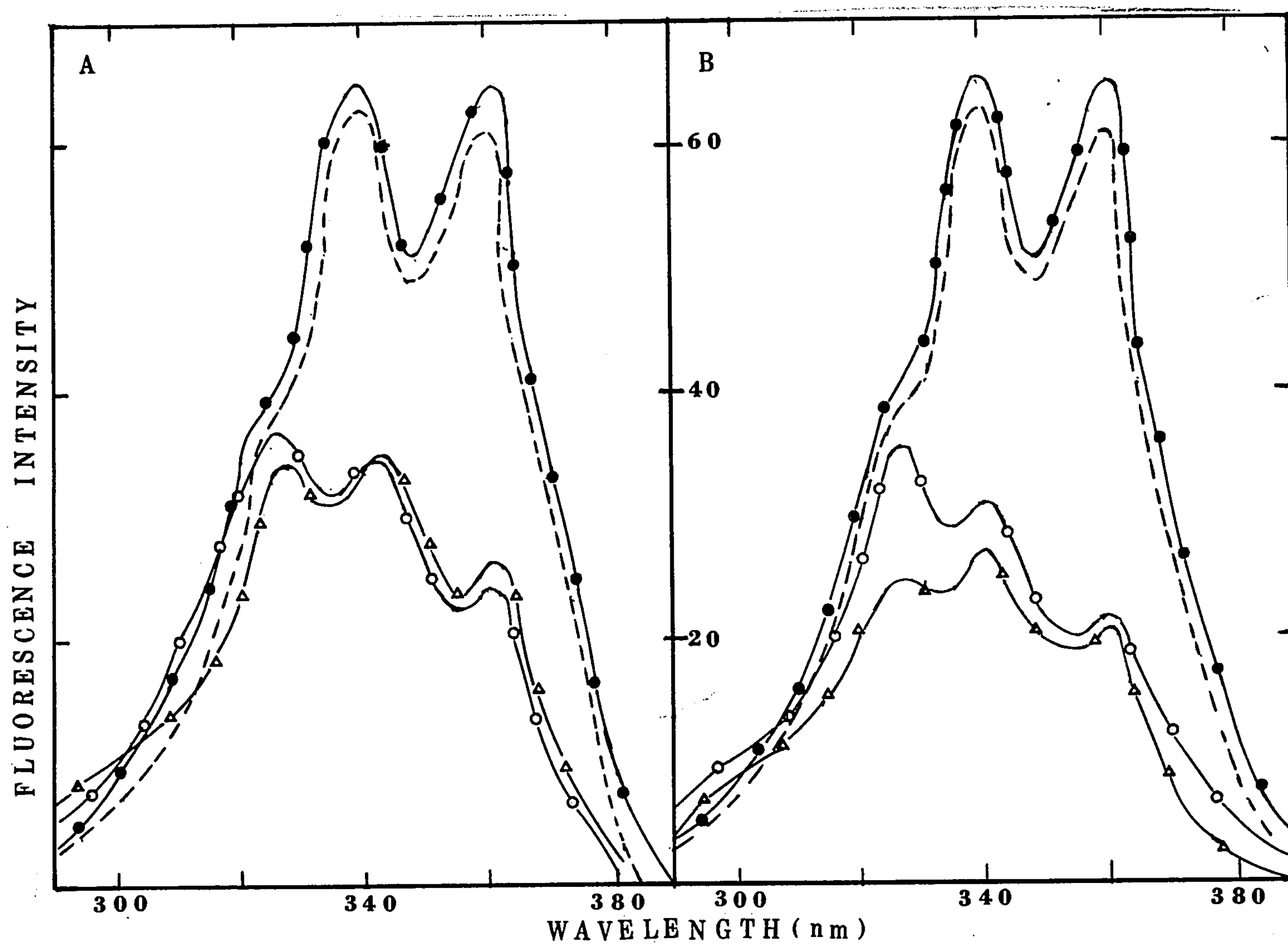


Figure 12.

TABLE V. Effect of Ciliary Membranes and Vesicles Derived from Ciliary Phospholipids on the Fluorescence Polarization of Filipin. [a]

Agent Added to Filipin	Fluorescence Polarization
Buffer	0.017
Tetrahymanol-containing membranes	0.020
Ergosterol-containing membranes	0.330
PLT vesicles	0.028
PLE vesicles	0.034
PLT-tetrahymanol vesicles	0.250
PLE-tetrahymanol vesicles	0.276
PLT-ergosterol vesicles	0.305
PLE-ergosterol vesicles	0.315

[a] The excitation and emission wavelengths were 325 and 480 nm, respectively. The concentration of filipin was 3 µM. The phospholipid and alcohol were present in vesicles at 7:3 molar ratio, and the total lipid concentration in the vesicles was 0.25 mM. The total lipid concentration in the membranes was 0.03 mM.

on the fluorescence polarization of filipin are shown in Table VI. These results are consistent with the CD and absorption data shown previously. Only vesicles containing cholesterol or ergosterol caused strong enhancement of the CD bands and fluorescence polarization intensity of filipin. Smaller enhancements are observed in the presence of vesicles prepared from lecithin, phosphatidylserine or digalactosyl diglyceride devoid of sterols. Epicholesterol-containing vesicles also gave small enhancements. The fluorescence polarization of filipin was enhanced strongly in the presence of cholesterol, especially when cholesterol was incorporated in the vesicles (Table VI). The binding affinity of filipin with aqueous suspensions of cholesterol devoid of phospholipid was lower than that with sonicated vesicles. No significant difference was found in the similar study of epicholesterol (Table VI).

The effect of vesicles on the excitation and emission spectra of amphotericin B is shown in Figure 13. In contrast to their effects on the fluorescence properties of filipin (Figure 11), lecithin vesicles and vesicles containing cholesterol and ergosterol produced nearly identical enhancements in the fluorescence intensity of amphotericin B. No significant changes in the fluorescence polarization of amphotericin B were observed in the presence of tetrahymanol- or ergoaterol-containing ciliary membranes.

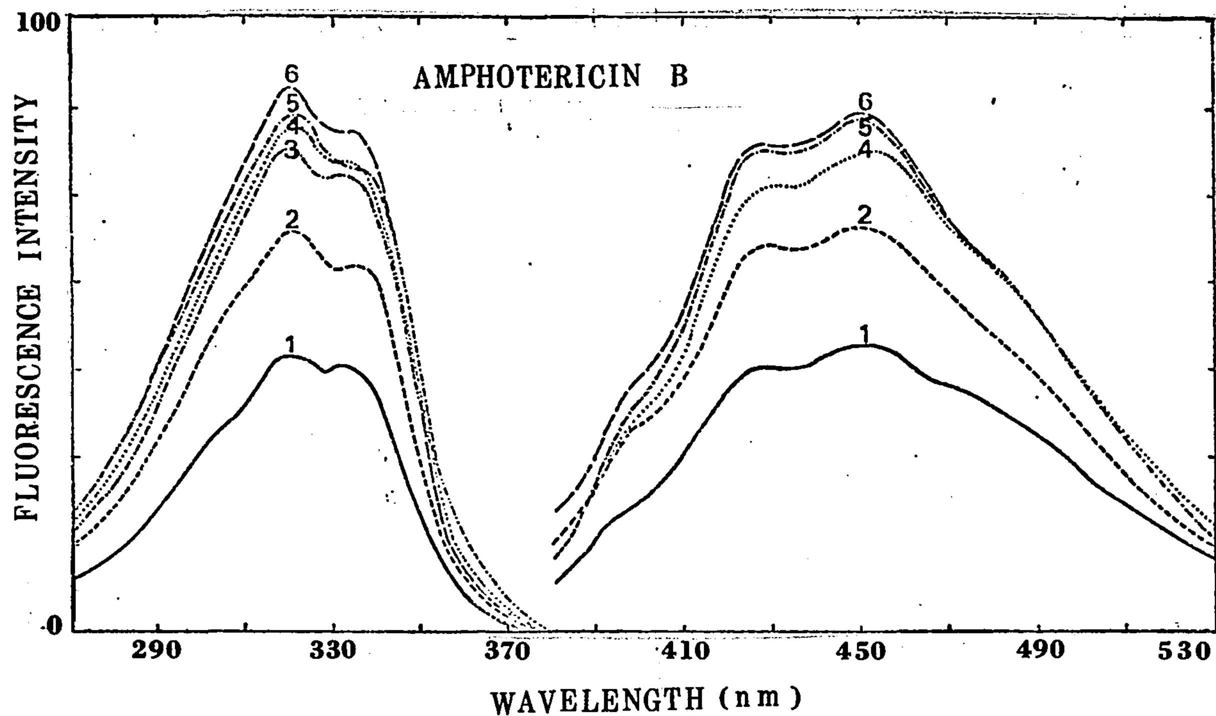


Figure 13. Fluorescence excitation (left) and emission (right) spectra of amphotericin B, and of amphotericin B in the presence of lecithin vesicles, lecithin-sterol vesicles at 7:3 molar ratio, and an aqueous suspension of cholesterol. The wavelengths of excitation and emission were 340 and 480 nm, respectively. The concentration of amphotericin B was 8.35 μM (except in curve 3). The total lipid concentration in the vesicles was 0.17 mM. The spectra shown represent amphotericin B in the presence of: 1, buffer; 2, lecithin-epicholesterol vesicles; 3, a suspension of 0.25 mM cholesterol in buffer (the amphotericin B concentration was 10.8 μM); 4, lecithin-cholesterol vesicles; 5, lecithin vesicles; 6, lecithin-ergosterol vesicles.

TABLE VI. Effect of Vesicles Derived from Phosphatidylserine, Digalactosyl Diglyceride, or Egg-Yolk Lecithin on the Fluorescence Polarization of Filipin. [a]

Agent Added to Filipin	Fluorescence Polarization
Buffer	0.017
Lecithin vesicles	0.045
Lecithin-cholesterol vesicles	0.315
Lecithin vesicles and Cholesterol*	0.216
Cholesterol aqueous suspension	0.170
Lecithin-epicholesterol vesicles	0.126
Lecithin vesicles and Epicholesterol*	0.122
Epicholesterol aqueous suspension	0.115
Phosphatidylserine vesicles	0.035
" -cholesterol vesicles	0.320
" -epicholesterol vesicles	0.091
Digalactosyl diglyceride vesicles	0.030
" -cholesterol vesicles	0.340
" -epicholesterol vesicles	0.080

[a] The excitation and emission wavelengths were 325 and 480 nm, respectively. The total lipid concentration in the vesicles derived from egg-yolk lecithin was 0.05 mM, and that in the phosphatidylserine or phosphatidylserine sterol vesicles was 0.15 mM. The total lipid concentration in the digalactosyl

(continued from TABLE VI)

diglyceride and digalactosyl diglyceride-sterol vesicles was 0.075 mM. The concentration of sterol in the vesicles or in aqueous suspensions were 0.025 mM, and the concentration of filipin was 4 μM. The molar ratio of lecithin to sterol was 1:1. The molar ratios of phosphatidylserine or digalactosyl diglyceride to sterol were 7:3.

* Sonicated lecithin vesicles were incubated for 24 hr at room temperature with equimolar amount of aqueous suspension of sterol (0.3% DMF).

D. Nuclear Magnetic Resonance

The effects of filipin on the chemical shift and integrated areas of the $N^+(CH_3)_3$ and $(CH_2)_nCH_3$ signals of lecithin in vesicles are reported in Table VII. At a total lipid concentration of 12.5 mM, changes in the integrated signals of the choline head group and methylene groups of lecithin were observed on interaction and lecithin-cholesterol vesicles with filipin at a molar ratio of lipid/antibiotic of 25:1 after 2 hr incubation as shown in Table VII. No nmr signals due to the polyene antibiotic were observed at concentrations of filipin as high as 0.5 mM in D_2O. The integrated area of the $N^+(CH_3)_3$ signal of lecithin vesicles was increased by 15% in the presence of filipin, whereas the area of the $(CH_2)_nCH_3$ signal increased by about 5%. The presence of cholesterol in vesicles at a lecithin/cholesterol molar ratio of 2:1 led to an 8% increase of the area of the $(CH_2)_nCH_3$ signal on introduction of filipin (at the cholesterol/filipin ratio of 8:1); in contrast, the area of the $N^+(CH_3)_3$ signal was decreased by 10%. The areas and chemical shifts of sonicated lecithin vesicles in D_2O agree with the results shown in the earlier reports (Chapman and Penkett, 1966; Darke et al., 1972).

TABLE VII. Effect of Filipin on the NMR Spectra of Lecithin and Lecithin and Cholesterol. [a]

		Lecithin			Lecithin-Cholesterol		
		without filipin	with filipin	% change	without filipin	with filipin	% change
$N^+(CH_3)_3$	cps	324	323	-0.3	323.5	323	-0.1
	area	82.5	95	+15	58.2	52	-10.3
$(CH_2)_nCH_3$	area	402.5	425	+5.7	209	225	+8.1

[a] The filipin concentration was 0.5 mM. The concentration of total lipid was 12.5 mM. The molar ratio of lecithin to cholesterol in vesicles was 2:1 and the molar ratio of cholesterol to filipin was 8. The chemical shift in cps was measured downfield from the internal standard of 0.13% silanor-D_2O-TSP (sodium-3-trimethylsilylpropionate-2,2,3,3,-d_4). The vesicles were sonicated for 20 min under N_2 at 4° and incubated with antibiotic for a minimum of 2 hr at room temperature in the dark.

II. Electron Microscopy

A. Filipin-Treated membranes

Electron micrographs of amphotericin B-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 advantages of negative staining for visualization of various lipid phase structures were discussed by Banham and Horne (1964), who suggested that this method preserved the structures as though they were in the presence of water. Filipin-treated vesicles prepared from lecithin alone or from a mixture of lecithin and epicholesterol, cholestanol, androstan-3β-ol and thiocholesterol looked essentially like the untreated vesicles (Figure 14), *i.e.*, no filipin-induced effect was observed. Figure 15 shows lecithin-ergosterol vesicles after treatment with filipin. "Pits" containing an electron-dense deposit of aqueous negative stain are present. A similar electron micrograph was found when lecithin-cholesterol vesicles were treated with filipin. The electron-dense center of each pit is surrounded by an electron-translucent annulus. The mean inner diameter of the annulus is 200±15Å and the mean outer diameter is 340±17Å. 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 (Figure 16, 17, and 18), resulting in

Figure 14. Untreated lecithin-ergosterol vesicles. Total lipid content in the vesicles was 1 mM. Lipid bilayers appear as white bands surrounded by black-stained aqueous spaces. This preparation and those shown in Figure 15 and 21 were stained with 2% (w/v) ammonium molybdate, pH 6.5. Marker=1000 Å.

