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FILIPIN AS A PROBE OF MEMBRANE STRUCTURE

City University of New York PH.D. 1980

FILIPIN AS A PROBE OF MEMBRANE STRUCTURE

by

ZENOWIJ MAJUK

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

1980

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

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ABSTRACT

FILIPIN AS A PROBE OF MEMBRANE STRUCTURE

BY

ZENOWIJ MAJUK

Advisor: Professor James Hogg

The polyene antibiotic filipin was used both as a membrane-perturbing agent and as an optical probe to study phospholipid-sterol interactions in natural and model membranes. Compared to the polyenes amphotericin B, lucensomycin, nystatin and pimaricin, filipin was the most effective polyene in disrupting ergosterol-containing liposomes. Filipin treatment resulted in the release of both large (horseradish peroxidase) and small (glucose) trapped markers from liposomes. Only glucose release was observed with the other polyenes.

Using filipin as a spectral probe, changes in the fluorescence polarization of filipin were used to monitor the lipid phase transitions of vesicles prepared from L-α-dipalmitoylphosphatidylcholine (L-α-DPL), L-α-dimyristoylphosphatidylcholine (L-α-DML) and sphingomyelin. Incorporation of cholesterol into the vesicles led to a broadening of the phase transition and to its disappearance at high concentrations of cholesterol. No marked differences indicative of preferential affinity of cholesterol for sphingomyelin over the lecithins

were observed in the phase transition curves, nor in the equilibrium dissociation constant for filipin binding to membrane-bound cholesterol.

The circular dichroism (CD) spectrum of filipin, when bound to various phospholipid vesicles, reveals differences in the bilayer microenvironment. Filipin alone in aqueous solution exhibits weakly negative ellipticities which in the presence of L-α-DPL and L-α-DML vesicles become reversed in sign and dramatically enhanced. At 53°C, a temperature above the phase transition temperature of L-α-DPL and L-α-DML vesicles, the positive CD spectrum of filipin reverts to one that closely corresponds to that of free filipin; conversely, when the temperature is lowered to room temperature, the spectrum becomes positive again. Incorporation of cholesterol into vesicles prepared from L-α-DPL or L-α-DML results in a dramatic sign reversal in the CD spectrum to produce a negative mirror image. With sphingomyelin and egg lecithin, the negative CD bands of pure filipin were only enhanced in intensity.

The interaction of the polyene antibiotic filipin with membrane-bound cholesterol in vesicular stomatitis, influenza, and Rauscher leukemia virions was studied. Exposure of intact and protease-treated vesicular stomatitis (VS) virions to filipin resulted in profound morphological alterations in the viral membrane; a series of ridges and depressions is observed in negatively stained preparations of particles that lack the glycoproteins,as well as in intact VS virions and in thin-sectioned virions. Morphological alterations induced by filipin in

influenza and Rauscher leukemia virions differed from those seen in VS virions. The infectivity of filipin-treated VS virions was reduced 500-fold, depending on the cholesterol/drug ratio. The stoichiometry of the filipin-cholesterol interaction is nearly identical in intact and protease-treated VS virions. The equilibrium dissociation constant is much larger for intact virions, and the initial rate of association of filipin with cholesterol in the intact virions is slower than with protease-treated particles. The fluidity of lipids in VS viral membranes, as probed by 5-doxylstearic acid was markedly reduced when both intact and protease-treated virions were treated with filipin. The results indicate that cholesterol is more accessible for interaction with filipin, and that the binding is stronger in protease-treated VS virions that lack surface glycoproteins, than in the intact virions.

The profound morphological changes caused by filipin in VS virions are accompanied by alterations in the permeability properties of the virions. Light-scattering measurements on VS virions indicate that they undergo swelling or shrinking much in the same manner as other membraneous vesicles. Treatment with filipin altered the kinetic and equilibrium permeability behavior of VS virions but the extent of leakage of osmotic shocking agent was less than in sterol-containing liposomes and ergosterol-containing Tetrahymena ciliary membrane vesicles. Removal of surface glycoproteins by proteases also altered the permeabilty behavior.

This work is dedicated
to my family

ACKNOWLEDGEMENTS

I want to acknowledge here the individuals who helped me during my years of graduate study.

First I want to thank Professors Maxwell Eidinoff, A.D. Baker, Julius Golubow, Fred Naider and Harvey Ozer who served with Professor James Hogg on my thesis committee. I would also like to thank Professors Frank Landsberger and Richard Compans for the productive and enjoyable times I spent at their laboratories at the Rockefeller University. In addition, I want to thank Professor Robert Bittman for the use of his facilities and help in the early stages of my work.

I would be remiss if I did not thank my friends Ewa Wajnberg, Lester May, Robert Hickey, Dan Hryb, Linda Malkis, Winston Chen, Maurice Masson and Susan Herrick for their moral support, practical assistance, comaraderie, and occasionally irreverent discussions. I would also like to thank Harvey Ozer for the kind special attention he gave me and my thesis.

Most of all, I want to express my gratitude to Professor James Hogg for enabling me to complete my doctoral training, for the guidance he gave me during the writing of the thesis, and for showing me the intricacies, pitfalls and rewards of research. On a personal level, I have enjoyed knowing and working with Jim Hogg and I will always remember his stories of Texas, his kindness, generosity and knowledge of biochemistry.

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Introduction

THE POLYENE ANTIBIOTIC FILIPIN AS A PROBE OF MEMBRANE STRUCTURE

General Introduction

Since the discovery of the first polyene antibiotic nystatin by Hazen and Brown in 1950 (1), nearly 100 other polyenes have subsequently been discovered and characterized to various degrees. As the amount of information regarding the antimicrobial properties of these antibiotics has grown, so has the number of review articles dealing with the biological and chemical properties of polyene antibiotics, including the extensive reviews of Kinsky (2), Hamilton-Miller (3), Norman et al. (4), and Kobayashi and Medoff (5). Among the clinically important polyenes are amphotericin B, nystatin and pimaricin. Amphotericin B is given parenterally for systemic fungal infections, while nystatin and pimaricin are applied directly to treat superficial or topical fungal infections. The polyene antibiotic filipin, on the other hand, is too toxic for clinical use, a liability it shares with other polyenes. Filipin causes severe damage to the membranes of non-fungal cells, but under carefully controlled conditions it is possible to use filipin's membrane-perturbing properties to study important aspects of membrane structure and function (3,4).

It was clear by the early 1960's that polyene antibiotics exerted their effects on cells containing membrane-bound sterols, therein producing membrane alterations that resulted in cell death. These membrane alterations resulted in the leakage of important cellular constituents such as potassium ions and small macromolecules (2). Further research showed that the targets of these antibiotics were sterols located within the plasma membrane of susceptible organisms (119). Target cells could be protected from the actions of the antibiotics by diverting the polyenes to exogeneously added sterols (120). Bacteria were unaffected by these drugs because they do not contain sterols. In experiments employing cholesterol-containing liposomes, it was observed that susceptibility to polyene antibiotics required the presence of sterols in the bilayer and that the binding of the drugs led to rearrangement of membrane components, followed by changes in liposomal permeability (3,4). In both biological membranes and liposomes, the interaction of filipin with cholesterol resulted in the appearance of pits, 150-250 A in diameter and in doughnut-shaped craters, neither of which were shown to go all the way through the membrane (6,7). Models of polyene antibiotic-cholesterol complexes have been proposed for filipin, amphotericin B, pimaricin, lucensomycin (etruscomycin) and nystatin by de Kruijff and Demel (8). Their detailed models were proposed on the basis of available information concerning the stoichiometry of the polyene-

cholesterol interaction, electron microscopic observations of the gross morphological alterations caused by the drugs, permeability modifications induced by the polyenes, and by the use of space-filling models.

The search for the molecular basis for the action of polyene antibiotics on the membranes of susceptible organisms has been aided greatly by the spectral properties of the antibiotics and by the use of liposomes as models of membranes. The initial observation by Lampen et al. (9) in 1960 showed that the addition of exogenous sterols to aqueous solutions of polyenes was accompanied by changes in the ultraviolet absorbance spectra of the drugs that resembled those when the drugs were bound to sterol-containing cells. Soon after, the field blossomed as other investigators began using the fluorescence, and ultraviolet absorption, as well as the circular dichroic properties of the polyenes to study the nature of the polyene-sterol interaction (4). It was fortuitous that, as the possibilities of using the spectral properties of the polyenes became apparent, liposomes came into use as models of biological membranes, especially after Bangham et al. characterized the osmotic properties of liposomes in 1967 (10). With the easy availability of artificial membranes that could be prepared from a variety of phospholipids and sterols, the biochemical mode of action of the polyenes could be studied in a defined lipid environment that

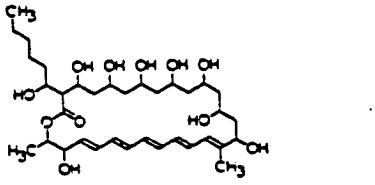
could be made to order. The interactions of the polyene antibiotics with sterol-containing liposomes were monitored with a variety of spectral techniques, including electron spin resonance (ESR)(109) and measurements of the initial rates of association (27). The results obtained with natural membranes and liposomes established that polyenes interact specifically and stoichiometrically with membrane-bound cholesterol, forming cholesterol-polyene complexes and thus disturbing membrane structure. As a result, the movement of ions across the membrane was facilitated, and in the case of filipin, the drastic rearrangement of the lipids resulted in membrane fragmentation.

Recently, the ability of amphotericin B and especially filipin to interact specifically with cholesterol and then to perturb the membrane has been used to probe eukaryotic membranes. Reviews by Bittman (11) and Norman et al. (4) list the many ways in which polyenes have been employed to study membrane structure and function. These reviews list how membrane-perturbing properties of filipin have been used to establish vitamin D-mediated Ca ion transport across the small intestine, phosphate transport into spermatozoa, and hormone-induced adenylate cyclase activation in eukaryotic membranes. Amphotericin B has been used to investigate the role of aldosterone in sodium ion transport, to study ion permeability in red blood cells, and to augment the penetration of non-polyene antibiotics into fibroblasts. Filipin

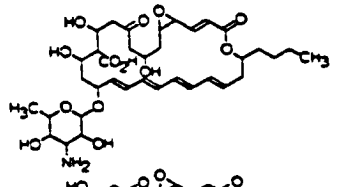
has been employed as a probe for examining phospholipid-cholesterol interactions and to investigate transbilayer distribution of cholesterol in the membrane of mycoplasma cells (11).

The polyene antibiotics are a subdivision of the class of macrolide antibiotics. They are characterized by a macrolide ring (a large ring of carbon atoms closed by an internal ester or lactone), and by an intense ultraviolet absorption spectrum. They are produced by the soil Actinomycetes, in particular, the genus Streptomyces. The structures of the five antibiotics of greatest biological interest are presented in Figure 1. Polyene antibiotics have in common a series of conjugated double bonds located on one side of the macrolide ring and by the presence of multiple hydroxyl groups (and carbonyl oxygens as in the case of pimaricin and lucensomycin). Polyene antibiotics are amphipathic due to the presence of the hydrocarbon chain and the hydrophilic hydroxyl groups. Except for filipin, the other polyenes have an aminosugar moiety (mycosamine) attached to the macrolide ring through a glycosidic bond and a free carboxyl group also attached to the ring, which confers upon these antibiotics amphoteric character. The conjugated trans double bonds are responsible for the intense ultraviolet absorption spectra. They are also the basis for the classification of polyenes on the basis of the longest chain of conjugated double bonds.

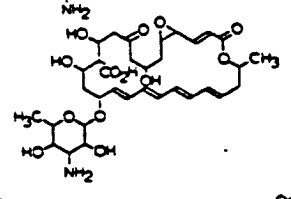
Figure 1



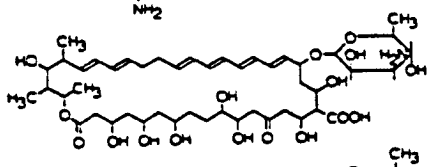
FILIPIN



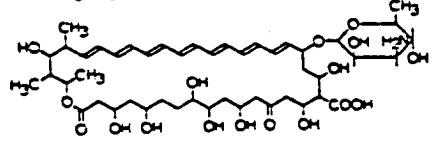
LUCENSOMYCIN (ETRUSCOMYCIN)



PIMARICIN



NYSTATIN



AMPHOTERICIN B

Accordingly, filipin is a pentaene, amphotericin B a heptaene, while nystatin, pimaricin and lucensomycin are all tetraenes. Filipin can be resolved into four components, I,II,III, and IV which constitute 4,25,53, and 18%, respectively of the original material. The various components in this filipin complex appear to differ in the number of hydroxyl groups. The structure in Figure 1 of filipin corresponds very closely to the molecular formulas of filipin III and IV. In most published investigations employing this polyene, filipin complex is used and is commonly referred to as filipin. Unless otherwise noted, filipin complex will be referred to as filipin in this dissertation. Further information regarding the polyenes can be found in the excellent review of Norman et al. (4).

Liposomes were used in these investigations because of the similarities in permeability properties of liposomes and biological membranes. As Bangham has originally shown, liposomes are concentric multilamellar structures separated by aqueous compartments that are formed spontaneously when purified phospholipids are dispersed in water (10). Because it is possible to trap solutes and proteins in the aqueous compartments, liposomes have been used as membrane models to study the effect of membrane-active agents (e.g. ionophores, steroids and anesthetics) by measuring the release of marker ions or molecules from liposomes (116).

The studies described in this dissertation were undertaken with the goal of using filipin both as an optical probe and a membrane-perturbing agent to study membrane structure and function. More specifically, I used filipin (1) in a comparison of the ability of several polyene antibiotics to disrupt ergosterol-containing liposomes, (2) to determine if differences do exist in the affinity of cholesterol for several important classes of phospholipids, (3) to monitor the phospholipid-cholesterol interaction by investigating changes in the circular dichroism spectra of filipin when bound to cholesterol-containing vesicles prepared from different phospholipids, (4) to investigate the state of cholesterol and the involvement of lipids in the physical and biological properties of lipid-enveloped viruses (76,77). Each of these related investigations is covered in a separate chapter that contains the necessary background information, a statement of the goal and a discussion of the experimental results.

CHAPTER 1

PERMEABILITY EFFECTS OF FIVE POLYENE ANTIBIOTICS ON ERGOSTEROL-CONTAINING LIPOSOMES

Introduction

The polyene antibiotics are a class of macrolide antibiotics some of whose members are clinically used as antifungal agents and also as probes of membrane structure. The binding of these drugs to susceptible organisms is known to perturb the membrane permeability barrier in such a manner that important cellular metabolites are released from the cells. This is then followed by either cell death or the inhibition of certain cellular activities (4). In addition, the membrane-perturbing properties of amphotericin B and nystatin have been used to potentiate the effects of other non-polyene drugs, presumably by making the cellular membranes more permeable to them (13,14).

As mentioned before (p. 2), the binding sites for these antibiotics are known to be sterols located within the lipid bilayer of both natural and model membranes. Some binding to phospholipids also occurs. Direct evidence for binding has been obtained by fluorescence and fluorescence polarization techniques, as well as by ultraviolet spectroscopy (15,16,-17,18,27,72). The binding of polyene drugs to sterols results in the formation of polyene-sterol complexes within the membrane, and in the

case of several polyene drugs, it is believed that these complexes come together to form aqueous transmembrane "pores" which can accommodate ions and small metabolites (8,19). Filipin, on the other hand, is thought to form complexes that fragment the membrane through some rearrangement of membrane components (8).

