INFORMATION TO USERS

This material was produced from a microfilm copy of the original document. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the original submitted.

The following explanation of techniques is provided to help you understand markings or patterns which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting thru an image and duplicating adjacent pages to insure you complete continuity.

2. When an image on the film is obliterated with a large round black mark, it is an indication that the photographer suspected that the copy may have moved during exposure and thus cause a blurred image. You will find a good image of the page in the adjacent frame.

3. When a map, drawing or chart, etc., was part of the material being photographed the photographer followed a definite method in "sectioning" the material. It is customary to begin photoing at the upper left hand corner of a large sheet and to continue photoing from left to right in equal sections with a small overlap. If necessary, sectioning is continued again — beginning below the first row and continuing on until complete.

4. The majority of users indicate that the textual content is of greatest value, however, a somewhat higher quality reproduction could be made from "photographs" if essential to the understanding of the dissertation. Silver prints of "photographs" may be ordered at additional charge by writing the Order Department, giving the catalog number, title, author and specific pages you wish reproduced.

5. PLEASE NOTE: Some pages may have indistinct print. Filmed as received.

Xerox University Microfilms
300 North Zeeb Road
Ann Arbor, Michigan 48106

THE STUDY OF MEMBRANE STRUCTURE USING FILIPIN III AS A PROBE

by

B. KUSUMA REDDY

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements 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 The Doctor of Philosophy.

January 11, 1974
date

Robert Bittman
Chairman of examining committee

1-17-74
date

Aaron Lustbader
Executive officer

Bernard J. Bulkin

Jan F. Hogg

Supervisory Committee

The City University of New York

ABSTRACT

THE STUDY OF MEMBRANE STRUCTURE USING FILIPIN AS A PROBE

by

B. KUSUMA REDDY

Adviser: Professor Robert Bittman

The organization of lipids in membranes has been investigated in recent years by various techniques. One approach is to observe the mode of action of agents which interact specifically with either phospholipids, sterols or phospholipid-sterol complexes. In this dissertation, membrane structure was investigated by using filipin III as a probe of the interaction of sterols with phospholipids in model membranes and in ciliary membranes of Tetrahymena pyriformis strain W grown in the absence and presence of sterols.

Model membranes were prepared from both egg lecithin and dipalmitoyllecithin, with and without sterols, and were fractionated on agarose gels. The interactions of filipin III with a homogeneous fraction of vesicles as well as with ciliary membranes isolated from Tetrahymena pyriformis

were studied by monitoring changes in absorbance, fluorescence and circular dichroism spectra.

When the absorption maxima of filipin in nonpolar solvents and in the presence of vesicles were compared with those of the polyene in aqueous solution, it was found that there were shifts toward longer wavelengths. In the presence of egg lecithin vesicles containing sterols, the ratio of peaks 323 nm/358 nm was changed significantly. This change was absent when thiocholesterol was incorporated in the vesicles. The decrease with time in the absorption maxima of filipin in the presence of vesicles prepared with and without sterols indicated that the binding of the probe to model membranes involved a reaction that occurred during the course of several hours.

There was an increase in the fluorescence intensity of filipin on interaction with vesicles prepared from egg lecithin but this increase was absent in dipalmitoyllecithin lamellae. These results indicated that the nature of the interaction depended on the presence or absence of the double bonds and on the phase of the fatty acyl chains. Filipin underwent an increase in fluorescence polarization on reaction with egg lecithin : cholesterol vesicles, suggesting that the mobility of filipin was reduced on binding.

In circular dichroism studies the antibiotic exhibited enhanced negative dichroic bands when bound to egg lecithin: cholesterol vesicles.

In the presence of ciliary membranes lacking sterols,

bathochromic shifts of filipin were absent but the peak ratio 323 nm/358 nm of the polyene was increased. On binding of filipin to ciliary membranes containing ergosterol, the extinction coefficients around 338 nm and 358 nm decreased, bathochromic shifts occurred, the peak ratio 323 nm/358 nm increased and the circular dichroic bands of the polyene were enhanced. The peak ratio 323 nm/358 nm of filipin was increased from 1 to 1.26 in the presence of tetrahymanol-containing ciliary membranes and to 1.87 in ergosterol-containing ciliary membranes in ethylene glycol-Tris buffer when the membrane preparations contained equivalent concentrations of tetrahymanol and ergosterol. Ciliary membranes containing ergosterol may have a higher affinity for filipin than those obtained in a medium deficient in this sterol.

Large changes in the circular dichroic bands of filipin occur when the lecithin-sterol interaction is strong. The changes in the circular dichroic properties of filipin in the presence of model membranes prepared with sterols and ciliary membranes of Tetrahymena reflect the strength of the lecithin-sterol interaction. These findings support the hypothesis that the polar lecithin phosphate group and the cholesterol hydroxyl group are hydrogen bonded because modifications at the 3 position of cholesterol produced marked changes in the fluorescence and circular dichroic properties of filipin.

ACKNOWLEDGMENTS

The author wishes to express her indebtedness and appreciation to Professor Robert Bittman, Professor Burton Tropp and Professor James F. Hogg for their continued interest and thoughtful suggestions during the course of this work and last but not the least to Mr. Thomas Dorsey for his unfailing moral support in the laboratory.

Thanks are also due to many members of the Department of Chemistry for their suggestions and for the excellent technical assistance rendered by Mr. Robert Wurman.

This thesis is dedicated to Lord Sri Venkateshwara of Tirumala Tirupati Devasthanam, Tirupati, Chittoor District, Hyderabad, Andhra Pradesh, India.

योगक्षेमं म वहंम्यहं

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

INTRODUCTION*

Polyene Antibiotics

The selective toxicity of the antifungal antibiotics has prompted their use as biochemical tools for the investigation and elucidation of the role of sterols in membranes. The mode of action of the polyene antibiotics has been explained by the well-known "sterol receptor" hypothesis. This hypothesis states that polyene antibiotics bind specifically to sterols. Considerable evidence indicates that polyenes bind to sterols present in the membranes of fungi and produce lethal changes in membrane permeability (6, 7). Studies with phospholipid dispersions indicated that some polyenes preferentially disrupt phospholipid structures containing cholesterol (8). Monolayer studies demonstrated that the polyene antibiotics, filipin and nystatin, penetrated monolayers of cholesterol or ergosterol but failed to penetrate monolayers of phospholipids (9). Bilayer experiments showed that films prepared from lecithin were rendered

---

*The laboratory work presented in this thesis was completed in July 1971. After the work described in this thesis was completed, publications appeared which indicated that spectral changes occurred when polyene antibiotics interact with cholesterol-containing membranes (1-5). Preliminary publication of a part of this work has appeared in the XXIII International Congress of Pure and Applied Chemistry, 1, 401 (1971).

unstable by filipin only when sterol was present (10). The ability of polyene antibiotics to reduce serum cholesterol levels upon oral administration in dogs indicated that a polyene could be used to study various aspects of cholesterol metabolism and function (11). The polyene antibiotics produced lesions in erythrocyte membranes which were similar to those produced by these antibiotics in lecithin-cholesterol liposomes (12). Addition of sterols to polyenes caused spectral changes in aqueous solutions of filipin and provided evidence that polyenes could interact with sterols in a stereochemically defined manner to produce a filipin-sterol complex (2, 13). Changes in fluorescence polarization were observed on the binding of polyene antibiotics to sterols and the binding constants could be correlated with the potencies of the polyene antibiotics (1). Therefore, it has been suggested that filipin could serve as a probe of lecithin-sterol interactions.

Models of Membranes

Many models of membrane structure have been proposed in recent years to study the molecular organization of biological membranes and the relation of structure to biological function.

In 1895 Overton observed that lipophilic substances could easily penetrate membranes, but polar molecules could not. These observations led to the concept that the cell membrane was a lipid barrier. In 1925 Gorter

and Grendel supported this idea (14) and were the first to propose the bilayer model (15). They proposed that the red cell was composed of a bimolecular layer of lipids (figure 1A). They extracted lipids from red blood cells and found that these lipids occupied a surface area twice that of erythrocyte cells. Their experiments in the light of today's knowledge were done poorly because the mean molecular area occupied by lipid molecules depends on the surface pressure. However, recent redeterminations by modern methods of extracting lipids and measuring surface pressure have supported their conclusions (16). In 1935 Davson and Danielli proposed a bilayer model for plasma membranes (figure 1B). Their model consisted of a bilayer of closely packed phospholipid molecules. The nonpolar paraffin chains are in contact with each other, whereas the polar ends are in association with proteins in an extended conformation (16).

Later Robertson introduced the unit membrane structure (17). This model was a refinement of the Danielli model because the thickness was defined and the protein existed on the exterior spread as a monolayer (figure 1C). These observations were largely based on electron microscope studies.

Kavanu proposed that natural membranes exist as protein-lipoprotein-lipid complexes (18). These complexes are capable of existing in many forms. The form having

a "closed" configuration has a bilayer of lipid and lies between two protein monolayers. The "open" form is arranged in the form of an hexagonal array of cylindrical micelles.

The membrane model as conceived by Lucy indicated that incorporation of sterols into biological membranes produced alterations in membrane structure (19). Modifications in the structure of sterols produced phase changes in these membranes.

Van den Heuvel suggested a model for lecithin-cholesterol complex. The molecular association of cholesterol and phospholipid is arranged in a manner such that the steroid nucleus is in Van der Waals interaction with the hydrocarbon chains of the phospholipids and also the hydroxyl group of the sterol is in close contact with the polar groups of the phospholipids (20).

The protein crystal model has been proposed by Vanderkooi (21). The membrane consists of lipids and proteins. The proteins are globular and bimodal (amphipathic) in nature. The lipid exists as a lamellar bilayer and fills in the spaces between the proteins.

Singer and Nicolson examined several models of gross structural organization of membranes and concluded that a fluid mosaic structure was the only membrane model consistent with the thermodynamics of hydrophobic interactions and the experimental data (22). In the mosaic model the proteins have an amphipathic structure

and are arranged in a manner such that polar groups face the aqueous phase and the nonpolar groups remain inside the hydrophobic environment of the membrane. The proteins are distributed randomly in the form of aggregates in the matrix of the bilayer of lipids and parts of the protein molecules are in association with the phospholipids. The protein-protein interactions in this model are not of a major consequence in the membrane structure. The phospholipids are arranged in a continuous bilayer structure where the ionic and polar head groups are in association with the proteins and the nonpolar fatty acyl chains are in a hydrophobic environment of the membrane. The phospholipids in this model, as indicated by calorimetric data are in a fluid rather than in a crystalline phase in all functional cell membranes.

Figure 1. Schematic representations of membrane models.

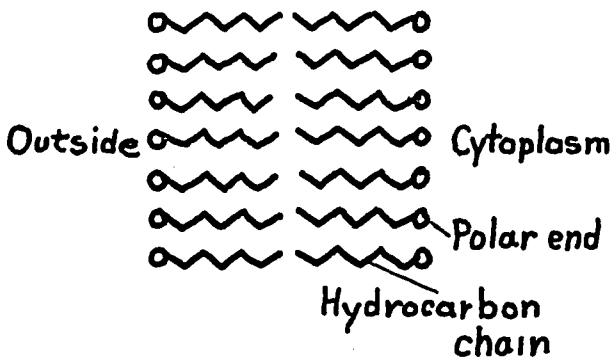
a. Hypothetical structure of the plasma membrane as visualized by Gorter and Grendel.

b. The bilayer model of Davson and Danielli. A layer of lipid is bounded on each side by monolayers of phospholipid molecules, the polar ends of which are associated with globular proteins.

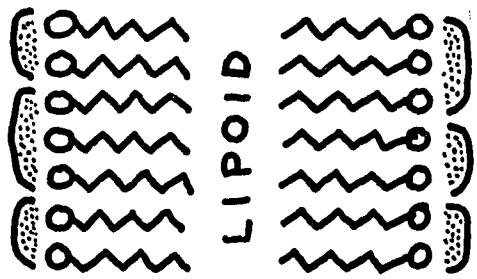
c. The molecular structure of the unit membrane as conceived by J.D. Robertson. This model defines the thickness of the lipid layer. Protein exists on the exterior of the membrane as a continuous monolayer.

d. The bilayer model of vesicles sketched schematically.

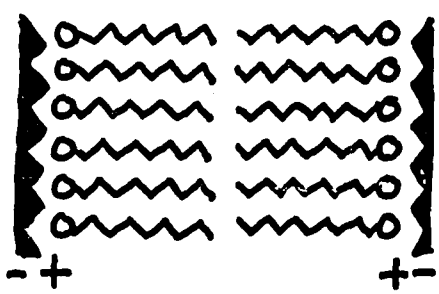
a


Outside
Cytoplasm
Polar end
Hydrocarbon chain

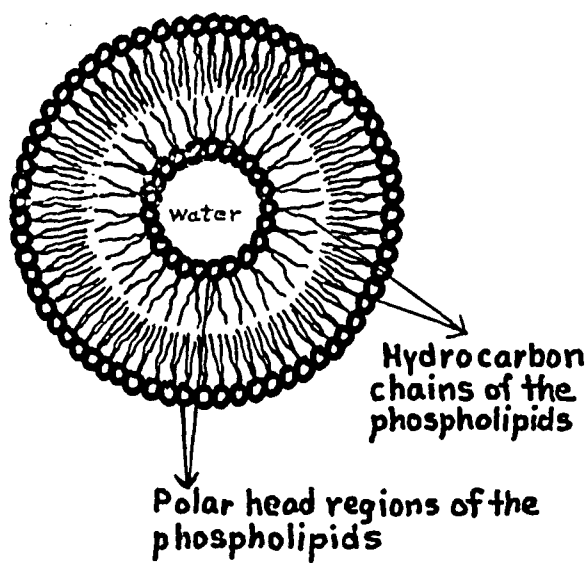
b


LIPOID

c


- +
+ -

d


water
Hydrocarbon chains of the phospholipids
Polar head regions of the phospholipids

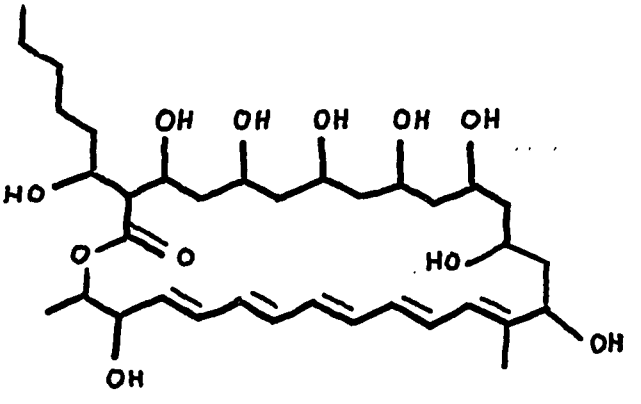
Chemistry of Filipin

The polyene antibiotic filipin is produced by Streptomyces filipensis which was isolated from the soil of the Philippines. The structure of filipin was established by Cedar and Ryhage (23). The proposed formula of filipin is illustrated in figure 2. It is a yellow crystalline compound with an empirical formula of $C_{35}H_{58}O_{11}$ (24, 25). The crystalline material consisting of the filipin complex has a specific rotation, $[\alpha]_D^{22}$, of -148.3 (c 0.89 in methanol). It has a characteristic uv spectrum with absorption maxima at 358, 338 and 323 nm and a shoulder at 305 nm. The light absorption of the antibiotic is due to the presence of the conjugated double bonds and also to the lactone group in the molecule.

The antibiotic is unstable to heat and light. It readily decomposes with loss of biological activity. The degradation product induced by heat has an altered uv spectrum and a complete loss of specific rotation (25, 26). Catalytic hydrogenation of filipin resulted in the loss of optical activity, suggesting that a racemic mixture of diastereoisomers were produced in the reduction of the polyene (26).

Filipin is a complex consisting of four components (27). Three of the four components constitute 96% of the complex. The antibiotic has been separated into four components by thin-layer chromatography on silica gel.

Figure 2. The structure of filipin as established by Cedar and Ryhage (23).


OH
OH
OH
OH
OH
HO
O
O
HO
OH
OH

They differ in their R_f values and are referred to as I, II, III and IV (27). Filipin II and III are the most potent and hemolytic constituents of the complex and account for the antibiotic's biological activity (28). Comparison of the antifungal activities of the individual filipin components indicated that the activity of the antibiotic was mainly due to the large amounts of filipin II and III present in the complex (27).

Recent mass spectral studies have indicated that filipin IV is isomeric with filipin III, and that filipin II and I contain one and two hydroxyl groups fewer than filipin III, respectively (29). Filipin III has the structure proposed by Cedar and Ryhage.

Monolayers

Substances such as long chain fatty acids and alcohols, when spread on the surface of water form a film at the air/water interface. The mean molecular area occupied by these molecules depends on the surface pressure of the monolayers. Plots of surface pressure against area of the film demonstrate that the molecular area depends on the nature of the substance and on temperature and pH. From studies on monomolecular layers of phospholipids, it is possible to observe the interactions between fatty acids of varying chain length and saturation, between fatty acids and cholesterol and between fatty acids and phospholipids (30).

The force-area and surface potential measurements indicated that the mean molecular area occupied by a lecithin molecule decreased with an increase in the length of the saturated hydrocarbon chain (31). The area per molecule in synthetic monolayers increased with increasing unsaturation in the fatty acyl chains (32). The mean area per molecule in mixed films of cholesterol with saturated lecithins followed a simple additivity rule (33). A condensing effect with cholesterol was evidenced in the presence of lecithins having one or more double bonds in one or both of the fatty acyl chains (30). Shah and Schulman postulated that in mixed monolayers of lecithin, cholesterol did not condense the monolayer nor did it have molecular inter-

action with the phospholipids, instead the sterol occupied the vacant spaces in the phospholipid monolayer. Therefore in this model the addition of cholesterol did not lead to an increase in the area (33). A more convincing interpretation of this effect was stated by Demel and coworkers (30). They also observed the same differences in the interfacial behavior of mixed cholesterol-saturated lecithin monolayers and attributed these differences to the increased Van der Waals forces between unsaturated lecithins and sterols. The ability of polyunsaturated lecithins to condense with cholesterol was reduced on increasing the temperature in cholesterol-containing monolayers. The temperature changes induced distortions in the double bonds. The distortion produced a reduction in the interaction of phospholipids with sterols. The mixing of the polyunsaturated phospholipids with the sterols may cause changes in the entropy of the hydrocarbon chains. This may lead to structure-breaking effects on the water adjacent to the monolayer. Thus the condensation phenomenon results from Van der Waal's interaction and alteration in the entropy of the fatty acyl chains and changes in the structure of water. All these effects, it has been experimentally observed, are governed by temperature.

Thin Lipid Membranes

Thin lipid membranes are also known as single bilayer membranes and as black lipid membranes. They are formed by applying a membrane-forming solution with a fine sable brush across a circular hole having a partition made from a teflon or polyethylene cup. An aperture of 1-2 mm in diameter, separates two aqueous phases (34). The sequence of events leading from the formation of a thick film to the appearance of a thin black membrane can be observed by reflected light.

In all membrane-forming solutions, an amphipatic component and a hydrocarbon solvent are present. Phospholipid mixtures containing lipids or lipids isolated from red cell membranes or synthetic lecithin, sphingomyelin, phosphatidyl inositol or dioleolecithin are dissolved in a hydrocarbon such as hexane, octane, heptane, decane or n-tetradecane. Addition of cholesterol to these membrane-forming solutions has also yielded stable structures.

It is generally recognized that thin lipid membranes are bilayers. There are several reasons for reaching such a conclusion. Results obtained from capacitance measurements (35), optical measurements (36) and electron micrographs (37) indicated that the thickness of the lipid film was 60 ± 20 Å.

The chemical composition of the bilayers has been determined by using radioactive constituents in the membrane-forming solutions. The analysis of the results indicated that the ratio of cholesterol to phospholipid is the same in membrane-forming solution as in the film, but the molar ratio of 700:1 of the hydrocarbon:phospholipid in the membrane-forming solution is altered to a molar ratio of 10:1 in the film (38). Therefore by this method of preparation, it is not possible to eliminate the hydrocarbon solvent in thin lipid membranes.

The bilayer is stable for hours and sometimes days under proper conditions. If appropriate precautions to exclude oxygen are observed, oxidation of the film leading to film rupture can be prevented (39).

The bilayer membrane has a low surface tension (1 dyne/cm). This is similar to that of the plasma membranes of sheep red cells (40, 41). The low surface tension of the plasma membrane was believed to be due to adsorbed proteins on the surface (41), but these studies showed that bilayer films also possess these properties.

The electrical resistance offered by black lipid membranes varies with the solvent used in the membrane-forming solution. The resistance of membranes formed from decane solutions was of the order $10^8 - 10^9\ \Omega\ cm^2$;

the resistance of membranes obtained from chloroform-methanol was between $10^5 - 10^6 \Omega$ cm^2. This indicated that the resistance of these lipid membranes was increased in a nonpolar environment. When ionic solutions at different concentrations were placed on either side of the membrane, a diffusion potential was produced which was used to calculate the transference numbers in these systems. The potential difference measurements indicated that cations were more permeable than anions when the membranes were prepared from erythrocytes (42).

The films were not permeable to urea, erythritol and glycerol (43). Lipid soluble organic substances such as indole (44) and large molecules like tobacco mosaic virus could pass through these membranes (45).

Comparison of the properties of black lipid membranes with natural membranes show striking similarities such as ion conductivity and water permeability. Electrical excitability could be induced in these films by addition of material obtained from cultures of Aerobacter cloacae (46). This material is generally referred to as EIM (excitability inducing material). When EIM was added to either or both sides of the film, electrical resistance was lowered. The electrical properties of these films are similar to the biological excitable systems (47). The ion selectivity of these lipid films was altered by antibiotics such as

valinomycin (48), gramicidin (48) and polyene antibiotics (10), and these results were consistent with the biological findings that these antibiotics exert similar effects in natural membranes.

Liposomes

Bangham, Standish and Watkins prepared a system of concentric shells of phospholipids often referred to as liposomes (49). They studied the properties of these model membrane structures. Liposomes share some common features with biological membranes in permeability properties. The model membrane system, in an aqueous medium, resemble mitochondria, erythrocytes and lysosomes. Lytic agents such as streptolysin S and certain steroids altered the permeability behavior of the liposomes. The permeability characteristics of the liposomes when exposed to these agents were similar to those of biological membranes (50). The dimensions of this system depend on various external factors such as water content, temperature, ionic strength and method of dispersion.

Egg lecithin contains a mixture of saturated and unsaturated long chain fatty acids and when dispersed in buffer, liposomes are formed (51). When viewed in the electron microscope after negative staining, liposomes appear to assume a variety of sizes and shapes. The liposomes exhibit positive, negative or zero birefringence, depending upon the surface charge present in the spherules.

The permeability of liposomes depends on the structure of the phospholipid (49) and the presence of charged components in the dispersion (52). The cation and anion permeability of liposomes can be altered by introducing charged substituents in the dispersion. For example,

when the liposomes contained stearylamine, the ratio of Cl^-/K^+ was increased, but when the liposomes contained stearic acid, the ratio decreased (52).

The permeability properties of the liposomes and thin lipid membranes present striking differences. Liposomes were more permeable to anions than cations, but the reverse was true for thin lipid membranes, when identical lipid composition was present in both the systems (51).

Vesicles

Stable aqueous phosphatidylcholine vesicles were obtained by prolonged ultrasonic irradiation under nitrogen followed by molecular sieve chromatography on large-pore agarose gels (53). A fraction of the separated vesicles was observed to be homogeneous. Rechromatography on agarose gel, sedimentation velocity experiments and electron microscopy indicated that this fraction consisted of a single species having a molecular weight of 2.1×10^6. Physical parameters such as intrinsic velocity, partial specific volume (54) and diffusion coefficient (55) of the homogeneous fraction of vesicles have been established. The structure of the vesicles is schematically sketched in figure 1D.

Natural Membranes

Ciliary Membranes

The ciliary membrane of Tetrahymena pyriformis is a three-layered cell membrane. In Tetrahymena, the axonemal

fibers can be removed from the cilia by treating the cells with 0.6 M potassium iodide to remove microtubules and to obtain ciliary membranes (56). Cilia obtained from cells grown in a proteose peptone medium with yeast extract contain a triterpenoid pentacyclic alcohol, tetrahymanol. Tetrahymena cells also grow with ergosterol in the medium. When this exogenous sterol is supplied, the organism is capable of incorporating the sterol into its ciliary membranes and ergosterol replaces tetrahymanol (57).

Effect of Filipin on Model Membranes

Monolayers

Force-area and surface pressure measurements demonstrated that filipin was able to penetrate monolayers of ergosterol and cholesterol (9). The polyene increased the surface pressure of sterol monolayers, until the sterols were compressed to an initial surface pressure above the collapse pressure of the antibiotic. At low antibiotic concentration (10^{-8}M), the polyene increased the surface pressure of cholesterol monolayers from an initial pressure of 2 dynes/cm to 31 dynes/cm (58). The inability of filipin to interact with monolayers prepared from bacterial lipids at low concentrations of filipin, but its ability to interact with monolayers prepared from neutral and total lipids from erythrocytes indicated that the polyene binds to monolayers containing sterols.

Reinvestigation of the preferential affinity of the polyene antibiotics with sterol monolayers at a wider range of antibiotic concentration showed that at low molar ratio of antibiotic to lipid, the initial surface pressure of sterol monolayers was significantly increased by filipin, etruscomycin, amphotericin B, pimaricin and nystatin (in decreasing order of effectiveness) (58). At high molar ratio of antibiotic to lipid it was demonstrated that filipin, nystatin and pimaricin interact with monolayers of lecithin in the absence of sterol (58). In mixed lecithin-cholesterol monolayers, the surface pressure

increase caused by filipin diminished as the phospholipid content was raised. The surface pressure of cholesteryl acetate monolayers was only slightly affected by filipin (58). This effect is consistent with the view that a free hydroxyl group in cholesterol is necessary for interaction with polyenes.

Perhydrofilipin, saponified filipin (alkali-treated) and uv-irradiated filipin are less potent than the parent antibiotic. The surface pressure changes produced by these derivatives on sterol monolayers was also small (59). The hydrogenation of filipin reduces the conjugated double bonds and irradiation destroys the conjugated chromophore of filipin. The alteration of these chromophores in filipin may affect the polyene-sterol interaction in monolayers.

Studies of filipin with pure sterol and mixed lecithin-sterol monolayers indicated that interaction of the polyene with the sterol was sterospecific (12). The highest pressure increases were obtained with cholesterol. Smaller increases were observed with androstan -3β-ol and ergocalciferol monolayers on interaction with filipin. These results indicated that deletion of the side chain (as in androstan-3 β-ol) or opening of the B ring (as in ergocalciferol) of cholesterol resulted in the reduction of the pressure increases. Equimolar mixtures of egg lecithin and sterol in monolayers gave pressure increases with filipin only with cholesterol or stigmasterol and with ergosterol at initial surface pressure of 8 dynes/cm. This indicated that in

mixed monolayers, a 3β-hydroxyl group and an intact sterol B ring are essential for filipin-sterol interaction. At the present time it is not known how the polyenes cause surface pressure increases. It is conjectured that it results from the accumulation of the polyene beneath the monolayers and subsequent reorganization of the constituents of the monolayer molecules when the antibiotic enters the monolayer (10).

Effect of Polyenes on Thin Lipid Membranes

Thin lipid membranes have been used as models to study the mechanism of polyene interaction in membranes. It was reported that the magnitude of the effect of filipin on thin lipid membranes largely depended on the molar ratio of phospholipids to sterols in the lipid bilayer (60). Extensive studies of bilayer permeability indicated that nystatin and amphotericin, unlike filipin, altered membrane permeability under conditions which did not alter the stability of the film. The polyene antibiotics lower the d.c. resistance of thin lipid membranes and the decrease in resistance was influenced by factors such as temperature, sterol content and the concentration of the antibiotic (61). Nystatin and amphotericin are anion selective but at high antibiotic concentration this selectivity was lost. The conclusions were arrived at independently by two groups of workers (60, 61). The ability of nystatin to enhance ion conductance across bilayers was diminished with an increase in temperature. Therefore, it has been proposed that polyenes form aggregates with sterols which in an unknown manner cause the formation of pores in membranes (12). An increase in temperature may lead to a decreased affinity of nystatin for sterol thus resulting in the reduction of pore formation which enhances ion conductance. The above effects of the antibiotic on the d.c. resistance of the bilayers appear only when cholesterol was present above a critical threshold concentration. The polyene antibiotics did not

affect the ion permeability properties of lecithin bilayer membranes, since their d.c. resistance was not altered (62). However, the d.c. resistance of equimolar lecithin-cholesterol bilayer films was reduced by filipin when added to one side of the film. The order in which the d.c. resistance was lowered by the components of the filipin complex parallels the order of their lytic action on lecithin-cholesterol bilayers. Transference number measurements indicated that calcium permeability was increased in lecithin-cholesterol bilayers when filipin was present (62).