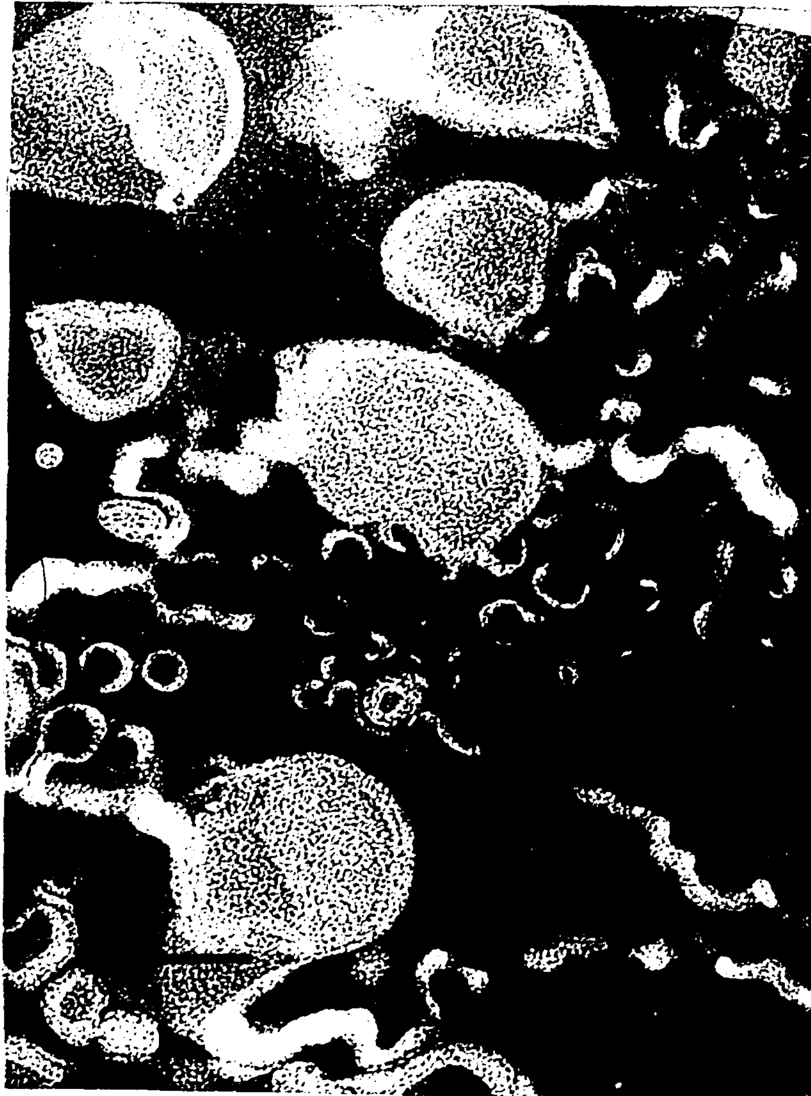


Figure 15. Lecithin-ergosterol vesicles treated with filipin at a final concentration of 12.7 µM. The total lipid content in the vesicles was 1 mM. The orderly array of lipid bilayers as presented in Figure 14 has been destroyed and annular hydrophobic bodies, "pits," appear within the thin film produced by lysis of the vesicle. Each annulus contains an electron-dense core. Marker=1000 Å.

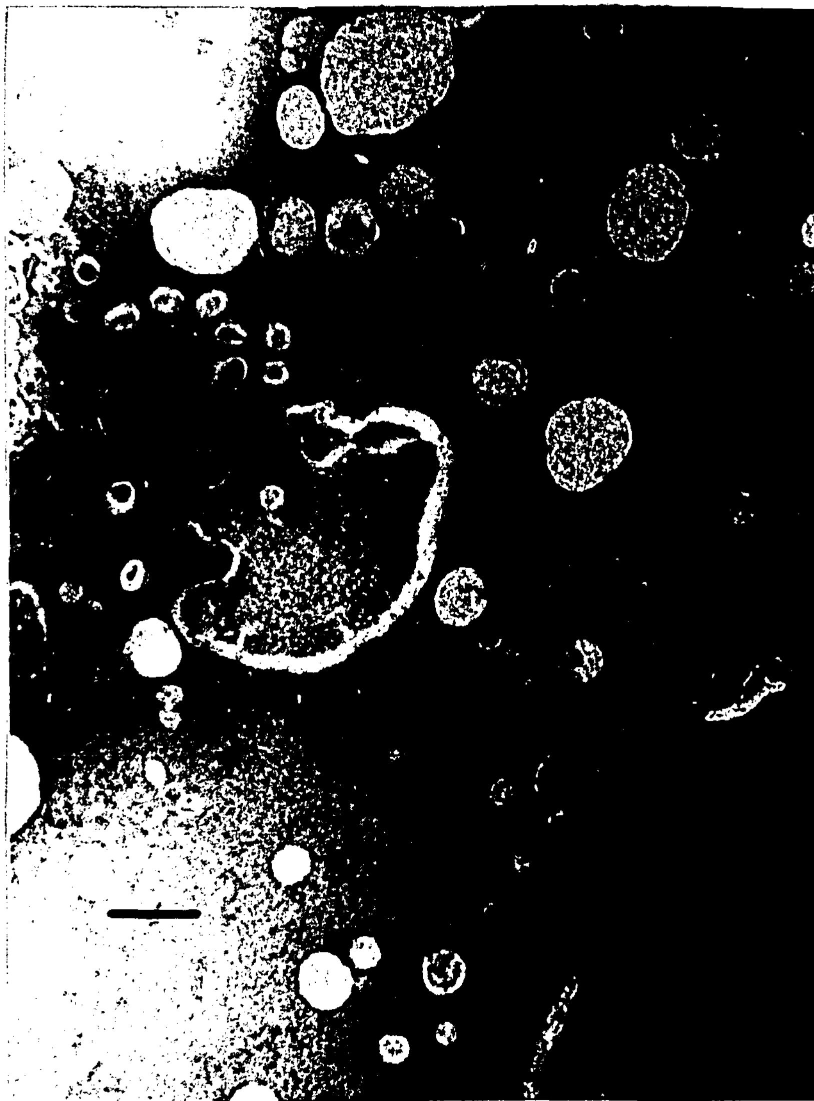


Figure 16. Lecithin-cholesterol vesicles treated filipin at a final concentration of 3.4 µM. The total lipid content in the vesicles was 1 mM. The orderly array of lipid bilayer as presented in Figure 18 has been destroyed. "Pits" appear within the thin film produced by lysis of the vesicles. Marker=1000 Å.

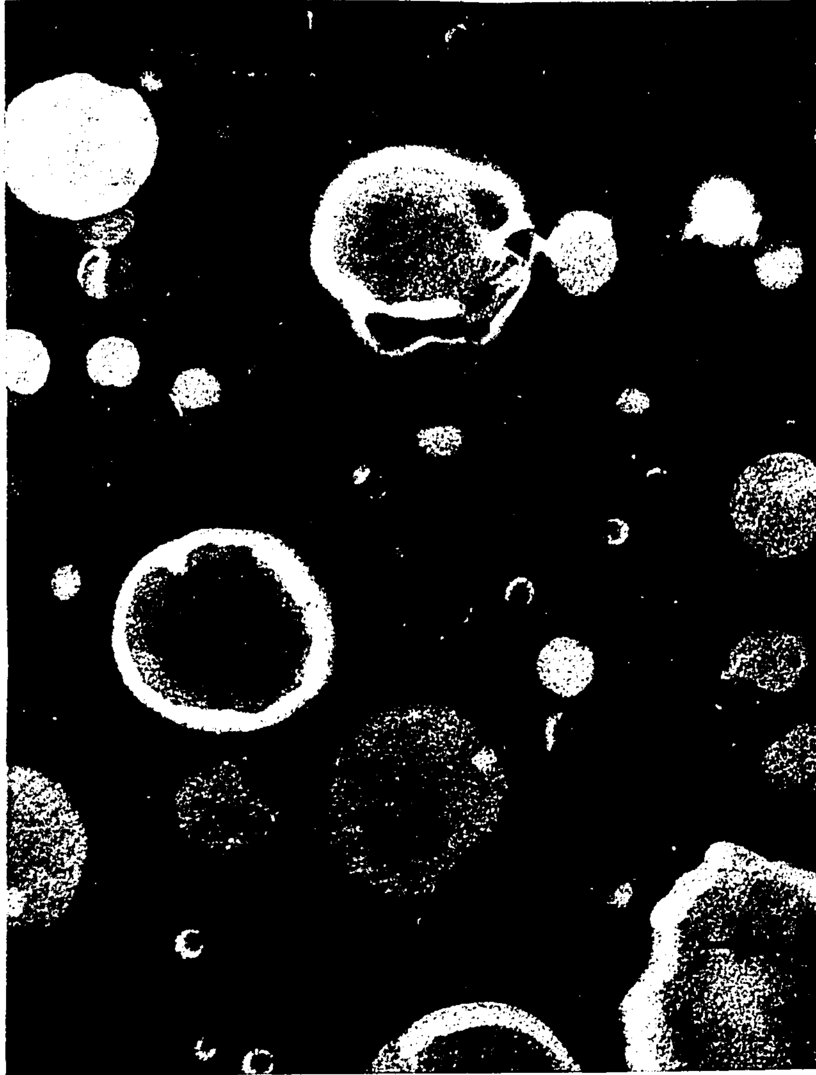


Figure 17. Lecithin-cholesterol vesicles treated with filipin at a final concentration of 1.9 μM. The total lipid content in the vesicles was 1 mM. Most vesicles were swollen, but few showed signs of complete disruption as presented in Figure 16. Marker=1000 Å.

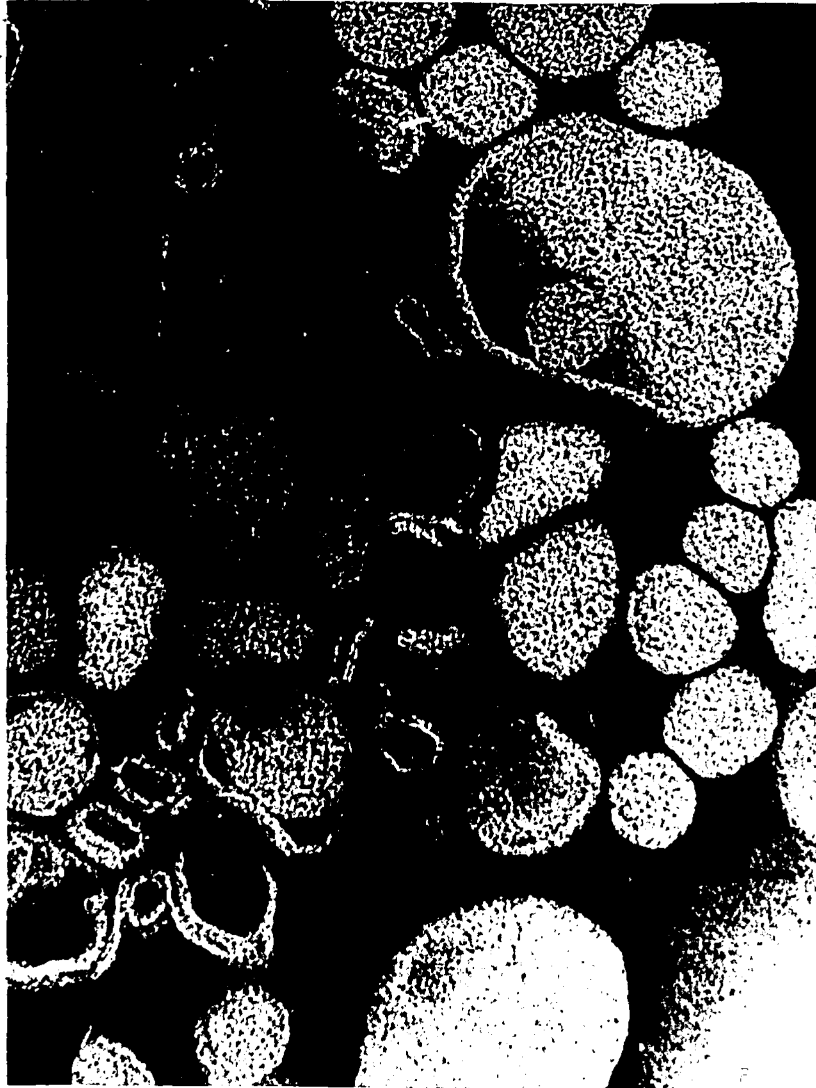


Figure 18. Lecithin-cholesterol vesicles treated with filipin at a concentration of 0.9 μM. The total lipid content in the vesicles was 1 mM. The molar ratio of lecithin to cholesterol was 7:3 (same as Figures 16 and 17). The orderly array of lipid bilayers was similar to untreated vesicles. Marker=1000 Å.

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 (Figure 17). Filipin 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 (Figure 18).

A preparation of ergosterol-containing membranes that is negative stained with phosphotungstic acid is shown in Figure 19. Following filipin treatment, membrane lysis and formation of electron-dense pits containing aqueous negative stain resulted (Figure 20). The pits are very similar to those observed in lecithin-ergosterol vesicles treated with filipin (Figure 15). The mean diameter of the pits in the lysed vesicles is almost identical to that in the Tetrahymena ciliary membrane. The mean inner diameter of pits in the ergosterol-containing ciliary membranes is 177 ± 32Å and the mean outer is 293 ± 32Å. Ciliary membranes containing tetrahymanol were not affected by amphotericin B or filipin.