Because of the differences in the permeability-altering properties of the various polyenes, several models of polyene-sterol complexes have been proposed by de Kruijff and Demel (8), Finkelstein and Holz (19) and Andreoli (122). The experimental justification for these models comes from the selective release of various markers from liposomes and cells (20). The size of the released marker, glucose for example, is used to gauge the maximum size of the membrane "pore".

The results of the present study suggest that the models of polyene-sterol complexes (8,19) may have to be modified somewhat in order to account for the movement of glucose out of ergosterol-containing liposomes after treatment with amphotericin B, nystatin, pimaricin and lucensomycin (etruscomycin). The relative degree of permeability alteration caused by these drugs on liposomes containing ergosterol was examined in this study. Ergosterol was used because it is the chief sterol found in the membranes of fungi, and because ergosterol has received less attention than cholesterol in the area of polyene antibiotic-sterol interactions, as pointed out be Nozawa et al. (33), and more recently by Sekiya et al. (117).

Changes in liposomal permeability were followed by measuring the

release of water-soluble markers from pre-loaded liposomes in response to alterations induced by the polyene antibiotics. The extent of marker release was measured by enzymatic assays performed on the markers, once they were released from the liposomes. Lysolecithin was incorporated into the liposomes for these studies in order to facilitate the passage of protein marker molecules through the membrane.* When incorporated into membranes at low concentrations, lysolecithin appears to expand the hydrocarbon chains (118), a property that has been used by Racker to facilitate the incorporation of proteins into liposomal membranes (21). Lysolecithin does not disrupt membranes or impair the ability of lecithin liposomes to act as osmotic barriers to glucose (22) or ions (21) when present at 10% of total lipids.

*In this way the liposome was adjusted for a more sensitive response to the less potent polyene antibiotics.

Methods and Materials

Glucose assay. The extent of glucose release was determined using a modification of the procedure developed by Kinsky (23,24) for measuring the release of glucose from multilamellar liposomes that had been treated with a variety of membrane-active agents, including filipin. In our modification, liposomes were first incubated with filipin for 20 minutes and then the extent of glucose release was measured. A dried lipid film containing egg yolk lecithin (EYL), ergosterol, dicetyl phosphate (DCP) (to provide a charge on the bilayer) and lysolecithin was dispersed in 300 mM glucose to prepare liposomes in which glucose was trapped. This was followed by an overnight dialysis of the liposomes against isotonic salt solution (75mM NaCl-75mMKCl) in order to remove untrapped glucose. The sterol concentration of the lipid dispersion was then measured using the modified Liebermann-Burchard method of Huang et al. (62). This was done to determine the amount of antibiotic to add in order to obtain a certain sterol/antibiotic ratio.

The liposome preparations were assayed for glucose release in two cuvettes containing approximately 0.80 ml of buffer (100 mM Tris, pH 7.5, 3.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, 64 mM NaCl) together with the appropriate quantities of $NADP^+$, ATP, and hexokinase. A five microliter aliquot of pre-loaded liposome suspension (total lipid concentration 34.7 mM) was added to each cuvette followed by the necessary amount of antibiotic dissolved in dimethyl formamide (DMF) to give a predetermined ratio of

sterol to drug. Amphotericin B was added as a solution in DMSO. Final volume was adjusted to 1.0 ml. All solutions contained 1 per cent (v/v) organic solvent (DMF or DMSO). The concentrations of ATP, $NADP^+$ and hexokinase in each cuvette were 2.0 mM, 1.0 mM and 42 µg/ml, respectively. After a 20 min incubation in the dark at room temperature to allow phosphorylation of the glucose, 25 µg of glucose-6-phosphate dehydrogenase (3.3 mg/ml) were added to the sample cuvette to initiate the reaction in which the glucose released as a result of drug treatment is oxidized. Under these assay conditions, essentially complete oxidation of all free glucose occurred within 1 to 2 minutes at room temperature. The increase in absorbance at 340 nm for the oxidation of glucose-6-phosphate by $NADP^+$ was followed for 5 min in order to insure that all released glucose had been measured. Liposomes that had been treated with DMF or DMSO (1 per cent by volume in buffer) were used as controls to determine the extent of spontaneous release of glucose in the presence of only the carrier solvent. These liposomes were assayed for glucose release under the same conditions as the antibiotic-treated liposomes.

Horseradish peroxidase assay. Liposomes were prepared by dispersing a dried lipid film containing egg yolk lecithin, DCP, ergosterol and lysolecithin in 0.05M phosphate buffer, pH 7.4, containing horse radish peroxidase (190 µg/ml). Unincorporated enzyme was removed by gel exclusion chromatography on Sephadex G-200. Following this, the ergosterol

concentration of the liposomes was measured in order to calculate the quantity of antibiotic to add to get a certain ratio of sterol to antibiotic. Release of trapped enzyme from the liposomes was measured by adding 5 µl of the liposomes to both reference and measuring cuvettes, each containing 0.975 ml of isotonic substrate media (o-dianisidine, 0.08 per cent, in 0.05M phosphate buffer, pH 5.8). Sufficient volumes of polyene antibiotics dissolved in DMF (except amphotericin B which was prepared in DMSO) were added to both measuring and reference cuvettes and then the lipid dispersions were incubated in the dark for 20 min at room temperature. Following this, 10 µl of a 0.30% (v/v) solution of hydrogen peroxide were added to the measuring cuvette in order to initiate the reaction in which o-dianisidine is oxidized to a colored product. Final volumes in the measuring and reference cuvettes were 1.01 ml and 1.00 ml, respectively. The increase in absorbance at 460 nm, due to the release of the peroxidase enzyme and the subsequent enzymatic reaction, was followed for approximately 40 min, the time necessary to reach a constant absorbance reading for these very low levels of the peroxidase. Liposomes treated with either DMF or DMSO (1% by volume) were used as controls in correcting for spontaneous leakage of the peroxidase.

Calculation of per cent marker release. The spectrophotometric methods used in these assays produce absorbancy values that provide a measure of the quantity of trapped marker molecules released upon incubation of

liposomes with the antibiotics. The drugs were added to both the measuring and reference cuvettes in order to cancel out the absorbancy of the polyene drugs themselves (the drugs have absorption maxima near 340 nm) and for any changes in light scattering of the liposomes caused by the antibiotics. The absorbancy values were then compared on a percentage basis to the total quantity of marker present in the liposomes, an amount determined by treating the liposomes with Triton X-100 in order to disrupt the membranes completely and thus release all trapped marker molecules. In determining the total amount of marker present in the preparations, Triton X-100 was added in sufficient quantity to give a final concentration of 1.0% (v/v) and 0.05% (v/v) for glucose and peroxidase-containing liposomes, respectively. The suspensions were then assayed for their respective marker molecules using procedures already described. Much higher concentration of Triton X-100 do not alter the degree of activity of the enzymes employed here. For the release of both glucose and peroxidase, the following equation was used to calculate the per cent marker released:

$$\text{per cent released} = \frac{A_{drug} - A_{control}}{A_{total}} \times 100$$

where

A_{drug} = absorbance obtained from the assay for marker released from drug-treated liposomes

$A_{control}$ = absorbance obtained from assay of marker released from solvent treated control liposomes

A_{total} = absorbance due to marker present in solution after liposomes were destroyed by Triton X-100

Materials. Glucose-6-phosphate dehydrogenase (yeast, type V), $NADP^+$ ATP and horseradish peroxidase were obtained from Sigma Chemical Co., St. Louis, Mo., while yeast hexokinase (A grade) was purchased from Calbiochem, San Diego, Calif. Triton X-100 and Tris buffer were obtained from Sigma Chemical Co. All other chemicals were of analytical reagent grade.

Lipids and sterols. Egg yolk lecithin was prepared from egg yolks by the method of Singleton et al. (25). The purity of lecithin was determined by thin-layer chromatography on silica gel plates using a solvent system consisting of (by volume) chloroform: methanol: water (65:25:4). Chromatographically pure egg yolk lysolecithin was purchased from Supelco, Inc., Bellefonte, Penn. Cholesterol and ergosterol were purchased from Sigma Chemical Co., and recrystallized twice from acetone.

Antibiotics. Filipin complex (MW 670) was kindly provided by Dr. G.B. Whitfield of the Upjohn Co., Kalamazoo, Mich., as lot no. 8393-DEG-11-8 and was purified according to the procedure described by Whitfield et al. (26). Amphotericin B (MW 923, lot no. 22-380-39568-001) and nystatin (MW 926,

lot no. 48975-026 were obtained from E. R. Squibb and Sons, Princeton, N.J. Pimaricin (MW 665.8, lot no. 3345C-49A) was obtained from American Cyanamid and lucensomycin was kindly supplied by Prof. Robert Bittman.

Results

Release of horseradish peroxidase. The results in Table 1 clearly demonstrate that only filipin of the five drugs tested was able to cause release of horseradish peroxidase (MW 40,000) from ergosterol-containing liposomes. The results are identical when the lysolecithin concentration is either 5 or 10 mole per cent. Although lysolecithin was incorporated into the membranes in order to make them more capable of enhancing or magnifying any leakage, however small, of marker molecules resulting from the membrane-disrupting effects of the drugs, the increased fragility of liposomes containing the higher lysolecithin content apparently did nothing to enhance the membrane-perturbing effects of the other antibiotics.

Using the same polyene antibiotics tested in this study, de Kruijff et al. (20) observed a similar pattern in the ability of these drugs to cause leakage of cytoplasmic glucose-6-phosphate dehydrogenase (MW 104,000) from cholesterol-containing Acholeplasma laidlawii cells. They observed a 50% and an 18% release of enzyme activity following filipin treatment of cholesterol and ergosterol-containing cells, respectively. The difference in the extent of release from cells containing either sterol is probably related to the greater affinity of filipin for membrane-bound cholesterol than ergosterol, as demonstrated in vesicles (27,109) and in cells (28,29). Affinity was not, however, measured directly in the case of cells.

Glucose release from liposomes. All of the polyenes tested here were capable of causing a release of glucose from ergosterol-containing liposomes in which lysolecithin was present (Table 2). Especially noteworthy however, is the significant release of glucose from liposomes treated with pimaricin, lucensomycin and nystatin. In a separate experiment, lucensomycin was found to increase glucose permeability from cholesterol-containing lysolecithin-free liposomes (Table 3). Filipin was able to cause a 45% increase in glucose release from these same cholesterol-containing liposomes (Table 3), a much larger increase than in ergosterol-containing liposomes. This is not unexpected in light of the preferential affinity of filipin for cholesterol over ergosterol (27,109). It should be noted, however, that tables 2 and 3 cannot be compared directly because of the presence of lysolecithin in the ergosterol-containing liposomes.

These results differ significantly from those reported by de Kruijff et al. (20) who observed that lucensomycin (etruscomycin), nystatin and pimaricin did not cause any release of glucose from cholesterol-containing liposomes at 25°C, as determined by directly measuring the amount of glucose released, or indirectly, by measuring turbidity changes (swelling or shrinking). Molecules or ions smaller than glucose (e.g., ribose, urea, K^+) were, however, released from the liposomes after treatment with nystatin or lucensomycin. Pimaricin was not able to increase the release

of these markers, even when the antibiotic and cholesterol concentrations were increased in order to maximize the amount of binding of pimaricin to membranes, as an examination of their data and indicates (20). The authors concluded that pimaricin was incapable of producing any permeability changes in cholesterol-containing cells and liposomes. Recently, however, Kolter-Brajtburg et al. (32) showed, in an extensive series of experiments concerning the relative potencies of polyene drugs, that pimaricin at high concentrations was indeed capable of causing a release of hemoglobin from red blood cells. They also observed that lucensomycin (etruscomycin) was more potent than even filipin in causing release of hemoglobin from red blood cells and K^+ from yeast cells.

In respect to the amphotericin B-glucose release experiments (Table 2), our results also differ from some of the observations reported by de Kruijff et al. (20). Again using cholesterol-containing liposomes and cells, they measured the turbidity changes that occurred when amphotericin B-treated membranes were exposed to different isotonic media, they found that the drug treatment did not increase permeability for glucose, but did do so for smaller markers e.g., K^+, urea and ribose. Yet, when glucose released from liposomes was measured directly (as in our experiments), they observed leakages which, however, were at slower rates than for filipin-treated membranes. Nevertheless, according to their own data

the final per cent release was almost identical to that for filipin. It thus appears that amphotericin B does increase glucose release in those experiments in which they measured release directly. Possibly a more pronounced leakage of glucose would have been observed if they had used approximately equimolar ratios of cholesterol to amphotericin B, as they did for their other drugs and as we did in our study.

Discussion

We have shown in this report that only filipin of all the polyene antibiotics tested here can induce leakage of both large (horseradish peroxidase, 30A Stokes radius) and small (glucose 8A diameter) marker molecules from ergosterol-containing liposomes. None of the other antibiotics (nystatin, amphoterican B, lucensomycin and pimaricin) that we examined were able to induce leakage of horseradish peroxidase from ergosterol-containing liposomes, a pattern followed in the results of de Kruijff et al. (20) in the leakage of glucose-6-phosphate dehydrogenase from cholesterol-containing A. laidlawii cells. These present results extend the body of evidence indicating that filipin is able to cause leakage of large molecules from sterol-containing liposomes (20, 30). This is believed to occur through the initial formation of sterol-filipin complexes throughout the membrane core, followed by the aggregation of these complexes to form large 150-250A aggregates. The formation of these large aggregates then results in fragmentation of the membrane. These aggregates are thought to be responsible for the appearance of the 150-250A or 250-300A diameter pits, and doughnut-shaped craters observed by freeze-fracture electron microscopy in cholesterol-containing liposomes, erythrocytes (17) and in ergosterol-containing cells (31).

The present study demonstrates that ergosterol-containing liposomes are sensitive to the membrane-perturbing effects of amphotericin B, pimaricin, nystatin and lucensomycin, as shown by the release of glucose from polyene-treated liposomes (lucensomycin was also able to induce leakage of glucose from cholesterol-containing liposomes as well). These observations contrast with the results of de Kruijff et al. (20), who found that, except for amphotericin B, none of these antibiotics were capable of causing the release of glucose from cholesterol-containing liposomes. Slow leakage was, however, observed for amphotericin B-membranes. The disparity between these two sets of observations may be rationalized by focusing on the sterol incorporated into the membrane. There is evidence to indicate that amphotericin B, nystatin, lucensomycin and pimaricin have more potent effects on membranes containing ergosterol than on those containing cholesterol. Kitojima et al. (31) have shown that these four antibiotics produce greater morphological alterations in ergosterol-rich fungal cell membranes than in cholesterol-containing whole red blood cells. Amphotericin B methyl ester has also been shown to cause a greater release of K^+ ions from ergosterol-containing mycoplasma cells and lecithin vesicles than from cholesterol-containing membranes (28). Using changes in the peak ratios in the ultraviolet absorption spectrum of amphotericin, de Kruijff et al. (20) showed that amphotericin B interacted preferentially with ergosterol as compared to cholesterol or other sterols. Marty and Finkelstein have shown

that nystatin and amphotericin B produced a greater effect on cation permeability in ergosterol-containing black lipid membranes than in those containing cholesterol (121). In addition, Karst and Jund have shown that nystatin causes much slower release of amino-isobutyric acid in fungal cells containing cholesterol compared to those containing ergosterol in their membranes (123). The apparent preferential affinities of these polyenes for ergosterol may result in a greater membrane modification that permits glucose to leak through the membrane.