The permeability of nonelectrolytes through bilayers was facilitated by polyenes. Amphotericin B selectively increased thiourea flux through bilayers prepared from cholesterol-dodecane films but not from dioleolecithin-dodecane films (63). Nystatin increased the flux of water, urea, ethylene glycol, glycerol, propionamide and erythritol through lipid bilayers prepared from ox brain (64). Amphotericin B strikingly increased the permeability of water when the membranes were formed from equimolar amounts of cholesterol and phospholipids (65). Bilayer membranes prepared from equimolar solutions of lecithin and cholesterol were disrupted by filipin complex, filipin II, filipin III, nystatin, etruscomycin and pimaricin (62). Filipin reduced the electrical resistance of the bilayer by a factor of 10 at this concentration and greatly reduced the survival time of the film. At concentrations

of 10^{-4}-10^{-2}M, filipin disrupted lecithin bilayers in the absence of cholesterol. These results were in agreement with those obtained in monolayer (58) and in liposome studies (8). Therefore, it has been proposed that the disruption of the model membranes observed at high antibiotic concentration may not be due to the direct interaction with cholesterol but to an effect caused by a low surface tension produced by amphipathic molecules (62).

Effect of Filipin on Liposomes

The effects of polyene antibiotics on liposomes was studied by Weissmann and Sessa (8). Markers such as chromate, phosphate and glucose were released from liposomes by the action of filipin, etruscomycin, amphotericin B and nystatin. Filipin and etruscomycin released markers irrespective of whether or not cholesterol was incorporated in the liposomes, but amphotericin B and nystatin were effective only when cholesterol was present. Further investigations by these workers revealed that the effect of filipin complex on artificial phospholipid liposomes largely depended on the polyene-lipid ratio (66). Filipin produced lysis of erythrocytes, causing leakage of hemoglobin. Incubation of filipin with liposomes containing egg lecithin-dicetyl phosphate-cholesterol prevented the hemolytic activity of the polyene on erythrocytes (66). Filipin III preferentially disrupted liposomes containing cholesterol when compared with those prepared with phospholipids alone. Filipin II disrupted liposomes indiscriminately whether or not cholesterol was present. Of all the components tested, filipin II and III were the most potent in antifungal and hemolytic activity. Filipin I, constituting only 4% of the entire complex, was more active than II or III in liposomes containing cholesterol and less active in the hemolysis of erythrocytes. Filipin IV was comparatively inert and was able to release anions only

from liposomes prepared with phospholipids alone, a characteristic which was also shared by uv-irradiated filipin. Incubation of all four components of filipin with sphingomyelin and cardiolipin liposomes prior to the addition of erythrocytes decreased hemolysis significantly. These results support the concept that filipin, like other polyene antibiotics interacts preferentially with sterol-containing membranes at low antibiotic to lipid ratios and with phospholipids at high ratios.

The disruptions produced by filipin on liposomes containing equimolar concentrations of lecithin and cholesterol were similar to the lesions observed in filipin-treated erythrocytes (66). With cholesterol-free liposomes, disruptions were not observed. Irradiated filipin and perhydrofilipin did not produce any morphological alterations in liposomes containing cholesterol. Electron micrographs of lecithin-cholesterol liposomes treated with perhydrofilipin resembled those produced by lysolecithin lysis. These observations suggest that reduction of the double bonds of filipin may convert the polyene to a derivative which produces lysis by a mechanism similar to that caused by lysolecithin. The disruptions produced by saponin on equimolar dispersions of lecithin and sterols are in regular geometrical pattern. The lecithin and sterol molecules arrange themselves in a hexagonal array. The disruptions caused by filipin are distributed randomly.

Lecithin-Cholesterol Interactions

Phospholipid-sterol interactions have been the subject of vigorous discussion for many years. The molecular organization of these two components has been extensively studied. The role of cholesterol in cellular membranes has been investigated to obtain information about the functional changes induced by lecithin-sterol interactions. Cholesterol-containing egg lecithin liposomes have less leakage of the sequestered marker ions than those prepared in the absence of the sterol (67). The initial rate of water permeability of liposomes containing egg lecithin and saturated lecithins was diminished on increasing the cholesterol concentration when the phospholipid preparations were above their crystalline to liquid-crystalline transition temperatures (68). The presence of cholesterol caused resistance to swelling in mitochondria (69), reduced the leakage of the essential constituents of the cells in Pythium (70) and decreased the water conductivity of thin lipid films (71).

Studies of the molecular association of lecithin with cholesterol indicated that in the presence of the sterol, the hydrocarbon chains of lecithin have a limited degree of freedom (72). Furthermore, the study of water-cholesterol-lecithin systems by X-ray diffraction indicated that cholesterol and lecithin molecules in the presence of water assumed a lamellar phase, thus giving rise to a palisade arrangement (72). These findings supported the

idea that cholesterol imposed restrictions on the movement of the fatty acyl chains in biological membranes.

The interactions of lecithin and cholesterol are altered to a large extent by the phase of the fatty acyl chains. X-ray diffraction studies have indicated that a single lamellar phase existed and was a function of the concentration of lipid and temperature (73). The rate of water permeability of liposomes containing saturated lecithins and cholesterol was enhanced when the phospholipid preparations were in the gel phase (68). Therefore, it appears that the molecular packing of the hydrocarbon chains is modified by cholesterol.

Lecithin failed to solubilize cholesteryl esters or sterols that lacked a 5, 6-double bond (74). These findings suggested that unsaturation in the steroid nucleus and the presence of a 3β-hydroxyl group were necessary for the solubilization of cholesterol by lecithin.

The effect of steroids on the permeability properties of egg lecithin liposomes toward glucose and glycerol were studied. Egg lecithin liposomes were prepared with 3β-hydroxy sterols, 3α-hydroxysterols and ketosteroids. The 3β-hydroxy sterols cholesterol, cholestanol, lathosterol, 7-dehydrocholesterol and B-norcholesterol reduced the permeability of liposomes toward glucose and glycerol. Liposomes containing the plant sterols ergosterol and stigmasterol, although possessing a 3β-hydroxyl group, were found to be less effective in reducing the permeability

of the sequestered marker molecules. When the liposomes contain a sterol lacking a side chain such as androstan-3β-ol or having a nonplanar sterol nucleus such as coprostanol, the permeability of either glucose or glycerol was not significantly reduced. Epicholesterol and androstan-3α-ol did not exert significant effects on the permeability of marker molecules. Cholest-5-en-3-one, cholest-4-en-3-one and cholestan-3-one did not alter the permeability of the lipid barrier. Therefore, it was concluded that the reduction of permeability depends on the presence of a planar sterol nucleus, 3β-hydroxyl group and an intact side chain (75). Many structural studies indicated a possible involvement of hydrogen bonding between lecithin and cholesterol in dry films (76, 77). Direct evidence of the interaction between lecithin and cholesterol was obtained in studies of the permeability of liposomes containing epicholesterol, thiocholesterol and cholesterol (68). The results of these studies support the idea that hydrogen bonds are involved in stablizing the lecithin-cholesterol complex.

Sterols have been recognized as important components of biological membranes. Cholesterol significantly reduced the mobility of the fatty acyl chains in the phospholipid bilayers containing egg lecithin (77A). The bilayer structure is modified when phospholipid preparations contain cholesterol. The effect of cholesterol on dipalmitoyllecithin-water dispersions was studied by

differential scanning calorimetry and by X-ray diffraction at varying ratios of the sterol and phospholipid (77B). The differential scanning curves indicate that with an increase in cholesterol concentration, the sharp gel-liquid-crystalline transition peak of dipalmitoyllecithin dispersions broadens and decreases. The endothermic peak virtually disappears when the ratio of cholesterol and dipalmitoyllecithin are equimolar. X-ray diffraction studies indicate that at 25° the long spacing in the low angle region, which is contributed by the lipid and water layers, is altered by the cholesterol content of dipalmitoyllecithin dispersions. At 7.5 mole % cholesterol there is a large increase in the long spacing, but on increasing the concentration of cholesterol the long spacing decreases to give a constant value of 64 Å at equimolar ratio of sterol and phospholipid. Dipalmitoyllecithin in the gel form has an ordered array of hydrocarbon chains having a tilt with an angle of 58° to the lipid-water surface. At low concentrations of cholesterol where the X-ray long spacing is increased, the hydrocarbon chains of dipalmitoyllecithin are aligned perpendicular to the lipid-water interface. On increasing the cholesterol concentration to 50 mole %, not only is there a decrease in the X-ray long spacing, but also there is a decrease in the amount of bound water. At 50 mole % of cholesterol in dipalmitoyllecithin dispersions, the lipid transition temperature is reduced, the heat of transition is decreased, the amount of water

bound to the lipid layers is increased and the X-ray long spacing is decreased. These findings suggest that cholesterol decreases the interaction between the adjacent fatty acyl chains and contributes to the fluidization of the hydrocarbon chains.

Biochemical Effects of Filipin

Of approximately 40 polyene antibiotics which have been studied, the most extensively investigated ones are filipin, nystatin and amphotericin B. Polyene antibiotics share some common structural features such as a macrolide ring, a conjugated system of double bonds (hydrophobic region) and a series of hydroxyl groups (hydrophilic region). Therefore, the antibiotics can be considered as amphipathic molecules.

1. Effects on Fungi

The mechanism of polyene action was studied by Wu in nearly 80 species of fungi (78). He found that filipin inhibited sporulation, growth and germination of yeast. Electron micrographs show that the yeast cell wall was damaged by filipin (26). The polyene antibiotic nystatin inhibited the growth and cell division in Candida and Saccharomyces and also inhibited respiration and glycolysis in yeast (79). Yeast cells grown in glucose, peptone and yeast extract under aerobic and anaerobic conditions were tested by Warburg's technique, and it was found that filipin was a strong inhibitor of respiration and glycolysis (78). The damage caused by filipin on yeast resulted in the leakage of nitrogen and phosphorus compounds into the medium, but this could be prevented by the addition of cholesterol simultaneously with the addition of the antibiotic to the incubation medium (78). The antibiotic binds to the free sterol in the medium, thus reducing the effective concentration of free polyene which can react with membrane-bound sterol.

2. Effects on Microorganisms

Tetrahymena pyriformis cells supplemented with ergosterol in the growth medium were lysed 20 times more effectively than cells in an unsupplemented medium (57). Acholeplasma laidlawii are pleuro-pneumonia-like organisms which grow equally well in the absence or in the presence of a sterol. Sterols are incorporated in the cell membrane when added to the growth medium. Cells grown in this manner were sensitive to filipin and produced spectral changes in the polyene (2). Acholeplasma cells that lack sterols are not affected by filipin (2).

3. Effect on Nucleotide Leakage

The discs isolated from cores of Solanum tuberosum and Beta vulgaris were treated with filipin. The material which absorbed at 260 nm was spontaneously released from the discs and was identified as nucleotides (80). The efflux of this material was greatly stimulated by filipin. The discs of Solanum tuberosum and Beta vulgaris contain free sterols and sterol esters. However, it is not known whether the mechanism involves adsorption, absorption or degradation of the antibiotic by materials in the disc.

4. Effects on Erythrocytes

Rat and human erythrocytes were lysed by filipin, nystatin and amphotericin (81). The extent of the lysis by filipin chiefly depended on the antibiotic to cell concentration ratio (82). The filipin derivatives,

perhydrofilipin and irradiated filipin, did not exhibit the same biological activity. Molar ratios of filipin to erythrocyte lipid of at least 0.1 were required for maximal hemolysis (83). Erythrocyte ghosts lysed by filipin displayed disruptions in the red cell membrane (83).

5. Effect on Calcium Transport

Vitamin D mediates calcium transport (84). Filipin was used as a probe for the biochemical function of transport. An increase in the calcium flux was observed in ileal segments of rachitic chicks in the presence of filipin (85). Thiourea and water flux were also increased. Electron micrographs revealed that prolonged exposure of ileal segments to filipin in rachitic chicks caused gross morphological alterations that were not observed with the ileal segments of vitamin D-treated chicks.

Transference number measurements indicated that filipin rendered lecithin-cholesterol bilayers cation selective and more permeable to calcium than other cations such as Mg^{2+} and La^{3+} (28). These results differ from the conclusions arrived at independently by Finkelstein and Cass (60) and by Andreoli and Monahan (61). They showed that after the introduction of the antibiotic, the lecithin-cholesterol bilayer was destroyed and the film did not exhibit ion selectivity.

Recently it has been reported that polyenes bind at the calcium affinity site of the cytoplasmic membrane of Debaromyces species (86), thus inhibiting the growth of the organism.

Probes Used to Determine the Structure and Function of Membranes

The interpretation of the structural and functional changes in membranes has been made possible recently by the use of spectroscopic probes. When the molecules under study have chromophores, they can be used as probes. Such molecules have been designated as "intrinsic probes." Indicators added externally are referred to as "extrinsic probes." Extrinsic probes have been used extensively to study membrane structure. The use of probes and analysis of the probe responses to the environment have aided in understanding the structural changes in cell membranes. Anilino-8-naphthalene sulfonic acid (ANS) and 2-toluidino-6-naphthalene sulfonic acid (TNS) are known to bind to proteins and have been used to study the binding characteristics of macromolecules. These fluorophores are almost nonfluorescent in aqueous media, but are highly fluorescent in a nonpolar environment. ANS has been used to study the state changes in the mitochondrial particles that accompany energization of the membrane (87), responses of Ca^{2+} binding to the mitochondrial membranes (88), changes in the sarcoplasmic reticulum under the influence of cations, pH, temperature and antibiotics (89) and the interaction of ions with mitochondrial membranes (90). When ANS binds to submitochondrial particles, its fluorescence increases (91, 92). The binding is accompanied by a hypsochromic shift, which indicated that the ANS site was not accessible to the aqueous

environment (93). The addition of phospholipase A lowered the fluorescence intensity of bound ANS (94). Therefore, the binding site has been considered to be, at least in part, phospholipid. These results have been confirmed by the findings that ANS binds to phospholipid micelles. Furthermore, local anesthetics react specifically with phospholipids and affect the fluorescence intensity of ANS in natural and model membranes (94).

Hemoglobin-free erythrocyte membranes interact with ANS. ANS binds to these membranes with enhanced fluorescence in the presence of cations (95). The volume changes observed in the erythrocytes in the presence of NaCl were due to the changes in membrane structure and resulted in the enhanced binding of ANS. It has been proposed that the introduction of the electrolyte results in the shielding of the electrostatic repulsive forces of the polar groups of the phospholipids in the erythrocyte membrane by the cations in the medium. This environment may induce alterations in the packing of the hydrocarbon chains of the phospholipids and thus increase the number of ANS binding sites in the membrane, resulting in the enhancement of the fluorescence intensity of the dye.

The fluorescence of ANS was enhanced by the skeletal muscle microsomes and phospholipids (96). The enhancement was a function of ionic composition and increased with cation concentration. La^{3+} increased the fluorescence intensity eight-fold when compared with ANS microsome mixtures in the

absence of the salt. Divalent cations produced four-fold enhancement and monovalent cations produced two-fold enhancement in ANS-microsome suspensions. The fluorescence enhancement in ANS-microsome suspensions and ANS-phospholipid dispersions was optimal below pH 6 and was accompanied by transitions which occurred at 35 - 40°. These changes occurred near the crystalline-liquid crystalline phase transition temperatures of the phospholipids of the microsomes and of phosphatidylcholine.

The interaction of leucocytes with ANS produced a marked rise in the fluorescence intensity (97). The enhancement was a linear function of protein concentration and occurred in about 2 seconds. When polystyrene spherules were added to untreated leucocytes, there was very little increase in the fluorescence intensity and in the oxidation of glucose, but when polystyrene spherules were added to pretreated ANS-containing leucocytes, there was an immediate enhancement of fluorescence intensity and oxidation of glucose. These results suggested that many cytoplasmic nonpolar sites of the membrane have been made available as the dye binding sites by the addition of ANS to the leucocytes. Therefore, the alteration in the cell structure appears to be related to the metabolic processes in the cell.

Studies of Phospholipid-Cholesterol Interactions Using Filipin as a Probe

The main theme of the problem presented in this dissertation is to study phospholipid-sterol interactions in model membranes. The solution to this problem would aid in understanding the relation between the structure and function of various lipid components found in natural membranes. Knowledge of the detailed molecular composition of cellular membranes and the structural relationship of lipid components would aid in interpreting the crucial role played by biological membranes in cellular phenomena. Recent studies have indicated that structural changes in the phospholipids and sterols lead to changes in the initial rates of osmotic shrinking of liposomes (68). Many functional changes are induced in biological membranes when the composition of the lipid constituents is altered. Ergosterol-grown cells are lysed by filipin twenty times more than tetrahymanol-containing cells in Tetrahymena pyriformis (57). When the mycelium of the Pythium species is grown in the presence of the sterol, the leakage of the cell components is decreased (70).

In this dissertation, phospholipid and sterol interactions were studied from two aspects, namely by altering the structures of the sterol and the phospholipids in model membranes, and by the incorporation of sterols in ciliary membranes of Tetrahymena pyriformis. The polyene antibiotic filipin was used as a membrane probe to study these interactions.

The interaction of phospholipids with sterols was investigated by monitoring changes observed in the absorption, fluorescence and circular dichroism spectra of filipin.

The two phospholipids studied were egg lecithin and dipalmitoyllecithin. Egg lecithin was in the liquid crystalline form, whereas dipalmitoyllecithin was in the gel form at the temperatures used. It was expected that the absorption spectrum of the polyene in the presence of these two types of phospholipid preparations would be different from each other as well as when compared with that of the free polyene. The difference spectra of free filipin and filipin bound to model membranes containing cholesterol would indicate the specific wavelength regions of the chromophore that are positively and negatively perturbed and the intensity of perturbation at varying concentration of the phospholipids in the presence and absence of sterols. Changes in the absorbance of filipin occur when the polyene is bound to model membranes containing sterols. Comparisons of the peak ratio 323 nm/358 nm in the presence of these model membranes would indicate whether the interaction of the polyene with model membranes is greatly influenced by the stereochemistry of the sterol. The ciliary membranes from Tetrahymena cells supplemented with ergosterol and from unsupplemented cells were studied to observe if there were any spectral changes in filipin in the presence of these membranes.

The presence of conjugated double bonds in filipin

produces a strong fluorescence in an apolar media (5). Experiments were planned to observe the binding of sterols (which were substituted derivatives of cholesterol) to phospholipids in vesicles by monitoring the changes observed in fluorescence when bound to filipin (5). The ciliary membranes of Tetrahymena when supplemented with ergosterol in the medium incorporates the sterol in its ciliary membranes (57). If the binding of filipin to ciliary membranes obtained from unsupplemented Tetrahymena and Tetrahymena supplemented with ergosterol produces changes in the fluorescence intensity, then the polyene could serve as a sensitive indicator of the relative strengths of phospholipid-ergosterol and phospholipid-tetrahymanol interactions in natural membranes.

The presence of many optically active centers in filipin suggested that CD spectra could monitor changes that are encountered in phospholipid-sterol interactions.

MATERIALS AND METHODS*

All solvents and chemicals used were of reagent grade. The solvent for filipin was DMSO. It was distilled prior to use. For the absorption, fluorescence and circular dichroism studies, solvents used were of spectral grade.

Buffers

The experiments were performed in the following buffers:

1. 5×10^{-3} M disodium phosphate, pH 7.0.
2. 5×10^{-3} M monosodium phosphate, 5×10^{-3} M disodium phosphate containing 5% DMSO by volume, pH 7.0.
3. 0.01M Tris, pH 7.4.
4. 0.01 M Tris, containing 2.5% DMSO by volume, pH 7.4.
5. 0.01 M Tris containing 5% DMSO by volume, pH 7.4.
6. 0.01 M Tris - 0.1 M NaCl, pH 8.5.
7. 0.01 M Tris - 0.1 M NaCl containing 2.5% DMSO by volume, pH 8.5.
8. 0.01 M Tris - 0.1 M NaCl containing 1.25% DMSO by volume, pH 8.5.
9. 0.01 M Tris - 0.1 M NaCl containing 1.25% ethylene glycol by volume, pH 8.5.

---

*The following abbreviations have been used in this dissertation: DPL, dipalmitoyllecithin; EYL, egg lecithin; CD, circular dichroism; DMSO, dimethyl sulfoxide; DMF, dimethylformamide; DTNB, 5, 5'-di-thiobis(2-nitrobenzoic acid).

Purity of Filipin

Filipin III used in these studies was from batch number U-25 (639) 8395-MEB-116. Its purity was analyzed by thin-layer chromatography. Silica gel H and Silica gel $H_{256 + 366}$ were purchased from Merck. Thin-layer chromatography plates were prepared from 15 g silica gel suspended in 15 ml of 0.2 M KH_2PO_4 and 15 ml of 0.2 M Na_2HPO_4. The glass plates were coated evenly with the slurry and air-dried for 2 hours at 130°. Silica gels 6060, 6061, F and aluminum oxide type 7 were used to prepare the plates and were obtained from Eastman Kodak Company. Filipin on thin-layer chromatography plates gave a single spot in methylene chloride : methanol (85:15 v/v) having an R_f value of 0.8.

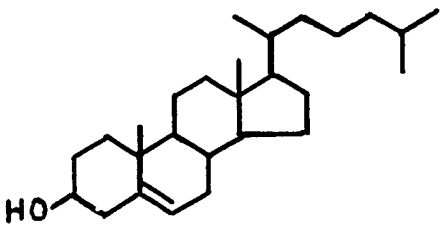
Purity of Phospholipids

DPL was purchased from General Biochemicals. EYL was prepared by a modification of the procedure of Singleton and coworkers (98). On thin-layer chromatography DPL gave a single spot in a solvent system consisting of chloroform and methanol (9:1, v/v) and (7:3, v/v). EYL also gave a single spot in chloroform : methanol (9:1, v/v).

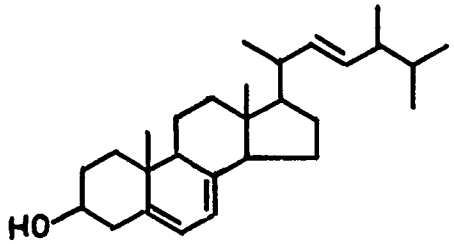
Source of Sterols

Cholesterol, cholesteryl acetate, epicoprostanol and β-cholestanol were purchased from Sigma Chemical Company. Thiocholesterol (Aldrich Chemical Company) was supplied by Dr. C. Huang. The structures of the sterols are given in figure 3. Cholesterol and other sterols were dissolved in

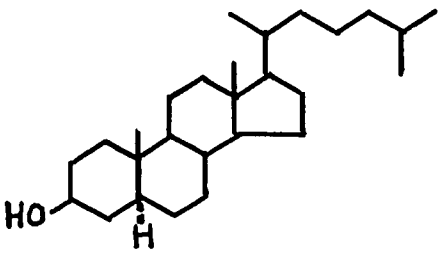
Figure 3. The structure of sterols and triterpenoid alcohol found in natural membranes and sterol derivatives incorporated in model membranes.



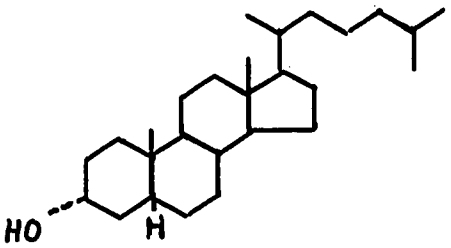
Cholesterol



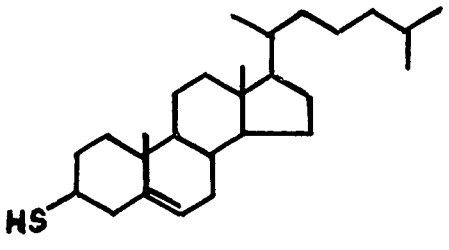
Ergosterol



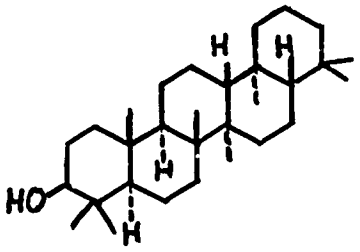
β-cholestanol



Epicoprostanol



Thiocholesterol



Tetrahymanol

chloroform and 30 μg was spotted on silica gel plates and developed in benzene. The dry sheet was sprayed with a saturated solution of antimony chloride in chloroform and kept in an oven at 100° for 2 minutes. Each sterol consistently gave a single orange spot on thin-layer chromatography.

Absorption Studies

The absorption and the difference spectra were recorded on a Cary 14 recording spectrophotometer. The temperatures were maintained at ± 0.1° by a Lauda K-2/R circulator (Brinkmann Instruments, Inc.). Cuvettes with an effective path length of 10 mm were employed. Four identical matching cuvettes were used to record the difference spectra. Two were placed in the sample compartment and the other two in the reference compartment. Buffer and membrane suspensions were placed in the reference compartment. One of the cuvettes in the sample compartment contained free filipin and the other contained filipin in the presence of the membranes. Only after a nondrifting baseline was obtained with the buffer were the ultraviolet and the visible difference spectra recorded.

Kinetic Studies

The rate constant of the slow reaction was calculated using the absorption change at 358 nm. The rate constant for the slow reaction was obtained from the slope of the plot of Δ absorbance vs. time on semilogarithmic graph paper. The slow reaction at a single wavelength was recorded at a temperature of 28°, unless otherwise specified.

The changes in absorbance were recorded immediately after mixing at the desired temperature. The infinity point for the reaction was obtained from the plots of the reciprocal of the observed absorbance vs. the reciprocal of the corresponding time according to the established procedures used by Rubalcava and coworkers (95). Free filipin did not undergo changes in absorption with time in a sealed cuvette when the antibiotic was stored in the dark at 4^{o} and flushed with nitrogen.

Fluorescence Studies

Fluorescence measurements were performed on three different instruments: A laboratory built fluorometer, a Farrand spectrofluorometer and a Hitachi Perkin-Elmer Model MPF-2A spectrofluorometer. The laboratory model was equipped with a 150 W xenon arc lamp. The light at 400 cps was passed through a Corning uv filter (C7-60) and focused by means of two lenses on the sample. The fluorescent light was then focused through two lenses on the entrance slit of a Farrand grating monochromator. The signal from the photomultiplier was fed into a lock-in amplifier which was chopped at 400 cps. The intensity was read on a lock-in amplifier. For these measurements, the light source, the sample and the spectrometer were set at a 90^{o} angle.

The Farrand and Hitachi Perkin-Elmer spectrometers use the same principle except that there are two monochromators which with other accessories are set and fixed in a commercially sold instrument.

The experimental conditions and the type of fluorometers used are given in the figure captions. Fluorescence polarization studies were performed by Dr. S. Levison of the Scripps Clinic and Research Foundation, La Jolla, California on an Aminco Bowman spectrofluorometer.

Circular Dichroism Studies

Circular dichroism measurements were performed on Cary 60 spectropolarimeter with a model 6001 circular dichroism accessory. The CD spectra of solutions were obtained in a tandem cell having two compartments of 10 mm path length each. CD spectra were recorded at a very low range, e.g., 0.02, 0.04 or 0.1, depending on the sensitivity required. The dynode voltage was not allowed to exceed 0.35. The absorbance of the preparation was kept below 1.5. Each spectrum recorded on the Cary 60 was analyzed by hand and was corrected for the contributions made by the vesicles alone. All CD measurements were made at room temperature. The molar ellipticity $[\theta]$ in degrees $.cm^2$ per dmole was calculated from the relationship:

$$[\theta] = \frac{\theta \times 100}{l \times M}$$

where θ is the observed rotation in millidegrees, l is the cell path length in dm, and M is the molarity of filipin. The molarity was calculated from the absorbance and the extinction coefficient of the filipin used in the binding studies. The extinction coefficient of filipin in 0.01 M Tris containing 2.5% DMSO by volume, pH 8.5, is 2.81×10^4 M^{-1} cm^{-1} at 338 nm at the time measurement was made.

Ultracentrifugation Studies

The ultracentrifuge experiments were performed at 20° in a Spinco Model E analytical ultracentrifuge equipped with an automatic camera and Schlieren optical system fitted with a phase plate. A double sector synthetic boundary aluminum/epon cell was used. The phase plate angle was 70° or 45° and the rotational speed was either 42,000 or 40,000 rpm.

Preparation of DPL Lamellae

DPL (130 mg, 1.81×10^{-1} mmole) was sonicated in 13 ml of 5×10^{-3} M monosodium phosphate, 5×10^{-3} M disodium phosphate buffer, pH 7.0, under nitrogen purge for 2 hours at 4°. A Branson model S-110 sonifier with a tap horn was used at power level 3 in a vessel through which ice water was circulated. The dispersion thus obtained was centrifuged at 14,000 rpm for 30 minutes. The supernatant was dialyzed extensively in the above mentioned buffer at 4°. The dispersion was turbid and had a milky appearance. The uv spectrum of the dispersion was recorded.

DPL lamellae were prepared with cholesterol in the same manner. DPL and cholesterol were in the molar ratio of 10:1 in these lamellae.