B. Amphotericin B-Treated Membranes

Amphotericin B-treated vesicles do not exhibit lysis as was observed with filipin treatment. It was found that for amphotericin B-treated vesicles: (1) the peripheral concentric lipid bilayers of all of the treated vesicles are disorganized to form a homogeneous electron-translucent

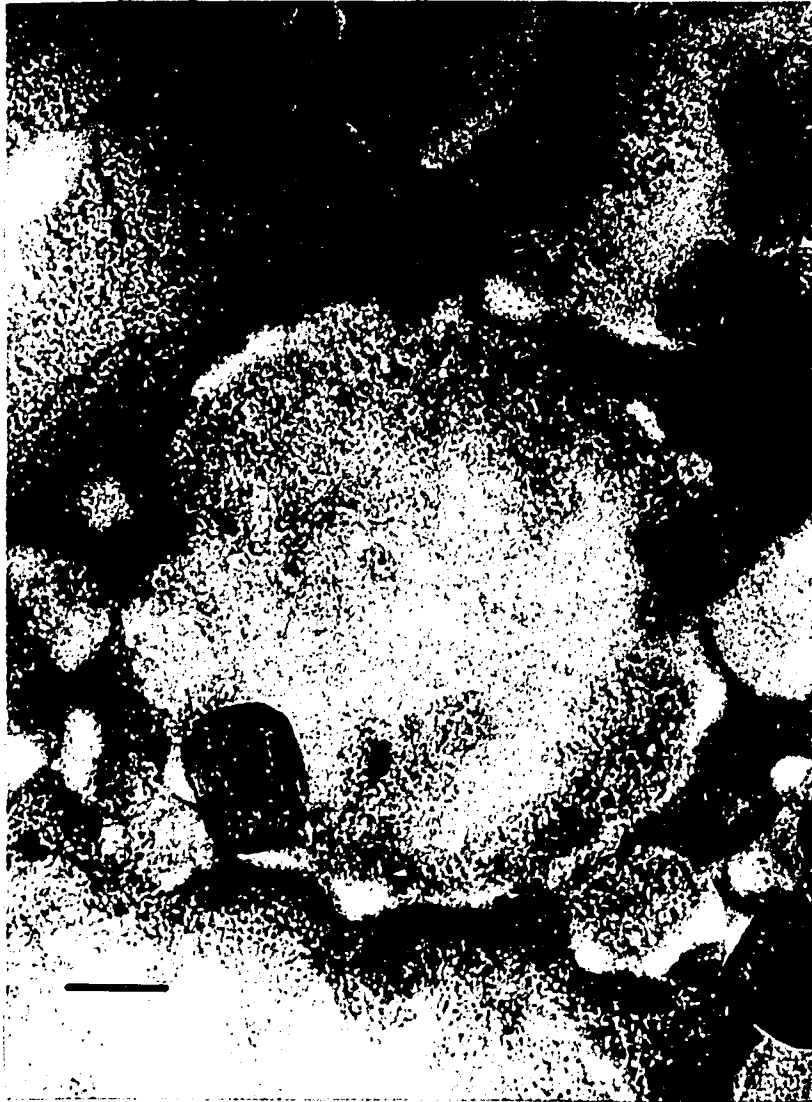


Figure 19. _Tetrahymena_ ciliary membrane fragment containing ergosterol. This preparation and the one shown in Figure 20 were stained with 2% (w/v) aqueous potassium phosphotungstate, pH 6.5. Marker=1000 A.

Figure 20. _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 15). Marker=1000 Å.

layer surrounding an inner electron-dense amorphous core; and (2) vesicle swelling was observed with vesicles prepared from lecithin alone, or lecithin and cholesterol or ergosterol. Figure 21 shows vesicles prepared from lecithin-ergosterol that were treated with amphotericin B. A homogeneous lipid boundary layer at the surface of the amhpotericin B-treated vesicles was observed. The effect of amphotericin B on lecithin-cholesterol vesicles is similar to that on lecithin-ergosterol vesicles. Diameter measurements were made to determine whether amphotericin B treatment caused vesicle enlargement. A summary of changes in vesicle diameter induced by amphotericin B treatment is presented in Table VIII. The major axis and minor diameter of each vesicle were measured. Table VIII lists the outer and inner core diameter along each axis. A t-test was performed to determine if statistically significant differences in mean diameter existed when amphotericin B-treated vesicles were compared to their controls. Statistically significant increases ($p < 0.05$) in mean outer and inner diameter were found when vesicles prepared from lecithin and from a mixture of lecithin and cholesterol or ergosterol were treated with amphotericin B. No statistically significant change was observed in lecithin-epicholesterol or lecithin-thiocholesterol vesicles that had been treated with amphotericin B. The increase in mean minor diameters is apparently a result of swelling induced by amphotericin B

Figure 21. Lecithin-ergosterol vesicles treated with amphotericin B 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 vesicles boundary bilayers has been disrupted to form a nonstratified lipoidal envelope. Marker=1000 Å.

TABLE VIII. Mean Diameters of Amphotericin B-Treated and Untreated Vesicles. [a]

	Lecithin Alone		Lecithin-Cholesterol		Lecithin-Ergosterol		Lecithin-Thiocholesterol		Lecithin-Epicholesterol	
Major Axis Outer Diameter	75	91*	105	180*	81	137*	124	122	95	100
Major Axis Inner Diameter	62	71*	86	156*	68	111*	88	95	85	74
Minor Axis Outer Diameter	48	67*	73	148*	52	97*	79	104*	66	73
Minor Axis Inner Diameter	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 B-treated vesicles. An asterisk indicates a statistically significant mean diameter increase ($p < 0.05$). For each entry the molar ratio of total lipid to amphotericin B 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.

treatment, yielding a more spherical body. Although there was no change in major diameter, the swelling results in an increase in minor diameter.

III. Equilibrium Binding Studies of Filipin III to Vesicles

In order to obtain quantitative information about the binding of filipin to vesicle-bound sterols, ultraviolet absorption and fluorescence polarization measurements were conducted at constant filipin concentration and varying lipid concentration. The ratio of bound filipin to total filipin concentration depends on the total lipid concentration of vesicles as shown in Figure 22. The concentration of bound filipin in the presence of vesicles was calculated from the absorbance at 358 nm (Figure 2).

$$C_B / C_T = \mathcal{E} - \mathcal{E}_F / \mathcal{E}_B - \mathcal{E}_F \qquad \text{(Eq.1)}$$

where C_T is the total concentration of filipin, C_B is the concentration of bound filipin, and $\mathcal{E}$, $\mathcal{E}_F$, and $\mathcal{E}_B$ are the extinction coefficients of the measured, free and bound forms of filipin. Eq.2 can be derived from Eq.1.

$$C_B = \frac{\mathcal{E}_F \cdot C_T - A/\ell}{\mathcal{E}_F - \mathcal{E}_B} \qquad \text{(Eq.2)}$$

where A is the measured absorbance and ℓ is the cell path-length.

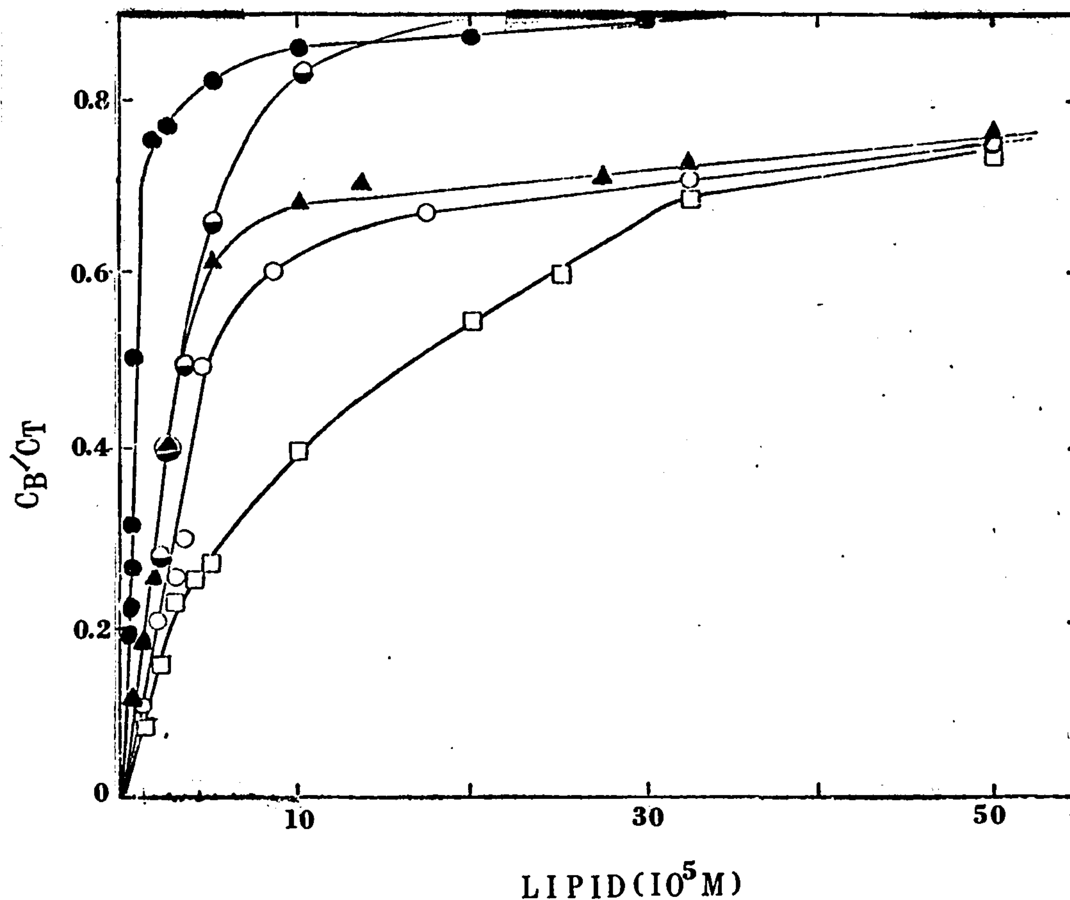


Figure 22. Dependence of the ratio of bound filipin / total filipin on the concentration of total lipid in vesicles. The total concentration of filipin was 12.5 μM. The concentration of bound filipin was determined from the absorbance at 358 nm as described in the text. Filipin and vesicles were allowed to stand overnight at 4° in the dark. The vesicles were sonicated for about 30 sec at 4° and were prepared from: □, lecithin; o, lecithin-epicholesterol in 7:3 molar ratio; lecithin-cholesterol in (▲), 7:1 molar ratio, and in(●) in 7:3 molar ratio; and ◒, lecithin-ergosterol in 7:3 molar ratio.

The extinction coefficient at 358 nm of bound filipin was calculated from the intercept of double-reciprocal plots of the absorbance of filipin in the presence of high lipid concentrations. Values of $\mathcal{E}_B$ (in the presence of various vesicles) were 4.5 x 10^4 (lecithin), 1.9 x 10^4 (lecithin-epicholesterol), 1.1 x 10^4 (lecithin-ergosterol) and 1.0 x $10^4 M^{-1} cm^{-1}$ (lecithin-cholesterol). Figure 22 shows that at low lipid concentration filipin binds to lecithin-cholesterol and lecithin-ergosterol vesicles, but higher lipid concentration are required to obtain appreciable conversion of filipin to the bound from in the presence of vesicles prepared from lecithin or from lecithin and epicholesterol.

A similar result is observed in fluorescence polarization studies (Figure 23). A binding isotherm can be calculated according to the method of Steinemann and Stryer (1973) from the absorbance data shown in Figure 22. Analysis of the fluorescence polarization data (Figure 23) by this method gave similar results. The concentration of unbound sterol was obtained by subtracting the concentration of bound filipin from the total sterol concentration, under the assumption that the molar ratio of filipin to sterol in the complex is 1. The equations can be derived if the concentration of cholesterol is much larger than the concentration of bound filipin, i.e., [cholesterol] $\gg C_B$.

For the equilibrium: cholesterol + filipin $\rightleftharpoons C_B$

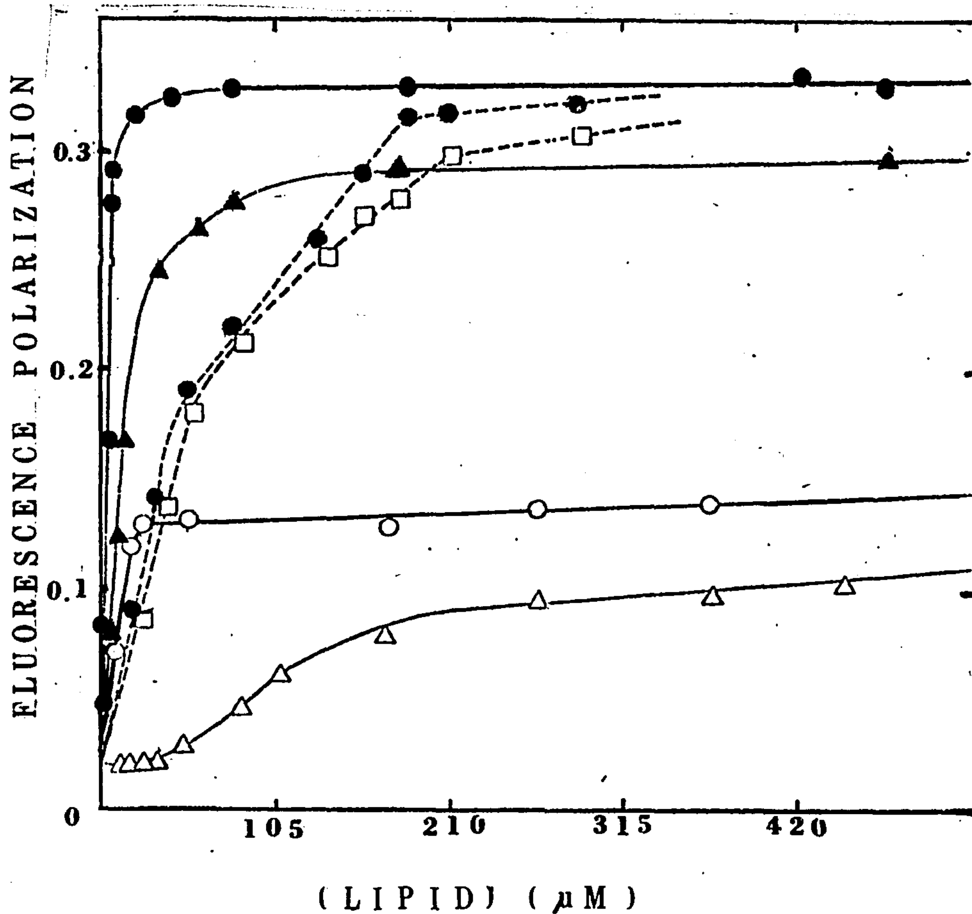


Figure 23. Dependence of fluorescence polarization of filipin on the concentration of total lipid in vesicles. The concentration of filipin was 5.2 μM. Filipin and vesicles were incubated for a minimum of 2 hr at room temperature in the dark. Vesicles were sonicated for about 30 sec at 4° and were prepared from: △, lecithin; ○, lecithin-epicholesterol in 7:3 molar ratio; and lecithin-cholesterol (▲) in 7:1 molar ratio, and (▲) in 7:3 molar ratio. The effects of aqueous suspensions of (□), epicholesterol, and (●), cholesterol are shown in dashed curves (data taken from Bittman and Fischkoff, 1972).

then,

$$K_D = \frac{C_F \cdot [Ch]}{C_B} \qquad \text{(Eq.3)}$$

where K_D is the dissociation constant, C_F and C_B are the concentration of free filipin and bound filipin, respectively, and Ch is the concentration of unbound cholesterol.