Because the results of the glucose release experiments do not agree with the permeability data of de Kruijff et al. (20), which are used as the experimental basis for the current model of polyene-sterol complexes, it may be necessary to modify or restrict the model to cholesterol only. The model of de Kruijff and Demel (8) is based on an analysis of space filling models, as well as on permeability changes induced by the interaction of polyenes with membrane-cholesterol. The amphotericin B-cholesterol complex is discussed next because it is the basis for the structures formed by the other polyenes, except for filipin. The binding of amphotericin B to cholesterol is thought to result in the formation of a transmembrane pore. Two half-pores are first formed, each composed of 8 cholesterol and 8 amphotericin B molecules, one on each side of the membrane; the pairing of these two half-pores creates the full transmembrane pore. The circular arrangement of sterol and drug molecules in each half-pore is maintained by hydrophobic attractions between the rigid bond system of the polyene and the rigid steroid backbone

of cholesterol that align the two components parallel to the fatty acid chains of the membrane phospholipids, with the polar portions of each molecule orientated towards the polar face of the bilayer, and conversely, the non-polar portions are embedded in the hydrophobic center of the membrane. The pore's interior is lined with hydroxyl groups belonging to amphotericin B. The size of the pore is 8 Å in diameter which is also the size of the glucose molecule. Both the space filling models and permeability studies of Finkelstein and Holz (19) also place the size of the pore at approximately 8 Å. The models proposed by de Kruijff and Demel for nystatin and lucensomycin are very similar but result in slightly smaller pores. For pimaricin, however, they proposed that half-pores are formed but because the length of the half-pore is less than the length of half the thickness of the bilayer, no conducting pores are formed, which explains the inability of the drug to induce any permeability changes in the experiments of de Kruijff et al. (20). The formation of 8 Å diameter pores for the other polyenes explains the leakage of ions and molecules smaller than glucose (8).

The close similarity in structure between ergosterol and cholesterol and their roles in maintaining the membrane's integrity suggests that similar mechanisms for the interaction of sterols with polyene antibiotics exist, as described by de Kruijff and Demel in their cholesterol model. But our permeability results suggest that their pore model, with its approximately 8 A diameter channel, may have to be modified in order to

account for glucose permeability in polyene-treated ergosterol-containing liposomes, possibly by enlarging the transmembrane opening by increasing the number of drug and sterol molecules in the complex. There is other evidence to indicate that in cholesterol-containing systems such a modification is needed. For example, the ability of amphotericin B to potentiate the effects of several classes of rifamycin antibiotics (MW 700) in fungal cells and transformed mouse fibroblasts (13,14) is thought to be due to the ability of amphotericin B to increase the membrane's permeability to these non-polyene antibiotics. In addition, in the recent study by Kolter-Brajtburg et al. (32) which we mentioned earlier, it was shown that nystatin has such a "permeabilizing" effect on mammalian cells. Furthermore, this same study reported that pimaricin, as well as nystatin, amphotericin B and lucensomycin was capable of causing K^+ leakage, cell death and hemolysis in yeast and red blood cells.

Table 1: Polyene antibiotic induced leakage of horse radish peroxidase from ergosterol-containing liposomes in which lysolecithin was incorporated. Liposomes were prepared in the manner described in the Methods section. Liposomes were composed of egg yolk lecithin, ergosterol and dicetyl phosphate in the molar ratio of 2:1:0.13. Lysolecithin was incorporated to the extent of 5 and 10 mole per cent. Total lipid concentration was 16.5 mM. Stock solutions of pimaricin and lucensomycin were prepared by dissolving accurately weighed quantities of antibiotic in dimethylformamide. Stock solutions of filipin, nystatin and amphotericin B were prepared by dissolving small amount of the antibiotic in dimethylformamide (DMSO in the case of amphotericin B). The concentrations were determined spectrophotometrically using the molar extinction coefficients for filipin ($\epsilon_{340} = 5.4 \times 10^4\ M^{-1}cm^{-1}$), nystatin ($\epsilon_{307} = 3.44 \times 10^4\ M^{-1}cm^{-1}$) and amphotericin B ($\epsilon_{387} = 4.0 \times 10^4\ M^{-1}\ cm^{-1}$). Amphotericin B solutions can also be prepared by dissolving weighed amounts of the drug in DMSO and using the molecular weight (923) to determine the concentration.

Table 1

Antibiotic	Ratio of sterol to antibiotic	Per cent peroxidase released	
		5%	10% Lysolecithin
Filipin	1.0	22.0	22.1
Pimaricin	1.0	0.0	0.0
Amphotericin B	1.0	0.0	0.0
Nystatin	1.0	0.0	0.0
Lucensomycin (Etruscomycin)	1.0	0.0	0.0

Table 2: Polyene antibiotic induced leakage of glucose from ergosterol-containing liposomes. Egg yolk lecithin, ergosterol and dicetyl phosphate were present in the ratio: 2:1:0.13. Lysolecithin was incorporated in the liposomes to the extent of 10 mole per cent. The total lipid concentration of the several liposome preparations used in these experiments was 34.7 mM.

Table 2

Antibiotic	Ratio of sterol to antibiotic	Per cent glucose released
Filipin	1.0	22.6 ± 3.8
Amphotericin B	1.0	23.4 ± 1.4
Nystatin	1.0	16.8 ± 1.3
Pimaricin	1.0	12.6 ± 0.5
Lucensomycin	1.0	11.7 ± 0.0

Table 3: Polyene antibiotic induced leakage of glucose from cholesterol-containing liposomes. No lysolecithin was incorporated. Egg yolk lecithin, cholesterol and dicetyl phosphate were present in the ratio 2:1:0.13. Total lipid concentration was 31.3 mM.

Table 3

Experiment	Antibiotic	Ratio of cholesterol to antibiotic	Per cent glucose release
1	Filipin	2.0	18.1
2	Filipin	1.0	44.9
3	Filipin	0.87	70.0
4	Lucensomycin	1.0	11.7

CHAPTER 2

FILIPIN AS A SPECTRAL PROBE OF THE LIPID PHASE TRANSITION IN PHOSPHOLIPID-CHOLESTEROL VESICLES

Introduction

The structure and function of biological membranes are intimately related to each other and to many membrane mediated phenomena. In addition to the membrane's role as a barrier in maintaining the internal milieu, it is also involved in intercellular communication, the transport of cellular metabolites, nerve excitation and immune recognition (34). Yet, surprisingly, the basic structure of membranes, the lipid bilayer, is relatively simple. According to Tanford, membranes are formed by the spontaneous drive of phospholipids in aqueous environments to achieve an energetically stable state, in which their polar end are exposed to water while the non-polar portions are buried in the hydrophobic core (35). Interspersed between the phospholipids are neutral lipids (mainly sterols) and glycolipids that contribute to the membrane's integrity and biochemical properties. In this "sea" of lipids, proteins are present, either buried deep within the hydrophobic region of the bilayer, or exposed to the surface, or perhaps spanning the membrane as drawn by Singer and Nicholson in their "fluid-mosaic" model (36). The membrane-bound proteins in this model funtion as hormone receptors, enzymes, ion pumps and as the cell's sensors involved in

intercellular communication.

The biophysical properties of membranes and their lipid components have been extensively studied in recent years to gain a fuller understanding of the membrane's architecture and biological functions. To this end, highly simplified lipid systems and a few natural membranes (mycoplasma cells and certain enveloped viruses) have been studied with a variety of techniques to examine the packing, mobility of lipids, and lipid-lipid, as well as lipid-protein interactions (37,38,39). From the important advances made, certain generalizations can be made.

First, the fluidity of the membrane at a given temperature depends in large part on the nature of the phospholipid head-group and the length and degree of saturation of the fatty acyl chains. As the temperature is increased, bilayers composed of a single species of phospholipid undergo a phase transition, a change in the phospholipids' state from one of a rigid crystal (or gel), characterized by high degree of order and efficient packing, to a more fluid liquid crystalline state. This phase transition is highly cooperative, as it involves space considerations and van der Waals interactions among adjoining fatty acyl chains, and occurs over a very narrow temperature range (T_m) in which the chains melting (40,41,42). When several types of phospholipids are present, a phase change occurs over a broad temperature range which is believed to result from a phase separation, with a clustering of phospholipids into patches of rigid lipids (higher melting phospholipids) and into more

fluid areas composed of lower meling phospholipids. The presence of these two extreme states has been shown in A. laidlawii cells over the whole of the transition, thus indicating the relevance of using simplified lipid systems as models of biological membranes (43). Phase separation also occurs in model membranes containing acidic pholpholipids in the presence of calcium ions, in which the rigid clusters are composed of calcium-chelated phospholipids (39,40,41). In addition to ions, proteins and changes in pH have also been observed to induce phase separation. The biological relevance of membrane fluidity is shown by the influence of membrane fluidity on the activity of membrane-bound enzymes (38,39).

The second important generalization to emerge is that incorporation of cholesterol into the membrane can modulate the fluidity of the bilayer by virtue of the sterol's ability to interact with phospholipids in such a way that the portion of their fatty acid chains closest to the polar head group end of the molecule is immobilized, while leaving the remaining portion of the chains relatively flexible and unable to undergo crystallization (42,44). When present in sufficient concentration, cholesterol abolished the highly cooperative gel-to-liquid crystalline phase transition of the phospholipids. Thus, cholesterol has the dual effect of restricting the motion of the hydrocarbon chain and preventing the formation of crystalline gel areas (42). In a sense, cholesterol imparts an intermediate state of fluidity to the lipid bilayer (42,44).

These cholesterol-induced effects are revealed in the permeability properties of lecithin-containing liposomes both above and below the T_m (44). In the case of other sterols, the consensus of opinion is that for a strong interaction, the sterol must have a planar steroid nucleus, a hydrocarbon chain at C-17 and a 3-β-hydroxyl group (41,44), all being the properties of the cholesterol molecule.

The cholesterol nucleus penetrates into the fatty acid region of the bilayer where it binds with the hydrocarbon chains via van der Waals interactions, while the sterol's hydroxyl group positions itself in the vicinity of the phospholipid's carbonyl groups, as shown by studies employing neutron and X-ray diffraction techniques (45,46). In this position, cholesterol does not affect the motions of the choline methyl groups nor does it interact with the phosphate portion of the phospholipid (44). Despite the close proximity of cholesterol's hydroxyl group to the phospholipid carbonyl groups, there appears to be no hydrogen bonding between the hydroxyl hydrogen and the carbonyl oxygens of lecithin, as judged by calorimetry and force-area curves (44). However, the possibility that hydrogen bonding does occur has been postulated on the basis of certain effects of cholesterol on the NMR spectrum of lecithin in bilayers (47) and by consideration of the packing between phospholipid and cholesterol molecules in the membrane (48). It has been recently proposed that another role cholesterol may play in the membrane is that

that of a spacer molecule, increasing the separation between phosphatidyl-choline headgroups, and in the process, decreasing the intermolecular interactions among phospholipid headgroups, as shown by the ^{31}P (^{1}H) nuclear Overhauser effect in ^{31}P NMR spectroscopy (49).

There is evidence that cholesterol is both heterogeneously distributed in the plane of the membrane as well as asymmetrically distributed across the membrane. Demel and de Kruyff in their review (44) describe how cholesterol can be either homogeneously or heterogeneously distributed in the plane of the membrane, depending on the lipid composition and the affinities of cholesterol for the various lipids. Another factor appears to be the difference in curvature and therefore differences in lipid packing between the outer and inner halves of the bilayer, which can lead to an asymmetric distribution across the bilayer.

A recent and fascinating development in the study of the cholesterol-phospholipid interaction is the indication that cholesterol may have a preferential affinity for sphingomyelin (SM) over that for other phospholipids. Results coming from van Deenen's laboratory (50,51,53) reveal that in mixtures of SM and phosphatidylcholines (PC), or SM and phosphatidylethanolamines (PE), that exhibit phase separation, cholesterol binds preferentially to SM, whether SM is the lower or the higher melting phospholipid. These conclusions were based on an analysis by differential scanning calorimetry of cholesterol's ability to selectively abolish the

phase transition of SM in a mixture of lipids. They also observed that cholesterol has a greater affinity for PC than PE (44). In experiments dealing with the exchange of cholesterol between liposomes, Nakagawa et al. (52) observed that cholesterol was preferentially transferred to liposomes containing SM, followed by liposomes containing various PCs (in order of decreasing melting points). The investigators attributed this order to the preferential affinity of cholesterol for SM. They could not explain the differences in the order of cholesterol transfer to the various phosphatidylcholines in their experiments (52) and the affinity order published by van Deenen's group (50,51,53), but perhaps the miscibility of the various phospholipids in the mixtures may have been a factor, as shown by the calorimetric results of Calhoun and Shipley (54). They observed no preferential affinity of cholesterol for either N-palmitoyl SM or dimyristoylphosphatidylcholine when the two were completely miscible in each other, that is, when there was no phase separation of phospholipids.

If it can be shown with certainty that cholesterol does indeed have a preferential affinity for sphingomyelin, then this special affinity could be used to explain in part some membrane-related aspects of aging and atherosclerosis. Cholesterol and SM are present at nearly constant ratios and at high concentrations in a variety of cell membranes (50), a situation that led Patton to propose a correlative relationship between

cholesterol and sphingomyelin in membranes (55), a circumstance he ascribes to the preferential affinity of cholesterol for SM. In a review article, Jackson and Gotto point to the increase in the SM content of cell membranes during the aging of animals and in the formation of atherosclerotic plaques, in which the cholesterol concentration is also high. They hypothesize that the increase in SM content of smooth muscle cell membranes is accompanied by an accumulation of cholesterol in order to maintain a state of proper fluidity in the membrane (56). This may be a general phenomenon in other cells.

Among the limited number of biophysical data available about SM in membranes, the most striking is that SM derived from animal membranes undergoes a phase transition close to the physiological temperature (37°C), while most other phospholipids are in their liquid-crystalline state. An examination of the structures of sphingomyelin and lecithin (Fig. 1) reveals a close overall similarity, such as the polar and non-polar regions of the molecules, and the choline headgroups common to both phospholipids. But the presence of an amide bond, a hydroxyl group and a trans double bond in SM is thought to enable SM to undergo inter- and intramolecular hydrogen bonding in SM bilayers (57). Another difference between the sphingomyelins and phosphatidylcholines of natural membranes is that the acyl chains in SM are longer and more saturated (58), which may explain the higher melting point of SM.

What we have tried to do in this investigation was to use the polyene antibiotic filipin as a spectrophotometric probe to examine the cholesterol-sphingomyelin interaction, hoping thereby to gain some insight into this interaction that is not available by other techniques. Filipin interacts strongly with membrane-bound cholesterol and in the process undergoes changes in its fluorescence and ultraviolet spectra which have been used to determine the binding affinities of filipin for sterol-containing vesicles (27), the accessibility of sterols to the bilayer surface (27,59), the phase transition temperature of phospholipids (60), and the interaction of filipin with natural membranes (61a). Using filipin, we measured the phase transition of phospholipid vesicles prepared from sphingomyelin and synthetic lecithins, with and without cholesterol. The phase transition results obtained with sphingomyelin-cholesterol vesicles were used to construct a phase diagram as had been done for the more thoroughly studied lecithin-cholesterol system. The dissociation constants for the interaction of filipin with cholesterol in vesicles prepared from sphingomyelin and dipalmitoyllecithin were measured in order to determine if preferential binding of cholesterol to filipin in sphingomyelin bilayers occurs relative to the corresponding complex in dipalmitoyllecithin bilayers.