Preparation of Vesicles with and without Sterols and DPL Lamellae

Phospholipid model membranes prepared from EYL or DPL have been used in the research described in this thesis. Preparations obtained from DPL referred to as lamellae are large membranous fragments. Model membranes derived from

EYL are referred to as vesicles. Vesicles are bilamellar structures having a diameter of about 250Å and a homogeneous shell-like structure.

EYL (300 mg, 3.83×10^{-1} mmole) was suspended in 10 ml of 0.01 M Tris-0.1 M NaCl, pH 8.5. The solution was ultrasonically irradiated under nitrogen using a Branson sonifier equipped with a solid tap horn at power level 3 or 4 for 3 hours in a jacketted vessel maintained at 4°. The undispersed phospholipid was removed by centrifugation at 105,000 x g for 1 hour at 4°. The clear supernatant was collected and filtered through an extensively washed 0.1 - μ MF4 Sartorius filter. The filtrate was subjected to molecular sieve chromatography on a Sepharose 4B column (2.5 x 45 cm) at 4°. The absorption of the column effluent was continuously monitored at 300 nm on an Optica spectrophotometer equipped with a flow cell or at 254 nm using a Uvicord II detector (LKB Instruments). The effluent solution was collected by an automatic fraction collector (Buchler Instruments). Fractions from the second peak of the elution pattern were collected and concentrated in a Sartorius collodion bag under vacuum. The concentrated vesicle preparation was equilibrated with fresh buffer by dialysis overnight at 4°.

The input molar ratio of EYL and sterols in vesicles containing cholesterol, thiocholesterol, cholesteryl acetate, β-cholestanol and epicoprostanol was 18:1. DPL lamellae were prepared in the same fashion as EYL vesicles.

The concentration of the vesicles and lamellae were

expressed in terms of phosphate, which was estimated by the Kodak-Elon method. This was performed by transferring 0.1 ml of the vesicle preparation to a large dry test tube. To this, 0.5 ml of water, 0.25 ml of 16 N H_2SO_4, a few carborundum chips and a few drops of n-butanol were added. The tube was heated in a digestion apparatus until dense white fumes appeared. The tube was allowed to cool and two drops of 20% H_2O_2 were added, and the tube was heated again until the sample was colorless. The solution was brought to a volume of 4 ml with water. Aliquots of 0.1, 0.2, 0.3, 0.4, 0.8 and 1.0 ml of phosphate standard solution was treated exactly the same way as the sample and brought to 4 ml with water. A standard solution of 1 x 10^{-3} mole was made by dissolving 0.136g of KH_2PO_4 in 3 ml of concentrated H_2SO_4 and diluting to 1 liter. The phosphate standard solution contained 2 μmoles of phosphate per ml. To all solutions 1 ml of ammonium molybdate solution (50 g of ammonium molybdate dissolved in 400 ml of 10 N H_2SO_4 and then made to 1 liter by addition of water) and 1 ml of Kodak-Elon (5 g of sodium bisulfite dissolved in 500 ml of water) were both added to the standard and sample solutions to make a final volume of 6 ml. The solutions were neutralized with 6 N NaOH. The absorbance of the solutions was read after 15 minutes at 660 nm. Neutralization of the solutions with alkali prior to making absorption measurements was essential to obtain accurate values.

Preparation of Filipin Solution

To make an aqueous solution, filipin was first dissolved in DMSO and then diluted in buffer. Before each experiment, filipin was freshly prepared; however, solutions prepared the previous day were used when they were stored at 4^{o} in the dark. In order to prevent oxidation, solutions were made in the dark with minimum exposure to air. The concentration of filipin was calculated from the extinction coefficient obtained in 0.01 M Tris - 0. 1 M NaCl containing 2.5% DMSO by volume, pH 8.5.

Ciliary Membranes

Ciliary membranes isolated from Tetrahymena pyriformis strain W were supplied by Dr. E. Kaneshiro of Prof. R. Conner's laboratory, Bryn Mawr College, Bryn Mawr, Pennsylvania. The preparation of these membranes has been described in reference 57.

RESULTS AND DISCUSSION

Column Chromatography of Vesicles and Lamellae

Figure 4 shows the elution pattern of DPL lamellae prepared with cholesterol. Fraction 5 which appeared as a peak was concentrated and rechromatographed. The elution pattern on rechromatography indicated that the lamellae were homogeneous.

Figure 5 shows the Sepharose 4B elution patterns of the vesicles and DPL lamellae.

Ultracentrifugal Analysis of EYL Vesicles and DPL Lamellae

The ultracentrifugal schlieren pattern of fraction II of EYL vesicles in Tris buffer indicated that the vesicles were homogeneous (figure 6A). In buffer containing 5% methanol by volume, pH 8.5, these vesicles were split into smaller species (figure 6B). The schlieren pattern of the vesicles was qualitatively unchanged in 0.01 M Tris containing 5% DMSO by volume, pH 8.5, when compared to that in Tris buffer alone (figure 7A). Ultracentrifugal studies on DPL lamellae indicated that the preparation was homogeneous (figure 7B).

Figure 4. The Sepharose 4B elution pattern of DPL: cholesterol lamellae in a molar ratio of 7:1. The column had dimensions of 9 x 30 cm. The volume of each fraction was 1.5 ml. The absorbance of the fractions were read at 256 nm on a Gilford spectrophotometer. The 0-0.1 absorbance slidewire was used to record the absorption of the elution pattern (——). Rechromatography of peak I after concentration is also shown (---). Effluent from the column was collected at the rate of 0.1 ml/min.

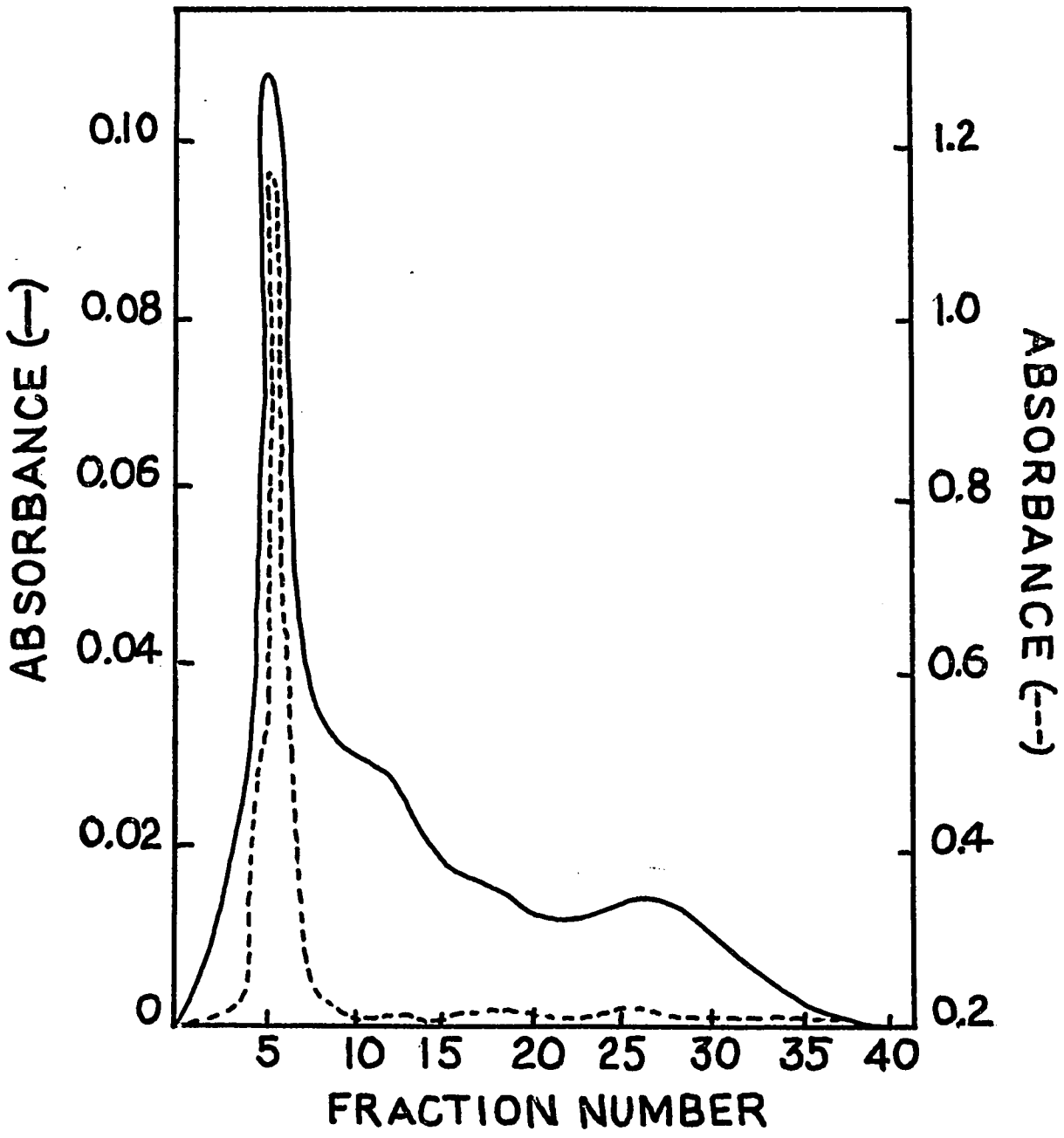

0.10
0.08
0.06
0.04
0.02
0
ABSORBANCE (—)
1.2
1.0
0.8
0.6
0.4
0.2
ABSORBANCE (---)
5
10
15
20
25
30
35
40
FRACTION NUMBER

Figure 5. The Sepharose 4B elution pattern of EYL vesicles containing sterols and DPL lamellae. The column dimensions were 2.5 x 45 cm. Each fraction had a volume of 3 ml. The absorption of the column effluent was monitored on a Uvicord II at 254 nm. The fractions between the arrows indicate fraction II. The flow rate was 0.2 ml/min.

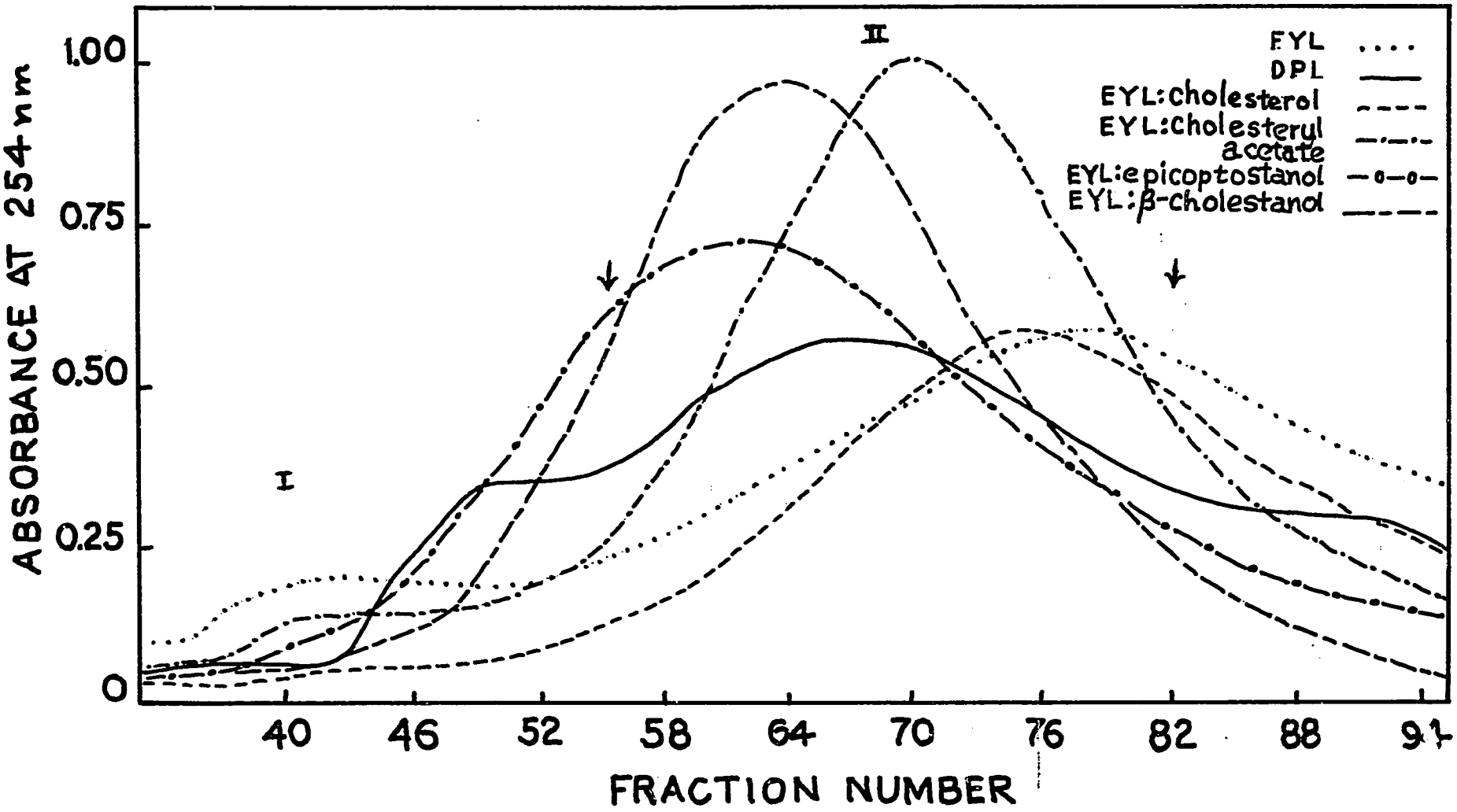

ABSORBANCE AT 254 nm
1.00
0.75
0.50
0.25
0
I
II
EYL
DPL
EYL:cholesterol
EYL:cholesteryl acetate
EYL:epicoptostanol
EYL:β-cholestanol
40
46
52
58
64
70
76
82
88
91
FRACTION NUMBER

Figure 6(A). The ultracentrifugal pattern of fraction II of EYL vesicles (1.91×10^{-2} mmole/ml) in 0.01 M Tris-0.1 M NaCl, pH 8.5. The time given in the figure refers to the profile obtained after the rotor attained a speed of 40,000 rpm at 4°. The phase plate angle was 70° throughout.

Figure 6(B). The ultracentrifugal pattern of fraction II of EYL vesicles (1.91×10^{-2} mmole/ml) in 5% methanol-0.01 M Tris-0.1 M NaCl, pH 8.5. The experimental conditions are similar to those described in figure 6(A).

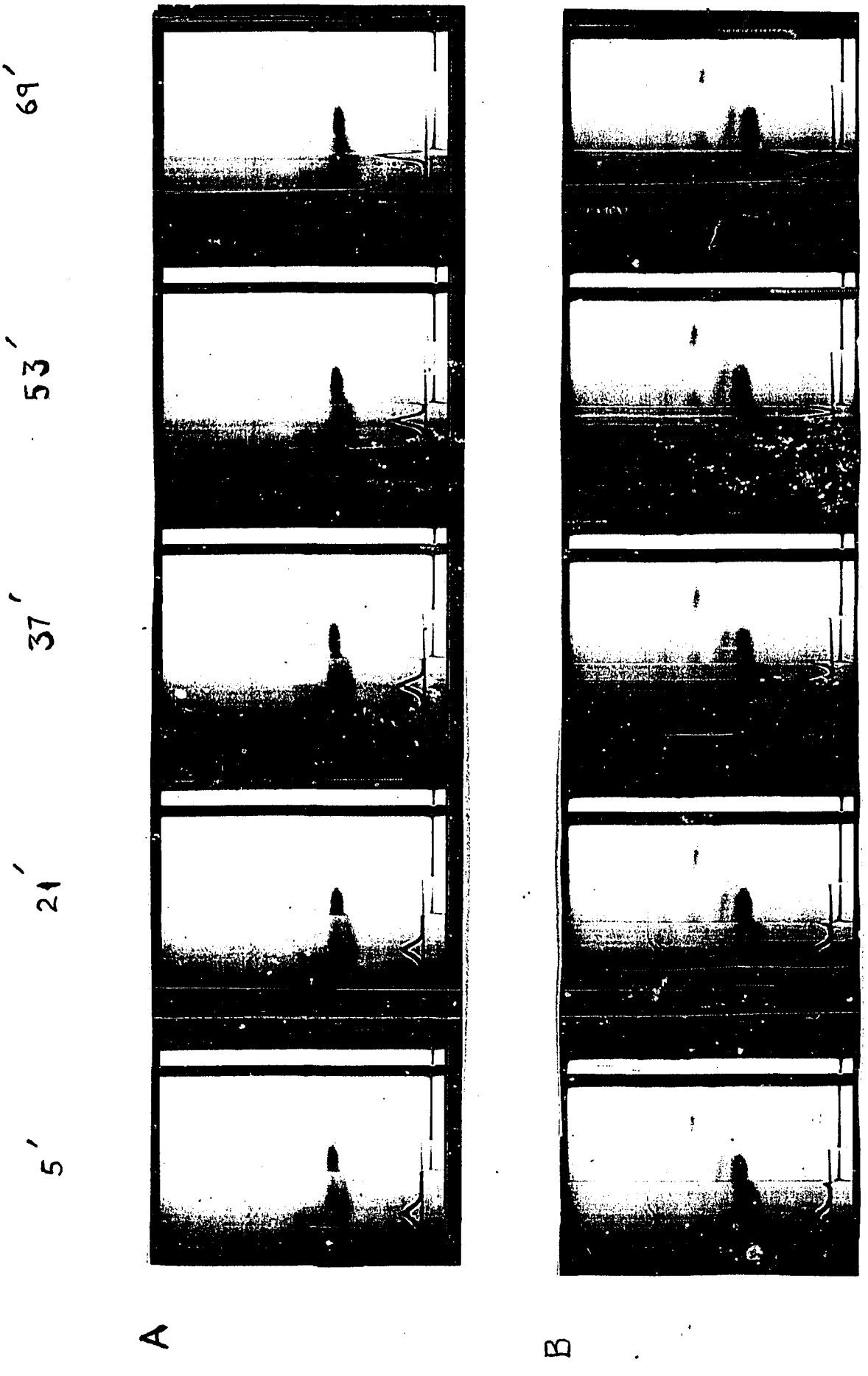
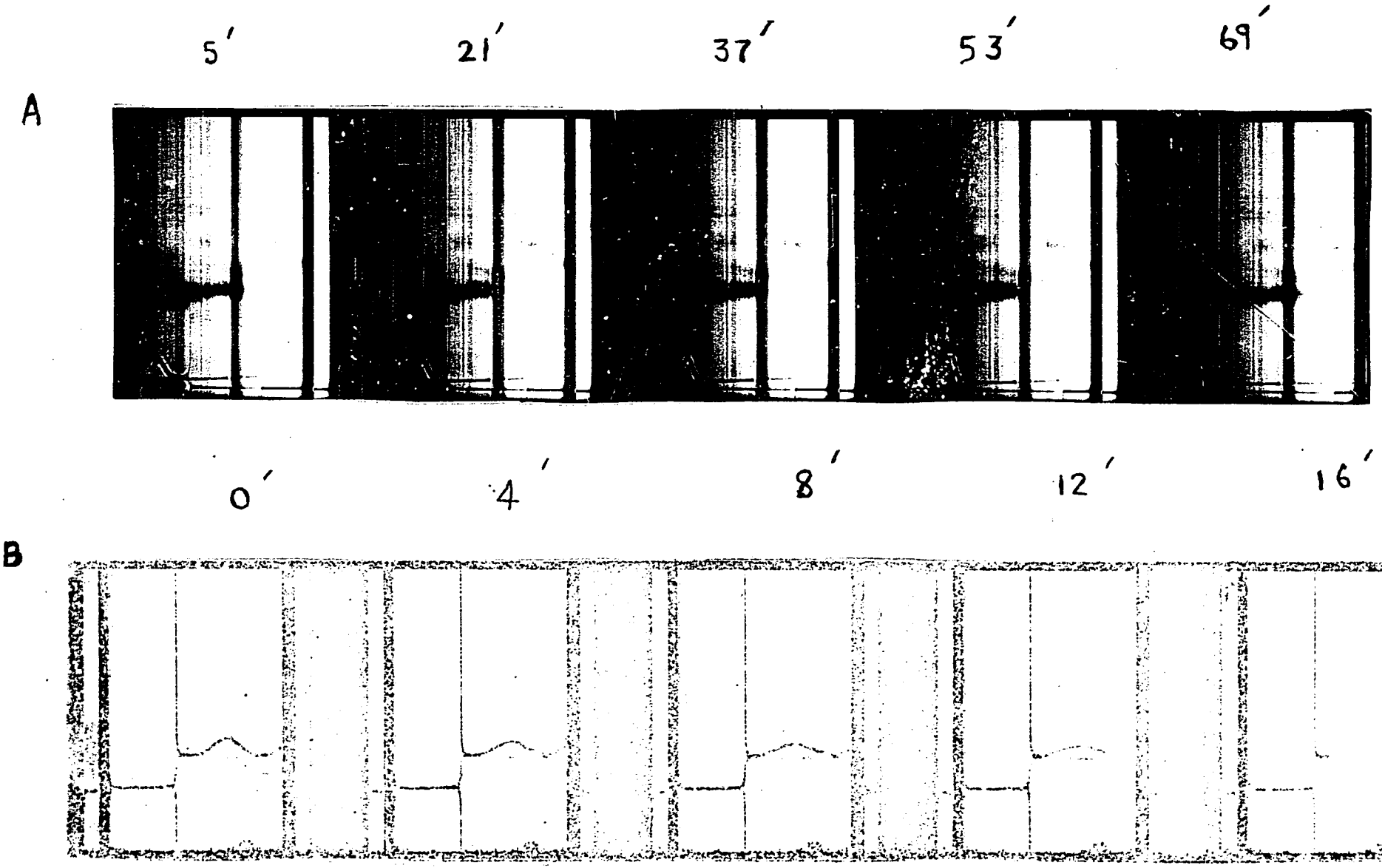

69'
53'
37'
21'
5'
A
B

Figure 7(A). The ultracentrifugal pattern of fraction II of EYL vesicles (1.91×10^{-2} mmole/ml) in 5% DMSO-0.01 M Tris-0.1 M NaCl, pH 8.5. The time given in the figure refers to the profile obtained after the rotor attained a speed of 40,000 rpm at 4°. The phase plate angle was 70° throughout.

Figure 7(B). The ultracentrifugal pattern of DPL lamellae (9.12×10^{-3} mmole/ml) in 0.01 M Tris, pH 7.4. The time given in the figure refers to the profile obtained after the rotor attained a speed of 40,000 rpm at 4°. The phase plate angle was 45° throughout.


5′
21′
37′
53′
69′
A
0′
4′
8′
12′
16′
B

Absorption Studies

Figure 8 shows the uv absorption spectra of filipin. The absorption bands are characteristic of compounds containing the polyene chromophore and are designated as 1, 2 and 3, commencing from the visible side of the spectrum. The extinction coefficient of filipin in pyridine is 6.02×10^4 M^{-1} cm^{-1} at 346 nm; in DMF the extinction coefficient is 7.92×10^4 M^{-1} cm^{-1} at 345 nm.

The spectra in figure 8 show that the filipin undergoes spectral changes in DMF, pyridine, aqueous solutions of pyridine and DMF, and in the presence of EYL vesicles when compared with the spectrum of filipin in 1.25% DMSO-Tris buffer. The shift of the absorption maxima in nonpolar solvents toward longer wavelengths suggested that in the presence of aqueous dispersions of phospholipids filipin was in a nonpolar environment and formed aggregates in aqueous solution.

Table 1 shows the effect of temperature on the absorption peak ratio 323 nm/358 nm of free filipin and filipin in the presence of an aqueous suspension of cholesterol, the ratio of peaks 323 nm/358 nm was increased. A 6° change in temperature did not alter the peak ratio 323 nm/358 nm of free or bound filipin immediately after mixing to a large extent.

Figure 8. The uv spectra of filipin in pyridine, DMF, 10% pyridine-90% water, 1.25% DMSO-0.01 M Tris-0.1 M NaCl, pH 8.5. The concentration of filipin in pyridine, DMF, 10% pyridine and 10% DMF was 2.0×10^{-5}M and in free and filipin bound to EYL vesicles in 1.25% DMSO-Tris was 2.1×10^{-5}M. The concentration of EYL vesicles was 1.1×10^{-2}M (phosphate).

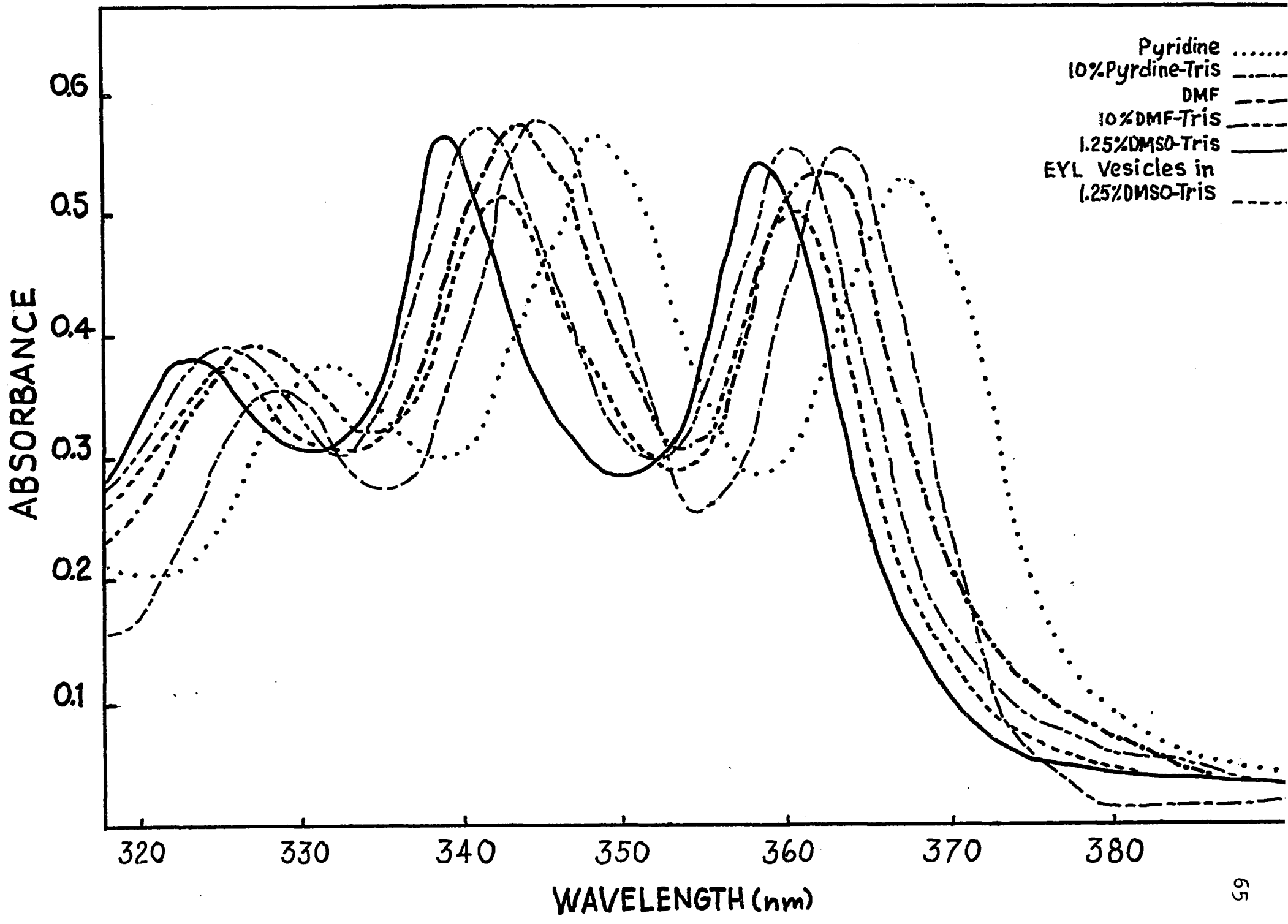

Pyridine
10%Pyrdine-Tris
DMF
10%DMF-Tris
1.25%DMSO-Tris
EYL Vesicles in
1.25%DMSO-Tris
ABSORBANCE
0.6
0.5
0.4
0.3
0.2
0.1
320
330
340
350
360
370
380
WAVELENGTH (nm)

Table 1: Effect of temperature on the absorption ratio of peaks 323 nm/358 nm of free and filipin bound to EYL vesicles, EYL:cholesterol vesicles and aqueous suspensions of cholesterol.