From Eq. 3: $C_F / C_B = K_D / [Ch]$ and $C_T / C_B = K_D / [Ch] + 1$ (Eq.4), where $C_T = C_F + C_B$. From a plot of C_T / C_B vs. $1 / [Ch]$ (Figure 24), K_D can be calculated from the slope. The y-intercept is close to 1.0, as expected from the derivation of Eq. 4. The standard deviation was minimized by the method of least squares. The dissociation constants that were obtained are shown in Table IX.

IV. Phase Transitions in Phospholipid Vesicles

The gel to liquid-crystalline phase transitions of vesicles of dipalmitoyllecithin, dimyristoyllecithin and sphingomyelin with and without cholesterol were studied by monitoring the fluorescence polarization of filipin embedded in the vesicles. The fluorescence polarization of filipin was strongly enhanced in the presence of vesicles derived from dipamitoyllecithin and sphingomyelin at room temperature (Figure 25). A smaller effect was found in the presence of vesicles derived from dimyristoyllecithin, slight effects were observed in vesicles derived from egg lecithin, phophatidylethanolamine and

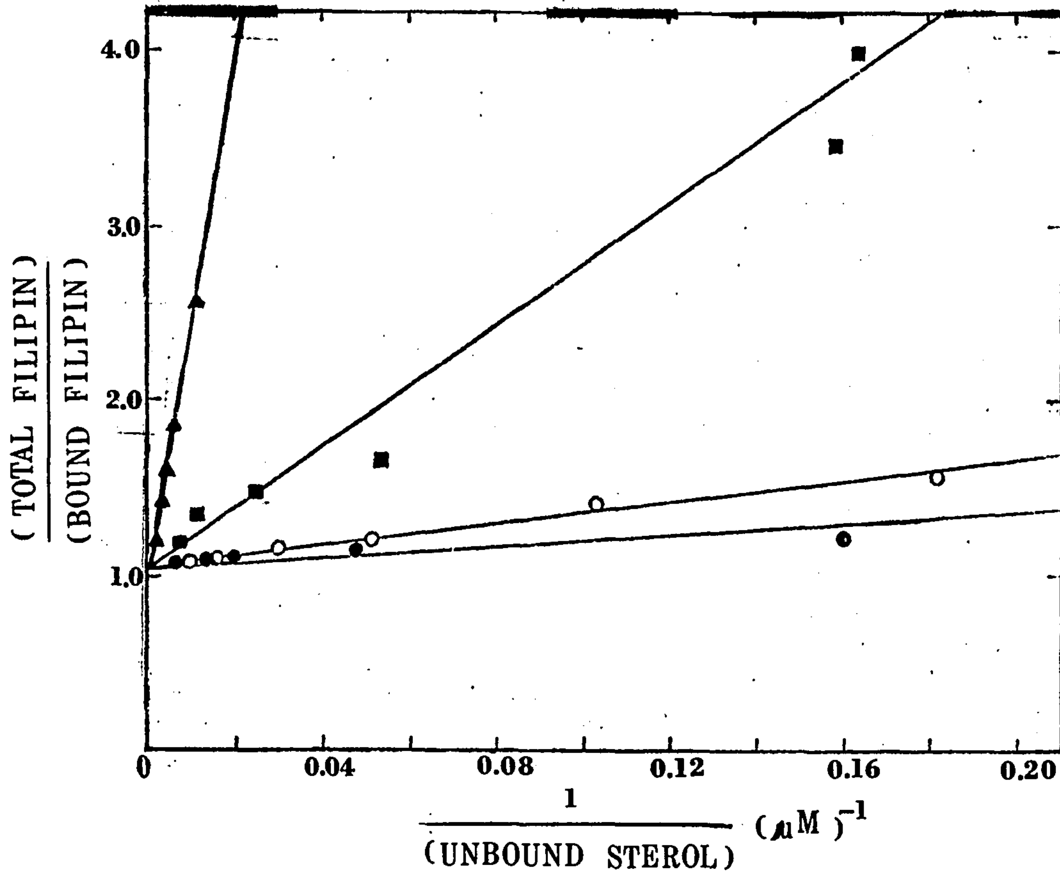


Figure 24. Determination of the binding constants of filipin with vesicles. The total concentration of filipin was 12.5 μM. The concentration of lipid (expressed in terms of sterol for sterol-containing vesicles and in terms of lecithin for vesicles prepared from lecithin alone) was varied. Data were obtained from Figure 22. The vesicles were prepared from:(▲), lecithin; (■), lecithin-epicholesterol;(○), lecithin-ergosterol; and (●), lecithin-cholesterol.

TABLE IX. Dissociation Constants (K_D) for the Binding of Filipin to Vesicles. [a]

Type of Vesicle	K_D (µM)
Lecithin	144 ± 24
Lecithin-Epicholesterol	13 ± 0.5
Lecithin-Ergosterol	2.47 ± 0.05
Lecithin-Cholesterol	0.80 ± 0.14

[a] For lecithin-sterol vesicles, K_D values are reported in terms of binding to the sterol (not to total lipid in the vesicle). K_D values were calculated from the slopes of the lines shown in Figure 24. The value of K_D given for lecithin-cholesterol vesicles is the average of the binding constants obtained from experiments using preparations of 7:3 and 7:1 molar ratios of lecithin to cholesterol. The molar ratio of lecithin to sterol in vesicles containing epicholesterol and ergosterol was 7:3.

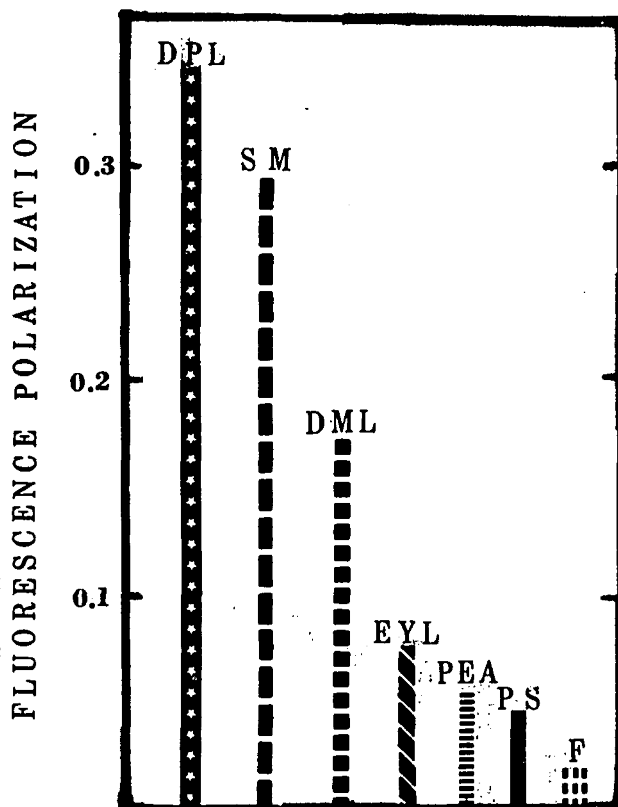


Figure 25. - Effect of phospholipid vesicles on the fluorescence polarization of filipin at 23°. The wavelengths of excitation and emission were 325 and 480 nm, respectively. The concentration of filipin was 4 µM. The total lipid concentration in the vesicles was 0.5 mM. The abbreviations are: dipalmitoyllecithin (DPL), dimyristoyllecithin (DML), sphingomyelin (SM), egg lecithin (EYL), phophatidylethanolamine (PEA), phosphatidylserine (PS) and free filipin (F). Vesicles were prepared by dispersing lipids into distill water above the transition temperature (Materials and Methods).

and phosphatidylserine . Figure 26 shows that the fluorescence polarization is decreased with increasing temperature. In the region 39-42° the decrease is pronounced with a midpoint of approximately 40.5^{o}. The effect of cholesterol on the fluorescence polarization of filipin in dipalmitoyllecithin and dimyristoyllecithin vesicles is shown in Figures 26 and 27. Addition of equimolar amounts of cholesterol abolishes the discontinuity in fluorescence polarization. A gradual decrease of fluorescence polarization is seen in cholesterol-containing vesicles with increasing the temperature. Figure 27 shows the decrease of fluorescence polarization of filipin in dimyristoyllecithin vesicles with increasing temperature. In the region 23-30° the decrease is pronounced with a midpoint of approximately 26.5^{o}. The transition temperature for vesicles derived from a mixture of equimolar amounts of dipalmitoyllecithin and dimyristoyllecithin was found to be 34-35° (Figure 28). This is about 7-8° higher than the main transition of dimyristoyllecithin and 6-7° lower than the main transition of dipalmitoyllecithin. The addition of an equimolar amount of cholesterol to the mixed phospholipids vesicles abolishes the discontinuity inthe fluorescence polarization of filipin (Figure 28). In vesicles prepared from equimolar amounts of egg lecithin and dipalmitoyllecithin, the midpoint was 30° (Figure 29). The phase-transition temperature for sphingomyelin vesicles was found to be approximately 39° (Figure 29). The light-scattering

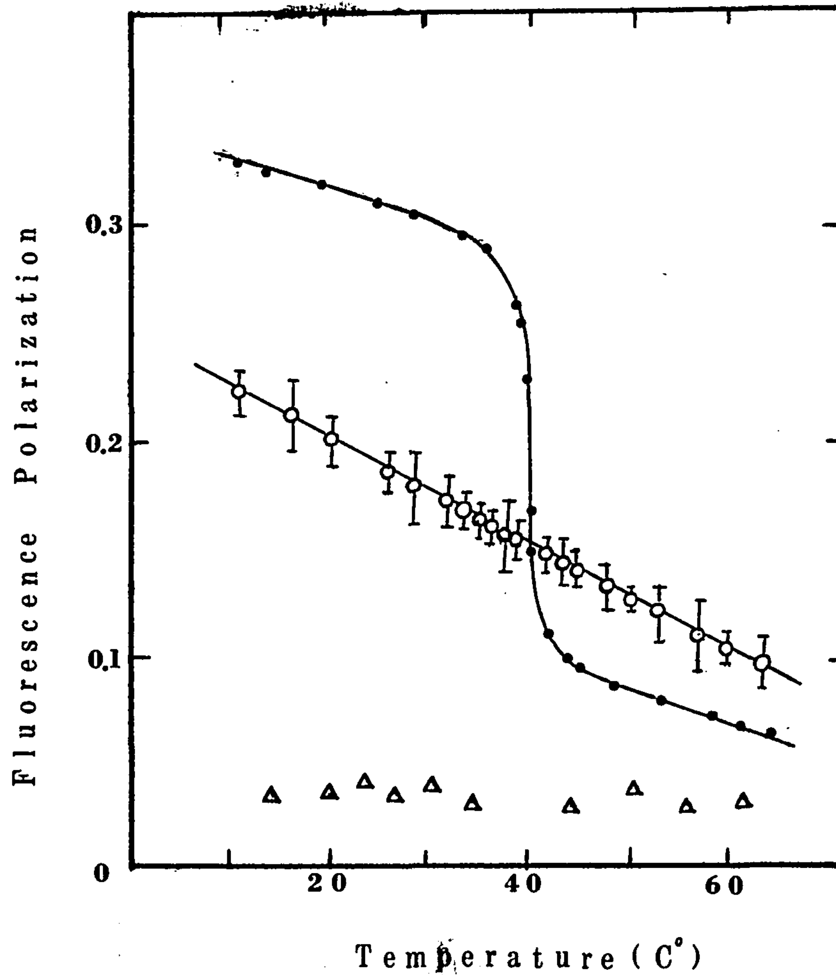


Figure 26. Effect of temperature on the fluorescence polarization of filipin in the presence of (•) dipalmitoyllecithin vesicles (0.5 mM) and (o) dipalmitoyllecithin-cholesterol vesicles (1 mM total lipid concentration, 1:1 molar ratio). The filipin concentration was 4 µM. The wavelengths of excitation and emission were 325 and 480 nm, respectively. Free filipin (△) did not show any alteration in fluorescence polarization with increasing temperature.

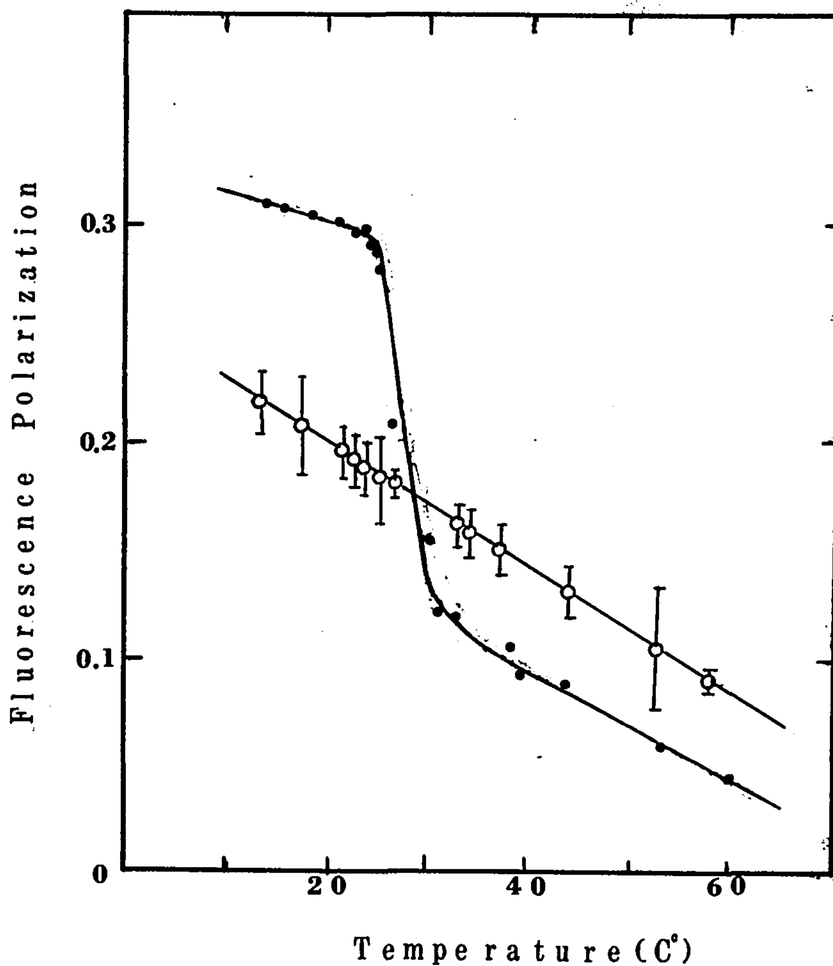


Figure 27. Effect of temperature on the fluorescence polarization of filipin in the presence of (•) dimyristoyllecithin vesicles (0.5 mM) and (o) dimyristoyllecithin-cholesterol vesicles (1 mM total lipid concentration, 1:1 molar ratio). The filipin concentration was 4 μM.