Materials and Methods

Lipids. Sphingomyelin, from bovine brain, was obtained from Lipid Products (Surrey, England). L-α-Dimyristoyllecithin and L-α-dipalmitoyllecithin were purchased from Calbiochem, San Diego, Calif. All phospholipids were chromatographically pure. Dicetyl phosphate was purchased from Sigma Chemical Co. Cholesterol, from Sigma, was recrystallized twice from ethanol. Stock solutions of lipids were prepared in chloroform and used within several days.

Preparation of vesicles. Vesicles were prepared as described in Chapter 1, except that distilled water at 60°C was used to disperse the dried lipid film. The lipid dispersions were sonicated under nitrogen for fifteen second bursts using a 20KHz Branson Sonifier, Model S-110 at power level 4. In some cases the vesicles were passed through a Sephadex G-50 column to remove unincorporated cholesterol. The cholesterol concentrations were then determined by the modified Liebermann-Burchard method of Huang et al. (62). A stock solution of filipin (10 μM) was prepared in DMF. Aliquots were removed from this stock solution and then added to distilled water. Then in turn this solution of filipin was mixed with an equal volume of vesicles and allowed to stand in the dark for one hour before fluorescence intensity measurements were taken. For the binding studies, filipin was dissolved in 0.5 ml of 2% aqueous water and incubated with the appropriate volumes of vesicles and enough water to give a final volume of 1.0 ml.

Fluorescence and ultraviolet absorption measurements. All fluorescence measurements were performed on a Hitachi-Perkin Elmer spectrofluorometer Model MPF-2A. Samples were excited at 325 nm and the emission was measured at 480 nm, at sensitivity 6 and with the slits set at either 14 or 16 nm. Polarization values were corrected for the light scattering of vesicles. The temperature of the cuvette holder was controlled by a thermostat and a thermister inserted into the cuvette itself was used to measure the temperature of the vesicles. The temperature was varied at the rate of about lo degrees per hour. Absorption measurements were carried out on a Cary Model 14 spectrophotometer at room temperature for the equilibrium binding studies.

Determination of the apparent dissociation constant (K_D) for the binding of filipin to vesicles. The K_D values for the binding of filipin to to sphingomyelin and dipalmitoyllecithin were calculated according to the spectrophotometric method devised by Bittman et al. (27). Filipin was incubated with vesicles in the dark for 20 min. The concentration of filipin was held constant while the phospholipid and cholesterol concentrations in the vesicles were varied. The concentration of filipin bound to vesicles, C_B, was obtained from the absorbance at 358 nm according to the following equation:

$$\frac{C_T}{C_B} = \frac{\epsilon_B - \epsilon_F}{\epsilon - \epsilon_F} = \frac{A_B - A_F}{A - A_F} = \frac{\Delta A_{max}}{\Delta A} \qquad \text{(equation 1)}$$

where C_T = total concentration of filipin

C_B = concentration of filipin bound to vesicles

ϵ_F = extinction coefficient of free filipin

ϵ_B = extinction coefficient of bound filipin

ϵ = extinction coefficient of filipin measured at 358 nm

The value for ΔA_{max} is gotten from the inverse of the intercept in a plot of 1/A vs. 1/total lipid. From the equilibrium expression for K_D:

Lipid (in vesicles) + filipin $\rightleftharpoons$ Lipid-filipin complex

$$K_D = \frac{C_F\,[\text{Lipid}]}{C_B} \qquad \text{(equation 2)}$$

where C_F is the concentration of free filipin and [Lipid] is the concentration of unbound lipid. In the binding of filipin to phospholipid vesicles, Lipid refers to the concentration of unbound phospholipid. Since $C_T = C_F + C_B$, equation 1 can be converted to

$$\frac{C_T}{C_B} = \frac{K_D}{[\text{Lipid}]} + 1 \qquad \text{(equation 3)}$$

The K_D can also be expressed in terms of unbound cholesterol for the binding of filipin to cholesterol in vesicles or in the membranes of enveloped viruses. In this case, [Lipid] in equation 3 is replaced by unbound cholesterol. From a plot of C_T/C_B vs. 1/Lipid , the value for K_D can be determined from the slope of the line which intercepts the Y-axis at approximately 1.0, as expected from equation 3.

Results

Figure 2 shows plots of the fluorescence polarization of filipin as a function of temperature when bound to vesicles prepared from dipalmitoyllecithin (DPL) and containing various amounts of cholesterol. The fluorescence polarization of filipin is approximately 0.02 in aqueous solution and increases dramatically in the presence of lipids (18). The sigmoidal shape of the curve for pure DPL is indicative of a cooperative phase transition occurring in the fatty acyl region of the bilayer. Similar curves have been observed using fluorescent and spin label probes (39). The abrupt decrease in the curve at the lower temperature (T_s) corresponds to the onset of the gel to liquid-crystalline transition. The second break at the higher temperature (T_f) corresponds to the completion of the transition. By drawing lines through the three distinct regions of the curve, values for T_s and T_f can be determined from the intersections of the lines and then used later in constructing a phase diagram (40,63). The phase transition temperature (T_m) for the melting of the lipid chains is determined experimentally from the midpoint of the line connecting T_s and T_f.

The value for the T_m of pure DPL obtained from Fig. 2 is 40.0 °C and is in good agreement with the values obtained by differential scanning calorimetry, fluorescence and electron spin resonance spectroscopy (64). In addition to DPL, I used filipin to examine the phase

transition of dimyristoyllecithin (DML) and found agreement with published results (66). Similar T_m values were obtained by W.C. Chen (60) using filipin.

Incorporation of cholesterol in DPL vesicles caused a broadening of the transition, a small decrease in T_m and at concentrations of cholesterol over 0.25 mole fraction to the disappearance of the transition, as shown in Figure 2. These results are consistent with the known effects of cholesterol on the phase transition of phospholipids revealed by a variety of physical techniques (41,42,44). Vesicles prepared from DML were similarly affected by cholesterol (results not shown).

The phase transition of sphingomyelin (SM) vesicles containing various amount of cholesterol are presented in Fig. 3. The value of 37.5°C obtained for the T_m of beef brain SM agrees quite well with the value of approximately 39°C obtained earlier by W.C. Chen (60), who used filipin, and with measurements using the fluorescent probe diphenylhexatriene and differential scanning (65,66). The small differences in the T_m for SM from other sources is probably due to the heterogeneity of the fatty acyl chains (58).

Incorporation of increasing amounts of cholesterol into SM vesicles results in broadening of the transition (as determined by drawing lines through the distinct regions of the curve in order to determine the

values of T_s and T_f, which can be used as a measure of transition broadening), a small decrease in T_m and finally, abolition of the transition associated with pure SM at mole fractions of cholesterol above 0.30, as shown in Fig. 3. The effect of cholesterol on SM vesicles follows the pattern observed in DPL vesicles (that is, a broadening of the transition as measured by the temperature difference between T_s and T_f and abolition of the transition at high cholesterol concentrations) and is consistent with calorimetric measurements on N-palmitoylsphingomyelin performed by Estep et al. (67). Their calorimetric scans on N-palmitoylsphingomyelin (T_m=40°C) show that at low concentrations of cholesterol the sharp endothermic peak associated with pure sphingomyelin is broadened and shifted to lower temperatures and that at moderate concentrations of the sterol, two peaks, one sharp and the other broad and smaller in intensity, appear, thus indicating a phase separation of lipids into two phases. The sharp peak they observed corresponds to a sphingomyelin-enriched phase which shifts to lower temperatures and finally disappears at approximately 25 mole percent cholesterol, while the broad peak, belonging to a cholesterol-rich phase, eventually broadens out to a point that it too is no longer discernible at 30 mole percent cholesterol. The interpretation of these results in terms of a phase separation was made possible in part because of the great similarity to the behavior of DPL-cholesterol mixtures for which there is a wealth of evidence implying that phase separation does occur (67).

A partial phase diagram for the SM-cholesterol system is illustrated in Fig. 4 in the manner described by Lee (39,41), in which the temperatures for the onset (T_s) and completion (T_f) of the gel to liquid-crystalline transition are plotted as a function of the mole fraction cholesterol. From this experimentally determined phase diagram for a binary mixture, it can be seen that the phase transition associated with pure SM is broadened and that the solidus curve (composed of T_s values) shifts downward. Normally, for a binary lipid mixture such as DPL-cholesterol, both the fluidus (composed of T_f values) and the solidus curves should move upwards until 0.5 mole fraction of cholesterol. Beyond this point, the two curves may be extrapolated to the higher melting point of cholesterol (68). Our inability to obtain reproducible and useful values for T_s and T_f at mole fractions of cholesterol higher than 0.30 where no phase transition could be detected and at high temperatures limited our ability to construct a complete phase diagram for SM and DPL, as has been done by the use of spin label or fluorescent probes (41,68). Perhaps the long exposure of filipin to ultraviolet light, particularly at high temperatures, contributed to our lack of success by introducing instability to the fluorescence emission of filipin.

The ability of filipin to undergo changes in its ultraviolet absorption upon binding to cholesterol-containing vesicles has been used previously (27) to determine the equilibrium dissociation constants (K_D) for the binding of filipin to these vesicles. We determined the K_D values for binding of filipin to cholesterol-containing SM and DPL vesicles in order to ascertain whether or not cholesterol preferentially interacts with SM as has been suggested by various investigators. The values for K_D are presented in Table I. They indicate little difference in the affinity of filipin for either type of vesicle, which suggests that there is no preferential affinity of cholesterol for SM over DPL, at least under our experimental conditions. If cholesterol did exhibit a stronger affinity for SM, then it would have been less able to withdraw itself from interacting with SM in order to bind to filipin. Filipin is capable of withdrawing cholesterol from interaction with lecithin, even to the extent of causing a reappearance of the pure lecithin phase transition that is abolished by cholesterol in lecithin-cholesterol mixtures, as Norman et al. (69) have pointed out. In addition, the data of Blau and Bittman (59) suggest equal accessibility of cholesterol in DPL and beef brain SM vesicles for interaction with filipin. This is based on the observation that there were no differences in the initial rates of filipin binding to cholesterol in these vesicles.

Discussion

The results of our filipin binding studies and measurements of the phase transitions of vesicles containing cholesterol do not indicate any preferential affinity for the sterol in either sphingomyelin or dipalmitoyllecithin vesicles. We observed limitations in our use of filipin as a fluorescent probe of the phase transitions for vesicles containing greater than 0.30 mole fraction cholesterol, and for high temperatures where the fluorescence polarization values for filipin became increasingly diminished in intensity and subject to so much fluctuations as to be of little use. The phase transitions of SM and DPL vesicles could no longer be observed at a cholesterol mole fraction of 0.30 or higher. This is in agreement with calorimetric studies that show the disappearance of the gel to liquid-crystalline transition between approximately 0.25 and 0.33 mole fraction cholesterol (67,69). Nevertheless, our results obtained through the use of filipin show that the phase transitions of both SM and DPL are broadened and that the onset of fluidization of the lipid chains decreased with increasing cholesterol concentration. These results are in good agreement with those recently obtained by Jacobs and Oldfied using deuterium NMR on dimyristoyllecithin-cholesterol mixtures (70). In this same study, they were unable to construct a phase diagram because of the very broad transitions they observed at high cholesterol concentrations.

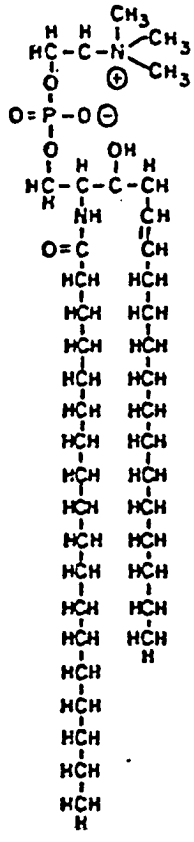
We observed several minor differences in the overall shape of the phase transition curves of DPL and SM (Fig. 2 and 3) in the presence of cholesterol, including that the transitions for DPL appeared to lose their characteristic sigmoidal shape at a lower concentration of cholesterol (the transition was no longer observed at .30 mole fraction cholesterol), whereas the intensity and the shape of the transition associated with pure SM remained relatively constant until about 0.30 mole fraction cholesterol; at higher mole fractions of cholesterol, the phase transition disappeared. This small difference may result from the ability of adjoining SM molecules to undergo hydrogen bonding with each other. As mentioned earlier, SM-SM interactions are thought to be made possible by the presence of an amide bond and a hydroxyl group in the headgroup region of SM (see Fig. 1). These interactions are believed to be responsible for the complex behavior observed by calorimetry for SM but not for lecithins (57) and for the lower permeability of SM liposomes to water and glucose as compared to egg lecithin liposomes (71).

The results of our binding study indicated no significant difference in the binding of filipin to cholesterol-containing DPL and SM vesicles, which can be interpreted to mean that cholesterol has no preferential affinity for interaction with either phospholipid. The ability of filipin

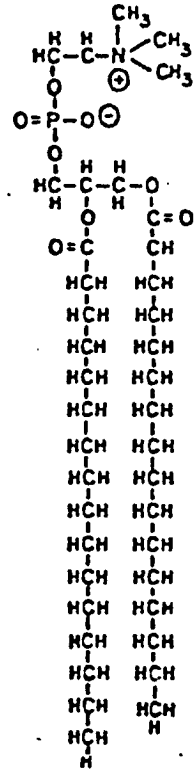
to remove cholesterol from interacting with lecithins, as shown by Norman et al. (69), could be the basis for future experiments to compare the relative strength of the cholesterol-phospholipid interaction. In these experiments, the ability of filipin to restore the phase transition of SM and DPL, first abolished by cholesterol would be compared by differential scanning calorimetry; the more strongly cholesterol interacted with certain phospholipid, the less able filipin would be to withdraw cholesterol from the interaction and thereby restore the phase transition.

Figure 1: Structures of sphingomyelin (I), L-α-dipalmitoyllecithin (II) and cholesterol (III).

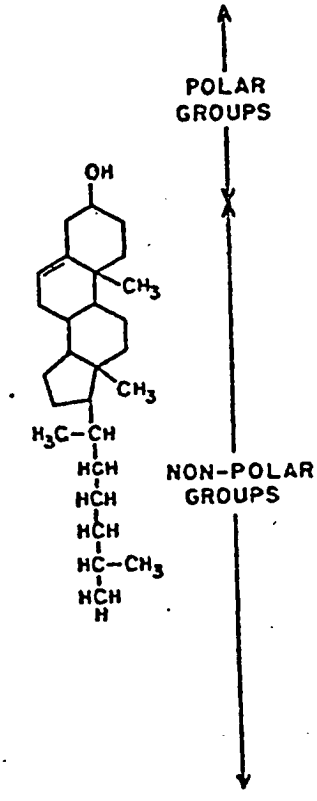
Figure 1



(I)



(II)



(III)

Figure 2: The effect of temperature on the fluorescence polarization of filipin in the presence of dipalmitoyllecithin vesicles containing: (★★★) 0 mole fraction cholesterol: (●●●) 0.10 mole fraction cholesterol; (○○○) 0.25 mole fraction cholesterol. All vesicles contained 4 mole per cent dicetylphosphate. The total lipid concentration in the vesicles was varied from 1.0 mM to 1.33 mM at a constant lecithin concentration of 0.96 mM. Filipin concentration was 2μM. All the fluorescence intensities were taken in water containing 0.05% dimethylformamide by volume.