Medium	Temperature (°C)	Ratio of the absorbance of peaks 323 nm/358 nm	
		Free	Bound
Cholesterol[1]	28	0.87	0.97
EYL vesicles[2]	17	0.73	0.87
EYL vesicles[2]	23	0.77	0.96
EYL:cholesterol vesicles[2]	17	0.72	1.2
EYL:cholesterol vesicles	23	0.77	1.3

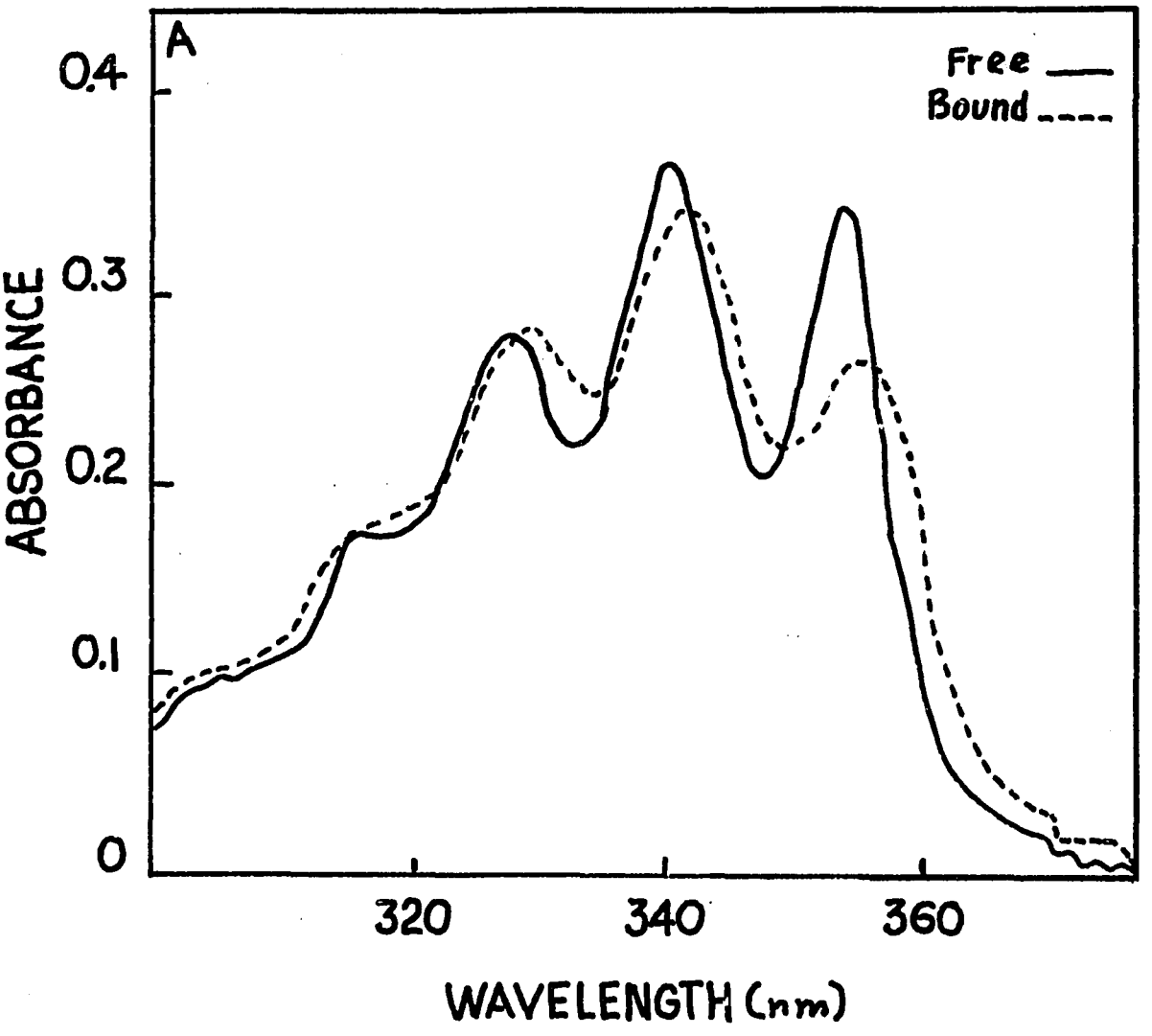
1. The concentration of filipin in 20% ethanol-2.5% DMSO phosphate buffer, pH 7.0, was 1.28×10^{-5}M. A stock solution of cholesterol (1 mg of cholesterol in 4 ml of ethanol) was prepared and aliquots were withdrawn and mixed with varying volumes of phosphate buffer. A sample containing 3.30×10^{-5}M of cholesterol and 1.3×10^{-5}M filipin in 20% ethanol-2.5% DMSO-phosphate buffer, pH 7.0 was used to examine the change in peak ratio 323 nm/358 nm of filipin on interaction with the sterol.

2. The filipin to phosphate ratio was 2.7×10^{-2}. The concentration of filipin was 1.8×10^{-5}M after mixing with the vesicles. The molar ratio of EYL: cholesterol in the vesicles was 18:1.

Figures 9A and 10A reveal bathochromic shifts, significant decreases in the extinction coefficients of peaks 1 and 2 and an increase in the ratio of the absorbance of peaks 323 nm/358 nm of the absorption maxima of filipin on binding to DPL lamellae and DPL:cholesterol lamellae. The alteration in the peak ratio 323 nm/358 nm of filipin is more pronounced when bound to sterol-containing lamellae than when bound to DPL alone, but bathochromic shifts of filipin seem to be larger in the presence of DPL lamellae than when bound to DPL:cholesterol lamellae. The difference spectra of filipin bound to the above two types of phospholipid preparations are different. Figure 9B shows relatively small perturbations in the 300-322-nm region when filipin was allowed to interact with DPL lamellae and displayed positive maxima differences at 315, 330, 347 and 365 nm. Sharp inflection points occurred between 360-365 nm, 350-355 nm, 340-342 nm, 335-337 nm, 325-327 nm and 312-315 nm. Distinct negative difference maxima occurred at 358, 337 and 320 nm. The ratio of peaks 323 nm/358 nm was altered from 0.81 in free filipin to 1.1 in filipin bound to DPL lamellae. The difference spectrum of filipin bound to DPL-cholesterol lamellae was more intense and qualitatively different in displacement. Figure 10B shows that the negative peaks are more intense at 360 and 339 nm than at 323 nm. A single and prominent positive peak was evident at 316 nm. Only one crossover point was present (between 318 and 320 nm).

Figure 9(A). The spectra of free filipin and filipin in the presence of DPL lamellae in 2.5% DMSO-phosphate buffer, 5 x 10^{-3}M, pH 7.1, at room temperature. The final concentration of filipin was 1.3 x 10^{-5}M and that of vesicles was 4.2 x 10^{-6}M (phosphate). The lamellae were prepared from DPL in phosphate buffer. The ratios of the absorbance of peaks 323 nm/358 nm are 0.81 and 1.1 in free and bound filipin, respectively.

Figure 9(B). The ultraviolet difference spectrum of free and bound filipin obtained from the spectra in figure 9(A).


A
Free
Bound
0.4
0.3
0.2
0.1
0
ABSORBANCE
320
340
360
WAVELENGTH (nm)

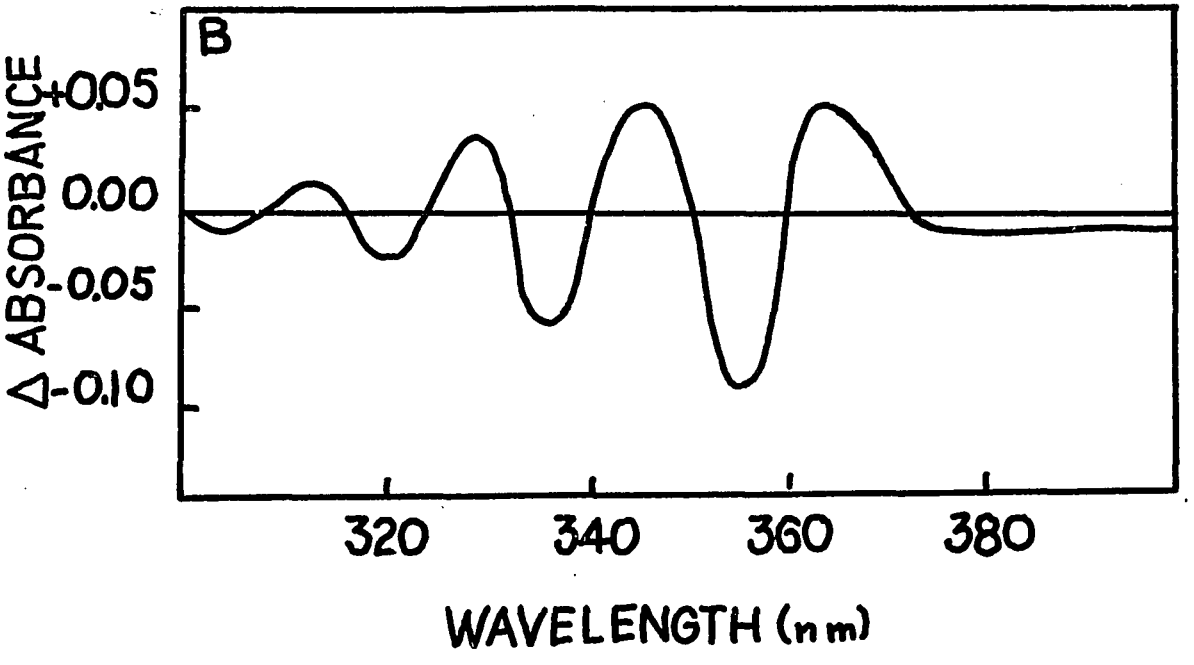
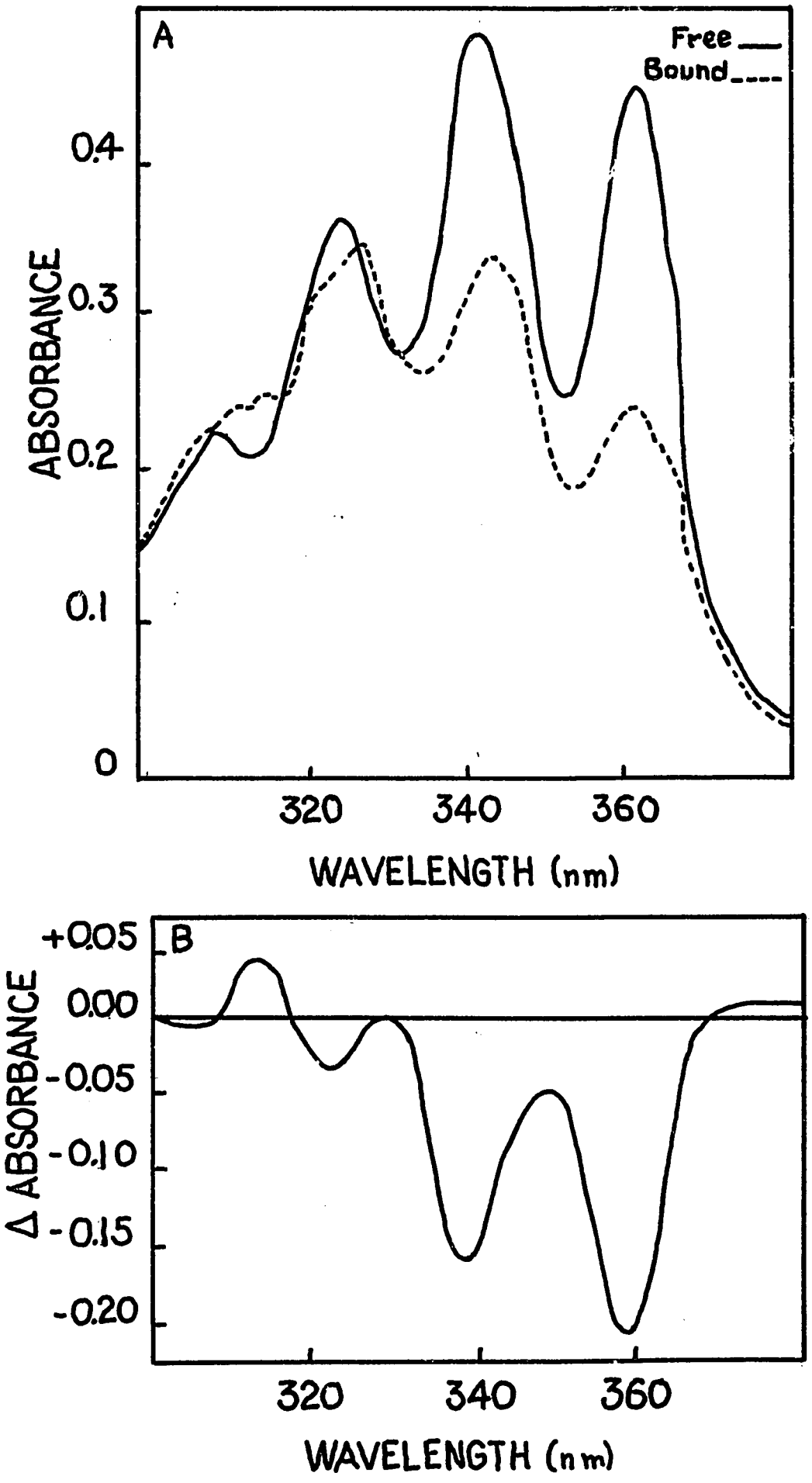

B
+0.05
0.00
-0.05
-0.10
Δ ABSORBANCE
320
340
360
380
WAVELENGTH (nm)

Figure 10(A). The spectra of free filipin and filipin in the presence of DPL:cholesterol lamellae in 2.5% DMSO-0.01 M Tris, pH 7.4, at room temperature. The final concentration of filipin was 1.9×10^{-5}M and the concentration of vesicles was 7.0×10^{-6}M (phosphate). The lamellae were prepared in 0.01 M Tris, pH 7.4, from DPL: cholesterol in 7:1 molar ratio. The ratio of the absorbance of peaks 323 nm/358 nm, was 0.81 and 1.4 in the free and bound form, respectively.

Figure 10 (B). The ultraviolet difference spectrum obtained from free and bound filipin in figure 10(A).


A
Free
Bound
ABSORBANCE
0.4
0.3
0.2
0.1
0
320
340
360
WAVELENGTH (nm)
B
Δ ABSORBANCE
+0.05
0.00
-0.05
-0.10
-0.15
-0.20
320
340
360
WAVELENGTH (nm)

The ratio of peaks 323 nm/358 nm was increased from 0.81 in free filipin to 1.41 in the bound form. Comparison of figure 9B with figure 10B suggests that the difference spectra arise from the absence (figure 9B) or presence of cholesterol (figure 10B) in the lamellae. Since the absorption spectra of filipin in the presence of cholesterol-phospholipid dispersions differed from the spectrum of free filipin, cholesterol-DPL dispersions at varying concentrations were allowed to interact with a fixed amount of filipin and their spectra were recorded. Figures 11A and 11B show that on increasing the dispersion concentration, the extinction coefficients at the absorption maxima decreased and the ratio of peaks 323 nm/358 nm increased from 0.86 to 1.14, 1.32 and 1.53. Subtle differences on increasing the dispersion concentration appeared in the difference spectra (figure 11B). The negative differences around 358-360 nm were larger and more intense than those around 300-325nm.

DPL lamellae were also studied. The absorption and difference spectra of filipin bound to DPL lamellae at varying concentrations were recorded (figures 12A and 12B). As the concentration of lamellae was increased, shifts of λ max became larger, the absorbance decreased and the ratio of the absorbance of peaks 323 nm/358 nm increased from 0.81 to 0.87, 0.92 and 0.95. The difference spectrum displayed negative absorbance changes, which were reduced on decreasing the concentration of the lamellae.

Figure 11(A). The spectra of free filipin, and filipin bound to DPL:cholesterol lamellae at room temperature in 1.25% DMSO-0.01 M Tris, pH 7.4. The concentration of filipin was 1.3×10^{-5}M and the concentration of the lamellae were: (1) 2.4×10^{-5}, (2) 4.8×10^{-5} and (3) 8.7×10^{-5}M (phosphate). The lamellae were prepared from DPL and cholesterol at a molar ratio of 7:1 in 0.01 M Tris-0.1 M NaCl, pH 7.4.

Figure 11(B). The ultraviolet difference spectrum (free filipin minus bound filipin) from the spectra obtained in figure 11(A).

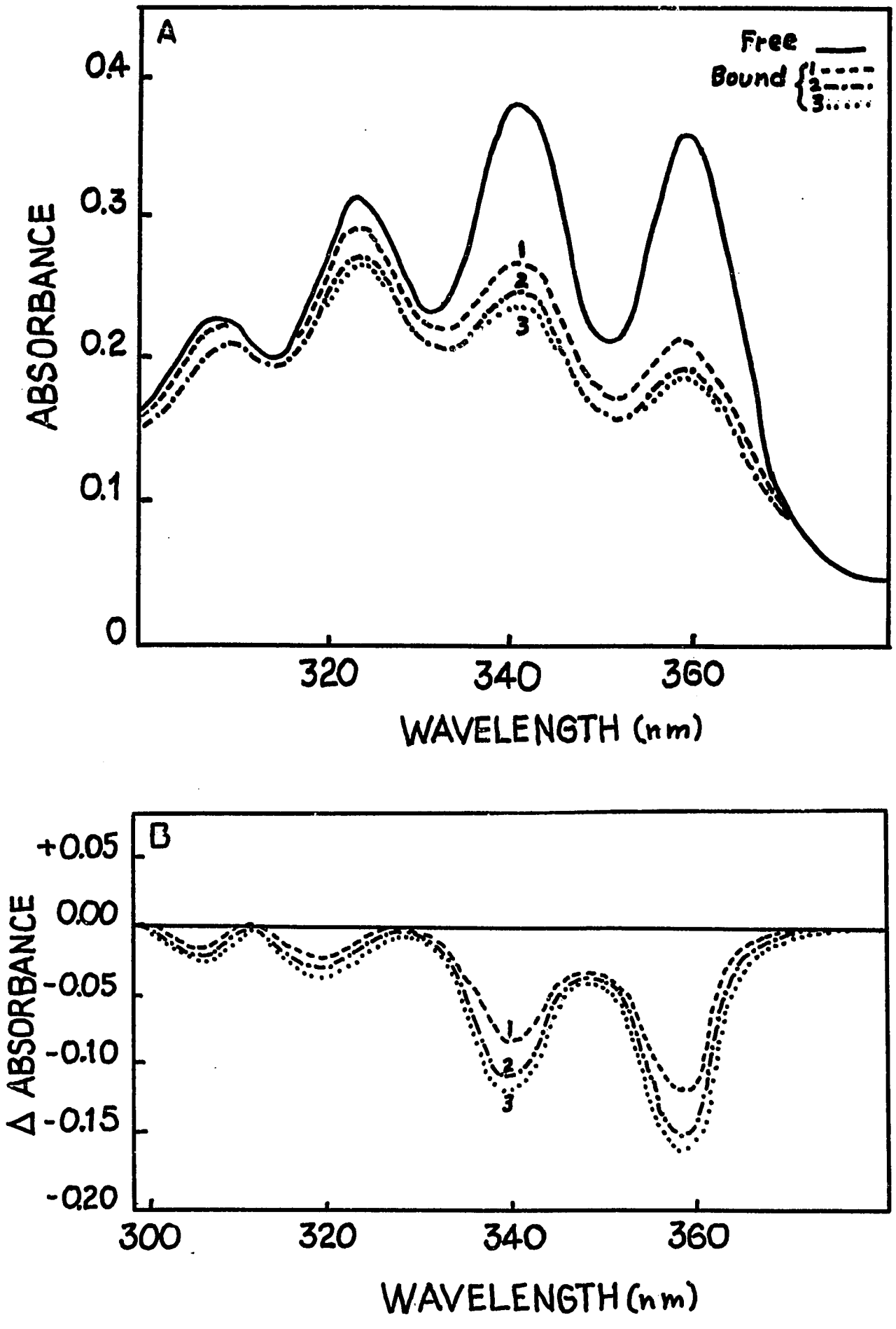
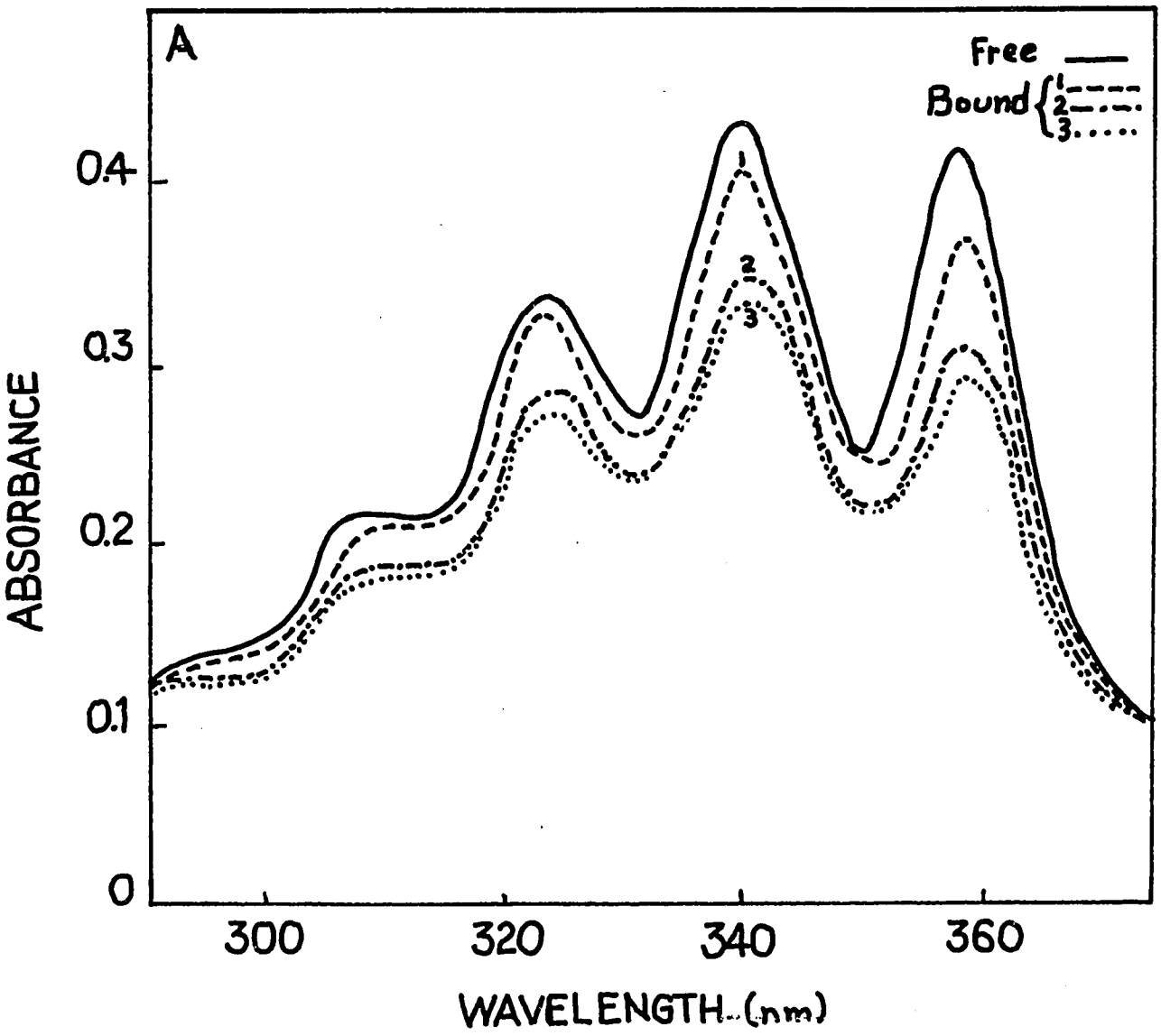
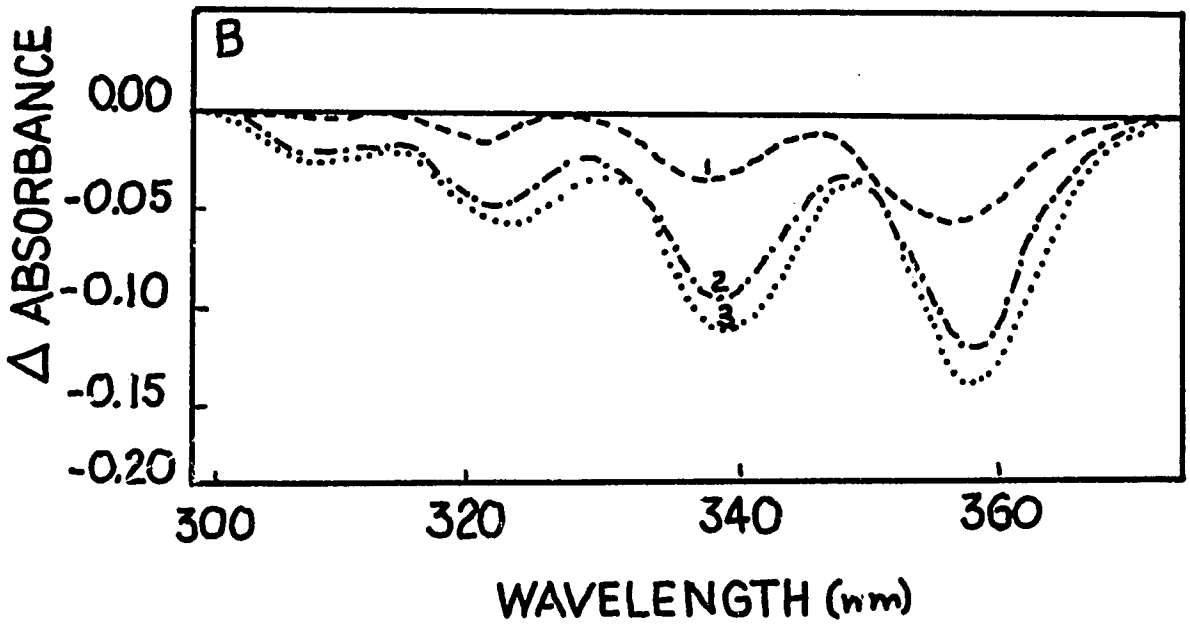

A
Free
Bound
1
2
3
ABSORBANCE
0.4
0.3
0.2
0.1
0
320
340
360
WAVELENGTH (nm)
B
+0.05
0.00
-0.05
-0.10
-0.15
-0.20
Δ ABSORBANCE
300
320
340
360
WAVELENGTH (nm)

Figure 12(A). The spectra of free filipin, and filipin bound to DPL lamellae in 2.5% DMSO-0.01 M Tris, pH 7.4, at room temperature. The concentration of filipin was 1.4×10^{-5}M and the concentrations of the lamellae were: (1) 1.5×10^{-5}, (2) 3.0×10^{-5} and (3) 4.5×10^{-5}M (phosphate). The dispersions were prepared from DPL in 0.01 M Tris-0.01 M NaCl, pH 7.4. The ratios of the absorbance of the peaks 323 nm/358 nm in free and bound filipin in (1), (2) and (3) were 0.81, 0.87, 0.93 and 0.95, respectively.

Figure 12(B). The ultraviolet difference spectra of free and bound filipin obtained from figure 12(A).


A
Free
Bound
1
2
3
ABSORBANCE
0.4
0.3
0.2
0.1
0
300
320
340
360
WAVELENGTH (nm)


B
Δ ABSORBANCE
0.00
-0.05
-0.10
-0.15
-0.20
300
320
340
360
WAVELENGTH (nm)

Bathochromic shifts, alteration in peak ratios and decreases in the extinction coefficients of the characteristic absorption maxima of filipin occurred on binding to EYL vesicles prepared with and without sterols (figures 13A and 13B). Red shifts were present in filipin bound to vesicles containing thiocholesterol. Decreases in the extinction coefficients in peak 1 of filipin in the presence of vesicles containing β-cholestanol and epicoprostanol were prominent. The ratio of peaks 323 nm/358 nm in free filipin and filipin bound to the vesicles was calculated from the spectra in figures 13A and B and is shown in Table 2. The data in Table 2 show that vesicles containing cholesterol, cholesteryl acetate, epicoprostanol and β-cholestanol alter the peak ratio 323 nm/358 nm of filipin, but EYL vesicles and DPL lamellae alter the ratio to a lesser extent, and the ratio is unaffected in the presence of thiocholesterol-containing vesicles.

Figure 14 shows the absorption spectra of free filipin and filipin in the presence of ciliary membranes obtained from unsupplemented and ergosterol-supplemented cells of Tetrahymena. The absorption spectra revealed that the peak ratio 323 nm/358 nm of filipin was altered from 1.0 in free filipin to 1.27 in the membranes containing tetrahymanol and to 1.86 in the membranes containing ergosterol. The absorption spectrum of filipin bound to ergosterol-containing membranes shows that the peak around 358-362 nm is split into 2 minor absorption maxima.

Figure 13(A). The spectra of free filipin, and filipin bound to EYL vesicles prepared with and without sterols. The concentration of filipin was 2.0×10^{-5}M and the concentration of the vesicles was 1.1×10^{-2}M (phosphate). The measurements were made in 1.25% DMSO-0.01 M Tris-0.1 M NaCl, pH 8.5, at 29°. The vesicles containing EYL and sterols were prepared in the molar ratio of 18:1.