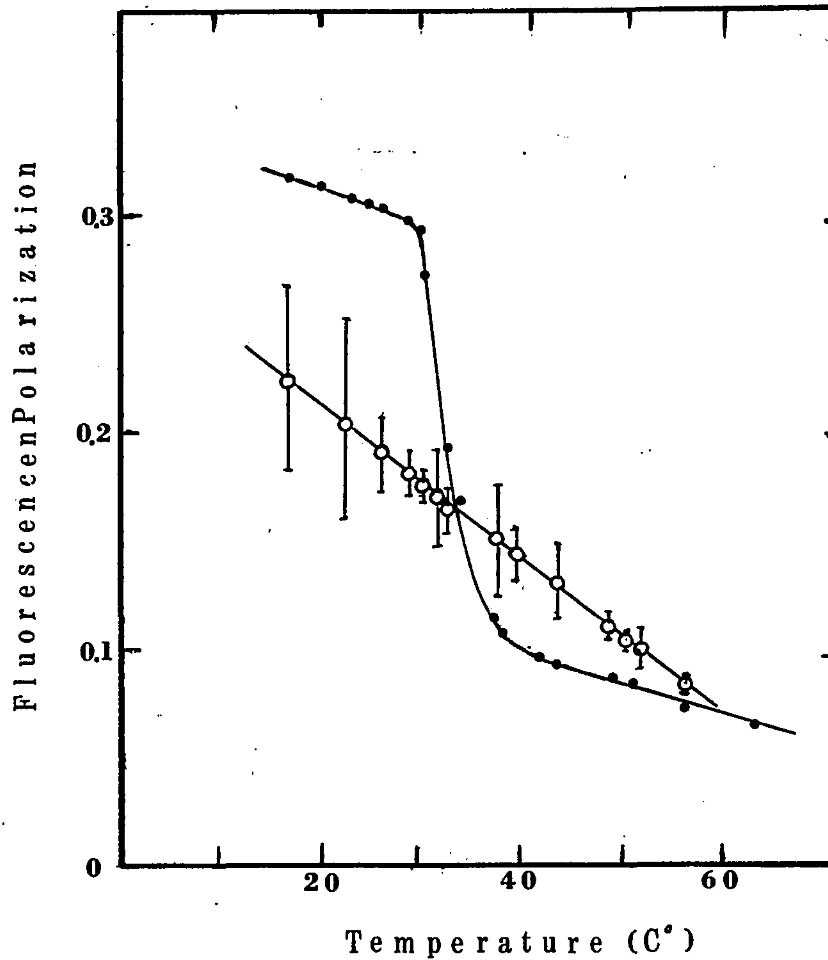


Figure 28. Effect of temperature on the fluorescence polarization of filipin in the presence of (•) dipalmitoyllecithin-dimyristoyllecithin vesicles (0.5 mM total lipid concentration, 1:1 molar ratio) and (o) dipalmitoyllecithin-dimyristoyllecithin-cholesterol vesicles (1 mM total lipid concentration, 1:1:2 molar ratio). The concentration of filipin was 4 μM.

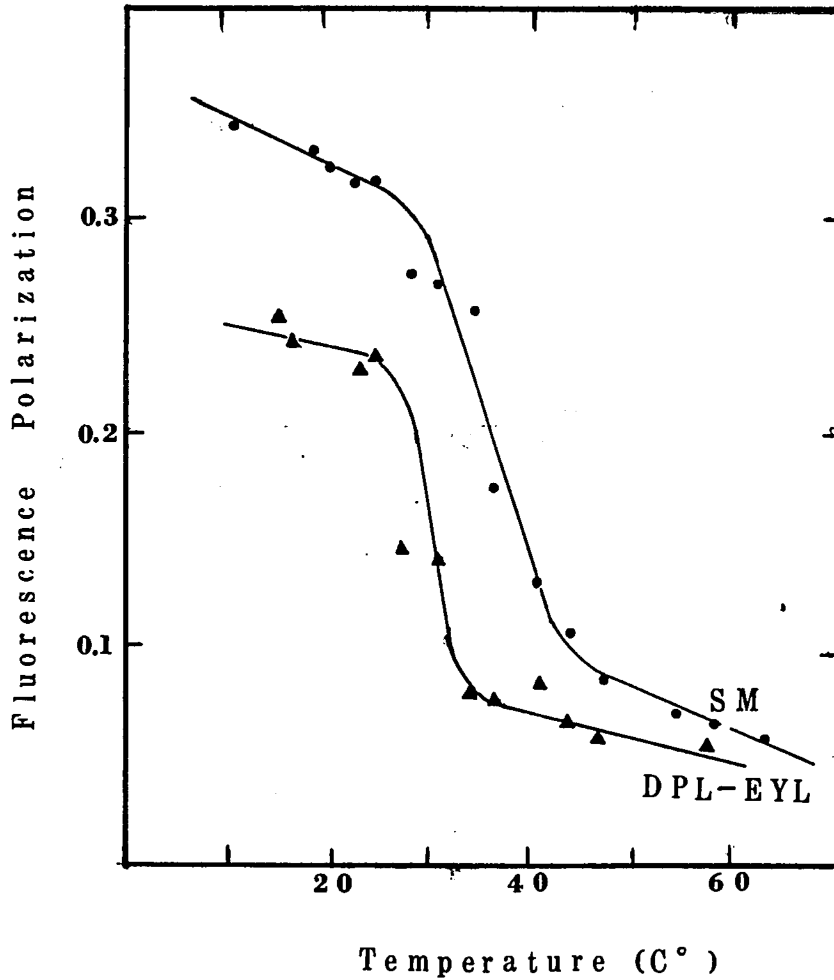


Figure 29. Effect of temperature on the fluorescence polarization of filipin in the presence of vesicles prepared from a mixture of (▲) dipalmitoyllecithin-egg lecithin(DPL-EYL) and from (●) sphingomyelin(SM). The molar ratio of DPL to EYL was 1:1. The total lipid concentration in the vesicles was 0.5 mM. The concentration of filipin was 4 μM.

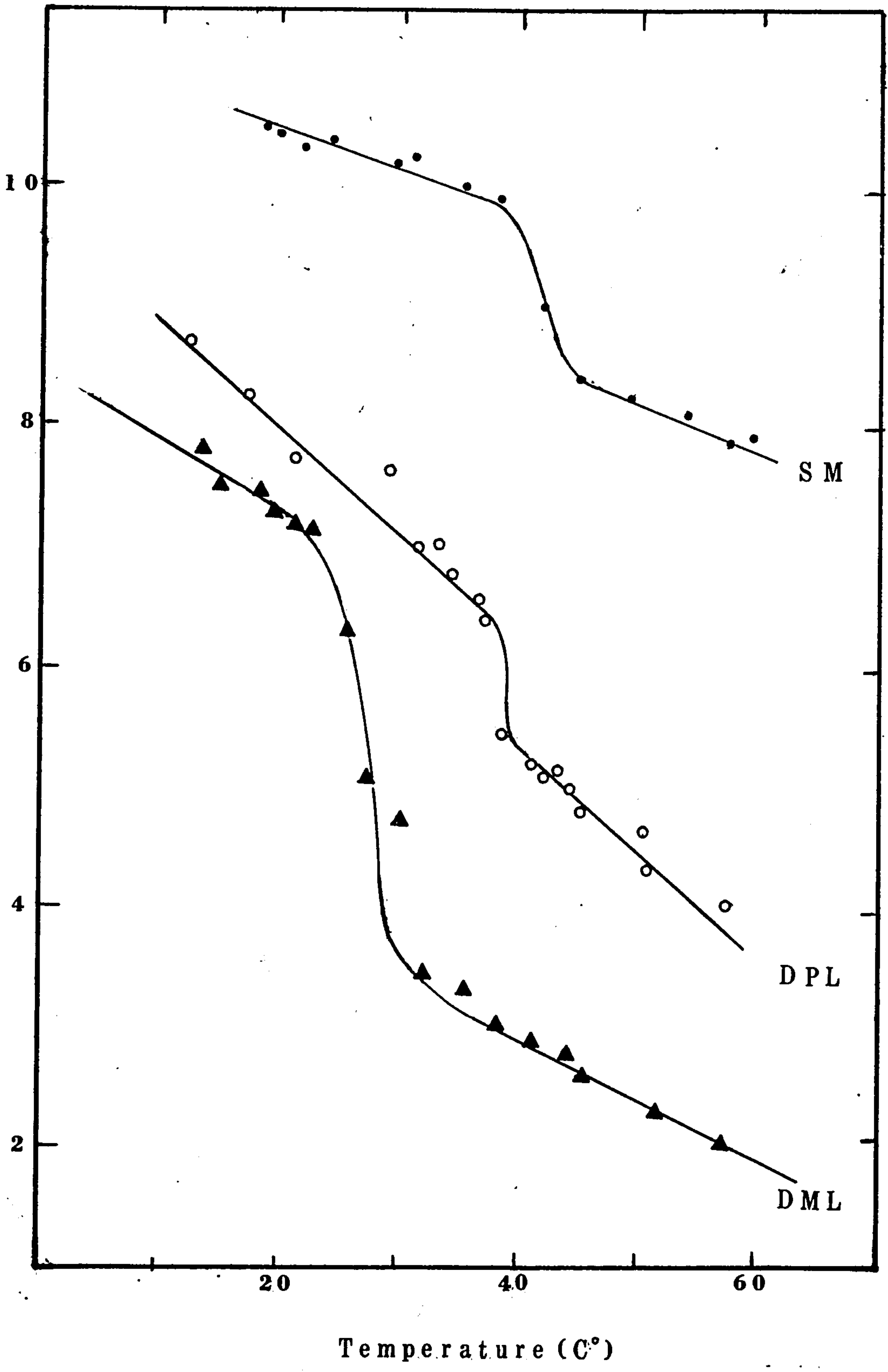
measurements on vesicles devoid of filipin were recorded at excitation and emission wavelengths of 325 and 480 nm, respectively. The intensity due to light scattering decreases as the vesicles undergo the gel to liquid-crystalline phase transition. Transition temperatures calculated from light-scattering measurements were found to agree with the results obtained from changes in the fluorescence polarization of filipin (Figure 30). The fluorescence polarization of free filipin does not change when the temperature is increased (Figure 26).

V. Aggregation of Polyene Antibiotics in Aqueous Solution

Many polyene antibiotics appear to be self-associated in aqueous media (Lampen et al., 1960; Schaffner and Mechlinski, 1972). The deviation of the absorbance of filipin from Beer's law in aqueous media or in the presence of lecithin-cholesterol vesicles (Figure 31) suggests self-association. Beer's law is obeyed when the concentrations of filipin are below 1 µM in aqueous buffer and below 10 µM in the presence of lecithin-cholesterol vesicles.

A plot of the absorbance peak ratios at 335 and 385 nm against the concentration of amphotericin B (inset, Figure 32) shows the deviation from Beer's law. The absorption spectra of monomer and aggregate are shown in Figure 32. The absorption maximum of the aggregate at an amphotericin B concentration of 10 µM is 335 nm, whereas the absorption maxima of the monomer at a concentration of 1 µM are 385 and 415 nm.

Figure 30. Effect of temperature on the relative light scattering intensities of phospholipid vesicles. Vesicles were prepared from (●) sphingomyelin(SM), (○) dipalmitoyllecithin(DPL) and (▲) d myristoyllecithin(DML). The total lipid concentration in the vesicles was 0.5 mM. Light scattering was measured with excitation and emission monochromators set at 325 and 480 nm, respectively, with both polarizers set at 0°. The light scattering intensities are given in arbitrary units. The concentration of filipin was 4 µM.