Figure 2

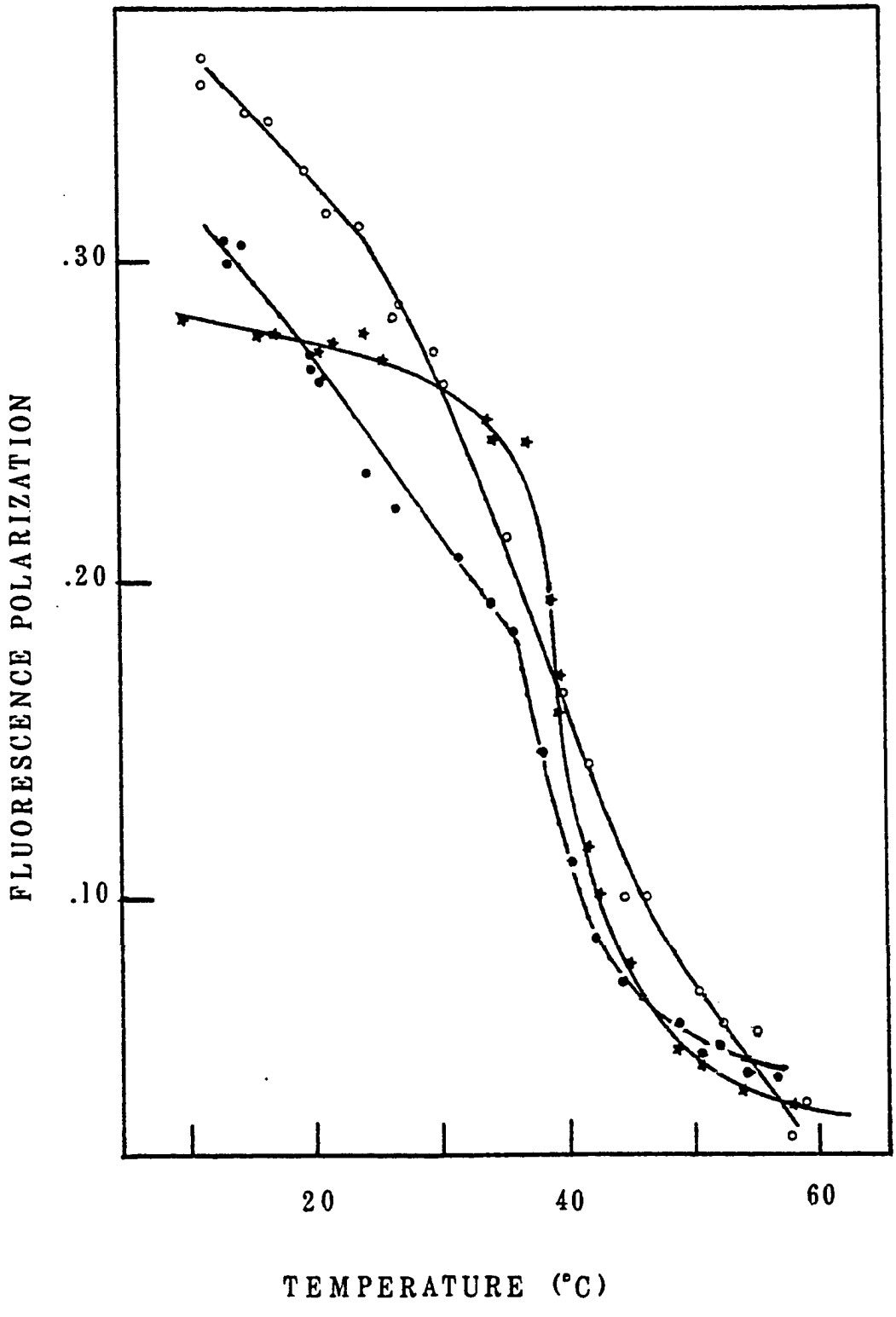


Figure 3: The effect of temperature on the fluorescence polarization of filipin in the presence of sphingomyelin vesicles containing: (★★★★) 0 mole fraction cholesterol; (●●●) 0.20 mole fraction cholesterol; (○○○) 0.30 mole fraction cholesterol. All vesicles contained 4 mole per cent dicetylphosphate. The total lipid concentration in the vesicles was varied from 1.0 mM to 1.4 mM at a constant filipin concentration of 2.0μM. All fluorescence intensities were measured in water containing 0.05 % dimethylformamide by volume.

Figure 3

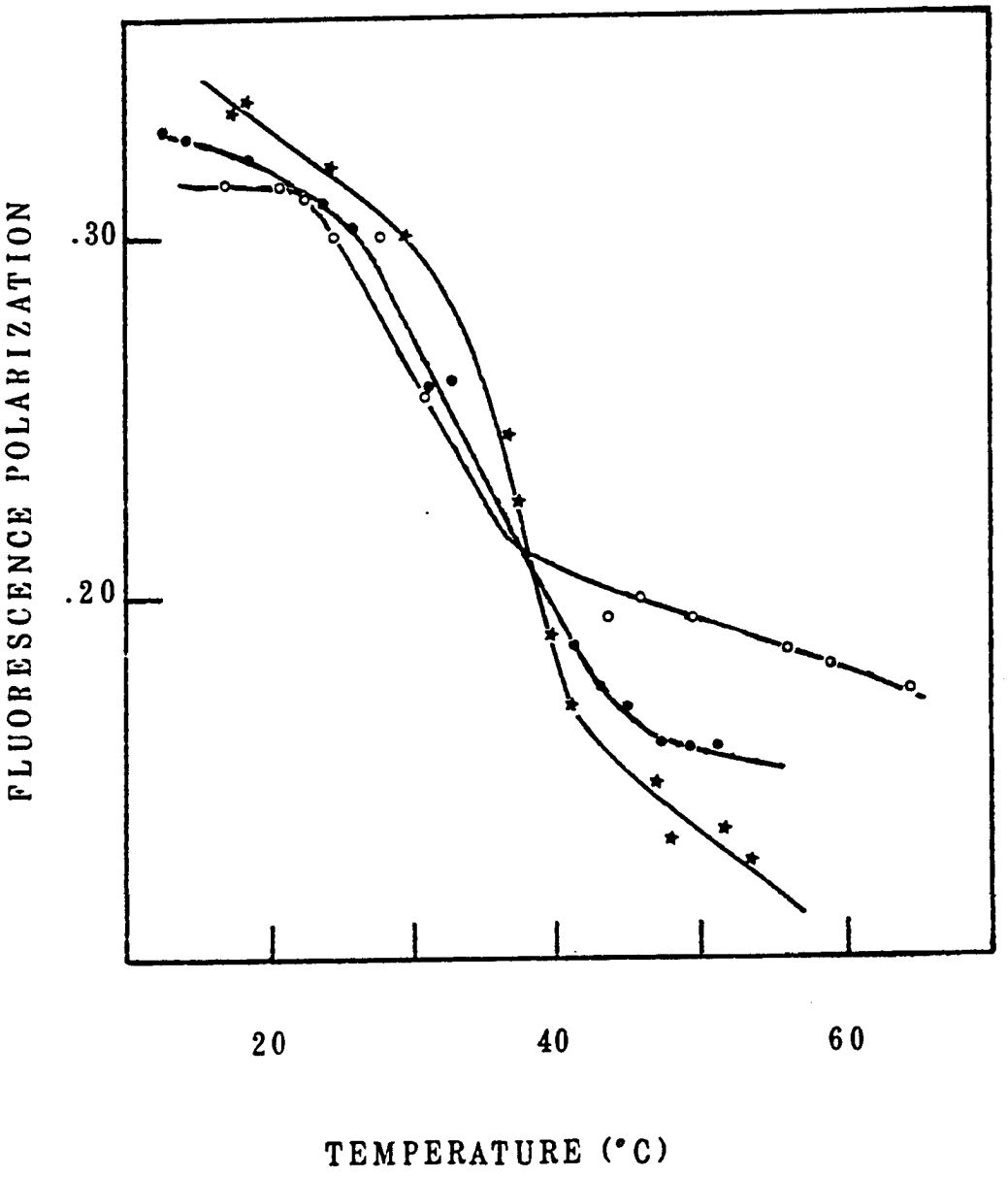


Figure 4: Phase diagram for the sphingomyelin-cholesterol system. Temperatures for the onset (T_s) and the completion (T_f) of the gel to liquid-crystalline transition were obtained from the plots of fluorescence polarization against temperature as described in the Results section. Solidus and fluidus curves were obtained from the values of T_s and T_f, respectively. All vesicles contained 4 mole per cent dicetyl phosphate. Filipin concentration was 2μM in all lipid preparations.

Figure 4

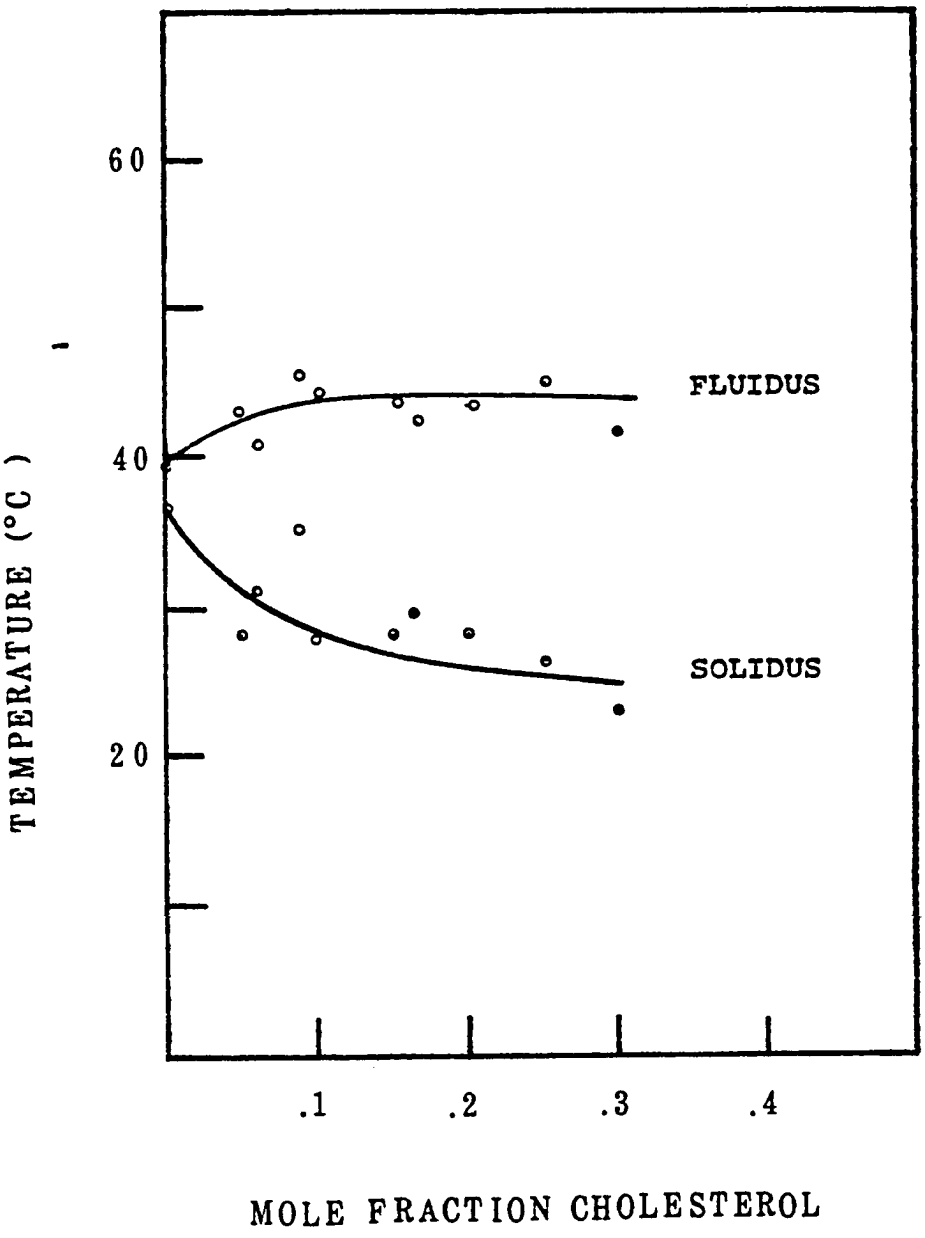


Table 1: Apparent dissociation constants (K_D) for the binding of filipin to vesicles prepared from sphingomyelin and dipalmitoyllecithin. Values for K_D are reported in terms of binding to the total lipid present in the vesicles and were calculated in the manner described in the Methods and Materials section. Cholesterol was present in a 4:1 molar ratio of phospholipid to cholesterol. All vesicles contained 4 mole per cent of dicetylphosphate. Total lipid concentrations were varied from 180μ M to 18μM at a constant filipin concentration of 20μM. All absorbance measurements were taken at the λ_{max} of the absorption peak at 358 nm of filipin in water containing 1.0% dimethylformamide by volume. Values for K_D were calculated from plots of C_T / C_B vs. 1/ [Lipid] (data not shown) as described in the Methods and Materials section. The value of K_D for SM-cholesterol vesicles is the average of two trials and one trial for DPL-cholesterol vesicles. The error limits were calculated on the basis of errors in the slope and the intercept in plots of C_T / C_B vs. 1/ [Lipid] .

Table 1

Type of Vesicle	Apparent Dissociation Constant (μM)
Sphingomyelin-cholesterol	3.38 ± 0.83
Dipalmitoyllecithin-cholesterol	2.14 ± 0.09

CHAPTER 3

INTERACTION OF FILIPIN WITH PHOSPHOLIPID-CHOLESTEROL VESICLES: A CIRCULAR DICHROISM STUDY

Introduction

The presence of five conjugated double bonds give filipin its characteristic fluorescence, ultraviolet absorption and circular dichroism spectra. These optical properties have greatly facilitated the study of filipin-lipid bilayer interactions and have made filipin a useful spectroscopic probe of events occurring in the bilayers of phospholipid vesicles. In addition to the changes in the fluorescence and the absorption spectra that occur upon filipin binding to vesicles (4,18,72), changes in the circular dichroism spectrum of filipin, as well as amphotericin B, have been used to study the interaction of polyene antibiotics with phospholipid vesicles, as Bittman et al. (72) and others(73,74) have done. Dr. B.K. Reddy, working with Prof. Bittman, observed that in the presence of sterol-containing vesicles, the negative CD bands of filipin were greatly enhanced and that the enhancement was maximized when the lecithin-sterol interactions were strong (61b). Similar enhancements were later observed by Bittman et al. (72), indicating that filipin could be used as indirect probe of the phospholipid-cholesterol interaction.

In this present study, the circular dichroic properties of filipin were examined when the polyene was bound to vesicles prepared from brain sphingomyelin, dimyristoyllecithin and dipalmitoyllecithin in order to determine if differences do exist in the interaction of cholesterol with the various phospholipids. This is a companion study of the work described in chapter 2 of this thesis, where the changes in the fluorescence polarization and the ultraviolet absorption spectrum of filipin were used to monitor the cholesterol-phospholipid interaction.

Materials and Methods

Lipids. The sources of egg yolk lecithin, cholesterol, dicetyl phosphate, L-α-dimyristoyllecithin and L-α-dipalmitoyllecithin are as described earlier. Some bovine sphingomyelin was obtained from Supelco, Inc., Bellefonte, Penn. In some experiments, synthetic phospholipids were obtained from Nutritional Biochemicals, Cleveland, Ohio. The source of epicholesterol was Sigma Chemical Company.