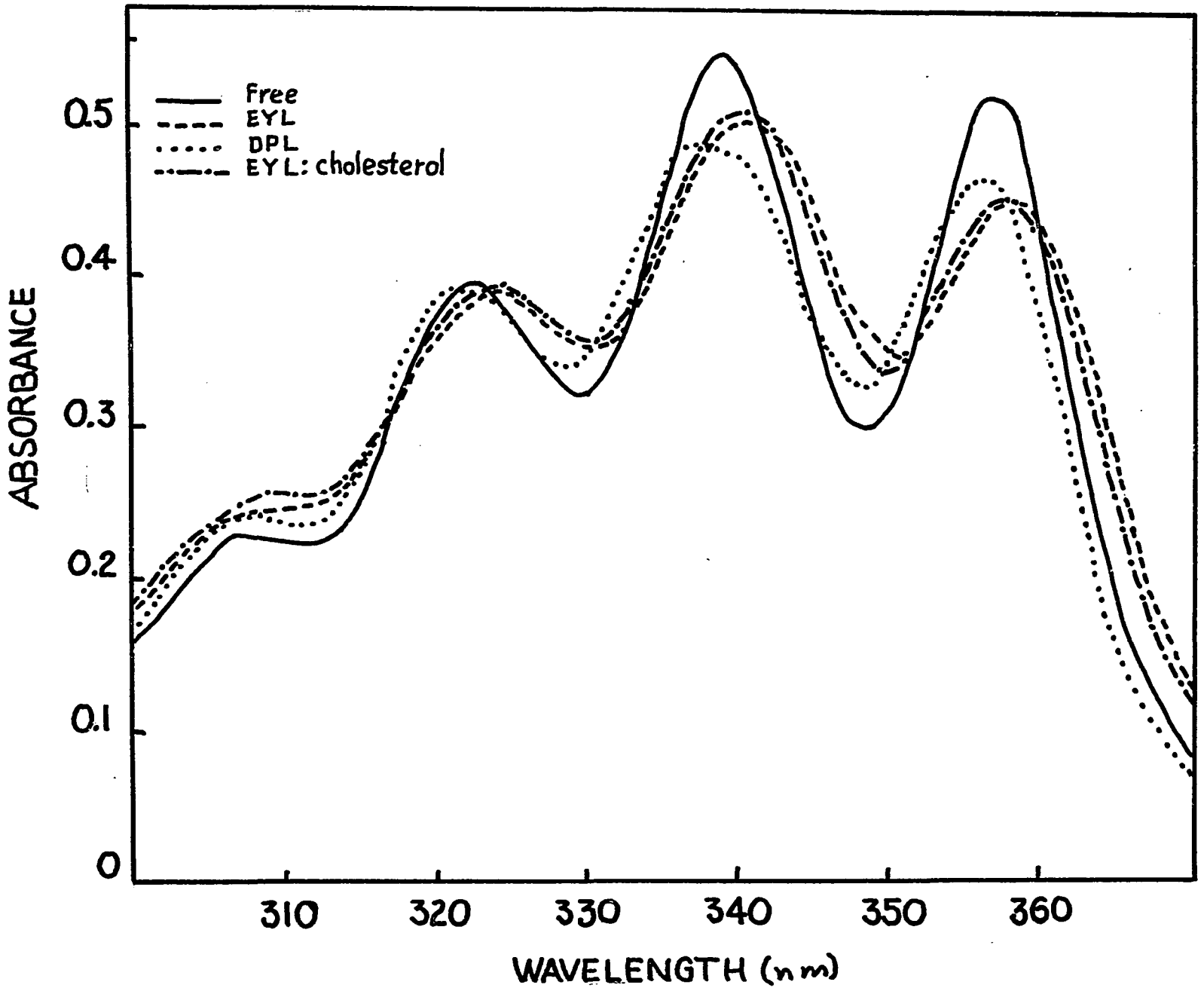

Free
EYL
DPL
EYL: cholesterol
ABSORBANCE
0.5
0.4
0.3
0.2
0.1
0
310
320
330
340
350
360
WAVELENGTH (nm)

Figure 13(B). The spectra of free filipin, and filipin bound to EYL vesicles prepared with and without sterols, and to DPL lamellae. The concentration of filipin was 2.0×10^{-5}M; that of vesicles was 1.1×10^{-2}M (phosphate), except for the concentration of vesicles containing thiocholesterol which was 8.0×10^{-3}M. All measurements were made in 1.25% DMSO-0.01 M Tris-0.1 M NaCl, pH 8.5, at 29°. The vesicles containing EYL and sterols were prepared in the molar ratio of 18:1.

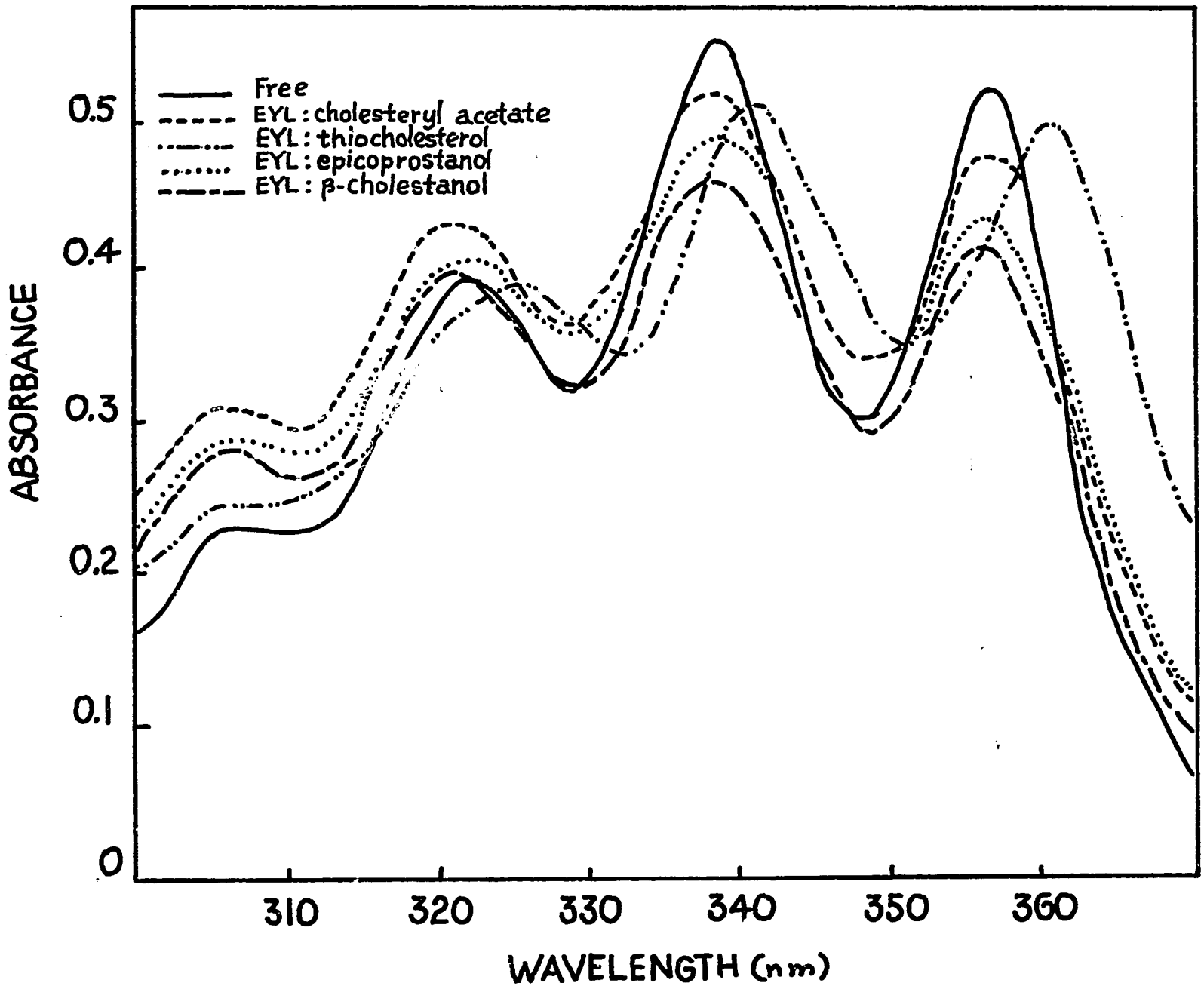
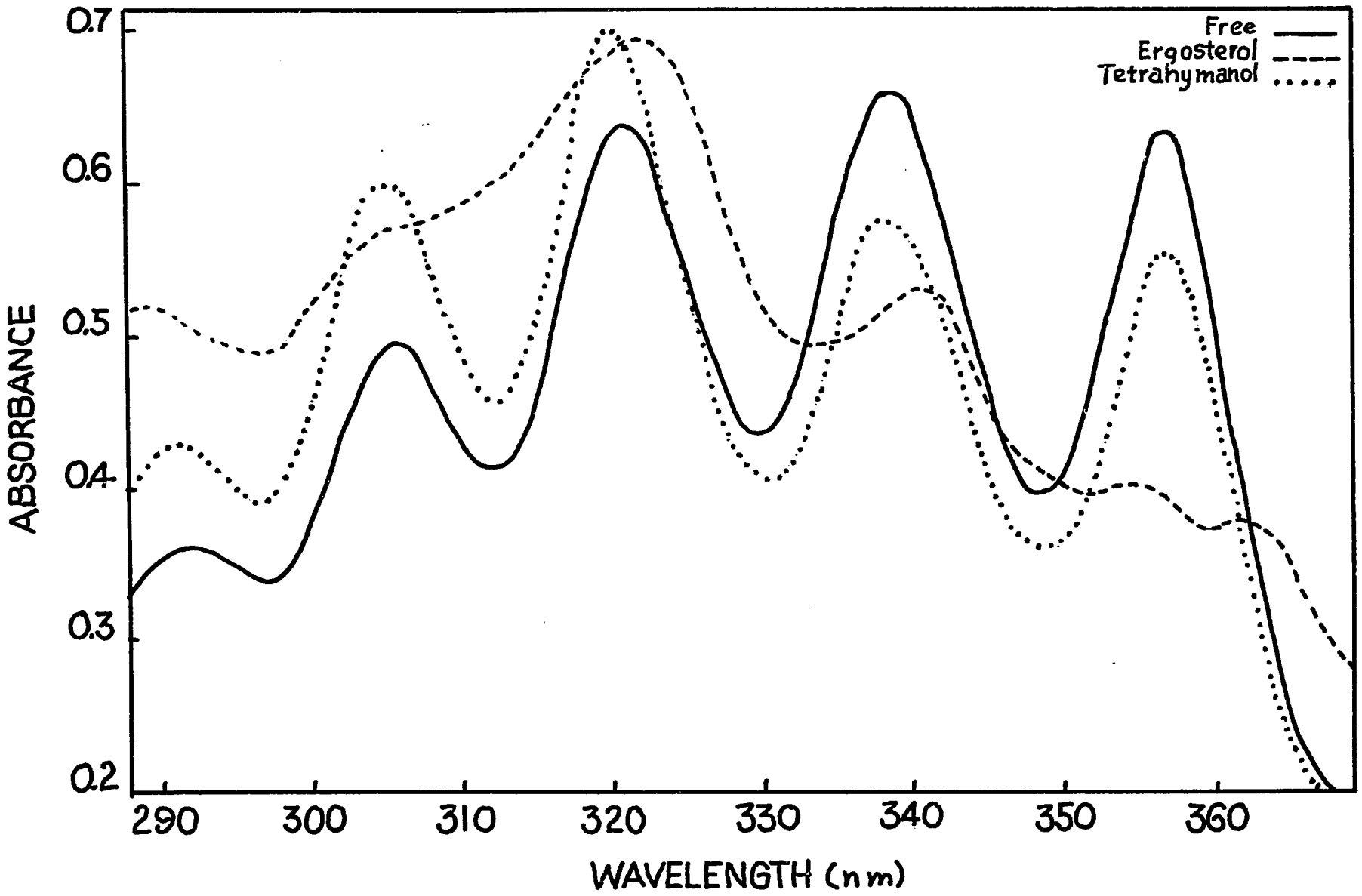

Free
EYL : cholesteryl acetate
EYL : thiocholesterol
EYL : epicoprostanol
EYL : β-cholestanol
ABSORBANCE
0.5
0.4
0.3
0.2
0.1
0
310
320
330
340
350
360
WAVELENGTH (nm)

Table 2: Effect of EYL vesicles (with and without sterols) at 29° on the absorbance ratio 323 nm/358 nm of filipin[1]

	Sample	Ratio of absorbance (peaks 323 nm/358 nm)
1.	Filipin	0.77
2.	Filipin + EYL vesicles	0.86
3.	Filipin + EYL: cholesterol vesicles	0.90
4.	Filipin + EYL: cholesteryl acetate vesicles	0.91
5.	Filipin + EYL: thiocholesterol vesicles	0.77
6.	Filipin + EYL: epicoprostanol vesicles	0.94
7.	Filipin + EYL: β-cholestanol vesicles	0.93
8.	Filipin + DPL lamellae	0.80

[1]The concentration of filipin was 1.98×10^{-5}M from (1) through (8). The ratio of filipin to phosphate was the same as given in figures 13 (A) and (B). The absorbance ratio of peaks 323 nm/358 nm varies from preparation to preparation.

Figure 14. The absorption spectra of filipin free (——), filipin bound to ergosterol-containing ciliary membranes (---) and filipin bound to tetrahymanol-containing ciliary membranes (····). The concentration of proteins in ergosterol-supplemented membranes was 120 µg/ml and the molar ratio of phosphate to sterol was 2.4. The concentration of phosphate in membranes isolated from unsupplemented cells was 125 µg/ml and the molar ratio of phosphate to tetrahymanol was 3.2. The concentration of filipin was 2.3×10^{-5}M. The ratio of peaks 323 nm/358 nm in free filipin and filipin bound to ergosterol-containing and to tetrahymanol-containing membranes are 1.0, 1.86 and 1.27, respectively. The spectra were recorded at 28° in 1.25% ethylene glycol-0.01 M Tris-0.1 M NaCl, pH 8.5. The spectra were corrected for light scattering of the membranes alone.


Free
Ergosterol
Tetrahymanol
ABSORBANCE
0.7
0.6
0.5
0.4
0.3
0.2
290
300
310
320
330
340
350
360
WAVELENGTH (nm)

The interaction of filipin with three systems has been described, namely free sterol in aqueous suspension, model membranes prepared with and without sterols and natural membranes. With free cholesterol, it was found that there was a spectral change in the peak ratio 323 nm/358 nm (Table 1). It has been established that this interaction is not affected by proteins, salt or urea (2). The interaction of filipin with a homogeneous system of vesicles and phospholipid dispersions prepared with and without sterols caused alterations in the peak ratio 323 nm/358 nm. A close examination of the absorption spectra of filipin bound to DPL lamellae prepared with and without cholesterol (figures 9, 10, 11 and 12) indicated that the alteration in the absorbance peak ratio 323 nm/358 nm was greater when sterol was present. For example, the peak ratio 323 nm/358 nm of filipin bound to lamellae containing DPL and DPL lamellae containing cholesterol was 0.95 and 1.1, respectively. This clearly demonstrated that the polyene binds to sterol bound to lamellae.

The effect of temperature on free filipin and filipin bound to EYL vesicles prepared with and without cholesterol was studied (Table 1). The absorption properties of free and bound filipin were altered by temperature. A comparison of the ratio of the absorbance of peaks 323 nm/358 nm of free and bound filipin indicated that at 17° the ratio of peaks 323 nm/358 nm increased in bound filipin and the same result was true at temperature of 23°. The increase

in the ratio of peaks 323 nm/358 nm was generally more prominent in the presence of sterol-containing EYL vesicles than in the presence of EYL vesicles.

The absorbance ratio of peaks 323 nm/358 nm of free filipin varies not only in the presence of sterols but also with changes in temperature and solvent. For example, in Table 1 the ratio for free filipin was 0.87 in 20% ethanol-2.5% DMSO-phosphate buffer and in figure 9 the ratio was 0.81 in 2.5% DMSO-Tris buffer at room temperature. In 1.25% ethylene glycol-Tris buffer, the ratio was 1.0 (figure 14), and the ratios at 22° and 29° in 1.25% DMSO-Tris buffer were 0.72 and 0.77, respectively (Table 1).

The studies reported in this dissertation by absorption indicated that cholesterol, cholesteryl acetate, epicoprostanol and β-cholestanol in EYL vesicles produced approximately the same changes in the peak ratio 323 nm/358 nm of filipin on binding (figure 13). These changes did not indicate a necessity for specific stereochemical orientation of liposomally bound sterol. The requirement was found to be essential in the polyene-sterol interaction in vesicles (2, 3). It was found that filipin had an optimal interaction with cholesterol. Absorption studies reported here have shown that for filipin to interact in the model membranes, it is not necessary to have a 3 β-hydroxyl group. The same results can be obtained with a substituted derivative of cholesterol in the 3 position and with epicoprostanol.

The discrepancies in the results reported here and those in reference 3 may be due to the differences in the molar ratio of sterols and phospholipids and also to the ratio of cholesterol/filipin. The studies reported in reference 3 on polyene interactions with liposomes used a molar ratio of phospholipid to cholesterol of 6:1 or 2:1 and a ratio of sterol to filipin of 4, whereas in this dissertation absorption studies were performed with vesicles having a molar ratio of phospholipids to sterols of 18:1 and at a ratio of sterol to filipin of 30. At these latter ratios of the phospholipid tc sterol and sterol to filipin, absorption is too insensitive a technique to distinguish between the structural requirements necessary for the sterol (bound in vesicles) to interact with filipin. However, fluorescence and circular dichroism studies indicated that even at this low concentration of the sterols, the polyene-sterol interaction showed the same structural requirements as observed in the absorption results reported in reference 3. No interaction of filipin occurred when oxygen was replaced by sulfur as in thiocholesterol-containing vesicles, although the thiol group was in the β configuration at the 3 position. The lack of interaction of filipin with EYL:thiocholesterol vesicles is consistent with the small enhancement of the fluorescence polarization of the polyene in the presence of these vesicles (1) and with the finding that the rate of permeability of these vesicles

to water was not markedly diminished when compared with the vesicles containing cholesterol (68). These results suggested that filipin is a useful probe to study lecithin-sterol interactions in membranes.

The interaction of filipin with membrane-bound sterol was accompanied by spectral changes, such as the decrease in the extinction coefficient, increase in the peak ratio 323 nm/358 nm and red shifts (figure 14). As shown in figure 14, when sterol was not present in the growth medium of Tetrahymena, changes in the spectrum of filipin were absent. Further evidence that filipin is a useful probe is proved by the alteration of the absorbance peak ratio 323 nm/358 nm and in the shifts in the spectrum on binding to sterols present in membranes.

Kinetic Studies

Figure 15 shows that on binding to EYL vesicles, filipin underwent decreases in absorption with time. Similar results were obtained when filipin interacted with EYL:cholesterol vesicles (figure 16). These observations were made after the polyene and the vesicles were mixed and the changes in absorption were recorded at different time intervals until a satisfactory end point was obtained. On mixing filipin and EYL vesicles, the absorbance of peaks 1 and 2 decreased rapidly, but after 20 minutes, the change was gradual. The absorbance of peak 3 was not altered very much. Cholesterol-containing vesicles in the presence of the polyene demonstrated a marked increase in the ratio of the absorbance of the peaks 323 nm/358 nm during the first 30 minutes of the reaction; thereafter the absorbance of peaks 1 and 2 declined at a very slow rate and came to a virtual stop after 195 minutes. The rate constants for the slow reactions between filipin and vesicles are given in Table 3.

A study of the kinetics of the interaction of different vesicles (derived from EYL with and without sterols and from DPL lamellae) with filipin was undertaken. The phosphate concentration of the vesicles and lamellae and the concentration of filipin were maintained constant. Table 4 gives the rate constants of reactions occurring on the binding of the probe to the different types of vesicles. With EYL, EYL:cholesteryl acetate, and EYL:epicoprostanol vesicles, a single reaction was evident. The slowest of these was

Figure 15. The absorption spectra of free filipin and filipin bound to EYL vesicles at various times after mixing. The observations were made at 28° in 1.25% DMSO-0.01 M Tris-0.1 M NaCl, pH 8.5. The concentration of filipin was 1.1×10^{-5}M and that of vesicles was 1.8×10^{-3}M (phosphate).

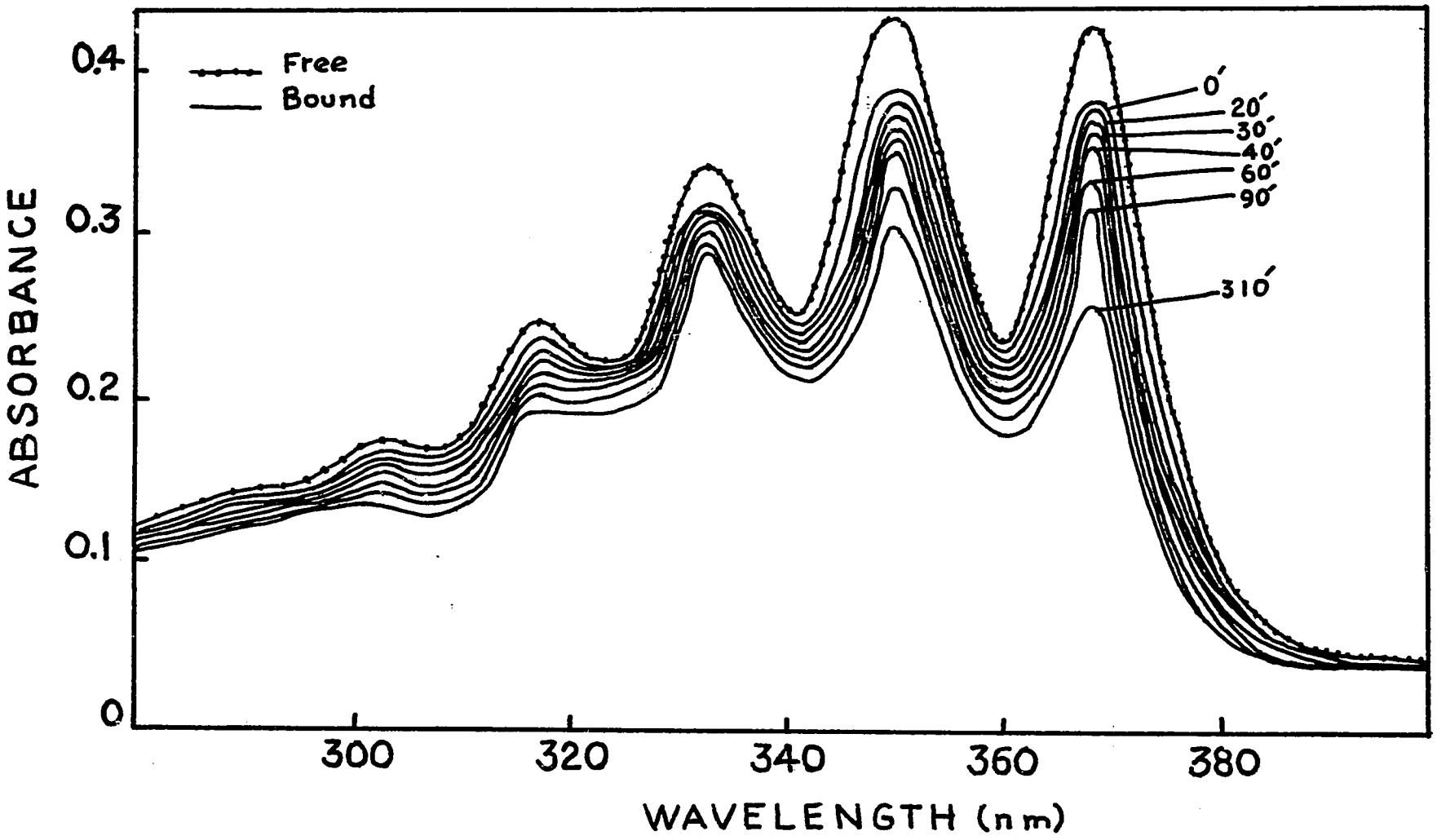

Free
Bound
0'
20'
30'
40'
60'
90'
310'
ABSORBANCE
0.4
0.3
0.2
0.1
0
300
320
340
360
380
WAVELENGTH (nm)

Figure 16. The absorption spectra of free filipin, and filipin bound to EYL:cholesterol vesicles at various times after mixing. The observations were made at 29° in 1.25% DMSO-0.01 M Tris-0.1 M NaCl, pH 8.5. The concentration of filipin was 1.5×10^{-5}M and the concentration of vesicles was 2.5×10^{-3}M (phosphate). EYL and cholesterol were in the molar ratio of 18:1.

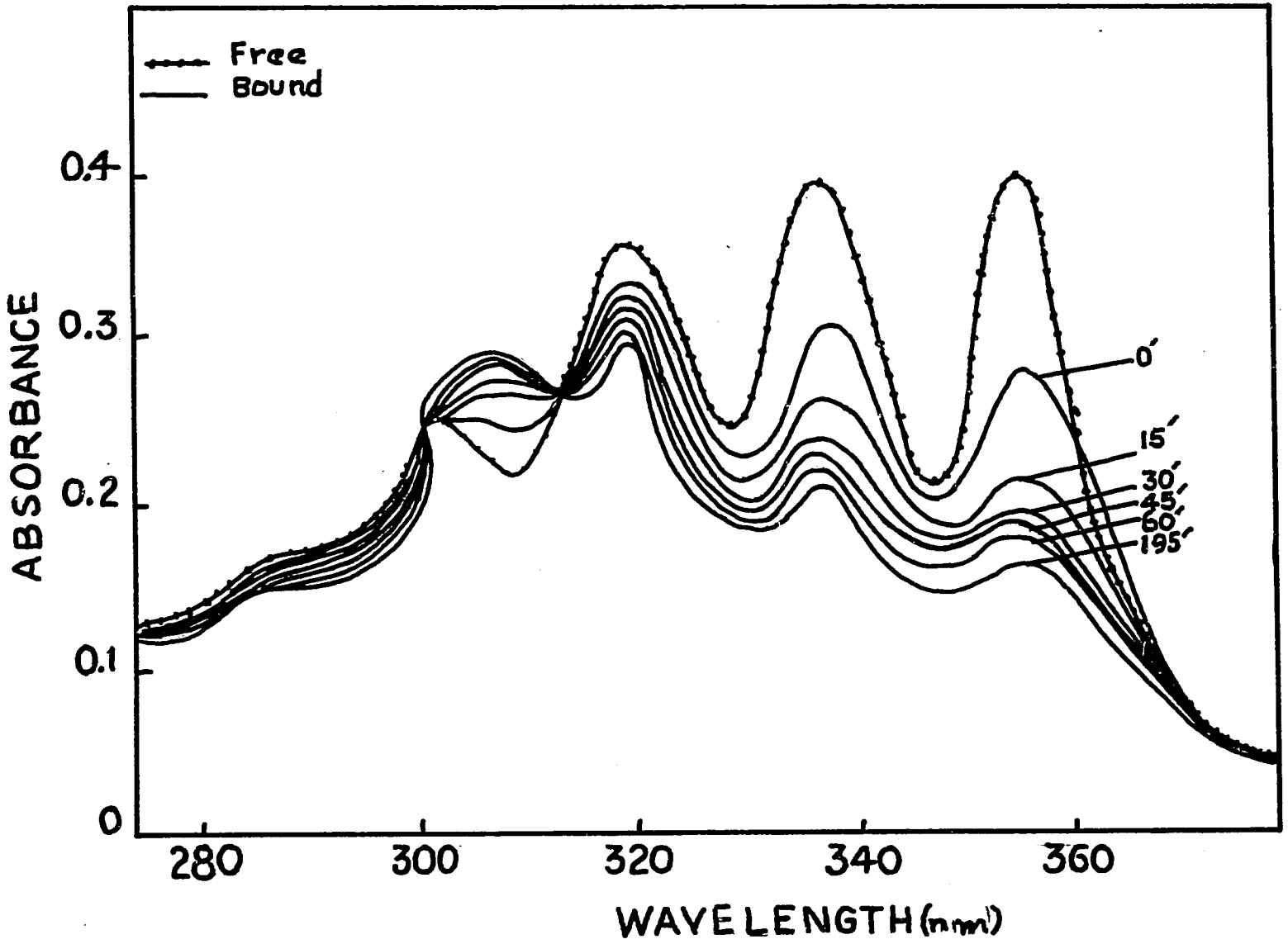

Free
Bound
0.4
0.3
0.2
0.1
0
ABSORBANCE
0'
15'
30'
45'
60'
195'
280
300
320
340
360
WAVELENGTH (nm)

Table 3: The rate constants of the slow reaction of filipin with vesicles

Type of Vesicles	Conc. of filipin	Conc. of lecithin	Ratio of F/P	Temp. (°C)	$k(sec^{-1})$
EYL	1.9×10^{-5}M	3.0×10^{-3}M	6×10^{-3}	28	8.9×10^{-5}
EYL: cholesterol[1]	1.5×10^{-5}M	2.5×10^{-3}M	6×10^{-3}	29	3.1×10^{-5}
EYL	1.1×10^{-5}M	1.5×10^{-3}M	7×10^{-3}	23	6.5×10^{-4}
EYL	1.1×10^{-5}M	1.8×10^{-3}M	6×10^{-3}	23	5.5×10^{-4}

[1] EYL and cholesterol were in the molar ratio of 18:1. The measurements were made in 1.25%-0.01 M Tris-0.1 M NaCl, pH 8.5, at 338 nm.