Light Scattering Intensity (0°—0°)
10
8
6
4
2
20
40
60
Temperature (C°)
SM
DPL
DML

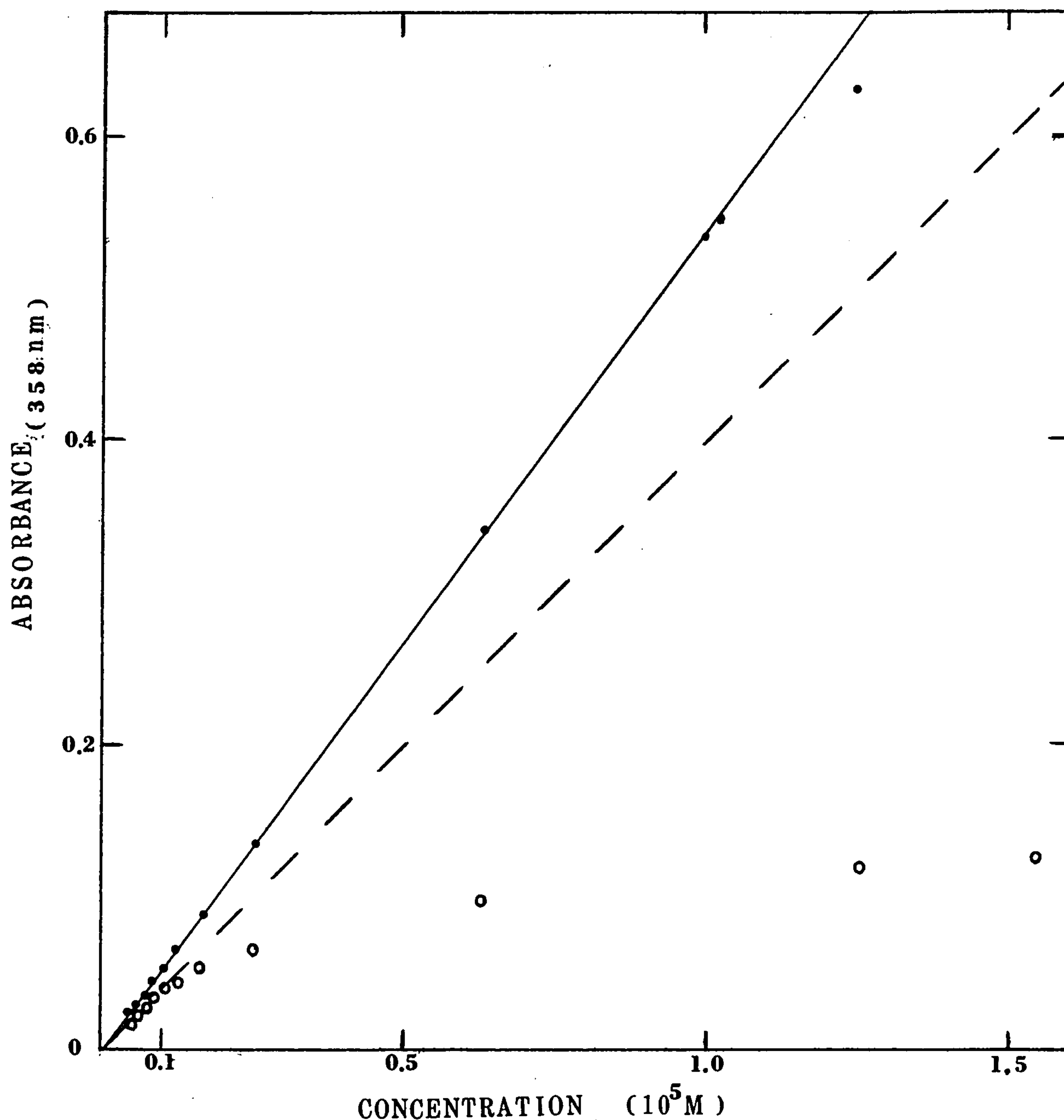
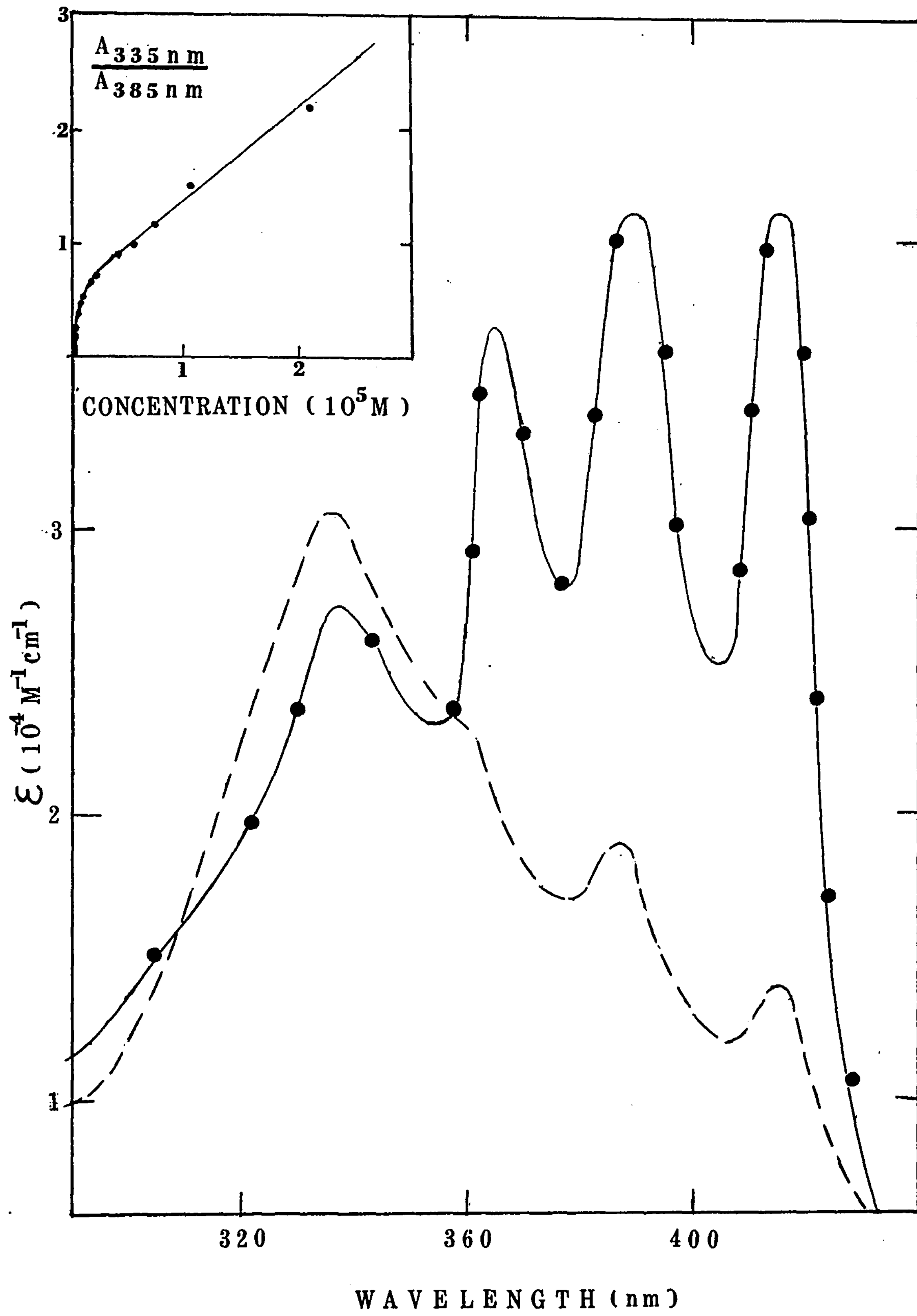


Figure 31. Deviation from Beer's law of (●) filipin, and (○) of filipin in the presence of lecithin-cholesterol vesicles in water. X-axis = concentration of filipin. Y-axis = absorbance peak of filipin at 358 nm. The pathlengths used were 1 to 10 cm. The molar ratio of lecithin to cholesterol was 7:3. The molar ratio of total lipid to antibiotic was kept constant at 40. The absorbance at 358 nm was corrected for light scattering of vesicles. The time of incubation for mixing the vesicles and antibiotic was at least 2 hr. The absorbance of free filipin was taken promptly after preparation of the antibiotic solution.

Figure 32. Absorbance spectra of amphotericin B in water at concentrations of (----) 10 μM and (—•—•—) 1 μM. Pathlengths of 1 cm and 10 cm were used. A plot of absorbance peak ratios vs. concentration of amphotericin B is shown in the inset. The spectra of amphotericin B were taken at room temperature promptly after the preparation of the antibiotic solution.



A_{335nm} / A_{385nm}
CONCENTRATION (10^5M)
ε (10^{-4} M^{-1}cm^{-1})
WAVELENGTH (nm)
320
360
400
1
2
3

The self-association of amphotericin B in aqueous solution can be disrupted by increasing the content of organic solvent, e.g., methanol (Figures 33 and 34), or increasing the temperature of the solution (Figure 35). The absorption spectra of amphotericin B in organic solvent and in aqueous solution at high temperature are similar to the absorption spectrum of the monomer (Figure 32). The ellipticity of amphotericin B at 347 nm in aqueous solution was decreased dramatically when the concentration of methanol increased (Figure 34A). Similar results were observed when DMF and dioxane were used. The ellipticities of amphotericin B are decreased in the presence of methanol, lecithin-cholesterol vesicles, lecithin-epicholesterol vesicles, and cholesterol aqueous suspension (Figure 36). The concentration-dependent feature of molecular ellipticity of amphotericin B is shown in Figure 36 (inset).

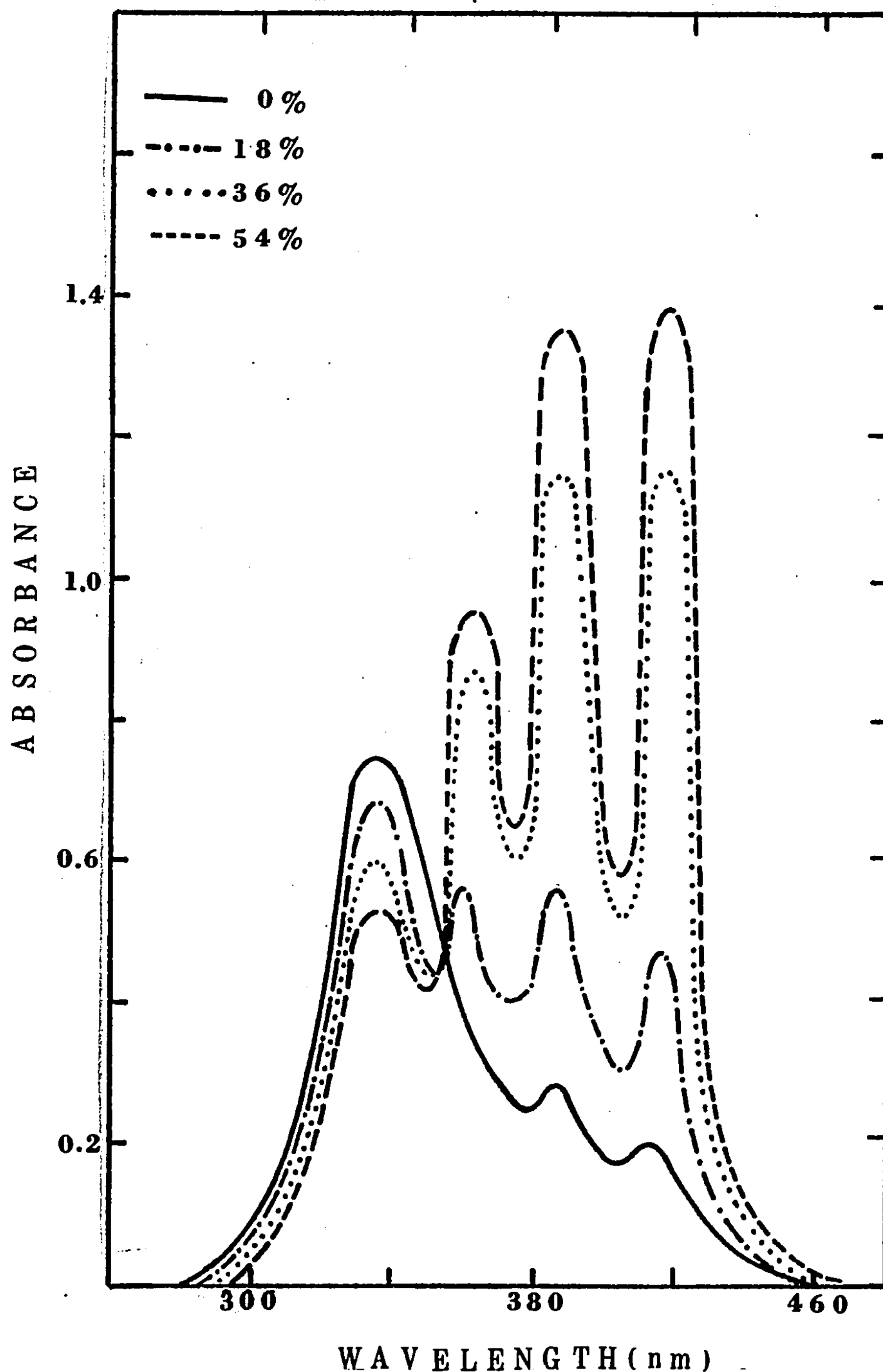


Figure 33. Effect of methanol on the absorption spectra of amphotericin B in water. No significant alteration on the spectra of amphotericin B was observed in the presence of methanol below 5% (v/v). The concentration of amphotericin B was 28 μM.

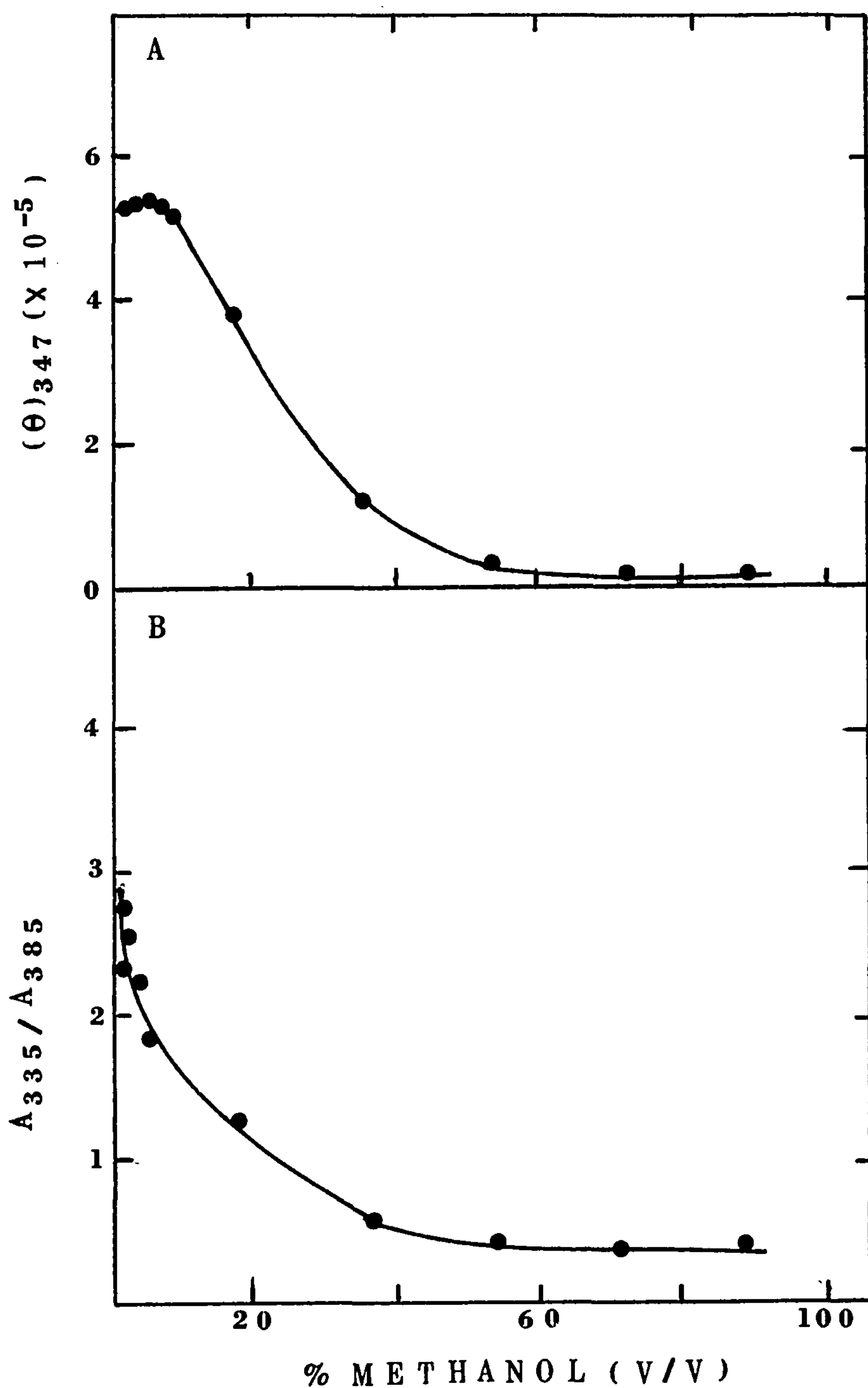


Figure 34. Effect of methanol on the molecular ellipticity $[\theta]_{347}$, and absorption peak ratios, A_{335} / A_{385}, of amphotericin B. The concentrations of amphotericin B were 45 μM in A and 28 μM in B.