Filipin. Stock solutions of filipin were prepared in dimethylformamide (DMF) as described earlier. The concentration of filipin (approximately 60μM) was obtained by diluting an aliquot of the stock solution in a volume of DMF or distilled water and using the absorbance at 338 nm together with the extinction coefficient of filipin in water (5.0×10^4 M^{-1} cm^{-1}) or in DMF (5.4×10^4 M^{-1} cm^{-1}). Stock solutions were kept for no more than two days at 4°C under nitrogen.

Preparation of vesicles. Required volumes of phospholipids, sterols and dicetyl phosphate, all dissolved in chloroform, were added to glass vials and the solvents removed first under a stream of nitrogen and then under a vacuum to produce a thin film of lipids. Two glass beads and distilled water, at a temperature above the phase transition of the particular phospholipid used, were then added. The thin film was

then immediately dispersed on a Vortex mixer for 60 sec. The beads were then removed and the dispersions were placed in a water bath whose temperature was kept above the phase transition temperature of the phospholipid. The liposomal dispersions were then sonicated for one minute under nitrogen in four 15 sec burst. (Longer times were used for more turbid dispersions.) The temperature of the vesicles during the sonication was monitored and kept just above the melting point of the phospholipids. The vesicles contained 4 mole per cent dicetyl phosphate, with a total lipid concentration of 2 mM. Vesicles were diluted with an equal volume of aqueous filipin solution containing 2 per cent DMF and then incubated in the dark at room temperature for two hours. Final concentration of DMF was 1% (v/v) and that of filipin was approximately 30 μM.

Circular dichroism measurements. Circular dichroic spectra were obtained using a Cary 60 spectropolarimeter equipped with a model 6001 circular dichroism attachment. A jacketed, 1 cm pathlength cell was used together with an external water-circulating constant temperature bath in the temperature studies. A digital thermister probe was placed close to the cell in order to measure the temperature of the vesicles. Correction for light scattering was made by running blanks. The dispersions were checked frequently for the presence of air bubbles or for any settling of vesicles.

Results

The CD spectrum of filipin in aqueous solution is shown in Figure 1. Filipin by itself exhibits weak negative ellipticities which correspond closely to the ultraviolet absorption peaks of the antibiotic. Negative maxima are observed at 323, 337, and 358 nm. In the presence of vesicles prepared from SM or EYL, there is a small red shift of about four nm, except for the peak at 358 nm, and little, if any change in the intensities of the negative CD peaks (Figure 1).

Under similar conditions, but in the presence of vesicles prepared from L-α-DPL or L-α-DML, the CD bands of filipin underwent a dramatic sign reversal and an enhancement in intensity resulting in a positive CD spectrum (Figure 2). Dr. B.K. Reddy has also observed a sign reversal in the CD spectrum of filipin bound to L-α-DPL vesicles (61b). The CD bands for L-α-DPL were consistently more positive than for L-α-DML vesicles. Mixed phospholipid vesicles prepared from EYL and L-α-DPL also gave rise to an intense positive CD spectrum (Figure not shown). These are the results of multiple experiments with different batches of lecithins and filipin. Freshly recrystallized filipin was used in the most recent experiments. There was some instability in the region of the trough between the positive CD peaks at 328 nm and 343 nm and in the intensity of the peak at 322 nm. This could have been due to minor fluctuations in the instrument such as a drifting base line or to the aging of filipin. If aging was a problem, it was not evident from the UV spectrum of the antibiotic which was recorded

prior to each day's series of experiments.

Incorporation of cholesterol, in equimolar amounts, into vesicles prepared from SM or EYL resulted in an enhancement of filipin's negative CD bands and a small shift to longer wavelengths as illustrated in Figure 3. When cholesterol was present in equimolar amounts in vesicles prepared from L-α-DPL or L-α-DML, there was a dramatic sign reversal of the positive dichroic bands to produce a negative CD spectrum that was nearly the mirror image of the one observed when filipin was incubated with sterol-free vesicles (Figure 4). In the presence of pure cholesterol micelles, the weakly negative CD spectrum of filipin was enhanced with a small red shift in the peaks (Figure not shown). Cholesterol was able to produce a negative CD spectrum when incorporated into L-α-DPL vesicles even when present in a molar ratio as low as 1 to 10 (sterol to phospholipid). The intensity, however, was diminished under these conditions. Incorporation of epicholesterol into L-α-DPL vesicles also resulted in a sign reversal of the CD bands, but to a lesser extent compared to cholesterol. When present in SM vesicles, epicholesterol was also less effective than cholesterol in enhancing the negative CD bands. These effects are most probably related to the weaker interaction of filipin with vesicles containing epicholesterol as has been revealed by fluorescence polarization measurements (27) and CD measurements in phospholipid-epicholesterol vesicles (72).

The effect of temperature, hence the physical state of the membrane bilayer, on the circular dichroism spectrum of filipin was next examined. Vesicles prepared from L-α-DML and L-α-DPL were used. Increasing the temperature of interaction from 10.2°C to 53.4°C results in a gradual decrease in the intensity of the positive dichroic bands and finally gives rise to weakly negative CD spectra resembling that of free filipin at 53°C (Figures 5 and 6). At temperatures just over 42°C, the sign of the CD bands were often still positive for L-α- DPL, though greatly reduced in intensity (figure not shown). These temperature extremes were chosen because they are below and above the phase transition temperatures (T_m) of the two phospholipids, namely 23 C° and 42°C for L-α- DML and L-α- DPL respectively. When the vesicles were returned to 10.2°C, the spectra were nearly identical to the original, thus indicating that the interactions are reversible. These spectral changes also suggest that the sign of the CD bands is independent of the physical state of the hydrocarbon chains belonging to the phospholipids. The CD bands were positive at the lowest temperature where the fatty acid chains are in a rigid crystalline state and remain positive, but less positive for L-α-DPL due to the dissociation of filipin, as the temperature is increased and the chains "melt", first for L-α-DPL at 42°C.

The consistent observation of weakly negative CD bands at higher temperatures (Figures 5 and 6) with L-α-DML and L-α-DPL vesicles is probably due to a lack of significant binding of filipin with the

phospholipids. By 53°C the spectra with both phospholipids resemble that of free filipin. That this is the case can be shown from an examination of the UV absorption spectra of filipin (Table 1). The ratio of filipin's absorbance peak at 323 nm to that at 358 nm can be used to gauge the degree of interaction between the phospholipids and filipin (60). Normally, the ratio is about 1.25 for bound filipin and 0.915 for free filipin. The data in Table 1 show that as the temperature is increased, the ratio in each phospholipid vesicle preparation approaches that of free filipin. This indicates that the effect of high temperature is to diminish the extent of antibiotic-lipid binding to a point where the CD spectrum begins to resemble that of free filipin in aqueous solution. Another possibility, not explored, is that the changes are due to changes in the state of aggregation of filipin as the temperature is altered.

We next studied the effect of temperature on cholesterol-containing vesicles. When cholesterol is incorporated into the phospholipid vesicles at a one to one molar ratio, filipin's negative CD bands are shifted to longer wavelengths and increasing temperatures have only a moderate effect on the intensity of the CD bands. In Figures 7 and 8, it can be seen that for cholesterol-containing EYL and L-α-DPL vesicles, an increase in temperature from 29°C to 53°C reduces the intensity of the peaks but does not entirely remove the enhancement due to the presence of the vesicles. This temperature stability most likely is a consequence of the stronger interaction of filipin with vesicles containing cholesterol than to those without (27).

Discussion

The results show that when the circular dichroism spectrum of the polyene antibiotic filipin is used as a probe to monitor the association of filipin with various phospholipids, significant differences appear as revealed in the dissimilarity of the antibiotic's dichroic spectrum. These differences may arise from the ability of filipin to exist in various conformations, depending on the lipid microenvironment in which the drug finds itself.

The weak negative CD bands of filipin observed in aqueous solution (Figure 1) probably are the result of a certain conformation that filipin attains. In this state, the electric and magnetic moments of the optically active centers, namely the pentaene system, partially cancel each other and thus give rise to an attenuated circular dichroism spectrum. It is also possible that aggregation of the filipin molecules may have something to do with the weak ellipticities. Filipin, as well as other polyene antibiotics, is believed to exist in aqueous solution as micellar or aggregated forms (72). However, recent evidence suggests that the weak dichroic bands of aqueous filipin solutions are due to a conformational effect and not to an aggregation of the drug molecules. LeMaitre and Moulki concluded that while two concentration-dependent circular dichroic bands between 283 and 305 nm arose from the aggregation of filipin, the effects of cholesterol, organic solvents and red

blood cell membranes on the remaining regions of the spectrum were best explained by alterations in the conformation (73). In an experiment involving the dilution of filipin by perhydrofilipin in filipin micelles, the stability of the CD spectrum indicated that the stacking of polyene molecules in an aggregate was not directly responsible for the observed CD phenomena, but that a certain conformation would be (72).

The observation that the CD spectra of filipin in the presence of EYL and SM vesicles or alone in aqueous solution are all weakly negative suggests that the conformational state of filipin is similar in all these situations. On the other hand, the strong positive dichroic bands observed with L-α-DML and L-α-DML vesicles would necessarily have to arise from a quite different conformation of filipin, one in which the magnetic and electric moments interact strongly with each other to produce a positive CD spectrum.

Incorporation of cholesterol into the lipid bilayers prepared from L-α-DML and L-α-DPL resulted in a dramatic sign reversal in the CD spectrum of filipin to produce a negative mirror image. With sphingomyelin and egg yolk lecithin vesicles the negative CD bands were only enhanced in intensity. Sign reversals in the CD spectra of other polyenes have been observed and shown to be dependent on the state of the bilayer and on the presence of cholesterol. Recently, Boudet et al. (74) observed that the CD spectrum of the polyene antibiotic amphotericin B in the presence of egg yolk lecithin, L-α-DPL and L-α-DML vesicles

was negative at temperatures above the phase transition temperature of the phospholipids. At temperatures below T_m, they observed a sign reversal to produce a positive mirror image in the CD spectrum of amphotericin B. The sign of the CD bands was independent of the presence of cholesterol and dependent only on the physical state of the phospholipid bilayer. This is just the opposite of what we observed with filipin. In another case, candicidin, a heptaene polyene antibiotic, also exhibited a sign reversal, going from a negative CD spectrum in the absence of cholesterol, to a positive spectrum in the presence of the sterol or red blood cells (75).

Figure 1: Circular dichroism spectra of filipin in water (—), in the presence of sphingomyelin (- - -) and egg yolk lecithin vesicles (• • •). Concentration of free filipin was 29.0μM and 31.2μM in the presence of vesicles. Both sphingomyelin and egg lecithin concentrations were 0.48 mM. Temperature was 29°C.

Figure 1

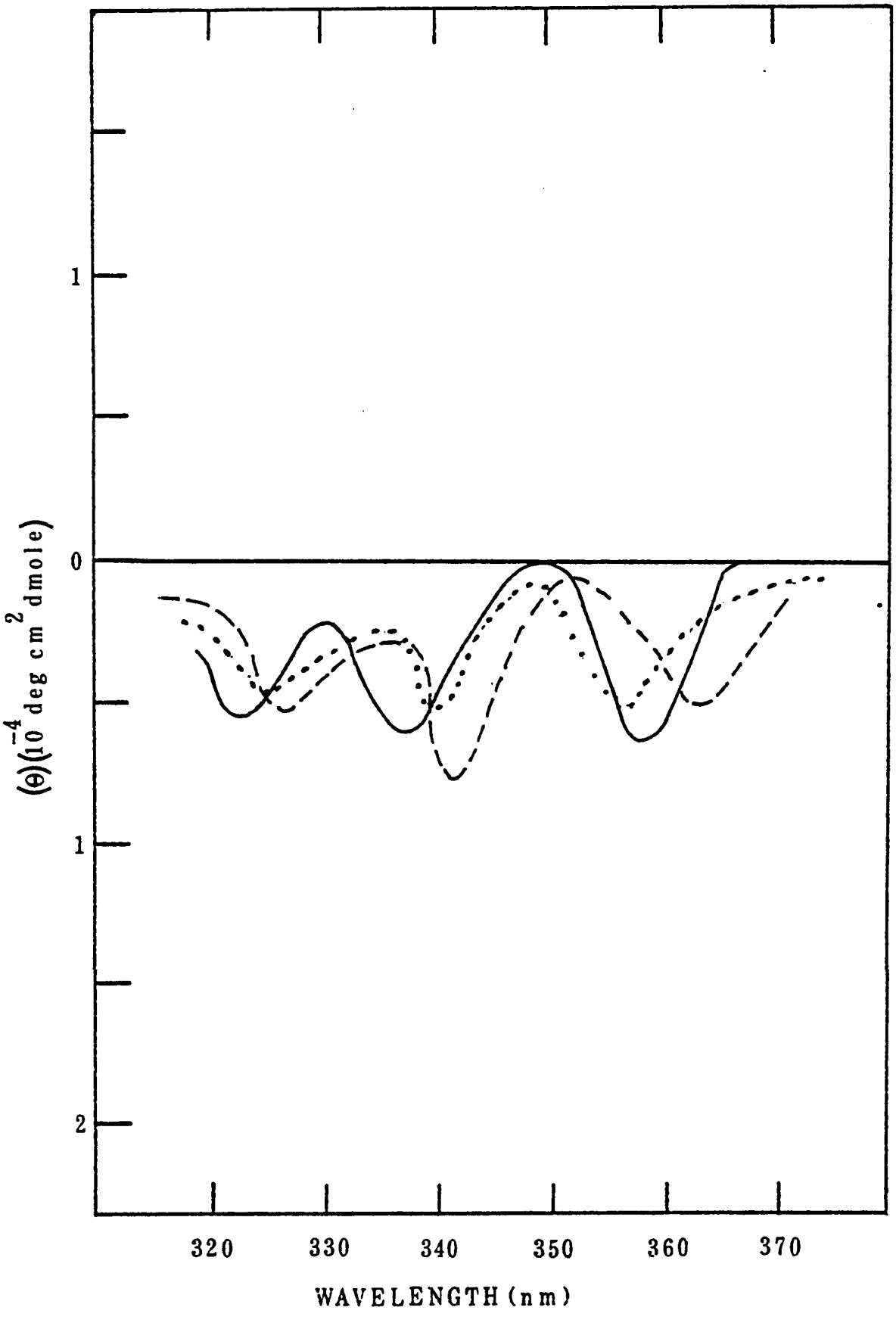


Figure 2: Circular dichroism spectra of filipin in the presence of L-α-DML (- - -) and L-α-DPL (——) vesicles. Filipin alone (• • •) was at a concentration of 29.0 μM and 26.8 μM in the presence of vesicles. The concentrations of L-α-DML and L-α-DPL were both 0.48 mM. All spectra were recorded at 29°C.