Table 4: The rate constants of the slow reaction of filipin with vesicles and with DPL lamellae

Sample	Rate constant (Sec^{-1})
EYL	7.7×10^{-4}
DPL	4.1×10^{-2}* 1.3×10^{-3}*
EYL:cholesterol[1]	7.6×10^{-3} 2.0×10^{-4}
EYL:cholesteryl acetate[1]	2.7×10^{-4}
EYL:epicoprostanol[1]	5×10^{-4}
EYL:β-cholestanol[1]	7.7×10^{-3}* 3.3×10^{-4}

[1]EYL and sterol were in the molar ratio of 18:1. The asterisk denotes the rate constant of the faster of the two reactions. All observations were made in 1.25% DMSO-0.01 M Tris-0.1 M NaCl, pH 8.5, at 358 nm and 28°. The concentration of filipin was 4.1×10^{-5}M and the concentration of EYL vesicles and DPL lamellae was 22.3×10^{-3}M. The ratio of filipin/phosphate in the vesicles and lamellae was maintained at 1.8×10^{-3}.

the reaction that occurred when filipin was mixed with EYL:cholesteryl acetate vesicles. Vesicles containing cholesterol and β-cholestanol exhibited two distinct reactions. When vesicles containing cholesterol were mixed with filipin, the first reaction occurred within 210 seconds, but with vesicles containing β-cholestanol, the first reaction was spread over 75 seconds.

The differences in the rates of the reactions of filipin with model membranes prepared with and without sterols may arise from variations in the molecular arrangement of the bilayer in the vesicles.

The interactions between EYL and epicoprostanol and EYL and cholesteryl acetate are different from the interactions between EYL and cholesterol. These differences are largely due to the structural differences of the sterol incorporated in the vesicles. Since hydrogen bonding is not possible between cholesteryl acetate and the polar group of lecithin, the association of EYL with cholesteryl acetate is expected to be weaker than that with cholesterol.

Tables 3 and 4 give the rate constants of the reaction between filipin and EYL:cholesterol vesicles. At a low ratio of the antibiotic to lipid, there were two distinct reactions, whereas at high ratio, there was only a single reaction present. The rate of the slow reaction at high ratio was much slower than the rate at low ratios. The analysis of the cholesterol to filipin ratio in these reactions revealed that when the ratio was 9:1 only one

reaction was observed, but when the ratio was 30:1, two reactions were observed.

The analysis of the amplitudes of the reactions indicated that two reactions were discernible in the plots of the logarithm of absorbance vs. time only when the hydroxyl group of the sterol-containing EYL vesicles was in the 3 β position and in DPL lamellae. The rate constants of these reactions were calculated from plots of the logarithm of Δ absorbance changes vs. time. When two reactions were observed, the rate constant of the first reaction was determined by extending the straight line of the slow reaction and then plotting the logarithm of Δ absorbance between the extrapolated line and the straight line obtained from the fast reaction vs. time. The % amplitudes of the first reaction between filipin and EYL:cholesterol vesicles, EYL: β-cholestanol vesicles and DPL lamellae were 65%, 30% and 60%, respectively, of the total reaction. The rate constant of the first reaction of each of the above reactions was derived from 9, 8 and 11 points and that of the second reaction was derived from 26, 10 and 9 points, respectively, in plots of logarithm of Δ absorbance vs. time.

Vesicles prepared with thiocholesterol have been shown to contain two types of kinetically distinguishable sulfhydryl groups. One class of kinetically distinguishable thiol group is on the external surface, and the second class of thiol group is masked in the interior of the vesicle (99). These results were obtained from the time dependence of the reaction of DTNB with EYL:thiocholesterol vesicles (99).

It is possible that two types of kinetically distinguishable hydroxyl groups may also be present in the cholesterol- and β-cholestanol-containing vesicles.

The reactions of filipin with EYL vesicles containing cholesterol most likely result from the affinity of filipin for the phospholipids and sterols. It is possible that filipin may form binary (filipin-sterol) and ternary (filipin-phospholipid-sterol) complexes. In the reaction between filipin and EYL vesicles containing cholesterol, when the absolute concentrations of the phospholipid and filipin were high, two rate constants were observed. When the concentrations were low, there was a single rate constant. At the higher concentrations, filipin/phospholipid ratio was lower when compared with that of the lower concentrations. At higher concentrations, the fast reaction may represent the formation of complexes with the sterols and phospholipid-sterol complexes on the external surface of the vesicle. As a consequence of the fast reaction, sterol and phospholipid-sterol complexes in the interior of the vesicle may be exposed. Unbound filipin may bind to such sterols and phospholipid-sterol complexes and the binding may be represented by the second reaction.

Fluorescence Studies

Figure 17 shows the excitation and emission spectra of filipin in aqueous solution. The excitation spectrum is similar to the absorption spectrum. The fluorescence emission spectrum of filipin has broad wavelength maxima at 455 and 476 nm, and a shoulder around 515-520 nm.

Figures 18 and 19 show the excitation and emission spectra of filipin in pyridine, 10% pyridine-90% water, DMF and 10% DMF-90% water. The corresponding absorption spectra are given in figure 8 (of results and discussion in absorption studies). Bathochromic shifts were observed in organic solvents in the absorption and excitation spectra. In pyridine and DMF, the intensities of the spectra were lowered approximately 20% in 10% pyridine-90% water and by 40% in 10% DMF-90% water compared to the spectra obtained in organic solvents.

Figure 20 shows the excitation and emission spectra of free filipin and filipin bound to DPL lamellae prepared with cholesterol. The corresponding absorption spectrum is given in figure 9. On binding of filipin to DPL: cholesterol lamellae, the absorbance ratio of peaks 323 nm/ 358 nm was increased from 0.81 (in free filipin) to 1.41. When filipin binds to DPL:cholesterol lamellae, the fluorescence intensity of filipin is quenched.

Figure 21 shows the emission spectra of filipin in the presence of DPL lamellae prepared with cholesterol. On binding to these lamellae, the polyene undergoes quenching which is proportional to the lipid concentration in the

Figure 17. The excitation and emission spectra of filipin (1.7×10^{-5}M) in aqueous solution. In the excitation spectrum, the emission wavelength was 480 nm and in the emission spectrum, the excitation wavelength was 358 nm. The emission slit width was 6 mm and the excitation slit width was 2 mm. The measurements were performed in 1.25% DMSO-0.01 M Tris-0.1 M NaCl, pH 8.5, at 28° on a Hitachi-Perkin Elmer spectrofluorometer.

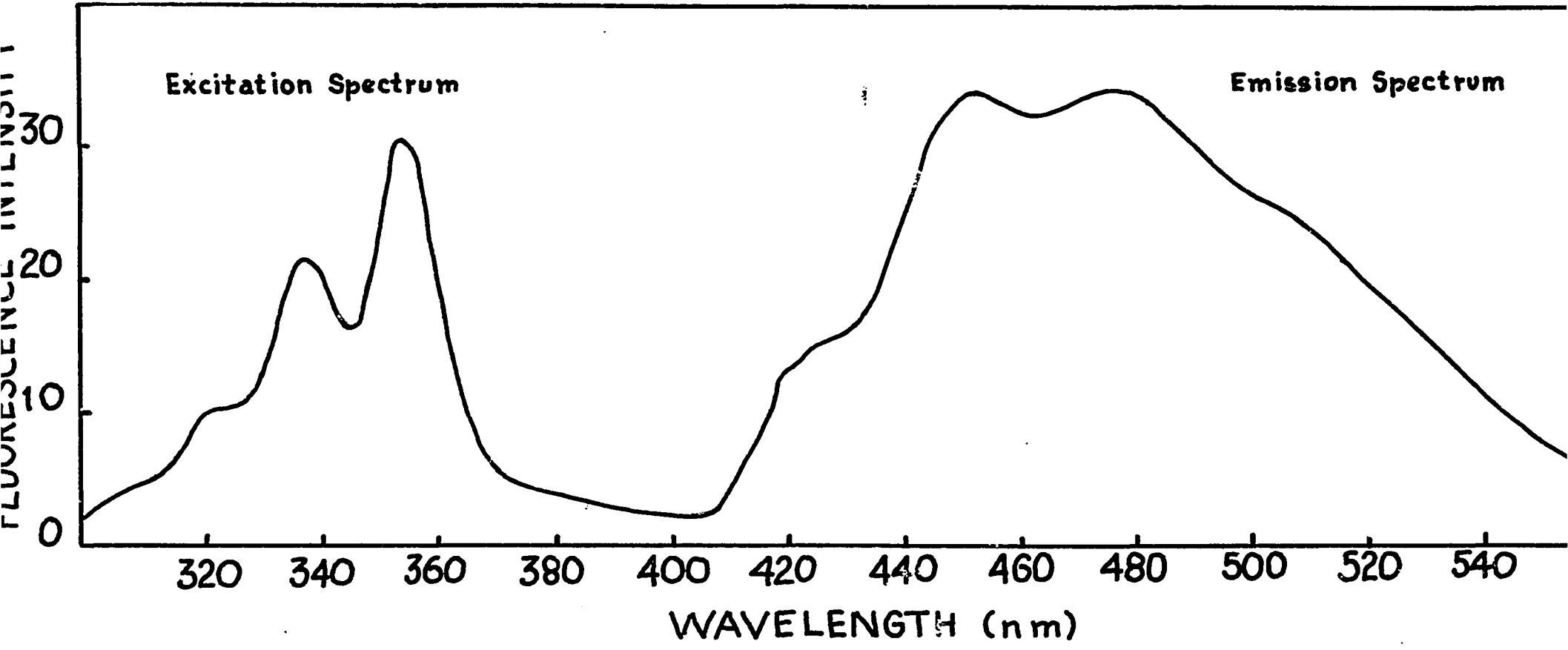

Excitation Spectrum
Emission Spectrum
FLUORESCENCE INTENSITY
0
10
20
30
320
340
360
380
400
420
440
460
480
500
520
540
WAVELENGTH (nm)

Figure 18. The excitation and emission spectra of filipin (2.1×10^{-5}M) in pyridine (——) and in 10% pyridine-90% 0.01 M Tris-0.1 M NaCl, pH 8.5 (---). In the excitation spectrum, the emission wavelength was 480 nm and in the emission spectrum, the excitation wavelength was 358 nm. The emission slit width was 6 mm and the excitation slit width was 2 mm. The spectra were recorded at 28° on a Hitachi-Perkin Elmer spectrofluorometer.

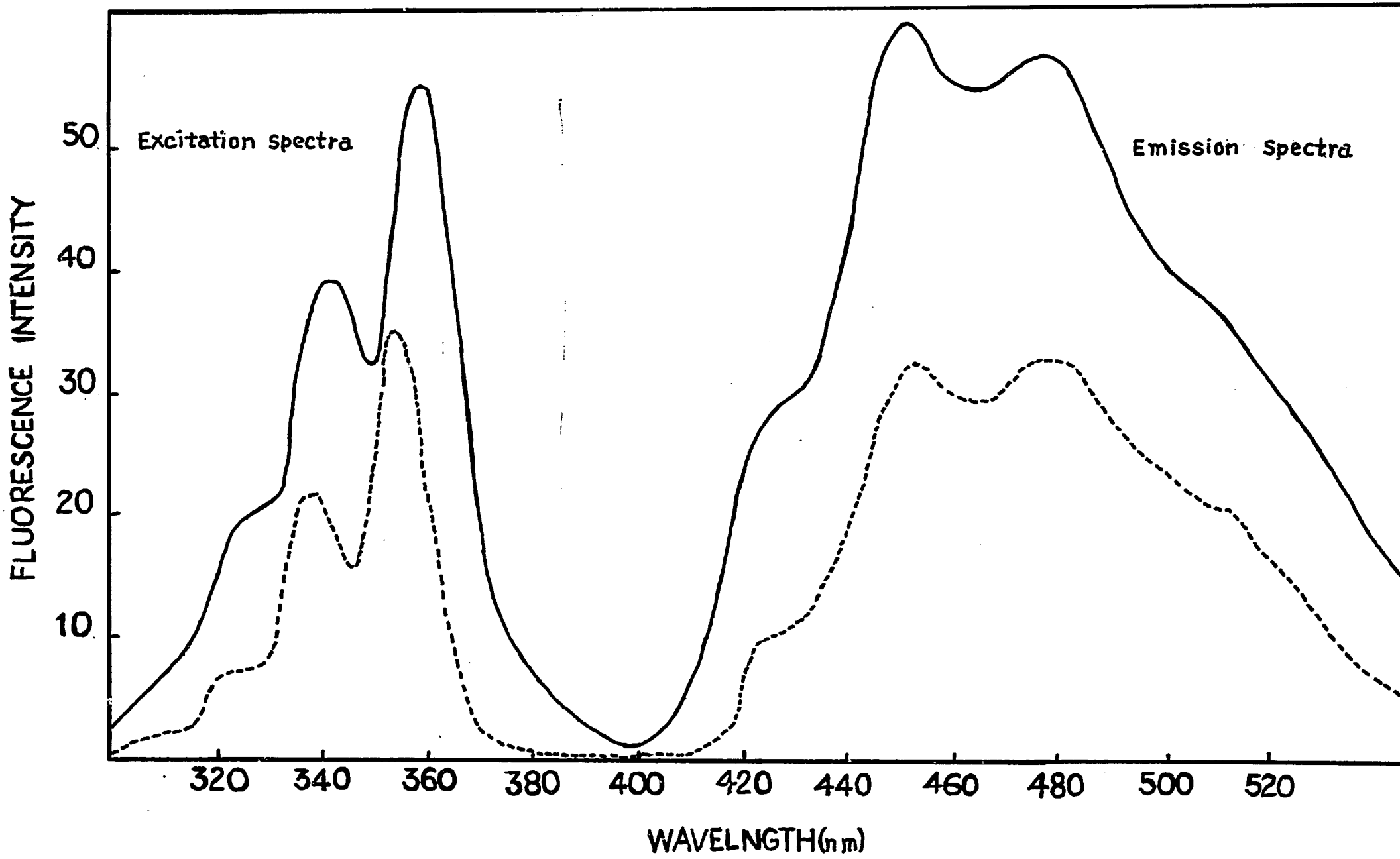

Excitation spectra
Emission Spectra
FLUORESCENCE INTENSITY
50
40
30
20
10
320
340
360
380
400
420
440
460
480
500
520
WAVELNGTH(nm)

Figure 19. The excitation and emission spectra of filipin (2.1×10^{-5}M) in DMF (——) and in 10% DMF-90% 0.01 M Tris-0.1 M NaCl, pH 8.5 (---). In the excitation spectrum, the emission wavelength was 480 nm and in the emission spectrum, the excitation wavelength was 358 nm. The emission slit width was 6 mm and the excitation slit width was 2 mm. The spectra were recorded at 28° on a Hitachi-Perkin Elmer spectrofluorometer.

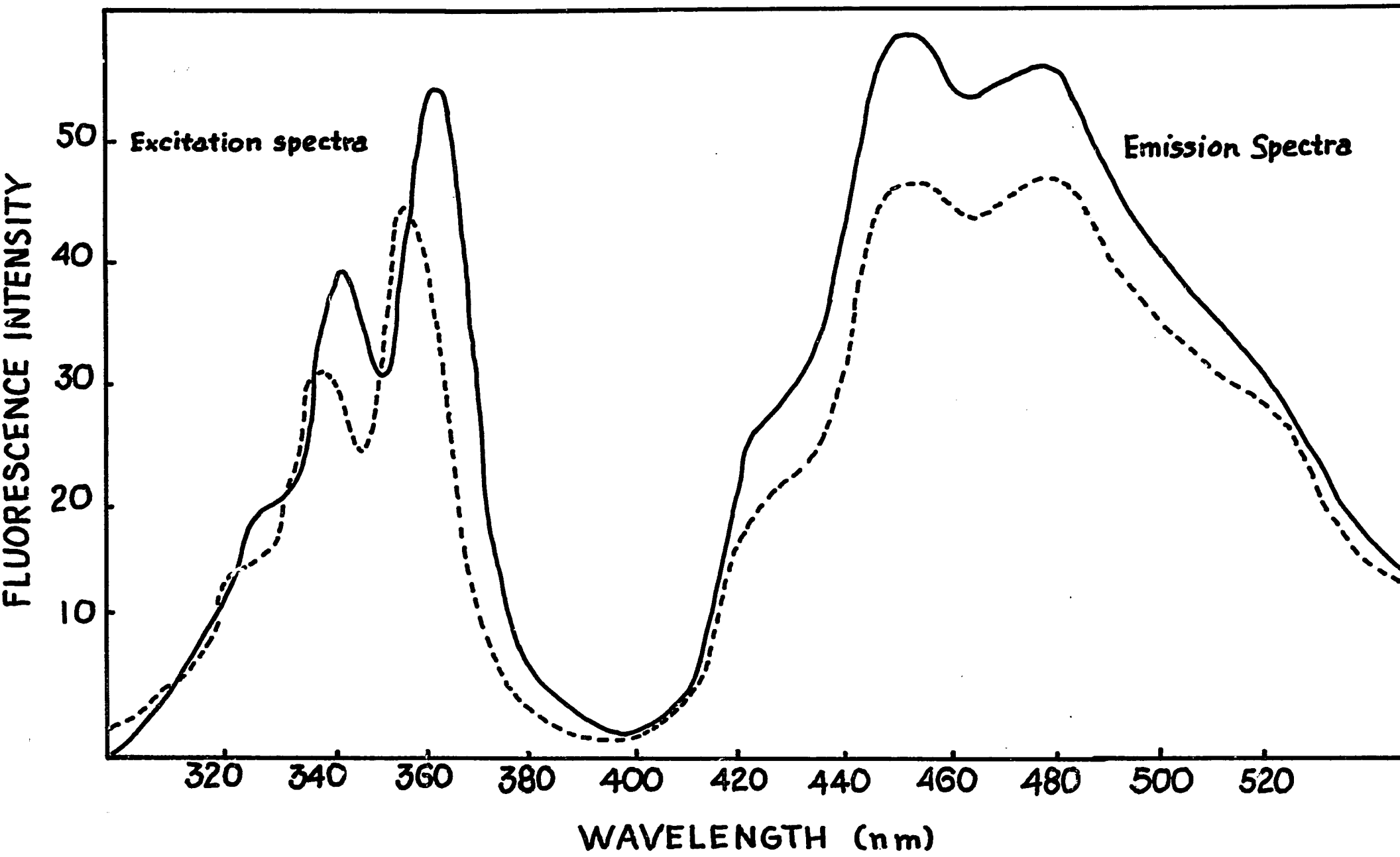

Excitation spectra
Emission Spectra
FLUORESCENCE INTENSITY
50
40
30
20
10
320
340
360
380
400
420
440
460
480
500
520
WAVELENGTH (nm)

Figure 20. The excitation and emission spectra of free filipin (——) and filipin in the presence of DPL:cholesterol lamellae (---) in 1.25% DMSO-0.01 M Tris, pH 7.4. The excitation spectra were recorded at an emission wavelength of 476 nm and the emission spectra were recorded at an excitation wavelength of 365 nm. The concentration of DPL lamellae containing cholesterol was 7×10^{-6}M (phosphate) and the concentration of filipin was 1.67×10^{-5}M. The corresponding absorption spectra of free and bound filipin are given in figure 9. The spectra were recorded at room temperature on a Farrand spectrofluorometer using a Corning glass filter CS7-31 and at 0.1 μ A sensitivity. DPL and cholesterol were in the ratio of 7:1 in the lamellae.

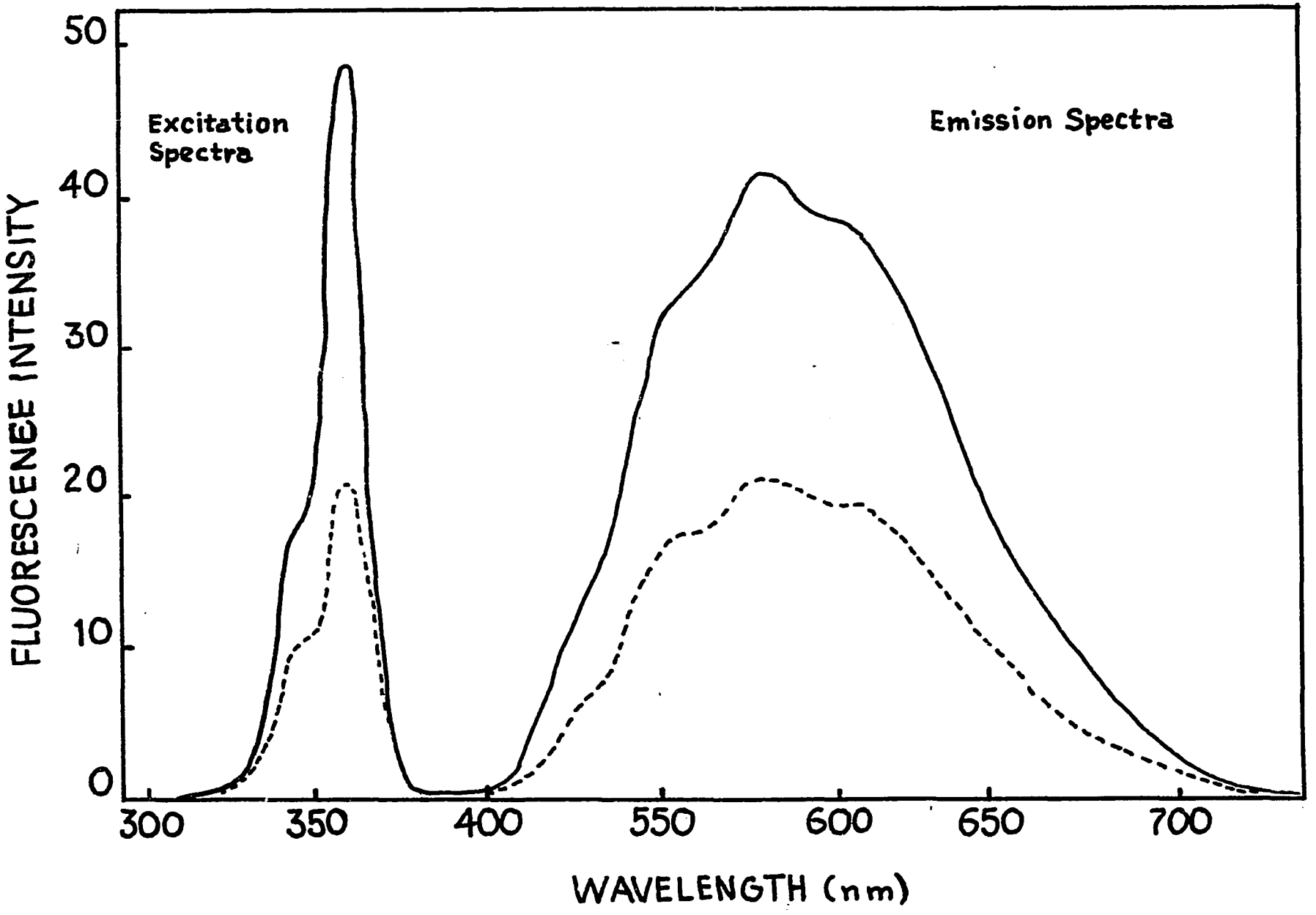
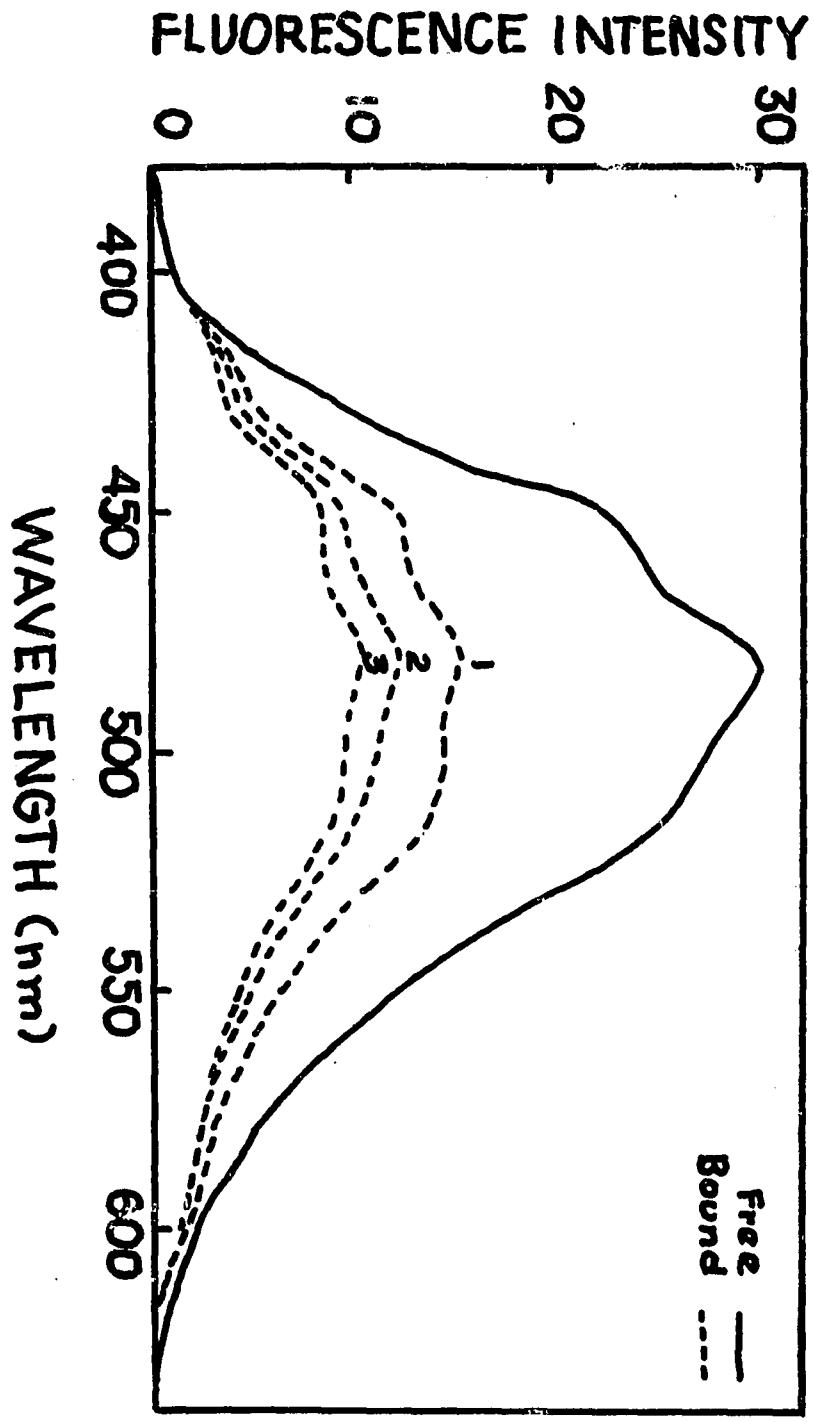

Excitation Spectra
Emission Spectra
FLUORESCENEE INTENSITY
50
40
30
20
10
0
300
350
400
550
600
650
700
WAVELENGTH (nm)

Figure 21. The emission spectra of free filipin (——) and filipin in the presence of DPL lamellae containing cholesterol (---) in 1.25% DMSO-0.01 M Tris, pH 7.4. The emission spectra were recorded at an excitation wavelength of 365 nm. The concentration of DPL lamellae were: (1) 2.4×10^{-5}, (2) 4.8×10^{-5} and (3) 8.7×10^{-5}M (phosphate) and the concentration of filipin was 1.9×10^{-5}M. The corresponding absorption spectra are given in figure 10. The spectra were recorded at room temperature on a Farrand spectrofluorometer using a Corning glass filter CS7-31 and at 0.1 μA sensitivity. DPL and cholesterol were in the ratio 7:1 in the lamellae.


FLUORESCENCE INTENSITY
0
10
20
30
400
450
500
550
600
WAVELENGTH (nm)
1
2
3
Free
Bound

lamellae; the corresponding changes in the absorption spectra are given in figure 10.

Figure 22 shows the fluorescence emission spectra of filipin bound to EYL vesicles prepared with and without sterols and to DPL lamellae lacking cholesterol. DPL lamellae prepared without cholesterol did not alter the fluorescence intensity of filipin, whereas EYL vesicles caused an enhancement of bound filipin. The changes in the fluorescence intensity largely depend on the stereochemistry of the sterol incorporated in the vesicles. The enhancement was nearly 100% with EYL vesicles containing thiocholesterol and cholesterol. The corresponding absorption spectra of filipin bound to vesicles containing sterols is given in figure 13. Although there was an increase in the fluorescence intensity of filipin in the presence of thiocholesterol-containing vesicles, filipin underwent a decrease in absorbance on binding to these vesicles without significant wavelength shifts. The fluorescent spectral characteristics of filipin bound to cholesterol- and thiocholesterol-containing vesicles were different. In the presence of EYL vesicles prepared with cholesteryl acetate and β-cholestanol, filipin had about identical degrees of enhancement; this was approximately 25% of the value obtained when filipin was in the presence of EYL vesicles.