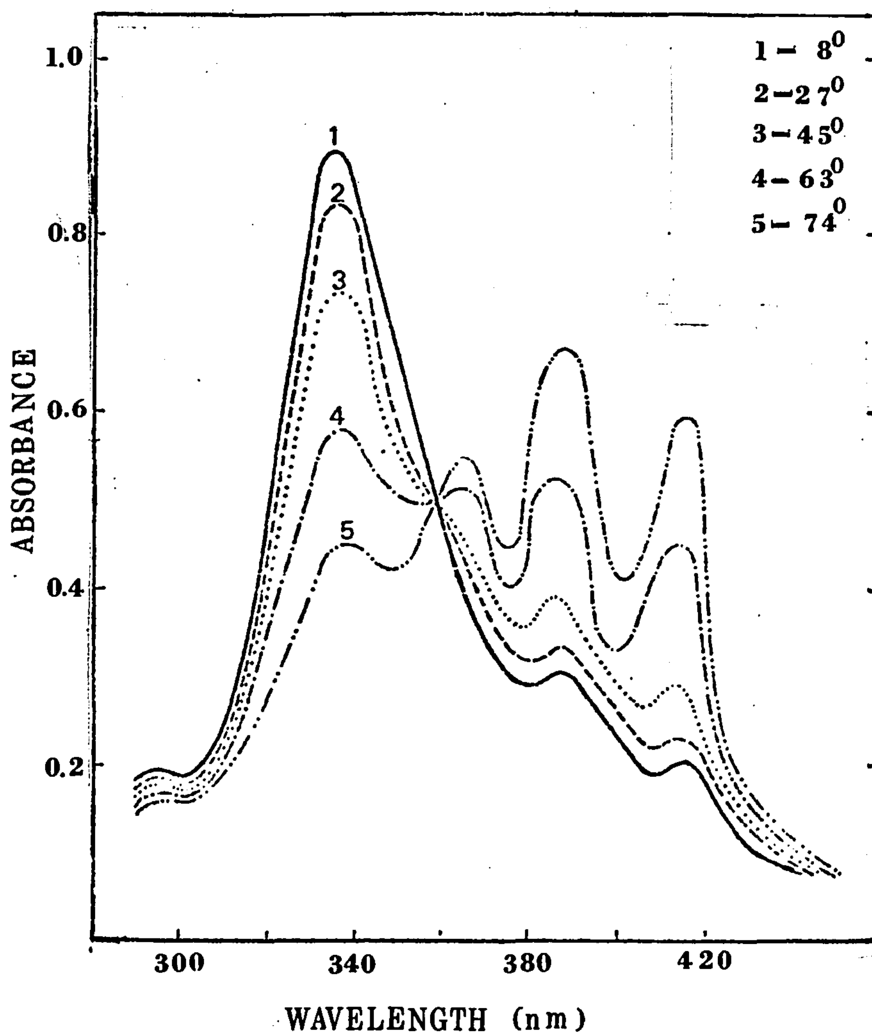


Figure 35. Effect of temperature on the absorption spectrum of amphotericin B in water. The concentration of amphotericin B was 30 μM. The spectra were taken in freshly preparation.

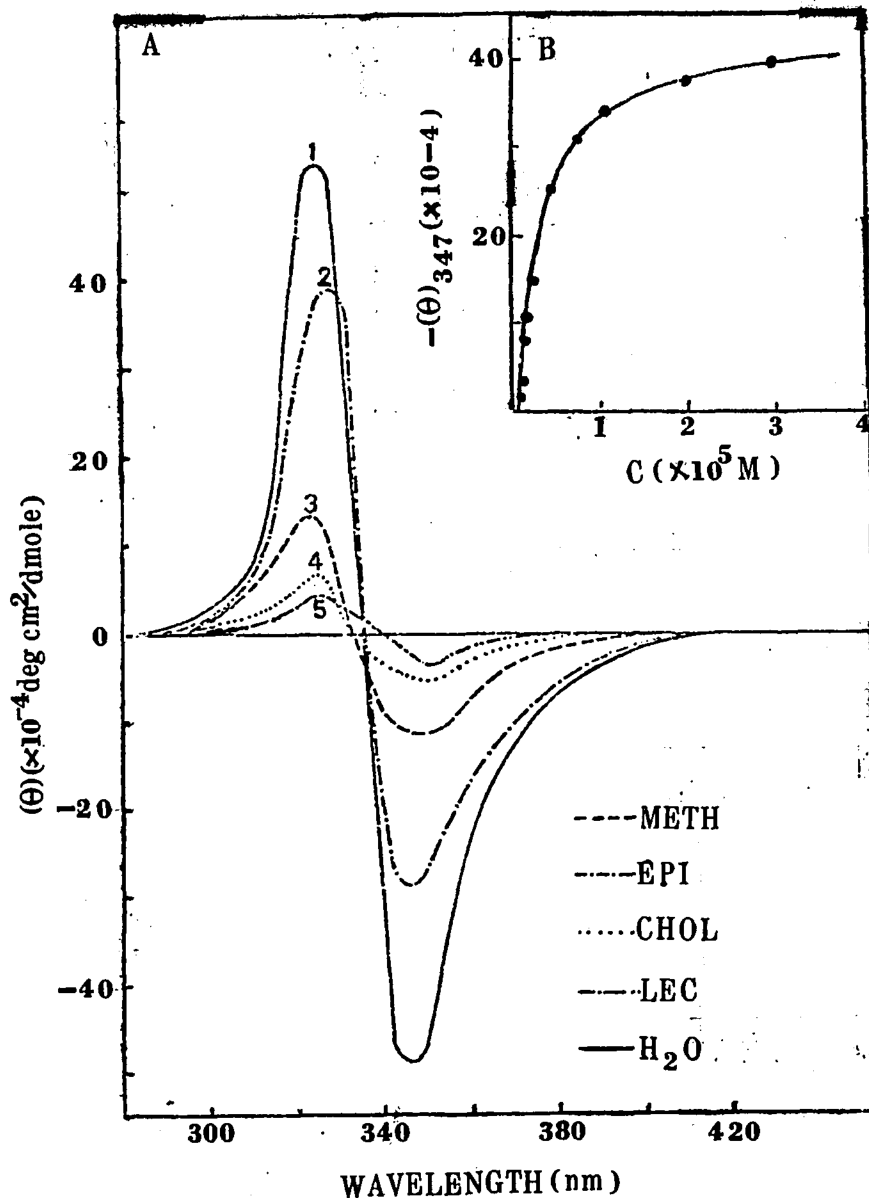


Figure 36. A. CD spectra of amphotericin B in the presence of: 1, H_2O; 2, aqueous suspension of epicholesterol; 3, 36% methanol in water (v/v); 4, aqueous suspension of cholesterol; 5, lecithin vesicles. The spectra were taken about 30 min after the preparation of the antibiotic solutions. The concentration of amphotericin B was 45 µM. The total lipid concentration in lecithin vesicles was 1.5 mM; the sterol concentration in the aqueous suspensions was 0.25 mM.
B. Concentration dependence of the molecular ellipticity of amphotericin B at 347 nm in H_2O. DMF was present in the aqueous suspensions of sterols at a concentration of 0.3% by volume.

DISCUSSION

A comparision of the results gathered by both spectrophotometric and electron microscopic techniques with those obtained from monolayer studies on the action of polyene antibiotics is of considerable interest. The interaction of filipin with steroids was found to be related both to the functional group at the 3 position and the hydrocarbon chain of the steroid (Kleinschmidt et al., 1972; Norman et al., 1972b; Bittman and Fischkoff, 1972). Formation of a steroid-filipin complex has been correlated with the ability of filipin to increase the permeability of the liposomes (Weissmann and Sessa, 1967; Sessa and Weissmann, 1968). From ultraviolet studies of the filipin-sterol complex, Norman et al. (1972b) concluded that for free steroid in water the interaction is primarily hydrophobic and the presence of a cholesterol ring structure and Δ-22 double bond produces an optimal interaction between filipin and steroids. Previous results indicated that the absolute concentration of filipin was important for the interaction with vesicles. For example, at 10^{-3}-10^{-5}M, filipin interacted equally well with liposomes whether or not cholesterol was present (Weissmann and Sessa, 1967). In contrast, other reports indicated a requirement for the presence of cholesterol for interaction at filipin concentrations of

10^{-5}-10^{-6}M (Kinsky _et al._, 1968; Norman _et al._, 1972a). At 10^{-2}-10^{-4}M filipin, the antibiotic is able to interact with monolayers of pure lipid in the absence of cholesterol (Demel _et al._, 1968). In an earlier investigation, it was demonstrated with nystatin, amphotericin B and pimaricin that both the rate and extent of hemolysis was dependent on antibiotic/cell ratio (Kinsky, 1963). The hemolytic action of filipin was also affected by the antibiotic/erythrocyte ratio (Kinsky _et al._, 1967a). From a plot of absorbance peak ratio differences against the molar ratio of lipid to filipin (Figure 3), the relative concentration of lipids, especially sterol concentration, are seen to be important parameters in the interaction.

Alteration in ultraviolet absorption or the ratios of extinction coefficients (Figure 2 and Tables I and II) suggests that filipin binds preferentially to vesicles containing cholesterol or to ciliary membranes containing ergosterol. Previous results of fluorescence polarization studies showed that filipin did not interact significantly with lecithin vesicles containing epicholesterol, androstan-3β-ol or thiocholesterol (Bittman and Fischkoff, 1972). In ultraviolet absorption studies, no spectral changes were evident in cells or membranes obtained from _Mycoplasma laidlawii_ grown in the absence of a sterol or in the presence of epicholesterol. Also, a significant increase in surface pressure was found when filipin was injected into

the subphase of lipid layer containing lecithin and cholesterol, or lecithin and stigmasterol; only a small increase in surface pressure of lecithin and epicholesterol monolayers was observed upon filipin treatment (Norman *et al.*, 1972b).

As cited in the Introduction, the ultraviolet chromophore of filipin molecule consists, in part, of five conjugated double bonds. Filipin has a highly characteristic ultraviolet absorption spectrum in water in the region between 280-380 nm (Figure 2). A slight shift in wavelength but not change in the ratios of extinction coefficients was found after transfer of filipin from the aqueous to an organic solvent such as DMF and dioxane. The micellar or aggregated form of filipin in aqueous solution probably was not altered significantly by the organic solvent. In circular dichroic studies, on the other hand, the molecular ellipticities of filipin were enhanced dramatically in organic solvents (Figure 5), and a significant wavelength shift was observed in the negative ellipticity bands of filipin as shown in Figure 5. This kind of alteration in rotational strength of filipin in organic solvents implies that solute-solvent interaction may have occurred.

Filipin in organic solvents follows Beer's law over a wide range of concentrations, whereas filipin in aqueous solution does not (Figure 31). Beer's law is obeyed when the concentration of filipin is below 10 μM. In the presence of lecithin-cholesterol vesicles, deviations

from Beer's law occurred at concentrations of filipin higher than about 1 μM, presumably because the association of filipin with lecithin-cholesterol vesicles caused a distortion in the chromophore of filipin, therefore reducing the extinction coefficient of filipin in aqueous solution (Figure 31).

The results cited above indicated that the interaction of the polyene with vesicles is sterol-dependent (spectral studies). When filipin is added to cholesterol in organic solvents, no change in spectrum occurs, but when filipin is added to cholesterol in aqueous solution, a dramatic alteration in the spectrophotometric properties is observed. Several investigators have indicated that this kind of alteration could be reversed by organic solvents (Lampen _et at_., 1960; Gottlieb _et al_., 1961; Bittman and Fischkoff, 1972), but could not reversed by sucrose, galactose, bovine serum albumin, 4M NaCl, 4M urea or variation of the pH from 2-9 (Norman _et al_., 1972b). These results suggest that hydrophobic forces are primarily responsible for the formation of the complexes.

Despite the existence of many optically active centers in the vicinity of the chromophore, filipin in buffer has a weak rotational strength. Two possible explanations may be reached: (a) the interaction between filipin molecules in the aggregate may lead to a partial cancellation of contributions to the circular dichroism from each of the chromophores, or (b) a conformational change of

the macrolide ring of filipin may have occurred on exposure to organic solvents such as DMF and adioxane, and cholesterol in aqueous suspension or vesicles. Results gathered by measuring the molecular ellipticities of filipin in a series of mixed micelles (or aggregate) composed of varying concentrations of filipin and perhydrofilipin could distinguish between these two explanations. It is assumed that the conformation of the filipin macrolide ring is the same in the pure filipin micelle (or aggregate) as in the mixed filipin-perhydrofilipin micelle (or aggregate). Therefore, if an increase in the molecular ellipticity of filipin upon dilution with perhydrofilipin were found, then the possibility that stacking of molecules in the self-associated filipin aggregate in aqueous solution gives rise to the diminished ellipticity can be established. On the other hand, if the molecular ellipticity were to remain unchanged upon dilution with perhydrofilipin in the mixed micelle, the second possibility could be a plausible explanation for the increase in molecular ellipticity observed in the presence of DMF, dioxane and aqueous suspensions of cholesterol. The results show that the molecular ellipticity of filipin is unaffected by dilution with perhydrofilipin. This suggests that cholesterol in aqueous suspension or in vesicles, and organic solvents such as DMF and adioxane induce a conformational change of the macrolide ring of filipin.