Figure 2

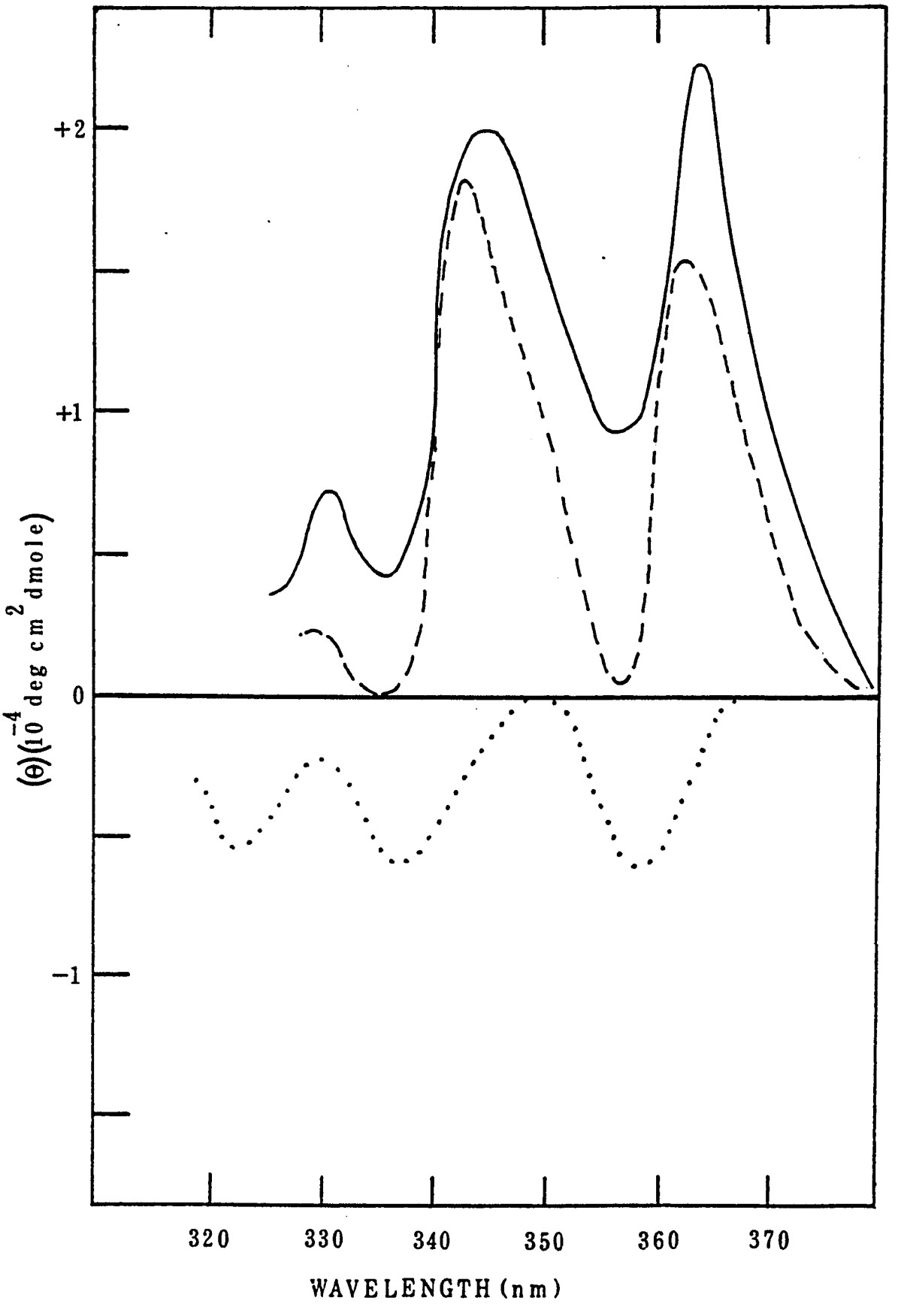


Figure 3: Circular dichroism spectra of filipin in the presence of egg yolk lecithin-cholesterol (——) and sphingomyelin-cholesterol (– – –) vesicles. Concentration of filipin was 26.8 μM. Concentrations of sphingomyelin and lecithin were both 0.48 mM. Molar ratio of phospholipid to cholesterol was 1:1. The temperature was 29°C.

Figure 3.

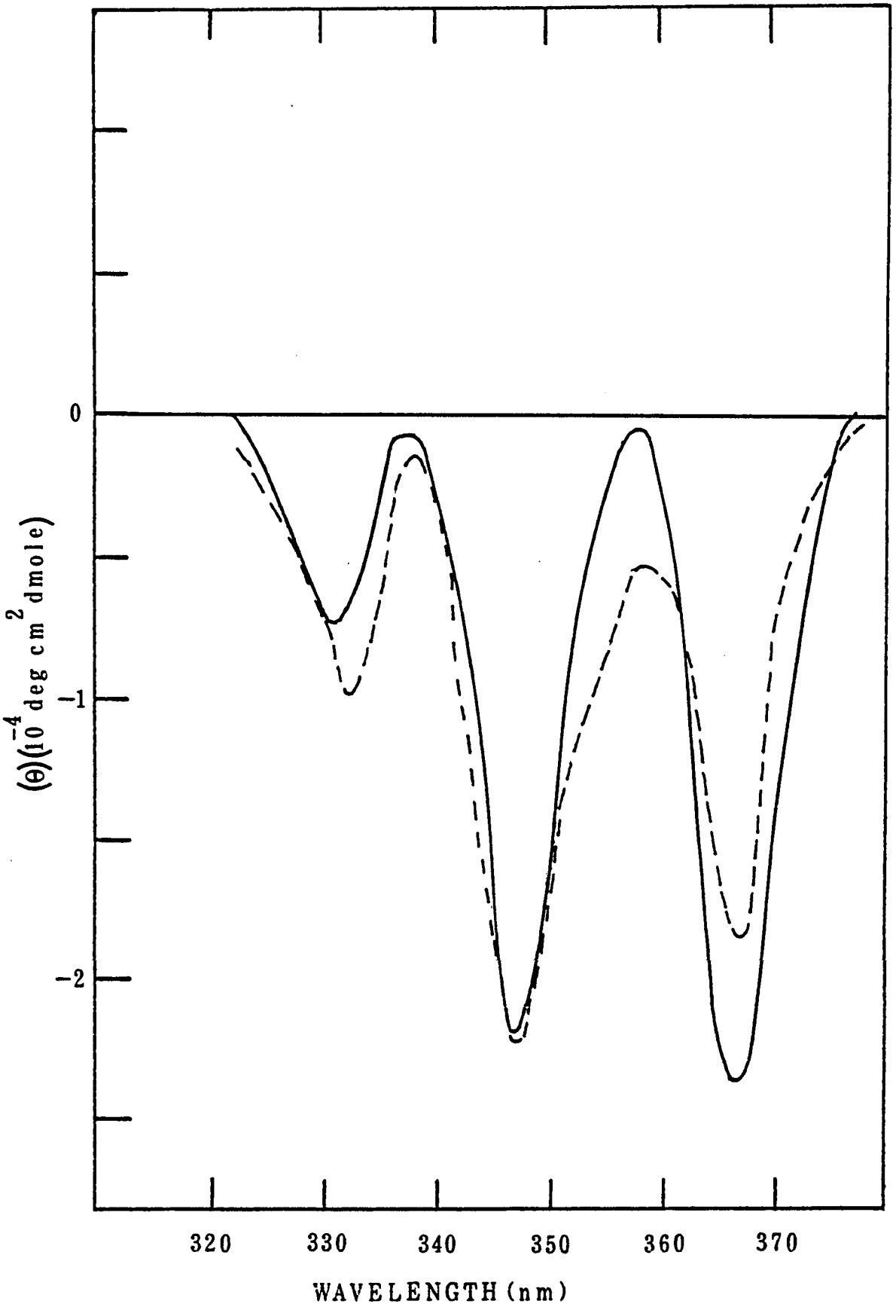


Figure 4: Circular dichroism spectra of filipin in the presence of lecithin vesicles. Positive spectra were obtained with L-α-DML (– – –) and L-α-DPL (——) vesicles. Negative CD spectra were obtained with L-α-DML-cholesterol (– – –) and L-α-DPL-cholesterol (——) vesicles. All phospholipid concentrations were 0.48 mM. Where cholesterol was present, it too was 0.48 mM. Filipin concentration was 26. μM. All experiments were run at 29°C.

Figure 4.

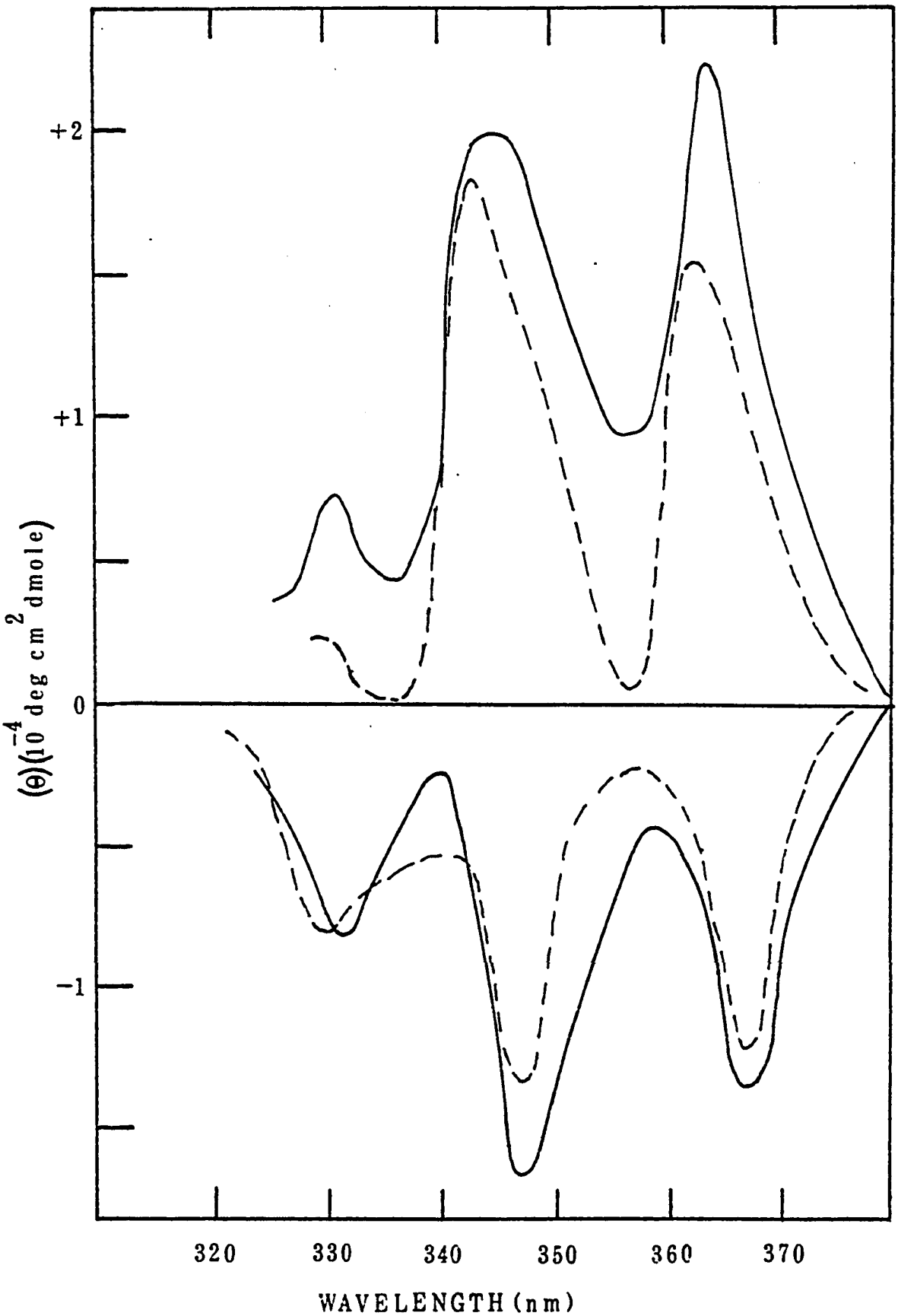


Figure 5: Effect of temperature on the circular dichroism spectra of filipin in the presence of L-α-DPL vesicles. Lecithin concentration was 0.48 mM while that of filipin was 30.9μM. The temperatures were 10°C (——), 33.4°C (• • •) and 53.4°C (– – –).

Figure 5

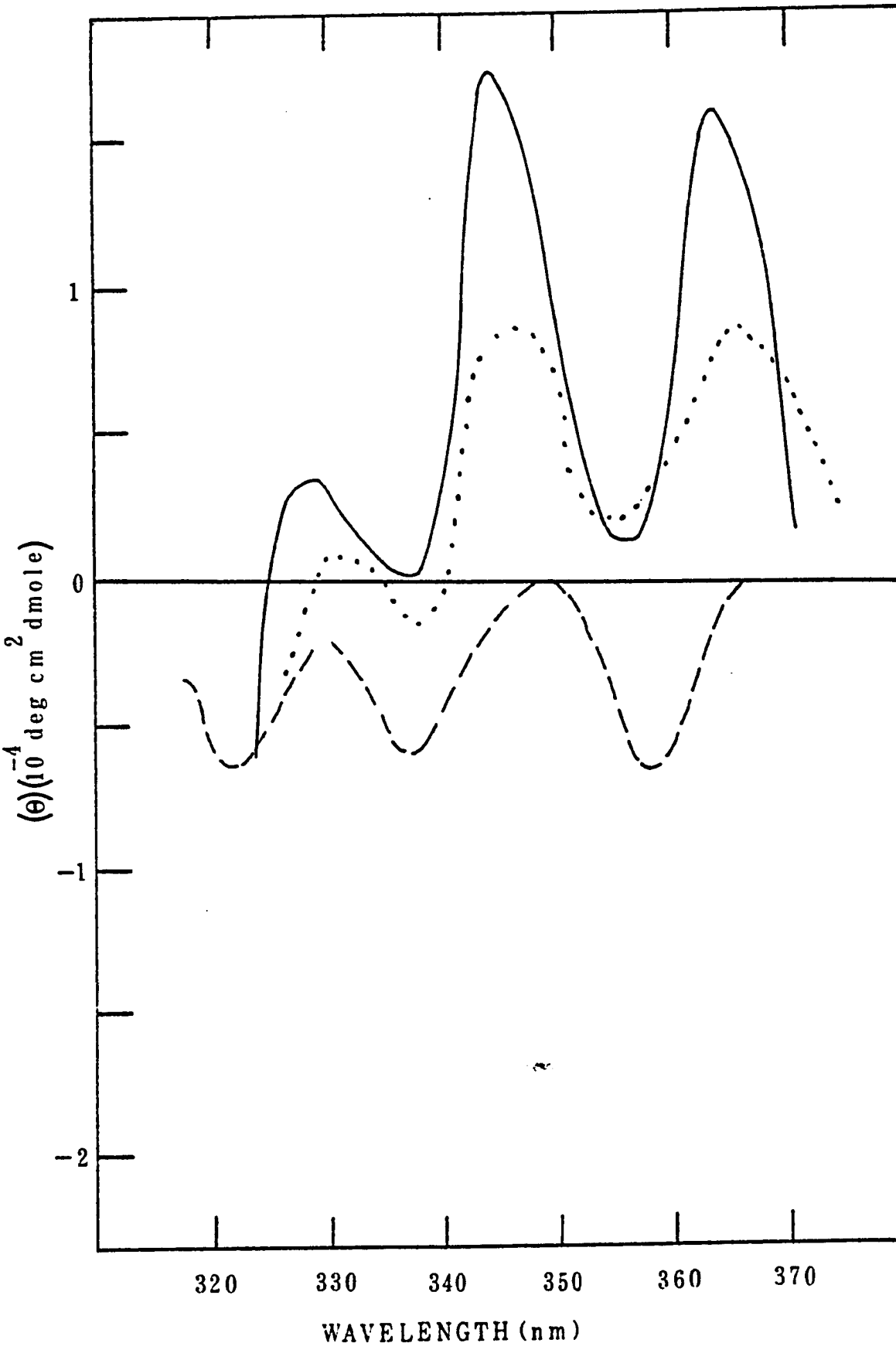


Figure 6: Circular dichroism spectra of filipin in the presence of vesicles prepared from L-α-DML at 10.2°C (——), 33.4°C (- - -) and 53.4°C (• • •). Filipin concentration was 30.9 μM while that of L-α-DML was 0.48 mM.