Table 5 gives the values of fluorescence intensity and fluorescence polarization of filipin bound to EYL:cholesterol vesicles. The polarization of free filipin does not vary

Figure 22. The emission spectra of free filipin and filipin bound to EYL vesicles (with and without sterols) and to DPL lamellae recorded immediately after mixing the antibiotic and vesicles. The emission spectra were recorded at 28° on a Hitachi-Perkin Elmer spectrofluorometer at an excitation wavelength of 358 nm and at an emission slit width of 2 mm. The concentration of the vesicles and lamellae was 1.13×10^{-2}M for all vesicles except those containing thiocholesterol; the latter had a concentration of 8×10^{-3}M (phosphate). The concentration of filipin was 2.0×10^{-5}M. The phospholipids and sterols were in a molar ratio of 18:1. All measurements were made in 1.25% DMSO-0.01 M Tris-0.1 M NaCl, pH 8.5. The corresponding absorption spectra are given in figure 13A and B.

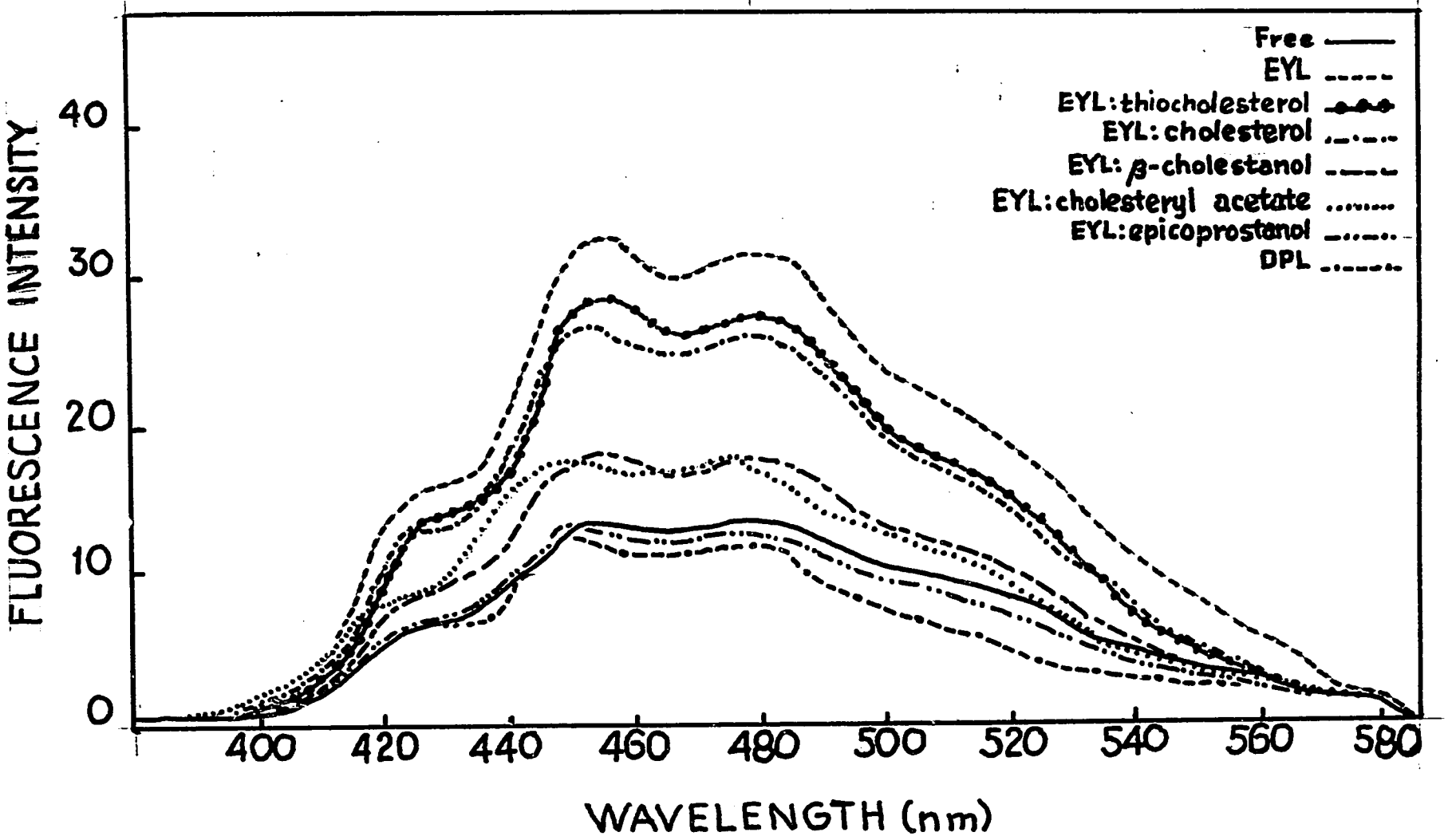

Free
EYL
EYL: thiocholesterol
EYL: cholesterol
EYL: β-cholestanol
EYL: cholesteryl acetate
EYL: epicoprostenol
DPL
FLUORESCENCE INTENSITY
0
10
20
30
40
400
420
440
460
480
500
520
540
560
580
WAVELENGTH (nm)

Table 5. The fluorescence polarization and fluorescence intensity values at varying filipin and EYL:cholesterol vesicle concentrations

Concentration of filipin x 10^8(M)	Fluorescence Polarization						Fluorescence Intensity					
	1	2	3	4	5	6	1	2	3	4	5	6
14.0	0.126	0.096	0.064	0.043	0.035	0.019	-	7400	6950	5935	5925	4740
8.4	0.126	0.096	0.062	0.043	0.034	0.018	4920	5005	4610	4015	3605	3230
5.8	0.126	0.096	0.062	0.043	0.033	0.017	3685	3790	3340	2935	2570	2300
3.1	0.124	0.100	0.064	0.040	0.036	0.019	2325	2380	1965	1810	1595	1390
1.9	0.129	0.105	0.064	0.040	0.026	0.018	1500	1476	1335	1145	1000	887
0.5	0.139	0.122	0.062	-	0.022	0.014	-	480	450	436	377	375

The numbers (1) through (5) refer to the concentration of phosphate in the vesicles: (1) 5 x 10^{-4}, (2) 2.7 x 10^{-4}, (3) 1.4 x 10^{-4}, (4) 0.7 x 10^{-4} and (5) 0.3 x 10^{-4}M. The number 6 refers to the values obtained with free filipin. The vesicles were prepared from EYL: cholesterol in the molar ratio 7:1. The measurements were made in 1.25% DMSO-0.01M Tris, 0.1M NaCl, pH 8.5, at room temperature at an excitation wavelength of 358 nm and emission wavelength of 476 nm.

markedly with the alterations in the concentration of the polyene. The polarization value of free filipin varied from 0.014 to 0.019 as the concentration of filipin was varied from 5 x 10^{-9} to 140 x 10^{-9}M. On binding to EYL: cholesterol vesicles, the fluorescence polarization reached a maximum value of 0.139 at 5 x 10^{-9}M filipin and 5 x 10^{-4}M lecithin phosphate, but decreased to 0.126 at 140 x 10^{-9}M filipin and 5 x 10^{-4}M lecithin phosphate. The fluorescence polarization was relatively constant when the filipin concentration was varied but fluorescence intensity depended on the filipin concentration (Table 5).

Since sterol-mediated spectral changes of filipin could be followed in the vesicles, it was anticipated that it would be possible to study the binding of filipin to sterols in natural membranes. Figures 23 and 24 show the results obtained when filipin was incubated with ciliary membranes. The excitation spectrum of filipin bound to ciliary membranes containing ergosterol shows that peak 1 decreases in absorption significantly similar to the situation observed in the absorption spectrum (figure 14). The emission spectra (figure 24) reveal that ciliary membranes containing ergosterol quench the fluorescence intensity of filipin, but those containing tetrahymanol do not influence the fluorescence significantly. There are no significant alterations either in the excitation or the emission spectra of filipin bound to tetrahymanol-containing membranes.

Figure 23. The excitation spectra of free filipin (——) and filipin bound to ergosterol-containing ciliary membranes (---). The concentration of protein in ergosterol-containing membranes was 120 μg/ml and the ratio of phosphate/sterol was 2.37. Filipin in the presence of tetrahymanol-containing ciliary membranes is also shown (-•-). The concentration of protein in tetrahymanol-containing ciliary membranes was 125 μg/ml and the ratio of phosphate/sterol was 3.2. The concentration of filipin was 3.2×10^{-5}M. The spectra were recorded at 25° at an emission wavelength of 480 nm, emission slit width of 4 mm, excitation slit width of 2 mm and sensitivity 6 on a Hitachi-Perkin Elmer spectrofluorometer in 1.25% ethylene glycol-0.01 M Tris-0.1 M NaCl, pH 8.5. The spectra have been corrected for membranes alone.

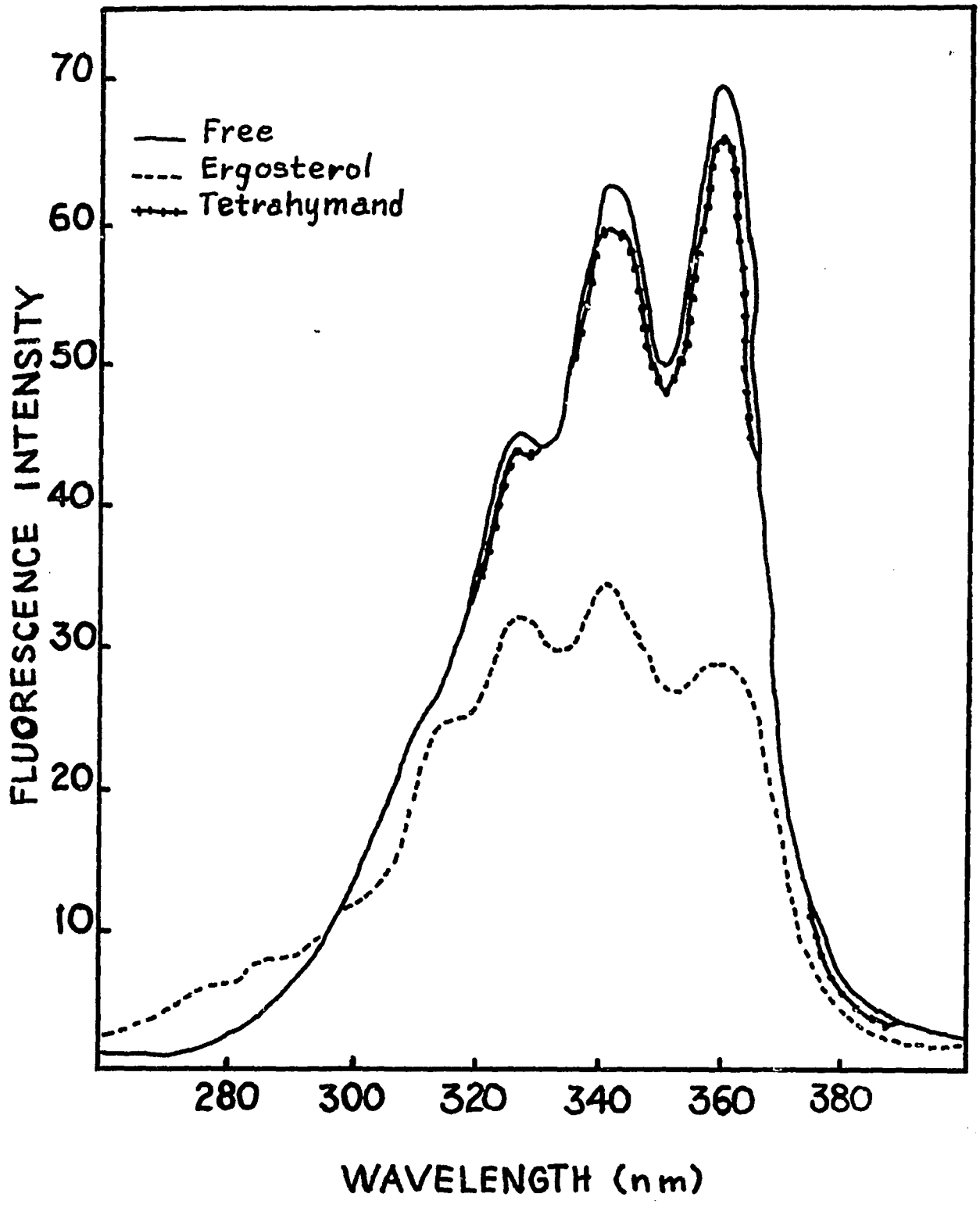
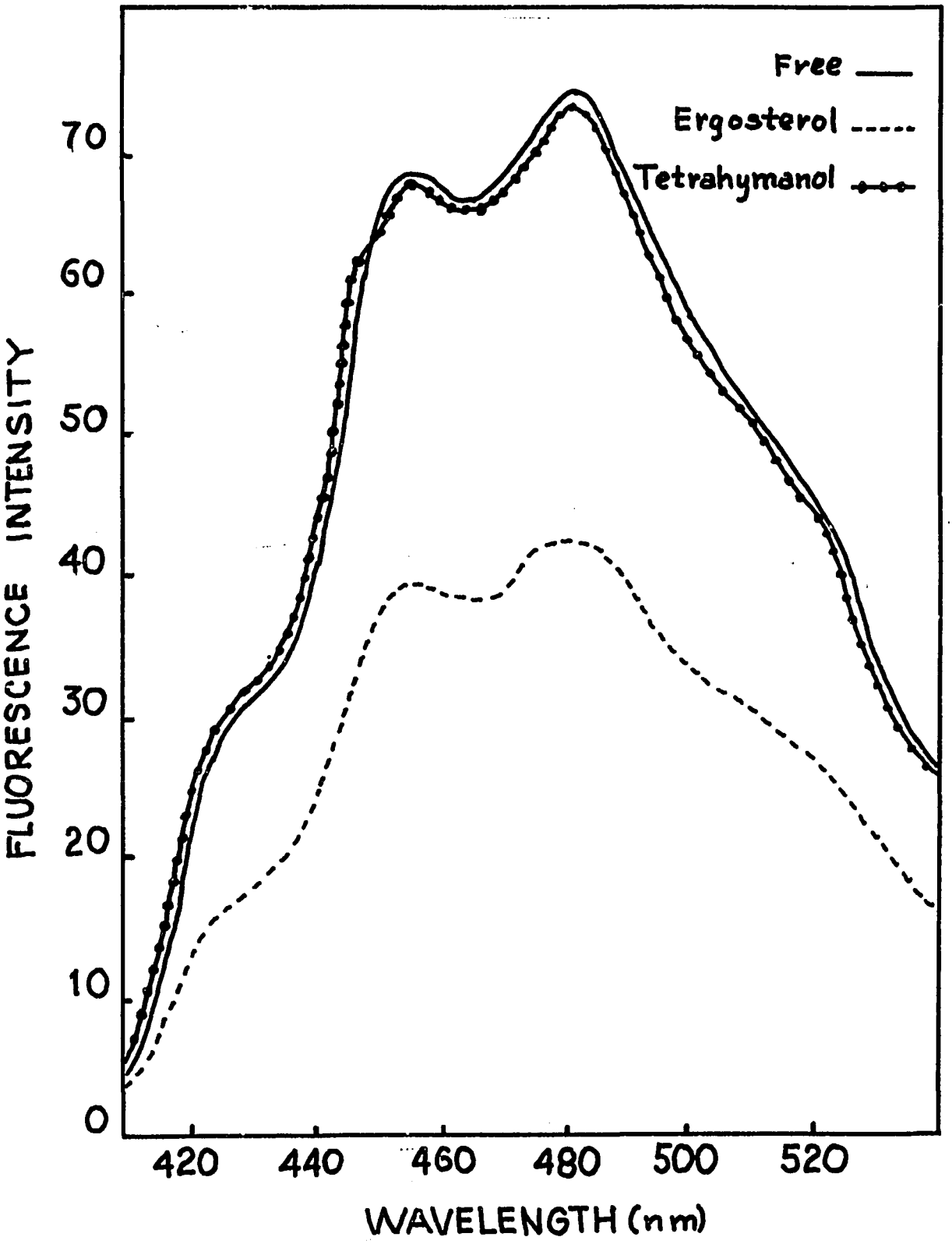

Free
Ergosterol
Tetrahymand
FLUORESCENCE INTENSITY
70
60
50
40
30
20
10
280
300
320
340
360
380
WAVELENGTH (nm)

Figure 24. The emission spectra of free filipin and filipin bound to ergosterol-containing ciliary membranes and to tetrahymanol-containing ciliary membranes. The excitation wavelength was 358 nm. All other experimental conditions are the same as those described in figure 23.


Free
Ergosterol
Tetrahymanol
FLUORESCENCE INTENSITY
0
10
20
30
40
50
60
70
420
440
460
480
500
520
WAVELENGTH (nm)

The fluorescence intensity of filipin was low in aqueous solutions but increased in solutions of low polarity. These results indicated that the effects may be due to the aggregation of the polyene in aqueous solutions (figures 18 and 19). It has been demonstrated that addition of free sterols, in contrast to the liposomal-bound sterols shown in figure 22 decreases the fluorescence intensity of the excitation spectrum of filipin and also that the fluorescence polarization is increased on the interaction of filipin with aqueous suspensions of sterols and with vesicles containing sterols (especially cholesterol, β-cholestanol and ergosterol) (1). These observations suggested that filipin could be used as a fluorescent probe to study lecithin-sterol interactions in model as well as in natural membranes.

The change in the fluorescence intensity of filipin depends on the type of lecithin and steroid incorporated in the model membranes. Recently published studies with cholesterol dispersions and phospholipid dispersions showed that the fluorescence intensity of filipin was not changed either in the presence of dispersions or liposomes, but with mixed phospholipid-cholesterol dispersions the fluorescence intensity of filipin was strongly enhanced (5). However, in contrast to these results, it has been shown that aqueous suspensions of cholesterol decrease the intensity of the fluorescence excitation spectrum of filipin (1), and the results reported in this dissertation show that

the phospholipid preparations obtained from EYL alter the fluorescence emission of the polyene. Addition of filipin to DPL lamellae containing cholesterol caused a marked quenching in the fluorescence intensity of filipin, and the quenching was proportional to the lipid concentration in the lamellae (figures 20 and 21). Lamellae containing DPL alone did not alter the fluorescence intensity of filipin (figure 22). These results are in agreement with the observations that significant increases in surface pressure were obtained when filipin interacted with monolayers prepared with DPL and cholesterol, but these changes in surface pressure were absent in DPL monolayers alone (58).

Circular Dichroism Studies

Figure 25 shows the CD spectra of filipin in pyridine, DMF and in an aqueous buffer. The absorption bands of filipin are optically active with well-defined negative dichroic bands in the uv range. Filipin in aqueous buffer solution has absorption peaks at 308, 323, 338 and 358 nm (figure 8, p. 65). The CD spectrum in buffer solution shows three distinct negative dichroic bands at 322, 340 and 358 nm. The absence in the CD spectrum of the shoulder present in the absorption spectrum indicated that the electric or the magnetic moments around 308 nm are very weak. The antibiotic in pyridine and DMF exhibited enhanced negative dichroic bands that are red-shifted. CD and absorption studies of filipin indicated that the absorption wavelength maxima of filipin in pyridine (330, 346 and 365 nm) and DMF (326, 342 and 361 nm) occur at longer wavelengths than those of the dichroic bands in pyridine (327, 344 and 363 nm) and in DMF (323, 340 and 358 nm).

Figure 26 shows the CD spectra of free filipin and filipin bound to EYL vesicles prepared with and without sterols and to DPL lamellae. The CD bands of the polyene were altered when filipin was bound to the phospholipid preparations. In the presence of EYL:cholesterol vesicles, the negative bands of filipin were enhanced three-fold whereas with β-cholestanol-containing vesicles small enhancements were obtained. In the presence of vesicles containing cholesteryl acetate, the bathochromic shifts

Figure 25. The CD spectra of filipin in 1.25% DMSO-0.01 M Tris-0.1 M NaCl, pH 8.5 (——), in pyridine (-•-•-) and in DMF (---). The spectra were recorded at room temperature. The concentration of filipin was 5.86×10^{-5}M.

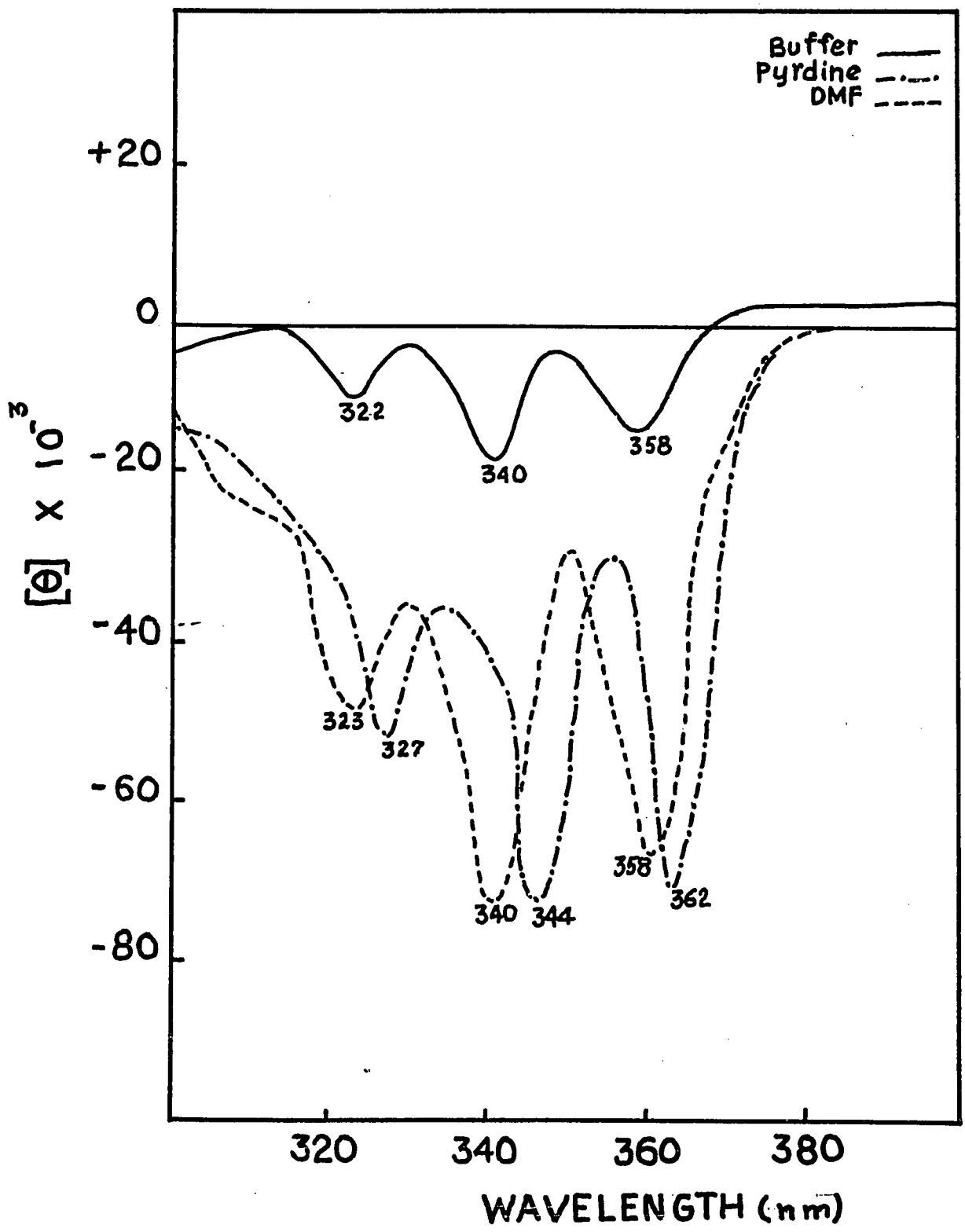
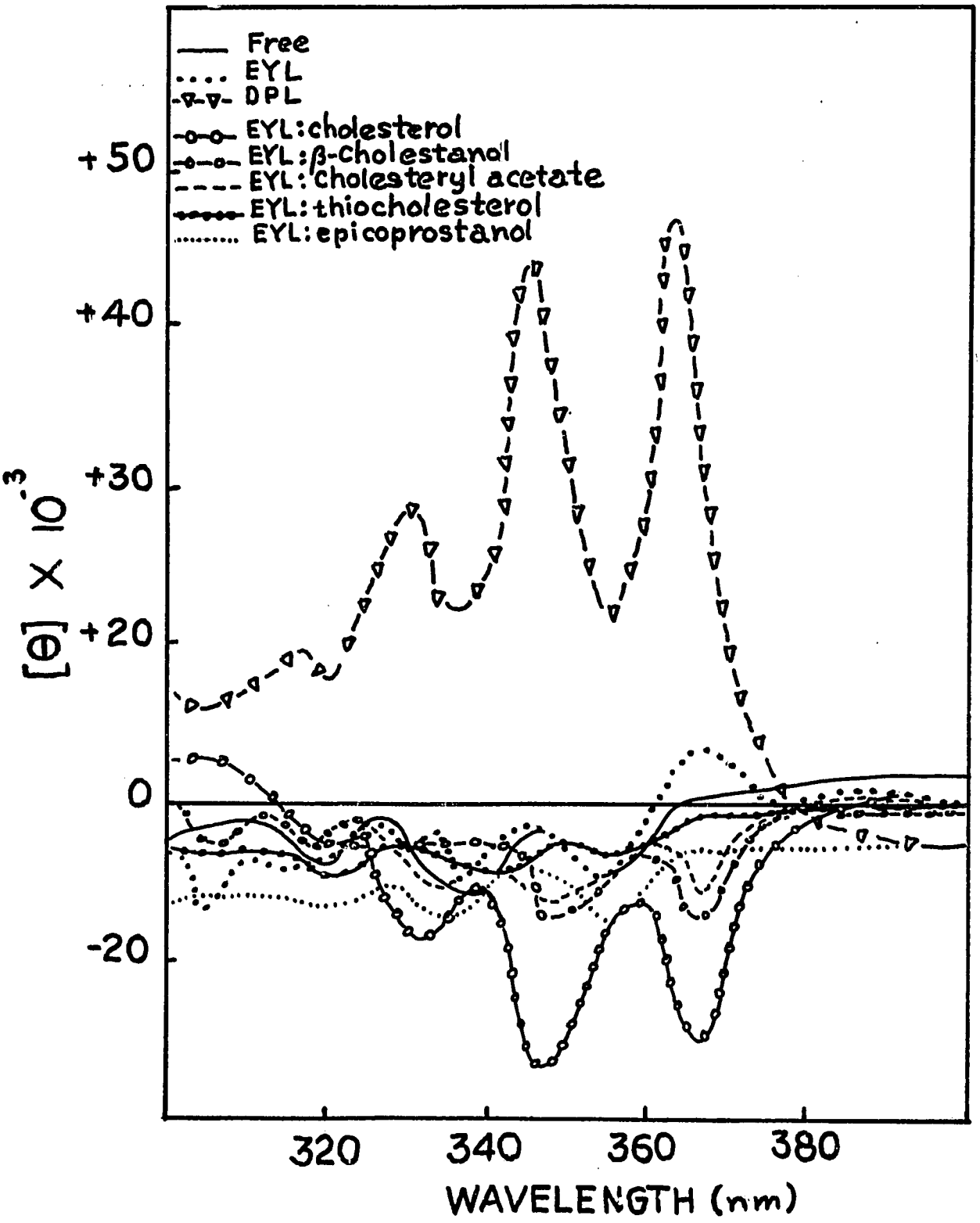

Buffer
Pyrdine
DMF
+20
0
-20
-40
-60
-80
[θ] x 10^-3
322
340
358
323
327
340
344
358
362
320
340
360
380
WAVELENGTH (nm)

Figure 26. The CD spectra of filipin bound to EYL vesicles (with and without sterols) and to DPL lamellae at room temperature. The molar ratio of EYL and sterols was 18:1. The phosphate concentration in the vesicles and lamellae was 11.2 x 10^{-3}M, with the exception of thiocholesterol-containing vesicles, where the concentration was 8 x 10^{-3}M. The concentration of filipin was 5.86 x 10^{-5}M. All measurements were made in 1.25% DMSO-0.01 M Tris-0.1 M NaCl, pH 8.5.