The fluorescence spectra (Figures 10 and 11) and polarization measurements (Tables V and VI) show that filipin

interacts significantly with cholesterol-, epicholesterol-, and ergosterol-containing vesicles which are derived from digalactosyl diglyceride, phosphatidylserine and ciliary phospholipids. A previous report indicated that the fluorescence polarization of filipin was strongly enhanced in lecithin vesicles containing cholesterol and ergosterol (Bittman and Fischkoff, 1972). The electron microscopy study indicates that filipin did not cause lysis of lecithin-cholestanol vesicles at polyene concentrations that caused lysis of cholesterol- and ergosterol-containing vesicles. Furthermore CD spectra showed that the molecular ellipticities of filipin did not increase significantly in the presence of lecithin-cholestanol vesicles. This is not in agreement with the results shown in ultraviolet absorption measurements of filipin (Table II), which show that the effect of lecithin-cholestanol vesicles on the polyene are as strong as the effect of lecithin-ergosterol vesicles. CD and EM studies also do not agree 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).

Bittman _et al._(1974b) found that the initial rate of association of filipin with aqueous suspensions of cholesterol devoid of phospholipid was lower than that with liposomes or vesicles, and the rate and degree of binding depends on both the absolute concentration of

cholesterol and on the mole percent of cholesterol in the bilayer. This finding is in agreement with the fluorescence polarization studies of filipin in the presence of aqueous suspensions of cholesterol devoid of phospholipid and cholesterol-containing vesicles (Table VI).

The results reports in the dissertation show that the spectral changes (uv and CD) observed in filipin when the polyene binds to ergosterol-containing ciliary membranes are very similar to those observed in the presence of lecithin-ergosterol vesicles (Figures 2, 6, and 7). Figures 19 and 20 show that when Tetrahymena ciliary membrane fragments containing ergosterol were treated with filipin, the pits are similar in size and morphology with those observed in filipin-treated lecithin-ergosterol vesicles (Figure 15). The good correlation between the appearance of morphological change induced by filipin in natural membranes and lipid bilayer vesicles suggests that the matrix provided for interaction of filipin with ergosterol may be similar in vesicles and ciliary membranes. Since the ratios of chloroform-methanol extractable phosphorus to sterol or triterpene alcohol are the same with or without ergosterol supplementation, ergosterol molecules may be inserted into the membranes at locations that would ordinarily would be occupied by tetrahymanol (Conner et al., 1971).

Spectral and electron microscopic results show that tetrahymanol-containing membranes do not interact strongly with filipin. Tetrahymanol in ciliary membranes may be incapable of interacting with filipin because of the structural dissimilarities between sterols and the triterpenoid alcohol. However, Nes (1974) suggested that tetrahymanol may play the sterol's role in Tetrahymena pyriformis W because of its sterol-like structure. An alternative is that the molecular arrangement of tetrahymanol in the ciliary membrane may not permit filipin-tetrahymanol interaction. Since filipin does interact with tetrahymanol in vesicles, as evidenced by absorption, fluorescence and CD studies (Figures 8, 12, and Table III), but does not interact appreciably with tetrahymanol-containing membranes, the orientation of tetrahymanol with phospholipid molecules in vesicles may be different from that in the natural membrane. Supplementation of ergosterol in the growth medium of Tetrahymena causes alterations in phospholipid fatty acid composition (Ferguson et al., 1971). Since lipid-lipid interactions play an important role in membrane function, changes in the length and the degree of unsaturation of fatty acid chains may affect the phospholipid-alcohol interaction and thus cause the sensitivity toward filipin of the two types of cells to differ. Corey-Pauling-Koltun molecular models indicate that tetrahymanol and ergosterol have a generally similar rigid cylindrical rod-

like shape, but ergosterol is about 5-6 Å longer than tetrahymanol and about 2 Å thinner than tetrahymanol at the C/D ring juncture. The gem-dimethyl group at C-4 of tetrahymanol may inhibit the hydrogen-bonding capacity or modify the dipole-dipole bonding properties of tetrahymanol relative to ergosterol.

Electron micrographs show that filipin-induced pit formation in ergosterol-containing lecithin vesicles is similar to that in ergosterol-containing *Tetrahymena* ciliary membranes. The preparation reported in this study of *Tetrahymena* membranes contained pits with a mean inner diameter of 177 Å. The results indicated here also show that cholesterol and ergosterol have the capacity to interact with filipin to induce pit formation in membranes, but cholestanol, thiocholesterol, androstan-3β-ol, and epicholesterol do not interact with filipin to produce lipid vesicle lysis. The molecular organization of pits in filipin-lysed lecithin vesicles containing cholesterol or ergosterol remains unsolved. The pits observed in negative-stained preparations are probably clumps of lipid within the hydrophobic layer of the membrane. Lucy (1969) suggested that the primary requirement for membranes fusion is that both of the membranes involved have a relatively high proportion of their lipid molecules in a micellar configuration, and not in bimolecular leaflet configuration. Dingle *et al.*(1968) suggested that a membrane must have a

high surface tension for fusion to occur. Since filipin increases the surface pressure of lipid monolayers containing cholesterol (Demel et al., 1968; Norman et al., 1972b), an increase in the surface tension might cause disruption of the lamellar structure and lead to a micellar arrangement (see Figures 16, 17, and 18). The pits produced by filipin are very similar to those observed when erythrocytes and liposomes are lysed in the presence of antibody complement (Borsos et al., 1964; Humphrey and Dourmashkin, 1969; Kinsky et al., 1970b; Kinsky, 1972), but different from those produced by saponin (Dourmashkin et al., 1962). Filipin-induced pits in freeze-etched human erythrocyte membranes are morphologically similar to the pits or surface rings in freeze-etched sheep erythrocyte membranes after immune lysis, and do not extend through the membrane as a hole (Verkleij et al., 1973; Tillack and Kinsky, 1973; Seeman, 1972; Iles et al., 1973). This similarity suggested that perhaps the terminal stages of polyene-induced and immune lysis may possess certain features in common.

The minimum concentration of filipin necessary to induce lysis in lecithin-cholesterol vesicles was found to be 3.4 μM (Figure 16). Filipin did not induce a lytic effect on cholesterol-containing lecithin vesicles at concentrations lower than 1.9 μM (Figure 18). Swelling of the vesicles after treatment with amphotericin B was observed in vesicles containing cholesterol, ergosterol, and thiocholesterol, or lecithin vesicles devoid sterol.

In confirmation of the spectral results (Figures 4 and 13), electron microscopy shows that amphotericin B is not as specific as filipin in its interaction with vesicles lipid components. The results from fluorescence polarization studies indicated that the interaction of amphotericin B with the various lecithin-sterol vesicles used does not display the selectivity exhibited by filipin (Bittman and Fischkoff, 1972). At low concentration of amphotericin B, the polyene undergoes alteration in extinction coefficients in the presence of membranes of A. laidlawii lacking cholesterol, as well as in the presence of those containing cholesterol (Norman et al., 1972a). Other investigations, however, have indicated that amphotericin B 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). The results shown in Table IV indicate that absorbance peak ratio differences of amphotericin B are higher in lecithin vesicles containing cholesterol than lecithin vesicles alone. Several investigations have suggested that interaction of amphotericin B with thin lipid membranes causes formation of pores of 5-10 Å diameter (Andreoli et al., 1969; Cass et al., 1970). Pores of such a small diameter cannot be detected by freeze-etching techniques. No morphological alterations were found on the freeze-fracture faces of amphotericin B-treated erythrocytes, lecithin-cholesterol liposomes and A. laidlawii cells grown in the presence of cholesterol (Verkleij et al., 1973).

Nuclear magnetic resonance study of lecithin vesicles was introduced by Chapman and Penkett (1966). The presence of cholesterol results in some restriction in the mobility of the fatty acid chains. This is consistent with spin-label studies (McConnell and McFarland, 1970). Cholesterol suppressed the motion of the fatty acid chains in phospholipid bilayers, but affected different regions of the chains differently, and did not greatly change the average fatty acid chain orientation. The interaction of filipin with lecithin or lecithin-cholesterol vesicles was investigated by nmr spectrometry (Table VII). It is possible that choline head group is not at the surface in lecithin vesicles since it appears to become more mobile after addition of filipin. The addition of filipin to lecithin or lecithin-cholesterol vesicles increases the mobility of the hydrocarbon chains of the phospholipids. The increase in mobility of the hydrophobic side chains of lecithin suggests that the interaction between lecithin and cholesterol is reduced in the presence of filipin. This is in agreement with the investigation by differential scanning calorimetry of the effect of filipin on the phase transition of lecithin and lecithin-cholesterol, which showed that filipin can reduce the lecithin-cholesterol interaction (Norman _et al_., 1972a).

Only sterol-containing organisms, _e.g._, fungi and protozoa, are polyene-sensitive. Some normally insensitive organisms which lack sterols (_Mycoplasma laidlawii_, _Pythium ultimum_)

are converted to polyene sensitivity by cultivating them in media supplemented with cholesterol (Weber and Kinsky, 1965; Schlosser and Gottlieb, 1966). These observation suggested that sterols in the membrane are the binding sites for polyene antibiotics. It is believed that the binding of polyene antibiotics to sterols causes impairment of membrane function, then leakage of essential metabolities, and eventually cause cell death (Lampen, 1966).

Difference in potencies among the polyene are thought to be attributable to their varing affinities for sterols (Bittman and Fischkoff, 1972). Stopped-flow studies revealed that the initial rate of association of filipin III with cholesterol is faster in sonicated aqueous dispersions (vesicles) than in unsonicated aqueous dispersion (liposomes) (Bittman et al., 1974b). No association reaction was observed in the stopped-flow apparatus with liposomes prepared from lecithin alone. For a given molar ratio of lecithin to cholesterol, the rate of binding increases with the concentration of cholesterol. This is in agreement with the results obtained from the ultraviolet absorption and fluorescence polarization studies that show that the degree of binding increases with cholesterol concentration (Figures 22 and 23). These results support the suggestion that the phospholipid to sterol ratio may be an important parameter in determining whether filipin interacts with membranes (Kinsky, 1970b). The results are in agreement with reports that filipin, at low concentration: (a) does not undergo

marked spectral changes in the presence of liposomes having very low cholesterol content (Norman et al., 1972b); (b) has no effect on monolayers (Demel et al., 1965), single bilayer membranes (Van Zutphen et al., 1966), and biological membranes (Kinsky et al., 1965) having low cholesterol content; (c) interacts with cholesterol-containing monolayers but not with cholesterol-lacking monolayers (Demel et al., 1968); and (d) increases the rate and extent of release of trapped ions and solute in cholesterol-containing liposomes (Kinsky et al., 1968; Sessa and Weissmann, 1968).

The binding of filipin to lecithin-epicholesterol vesicles is weaker than that to lecithin-cholesterol vesicles (Figure 24 and Table IX). This result is consistent with the observation that mixed lecithin-epicholesterol monolayers gave only small surface pressure increases (Norman et al., 1972b), lecithin-epicholesterol vesicles were not lysed by treatment with filipin at concentrations that lysed lecithin-cholesterol vesicles (Bittman et al., 1974a) and qualitatively less filipin appears to be bound to vesicles and A. laidlawii membranes containing epicholesterol than to those containing cholesterol (Bittman and Fischkoff, 1972; Norman et al., 1972b). That filipin binds somewhat more strongly to lecithin-cholesterol vesicles than to lecithin-ergosterol vesicles (Table IX) is consistent with the report that cholesterol antagonized the inhibitory action of filipin in Candida albicans more effectively than ergosterol (Zygmunt and Tavormina, 1966).

Several physical techniques have been applied to the study of thermotropic transitions of phospholipids. It has been established that the temperature of the gel to liquid-crystalline transition (T_c) depends on the length and unsaturation of the acyl chains and the chemistry of the polar head group of phospholipids. The most extensively studied phospholipid is dipalmitoyllecithin, which exhibits a T_c of about 41°. This transition is accompanied by a change in bilayer volume as shown by dilatometry (Träuble and Haynes, 1971), a decrease in the proton nmr line width (Chapman et al., 1967), and a decrease in the fluorescence polarization of various probes (Lussan and Faucon, 1971; Papahadjopoulos et al., 1973). Using filipin as a fluorescence label, the phase transition in dipalmitoyl-lecithin vesicles is seen to be accompanied by a sharp decrease in the polarization as the temperature increases from $39-42^o$, with a midpoint at approximately 40.5^o. This value agrees with the transition temperature obtained by use of spin labels and fluorescence probes (Papahadjopoulos et al., 1973; Sackmann et al., 1973). Similar agreement has been obtained in dimyristoyllecithin, sphingomyelin and dipalmitoyllecithin-dimyristoyllecithin vesicles (Phillips et al., 1970; Long et al., 1971; Shimshick and McConnell, 1973).

Light-scattering intensities of vesicles using 0°-0° polarized light were reduced sharply on passing through T_c. The effect of cholesterol on the properties of phospholipid

is reflected in the fluorescence polarization of filipin. The finding that the phase transitions of the phospholipids are broadened (Figures 26, 27, and 28) agrees with Raman investigations that showed broadening of the dipalmitoyllecithin signals in multilayers on addition of cholesterol (Lippert and Peticolas, 1971).

The concentration dependence of the molecular ellipticities of amphotericin B indicated that self-associated amphotericin B molecules in close proximity may enhance each other in rotational strength (Figure 36). Since the degree of association can be reduced by increasing the percentage of organic solvent such as methanol (Figures 33 and 34), or by increasing the temperature of the solution (Figure 35), no covalent bonds are involved in the self-association of amphotericin B in aqueous media. The absorption spectrum of amphotericin B methyl ester is similar to that of the monomer in aqueous solution (Schaffner and Mechlinski, 1972). The methyl ester of amphotericin B possesses the same fungicidal activity as the free acid but has higher solubility in aqueous media. Also, the absorbance peak ratio, A_{385}/A_{335}, of amphotericin B was found to increase by lowering the pH from 7 to 2 (Norman _et al._, 1972a). The information available from the above investigations suggests that the carboxyl group in amphotericin B may be involved in the aggregation but has no effect on antifungal activity. At similar concentrations of polyene in aqueous solution, amphotericin B has a higher inclination to form aggregates than does filipin (Figure 32).

The reduction of molecular ellipticities and alteration of the extinction coefficients suggest that the dissociation of amphotericin B aggregates is increased in the presence of vesicles or organic solvent. The concentration-, pH-, temperature-, and solvent-dependent features of amphotericin B indicate that the stacking of amphotericin B in aqueous solution is pronounced.

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