Free
EYL
DPL
EYL:cholesterol
EYL:β-Cholestanol
EYL:Cholesteryl acetate
EYL:thiocholesterol
EYL:epicoprostanol
+50
+40
+30
+20
0
-20
[θ] X 10^-3
320
340
360
380
WAVELENGTH (nm)

found with cholesterol- and β-cholestanol-containing vesicles were apparent with filipin, but the enhancements of the CD bands were negligible. With vesicles containing epicoprostanol, small enhancements were also observed, but wavelength shifts were absent. The CD spectrum of filipin bound to EYL vesicles had a small positive peak around 363-365 nm and a negative peak at 305 nm. The negative dichroic bands of filipin underwent a sign inversion in the presence of DPL lamellae, giving bands of positive ellipticity with conspicuous red shifts when compared with those of free filipin. There is almost a seven-fold enhancement in ellipticity compared to the ellipticity of free filipin.

Figure 27 shows the CD curves obtained from the titration of filipin-EYL:cholesterol complex with free filipin. Figure 27 reveals many facets of the binding. Upon addition of free filipin, the molecular ellipticities around 335, 347 and 367 nm were gradually decreased. The circular dichroic band was enhanced at 321 nm and red-shifted, and a positive dichroic band appears. An effort was made to construct Scatchard plots (100) from these data, but useful information about the association constant and the number of binding sites was not obtained.

Figure 28 shows that ergosterol-containing ciliary membranes alter the CD spectrum of filipin. There is a very large increase in the negative dichroic bands of filipin on binding to these membranes. Tetrahymanol-

Figure 27. The CD Curves obtained by the titration of filipin-EYL:cholesterol complex with free filipin. The concentrations of phosphate in the vesicles in curves 1 to 8 were 1.8×10^{-3}, 1.5×10^{-3}, 1.3×10^{-3}, 1.1×10^{-3}, 0.9×10^{-3}, 0.8×10^{-3}, 0.5×10^{-3} and 0.13×10^{-3}M, respectively, at 0.04 range and at time constant 1 in 1.25% DMSO-0.01 M Tris-0.1 M NaCl, pH 8.5. The concentration of filipin was 4.26×10^{-5}M. The molar ratio of EYL:cholesterol was 18:1. The spectra were recorded immediately after addition of free filipin to the complex.

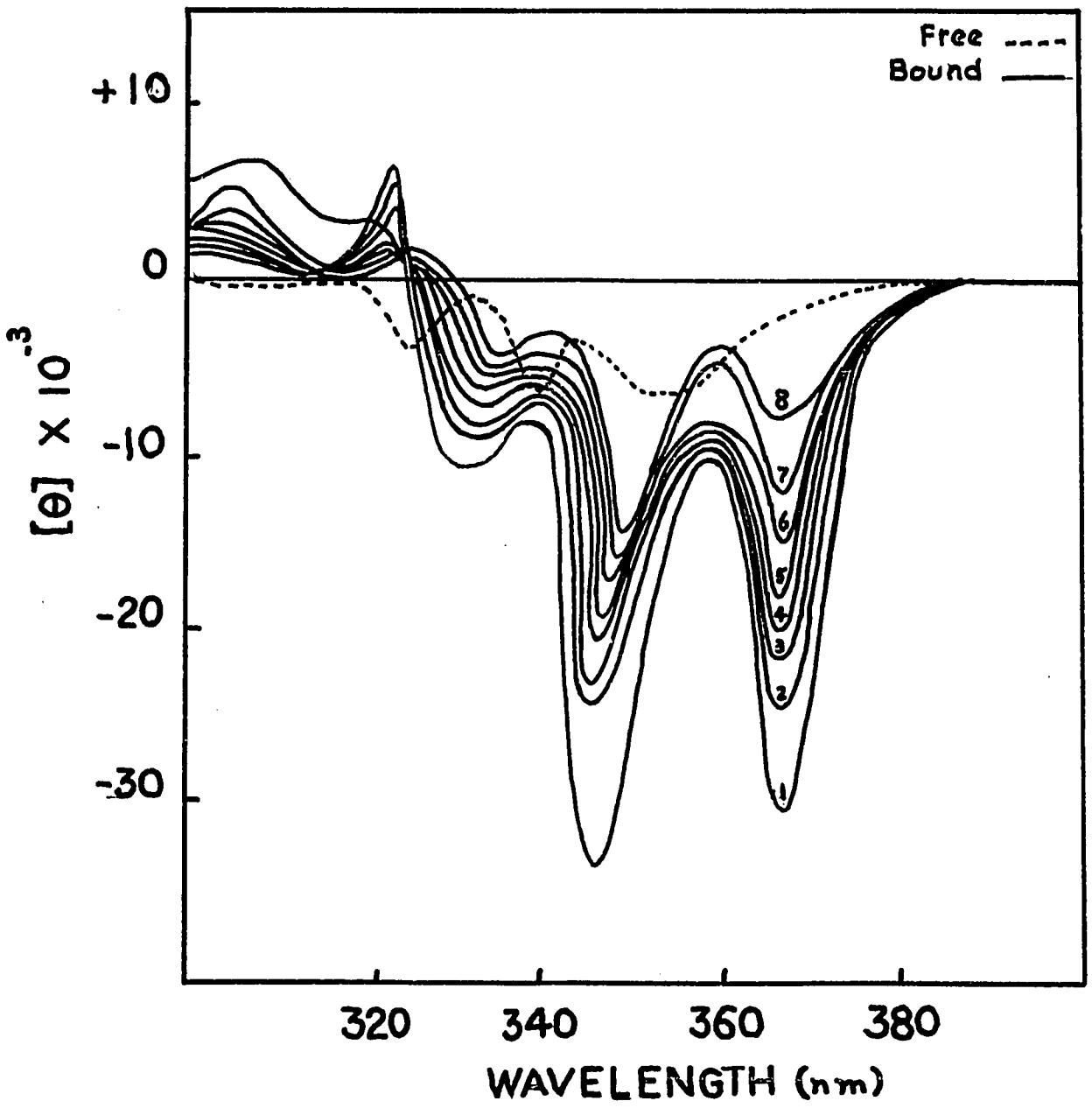
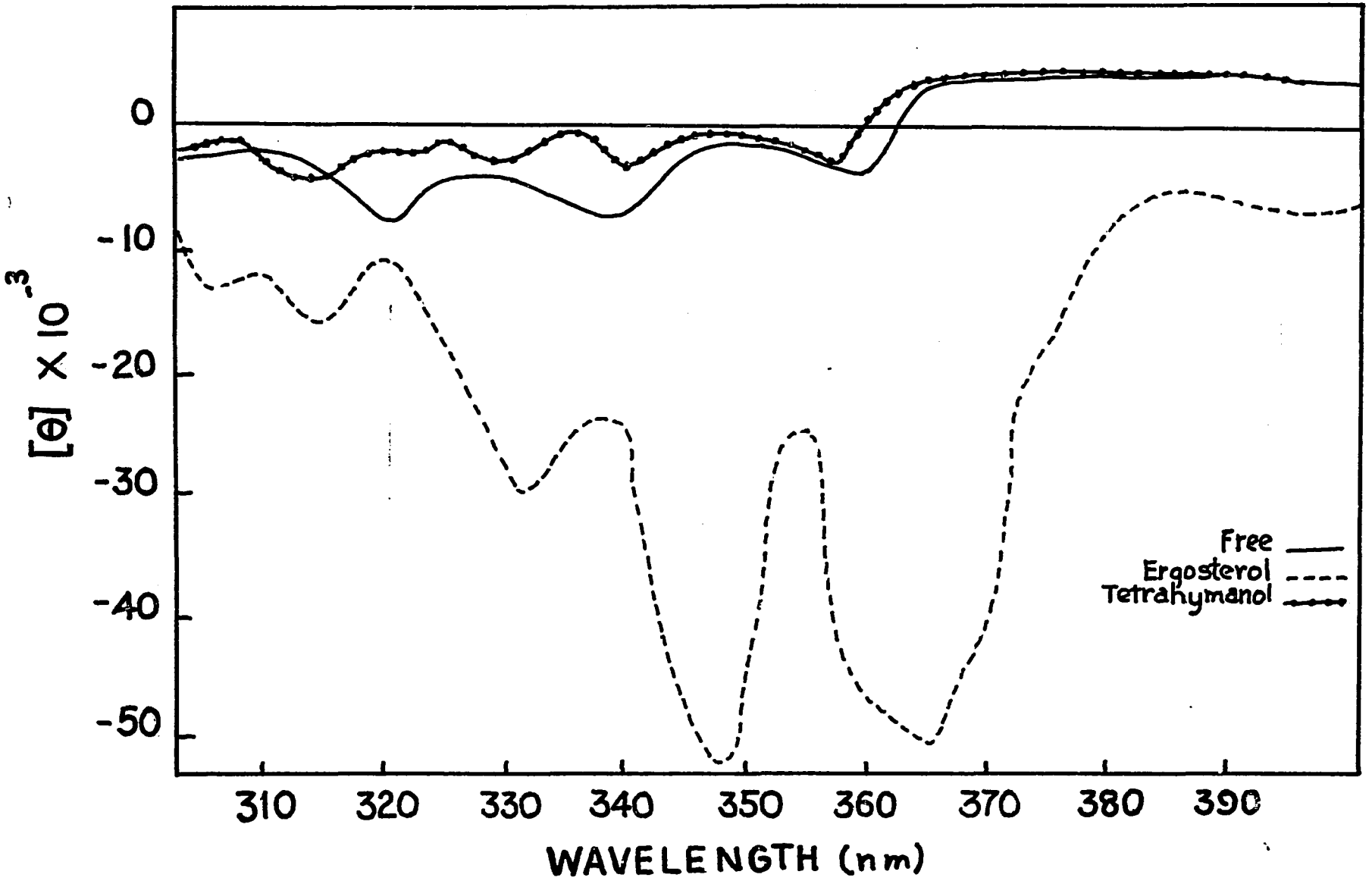

Free ----
Bound ——
+10
0
-10
-20
-30
[θ] X 10^3
8
7
6
5
4
3
2
1
320
340
360
380
WAVELENGTH (nm)

Figure 28. The CD spectra of free filipin (——) and filipin in the presence of ciliary membranes containing ergosterol (---) and tetrahymanol (-•-), recorded at room temperature. In ergosterol-supplemented membranes the concentration of protein was 120 μg/ml and the ratio of phosphate to sterol was 2.4. The concentration of protein in tetrahymanol membranes was 125 μg/ml and the ratio of phosphate to tetrahymanol was 3.2. The concentration of filipin was 2.3×10^{-5}M. The spectra were recorded in 1.25% ethylene glycol-0.01 M Tris-0.1 M NaCl, pH 8.5. The spectra were corrected for membranes alone.


0
-10
-20
-30
-40
-50
[θ] X 10^-3
310
320
330
340
350
360
370
380
390
WAVELENGTH (nm)
Free
Ergosterol
Tetrahymanol

containing ciliary membranes have no such effects on filipin; the CD spectrum remains practically unaltered.

The decrease in molecular ellipticity of free filipin with time suggested that filipin in an aqueous solution may exist as an aggregate. It is known that at high concentrations, probes such as ANS self-associate to form dimers and larger aggregates and exhibit extrinsic Cotton effects(101). Since solutions of filipin having an absorbance of 0.8-1.0 were used in these studies, it is possible that the decreases in the molecular ellipticities could be attributed to the ligand-ligand interactions. These results are in contrast to absorption studies reported on p. 48. Therefore, it appears that CD is inherently a more sensitive technique than absorption.

Filipin has a weak CD spectrum, but in the presence of DPL lamellae and EYL vesicles containing sterols, the electrical and magnetic moments are altered. In free filipin the contributions to the CD of neighboring filipin chromophores partially cancel each other. These effects may be caused by the tendency of filipin to aggregate in an aqueous solution. However, in the presence of vesicles containing sterols, these moments may be realigned, thus resulting in an enhanced CD spectrum. The degree of realignment may be attributed to stereochemistry of the sterol. The magnitude of the enhancement in the CD studies of bound filipin depends on the type of sterol incorporated in the vesicles (figure 26). Maximum enhancement and CD

spectral shifts in filipin were observed when the polyene was bound to EYL vesicles containing cholesterol. It appears that large enhancements occur when lecithin-sterol interactions are strong. The observations made in fluorescence polarization studies in filipin bound to cholesterol-containing vesicles revealed that there was a firm orientation of the polyene during the lifetime of the excited state which reflected the properties of the molecular interactions in the lecithin-sterol complex (1). The CD studies also showed that alteration of the binding properties of filipin reflected the nature of the molecular interactions of lecithin and sterol. These alterations largely depend on the stereochemistry of the sterol. Replacement of cholesterol in EYL vesicles by either β-cholestanol, epicoprostanol or cholesteryl acetate reduced the intensity of the CD bands of filipin. In β-cholestanol-containing vesicles, the ellipticities of the CD bands of filipin were much lower than those in cholesterol-containing vesicles but were of a higher magnitude than those in cholesteryl acetate-containing vesicles. Substitution of the 3 β-hydroxyl group by a 3 α-hydroxyl group, saturation of the 5, 6-double bond and alteration of the A:B ring junction in cholesterol (as in epicoprostanol) not only diminished the enhancement but also abolished the spectral shifts observed in the CD spectra of filipin bound to cholesterol. These results indicated that the 5, 6-double bond and a 3 β-hydroxyl group are essential to produce

strong lecithin-sterol interactions. It was also observed that replacement of a 3 β-hydroxyl group with a 3 β-thiol group in the sterol (as in thiocholesterol) diminished the lecithin-sterol interactions significantly. The CD bands of filipin bound to these vesicles were not altered when compared with those containing cholesterol. These results are consistent with those obtained in fluorescence polarization studies (1). Thiocholesterol-containing vesicles produced small enhancements of filipin in fluorescence polarization studies, indicating that the complex formed between lecithin and thiocholesterol was of a weaker nature than that formed between lecithin and cholesterol. The molecular interactions between lecithin and sterol in these two types of vesicles are different and are reflected in the properties of the CD spectra of bound filipin. DPL lamellae, although its structure is not known, appears to bind to filipin. EYL vesicles, on binding to filipin, do not cause any appreciable changes in the CD spectrum of filipin. These results indicated that the structure of EYL vesicles and DPL lamellae are different because they affect filipin differently.

The enhancement of the CD bands (figure 27) demonstrated that the electrical and magnetic moments do not partially cancel each other in filipin bound to ergosterol-containing ciliary membranes as they do in free filipin. In tetrahymanol-containing ciliary membranes, filipin does not undergo any appreciable change. Comparison of the CD

spectral properties of filipin bound to EYL:cholesterol vesicles and filipin bound to ergosterol-containing ciliary membranes indicated that the interaction of ergosterol with membrane phospholipids appear to be similar to the interactions present between lecithin and cholesterol in model membranes.

Data from the absorption studies indicated that at a EYL:sterol molar ratio of 18:1 filipin is bound, and approximately the same changes were obtained when the sterol was cholesterol, cholesteryl acetate, epicoprostanol or β-cholestanol. In contrast, absorption studies indicated that significant alterations in peak ratio 323 nm/358 nm of bound filipin did not largely depend upon the stereochemistry of the sterol. The binding was more favorable when a sterol having a 3 β-hydroxyl group was incorporated in the liposomes as reported by Norman and coworkers (2). Absorption studies by the latter workers showed that when filipin binds to liposomes containing 5 and 10 mole % of cholesterol, the peak ratio 323 nm/358 nm at pH 8.5 remained constant. Significant increases in peak ratio of filipin were observed only on increasing the mole % of cholesterol from 16% to 50% in liposomes. The results reported in this study were obtained with liposomes containing 5 mole % of cholesterol and agree with those reported by Norman and coworkers (2). The fluorescence studies reported in this dissertation indicated that cholesterol- and thiocholesterol-containing vesicles

produced the same enhancement in fluorescence on binding to filipin when the phospholipid and sterols were in a molar ratio of 18:1. In contrast to these results, fluorescence polarization studies indicated that at a molar ratio of 1:1 between lecithin and sterol, vesicles containing cholesterol on binding to filipin produced a large enhancement whereas the enhancement produced in the presence of vesicles containing thiocholesterol was smaller (1). The CD studies, although performed at a ratio of phospholipids to sterols of 18:1, demonstrated that a free 3 β-hydroxyl group was essential for the interaction of lecithin with sterols. This is consistent with the view that the antibiotic produces maximum change when the lecithin-sterol interactions are strong (1). The results of the CD studies are in agreement with those found by others using higher concentrations (1, 2). Therefore, the conclusion is made that the CD technique is more sensitive and accurate than all the techniques that have been studied to investigate interactions of phospholipids and sterols in membranes using filipin as a probe.

References

1. Bittman, R., and Fischkoff, S.A., Proc. Nat. Acad. Sci., USA, 69, 3795 (1972).
2. Norman, A.W., Demel, R.A., DeKryuff, B., and Van Deenen, L.L.M., J. Biol. Chem., 247, 1918 (1972).
3. Norman, A.W., Demel, R.A., DeKruyff, B., Geurts Van Kessel, W.S.M., and Van Deenen, L.L.M., Biochim. Biophys. Acta, 290, 1 (1972).
4. Schroeder, F., Holland, J.F., and Bieber, L.L., Biochemistry, 11, 3105 (1972).
5. Drabikowski, W., Lagwinski, E., and Sarzala, M.G., Biochim. Biophys. Acta, 291, 61 (1973).
6. Lampen, J.O., Symp. Soc. Gen. Microbiol., 16, 111 (1966).
7. Gottlieb, D., Carter, H., Sloneker, J.H., and Ammann, A., Science, 128, 361 (1958).
8. Weissmann, G., and Sessa, G., J. Biol. Chem., 242, 616 (1967).
9. Demel, R.A., Van Deenen, L.L.M., and Kinsky, S.C., J. Biol. Chem., 240, 2749 (1965).
10. Van Zutphen, H., Van Deenen, L.L.M., and Kinsky, S.C., Biochem. Biophys. Res. Commun., 22, 393 (1966).
11. Schaffner, C.P., and Gordon, H.W., Proc. Nat. Acad. Sci., USA, 61, 36 (1968).
12. Kinsky, S.C., Annu. Rev. Pharmacol., 10, 119 (1970).
13. Lampen, J.O., Arnow, P.M., and Safferman, R.S., J. Bacteriol., 80, 200 (1960).

14. Gorter, E., and Grendel, P., J. Exp. Med., 41, 439 (1925).

15. Danielli, J.F., and Davson, H., J. Cell Comp. Physiol., 5, 495 (1934-1935).

16. Maddy, A.H., in Information and Fate of Cell Organelles, ed. Warren, K.B., Academic Press, New York, 1967, p. 253.

17. Robertson, J.D., Biochem. Soc. Symp., 16, 29 (1959).

18. Kavanu, J.L., Nature (Lond.), 198, 525 (1963).

19. Lucy, J.A., J. Theoret. Biol., 7, 360 (1964).

20. Vandenheuvel, F.A., J. Amer. Oil Chem. Soc., 40, 455 (1963).

21. Vanderkooi, G., Ann. N.Y. Acad. Sci., 195, 6 (1972).

22. Singer, S.J., and Nicolson, G.L., Science, 175, 720 (1972).

23. Cedar, O., and Ryhage, R., Acta. Chem. Scand., 18, 558 (1964).

24. Gottlieb, D., Ammann, A., and Carter, H.E., U.S. Dept. Agriculture Plant Disease Reporter, 39, 219 (1955), [Chem. Abstr., 49, 9861 (1955)].

25. Whitfield, G.B., Brock, T.D., Ammann, A., Gottlieb, D., and Carter, H.E., J. Amer. Chem. Soc., 77, 4799 (1955).

26. Sloneker, J.H., Ph.D. Thesis, Univ. of Illinois, (1958); Diss. Abstr., 1911, 2744 (1959).

27. Bergy, M.E., and Eble, T.E., Biochemistry, 7, 653 (1968).

28. Van Zutphen, H., Demel, R.A., Norman, A.W., and Van Deenen, L.L.M., Biochim. Biophys. Acta, 241, 310 (1971).

29. Pandey, R.C., Narsimhachari, N., Rinehart, K.L., and Millington, D.S., J. Amer. Chem. Soc., 94, 4306 (1972).

30. Demel, R.A., Van Deenen, L.L.M., and Pethica, B.A., Biochim. Biophys. Acta, 135, 11 (1967).

31. Van Deenen, L.L.M., Pure Appl. Chem., 25, 25 (1971).

32. Demel, R.A., Van Kessel, W.S.G., and Van Deenen, L.L.M., Biochim. Biophys. Acta, 266, 26 (1972).

33. Shah, D.O., and Schulman, J.H., J. Lipid Res., 8, 215 (1967).

34. Mueller, P., Rudin, D.O., Tien, H.T., and Wescott, W.C., J. Phys. Chem., 67, 534 (1963).

35. Hanai, T., Haydon, D.A., Taylor, J., Proc. Royal Soc. (London). Ser. A, 281, 377 (1964).

36. Tien, H.T., J. Theoret. Biol., 16, 97 (1967).

37. Henn, F.A., Decker, G.L., Greenwalt, J.W., Thompson, T.E., J. Mol. Biol., 24, 51 (1967).

38. Henn, F.A., and Thompson, T.E., J. Mol. Biol., 31, 227 (1968).

39. Huang, C., Wheeldon, L., and Thompson, T.E., J. Mol. Biol., 8, 148 (1964).

40. Thompson, T.E., in Cellular Membranes in Development ed. Locke, M., Academic Press, New York, 1961, p. 83.

41. Harvey, E.N., and Danielli, J.F., Biol. Rev. Cambridge Phil. Soc., 13, 319 (1938).

42. Andreoli, T.E., Bangham, J.A., and Tosteson, D.C., J. Gen. Physiol., 50, 1729 (1967).

43. Vreeman, H.J., Konikl. Ned. Akad. Wetenschap. Proc. Ser. B, 69, 542 (1966), [Chem. Abstr., 66, 16515 v (1967)].

44. Bean, R.C., and Shepherd, W.C., Fed. Proc., 26, 862 (1967).

45. Petkau, A., Chelack, W.S., Parker, W.L., and Stackiw, W., Abstr. Biophys. Soc., 148 (1966).

46. Mueller, P., Rudin, D.O., Tien, H.T., and Wescott, W.C., Circulation, 26, pt. 2, 1167 (1967).

47. Mueller, P., and Rudin, D.O., Nature, 213, 603 (1967).

48. Mueller, P., and Rudin, D.O., Biochem. Biophys. Res. Commun., 26, 398 (1967).

49. Bangham, A.D., Standish, M.M., and Watkins, J.C., J. Mol. Biol., 13, 238 (1965).

50. Bangham, A.D., Standish, M.M., and Watkins, J.C., J. Mol. Biol., 13, 253 (1965).

51. Bangham, A.D., Progr. Biophys. Mol. Biol., 18, 29 (1968).

52. Papahadjopoulos, D., and Watkins, J.C., Biochim. Biophys. Acta, 135, 639 (1967).

53. Huang, C., Biochemistry, 8, 344 (1969).

54. Huang, C., and Charlton, J.P., J. Biol. Chem., 246, 2555 (1971).

55. Huang, C., and Lee, L., J. Amer. Chem. Soc., 95, 234 (1973).

56. Gibbon, I.R., Proc. Natl. Acad. Sci., USA, 50, 1002 (1963).

57. Conner, R.L., Mallory, F.B., Landrey, J.R., Ferguson, K.A., Kaneshiro, E.S., and Ray, E., Biochem. Biophys. Res. Commun., 44, 995 (1971).

58. Demel, R.A., Crombag, F.J.L., Van Deenen, L.L.M., and Kinsky, S.C., Biochim. Biophys. Acta, 150, 1 (1968).

59. Kinsky, S.C., Demel, R.A., and Van Deenen, L.L.M., Biochim. Biophys. Acta, 135, 835 (1967).

60. Finkelstein, A., and Cass, A., J. Gen. Physiol., 52, 145 S (1968).

61. Andreoli, T.E., and Monahan, M., J. Gen. Physiol., 52, 300 (1968).

62. Van Zutphen, H., Demel, R.A., Norman, A.W., and Van Deenen, L.L.M., Biochim. Biophys. Acta, 241, 310 (1971).

63. Lippe, C., J. Mol. Biol., 35, 635 (1968).

64. Cass, A., and Finkelstein, A., Biophys. J., 9, A-80 (1969).

65. Andreoli, T.E., Dennis, V.W., and Weigl, A.M., J. Gen. Physiol., 53, 133 (1969).

66. Sessa, G., and Weissmann, G., J. Biol. Chem., 243, 4364 (1968).

67. Weissmann, G., and Sessa, G., J. Biol. Chem., 242, 616 (1967).

68. Bittman, R., and Blau, L., Biochemistry, 11, 4831 (1972).

69. Graham, J.M., and Green, C., Eur. J. Biochem., 12, 58 (1970).

70. Child, J.J., Defago, G., and Haskins, R.H., Can. J. Microbiol., 15, 599 (1969).

71. Finkelstein, A., and Cass, A., Nature, 216, 717 (1967).

72. Lecuyer, H., and Dervichian, D.G., J. Mol. Biol., 45, 39 (1969).

73. Rand, R.P., and Luzzati, V., Biophys. J., 8, 125 (1968).

74. Neiderhiser, D.H., and Roth, H.P., Biochim. Biophys. Acta, 270, 407 (1972).

75. Demel, R.A., Bruckdorfer, K.R., and Van Deenen, L.L.M., Biochim. Biophys. Acta., 255, 321 (1972).

76. Zull, J.E., Greanoff S., and Adam, H.K., Biochemistry, 7, 4172 (1968).

77. Long, R.A., Hruska, F., Gesser, H.D., Hsia, J.C., and Williams, R., Biochem. Biophys, Res. Commun., 41, 321 (1970).

77.A. Chapman, D., and Penkett, S.A., Nature, 211, 1304 (1966).

77.B. Ladbrooke, B.D., Williams, R.M., and Chapman, D., Biochim. Biophys. Acta, 150, 333 (1968).

78. Wu, L., Ph.D. Thesis, Univ. of Illinois (1959); Diss. Abstr., 2005, 1540 (1959).

79. Stewart, G.T., Brit. Med. J., 1, 658 (1956).

80. Mudd, J.B., and Kleinschmidt, M.G., Plant Physiol., 45, 517 (1970).

81. Kinsky, S.C., Avruch, J., Permutt, M., Rogers, H.B., and Schonder, A.A., Biochem. Biophys. Res. Commun., 9, 503 (1962).

82. Kinsky, S.C., Archiv. Biochem. Biophys., 102, 180 (1963).

83. Kinsky, S.C., Luse, S.A., and Van Deenen, L.L.M., Fed. Proc., 25, 1503 (1966).

84. Adam, T.H., and Norman, A.W., J. Biol. Chem., 245, 4421 (1970).

85. Wong, R.G., Adams, T.H., Roberts, P.A., and Norman, A.W., Biochim. Biophys. Acta, 219, 61 (1970).

86. Berdicevsky, I., and Grossowicz, N., Isr. J. Med., 1, 1094 (1971).

87. Chance, B., and Mukai, Y., in Probes of Structure and Function of Macromolecules and Membranes, eds., Chance,

B., Lee, C.P., and Blasie, J.K., Academic Press, New York, 1971, Vol. 1, p. 239.

88. Chance, B., and Mela, L., in Probes of Structure and Function of Macromolescules and Membranes, eds., Chance, B., Lee, C.P., and Blasie, J.K., Academic Press, New York, 1971, Vol. 1, p. 261.

89. Vanderkooi, J., and Martonosi, A., in Probes of Structure and Function of Macromolecules and Membranes, eds., Chance, B., Lee, C.P., and Blasie, J.K., Academic Press, New York, 1971, Vol. 1, p. 303.

90. Gomperts, B., and Stock, R., in Probes of Structure and Function of Macromolecules and Membranes, eds., Chance, B., Lee, C.P., and Blasie, J.K., Academic Press, New York, 1971, Vol. 1, p. 303.

91. Hansen, M., and Smith, A.L., Biochim. Biophys. Acta, 81, 214 (1964).

92. Azzi, A., Chance, B., Radda, G.K., and Lee, C.P., Proc. Natl. Acad. Sci., USA, 62, 612 (1969).

93. Turner, D.C., and Brand, L., Biochemistry, 7, 3381 (1968).

94. Azzi, A., and Vainio, H., in Probes of Structure and Function of Macromolecules and Membranes, eds., Chance, B., Lee, C.P., and Blasie, J.K., Academic Press, New York, 1971, Vol. 1, p. 209.

95. Rubalcava, B., Muñoz, D.M., and Gitler, C., Biochemistry, 8, 2742 (1969).

96. Vanderkooi, J., and Martonosi, A., Arch. Biochem.

Biophys., 133, 153 (1969).

97. Romeo, D., Cramer, R., and Rossi, F., Biochem. Biophys. Res. Commun., 41, 582 (1970).

98. Singleton, W.S., Gray, M.S., Brown, M.L., and White, J.L., J. Amer. Oil Chem. Soc., 42, 53 (1965).

99. Huang, C., Charlton, J.P., Shyr, C.I., and Thompson, T.E., Biochemistry, 11, 4831 (1970).

100. Scatchard, G., Ann. N.Y. Acad. Sci., 51, 660 (1949).

101. Daniel, E., and Yang, J.T., Biochemistry, 12, 508 (1973).