Figure 6

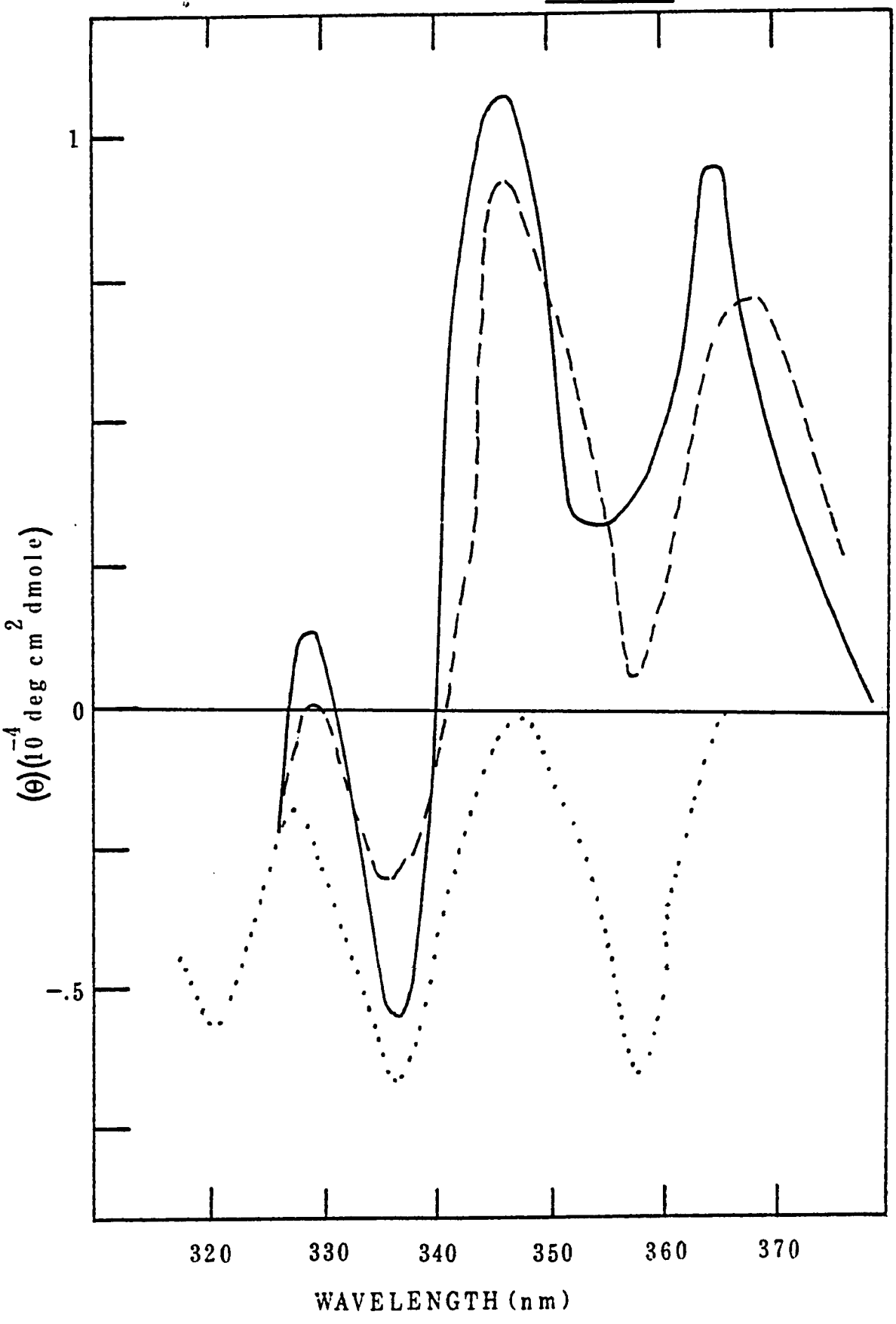


Table 1: Effect of temperature on the ultraviolet absorbance peak ratio of filipin bound to vesicles. The concentration of filipin was 30.9μM. Lecithin concentrations were 0.48 mM with 4 mole percent dicetyl phosphate. Absorbance measurements were run at each temperature on a Cary 14 spectrophotometer. Spectra were corrected for light scattering due to vesicles alone. Dimethylformamide concentration was 0.30 %.

Table 1

Type of Vesicle	Ratio of absorbance peaks at 323 nm to 358 nm at a temperature of :			
	29°C	34°C	44°C	53°C
None	0.914	0.915	0.900	0.920
L-α-DML	1.28	1.24	1.05	0.986
L-α-DPL	1.20	1.21	1.23	1.08

Figure 7: Effect of temperature on the circular dichroism spectra of filipin in the presence of egg yolk lecithin-cholesterol vesicles at a filipin concentration of 26.8μM. The concentration of both lecithin and cholesterol was 0.48 mM. The spectra were obtained at 29°C (——) and at 53°C (- - -). The spectrum of filipin alone was obtained at 30.7μM at 29°C (• • •).

Figure 7

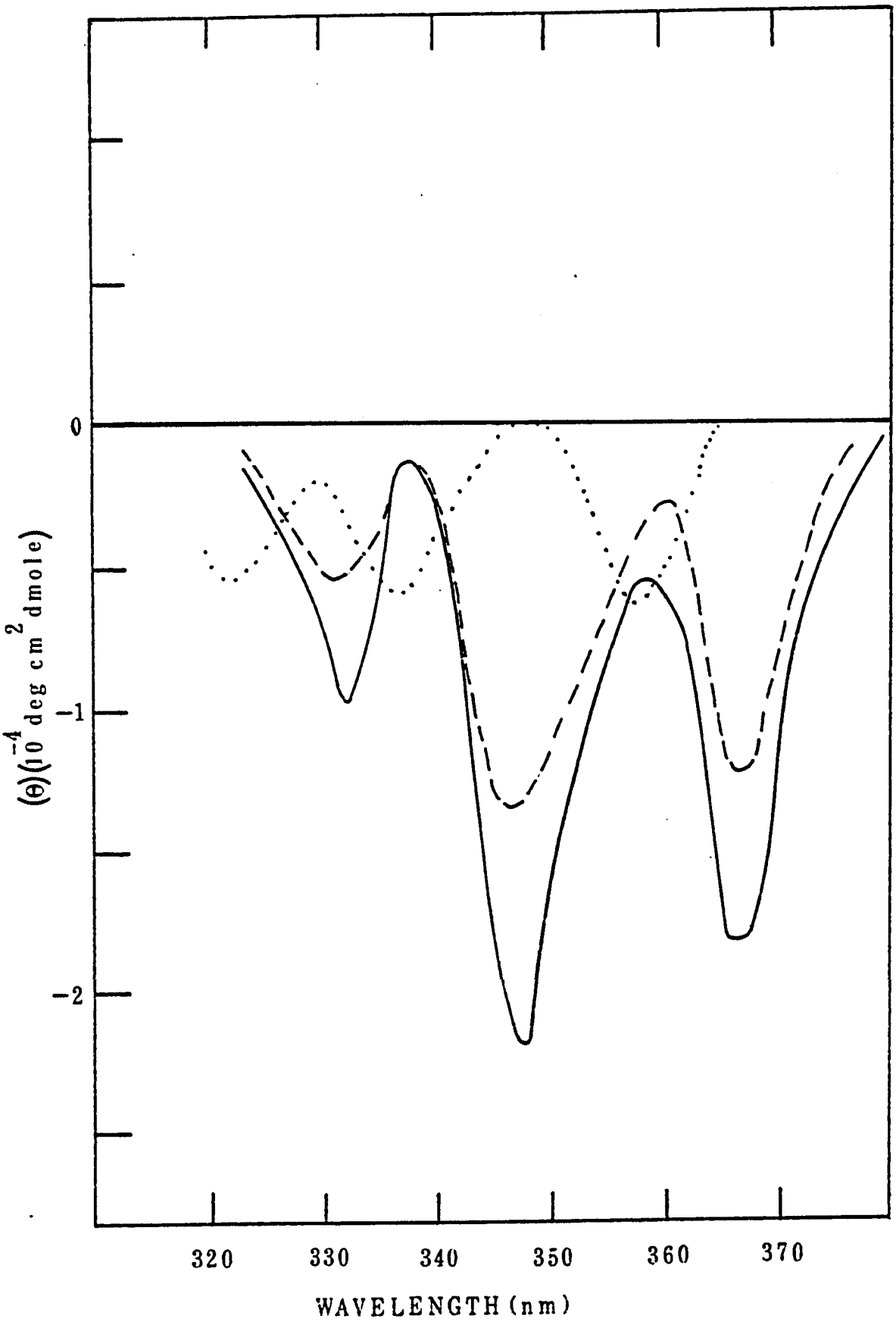
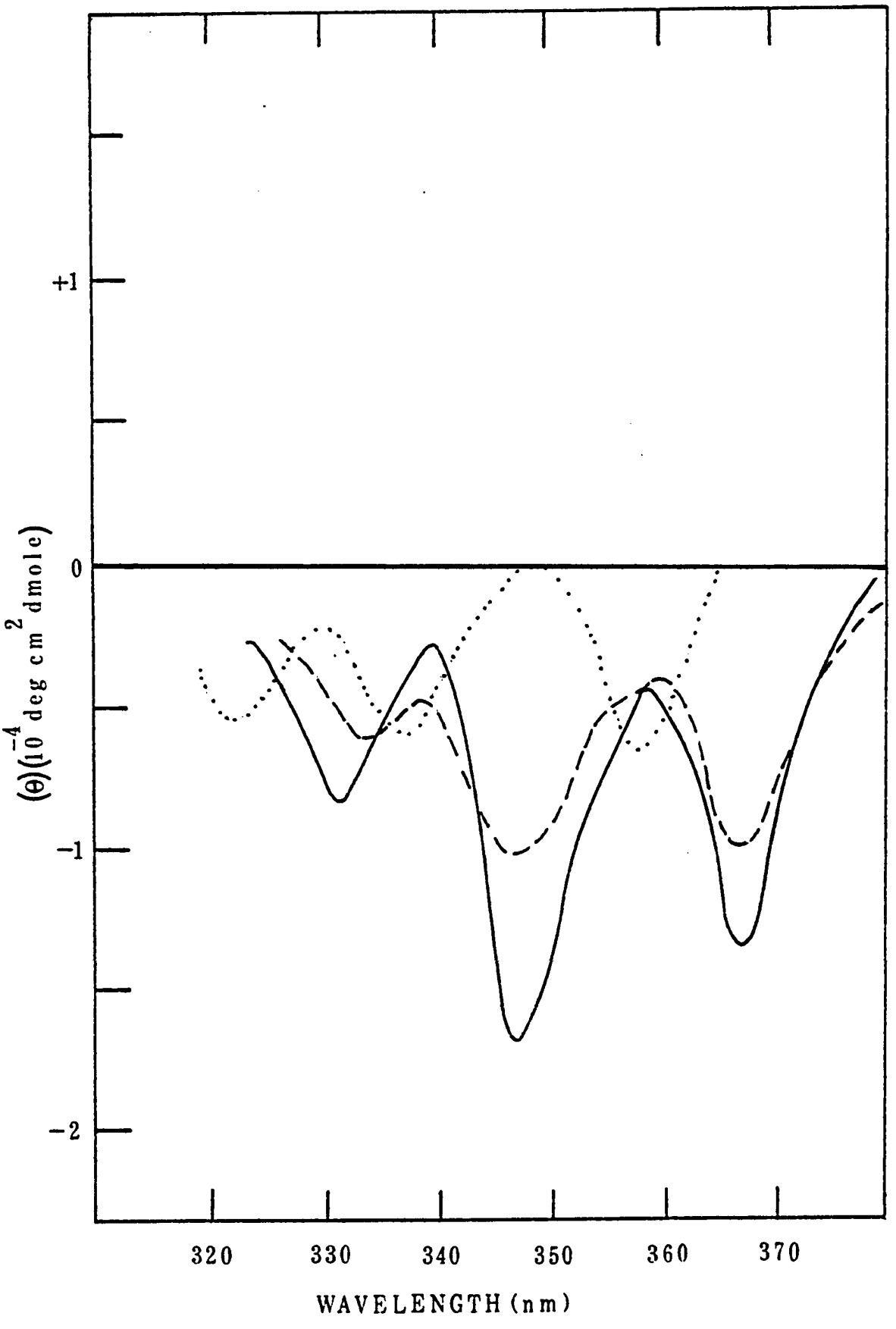


Figure 8: Effect of temperature on the circular dichroism spectra of the polyene antibiotic filipin in the presence of L-α-DPL-cholesterol vesicles at a filipin concentration of 26.8 μM. The concentrations of both lecithin and cholesterol were 0.48 mM. The spectra were obtained at 29°C (——) and at 53°C (– – –). The spectrum of filipin alone (• • •) was obtained at a concentration of 30.7 μM at 29°C.

Figure 8



CHAPTER 4

FILIPIN AS A PROBE OF THE VESICULAR STOMATITIS VIRUS MEMBRANE: PROPERTIES OF THE FILIPIN-CHOLESTEROL INTERACTION AND THE EFFECT ON MEMBRANE MORPHOLOGY, PERMEABILITY, INFECTIVITY, AND FLUIDITY[1]

Introduction

Viruses are forms of life with very simple needs. These are, according to Baltimore, a nucleic acid material to be transmitted to the next generation and a messenger RNA to oversee the synthesis of viral proteins. Viruses differ in the strategies they use for transcription and replication, but all utilize the host cell's enzymatic machinery for replication (78). Viruses differ greatly in size and complexity, from the large lipid-enclosed pox virus which has enough DNA to code for 30 proteins (some of them enzymes), to the much smaller plant viruses, some of which are essentially small particles of RNA (37a). Lipid-enclosed viruses are found most often in animals and are of particular interest both because of their ability to cause disease and because they can be used as models of eukaryotic cell membranes. Many of these animal viruses derive

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[1] A portion of the work described in this chapter has been published: Majuk et al. (76), and Bittman et al. (77).

their lipid-containing envelope from preexisting cellular membranes and these viral membranes of performing certain membrane-dependent functions, such as cell fusion. Also, these viral membranes contain enzymes and receptors; thus they can be thought of as carrying segments of eukaryotic membranes (37a,79).

There are at least 10 different families of enveloped viruses, all of which contain RNA, except for the DNA-containing Herpes and pox viruses. Only a few members of each family have been studied in any great detail. Enveloped viruses fall into three categories: (1) Those that obtain their membranes by budding from the host cell's plasma membrane or endoplasmic reticulum, and (2) Those viruses which obtain their membrane from the nuclear membrane (Herpes virus) or (3) Those that assemble their envelope by de novo synthesis in a specific region of the cytoplasma (pox virus). A great deal of current membrane research is focused on the membranes of four enveloped viruses that mature by budding from the plasma membrane of the host cell. They have been extensively studied because of the simplicity of their membrane and their ease of production (37a,80). They are the vesicular stomatitis virus (rhabdovirus), Sendai virus (paramyxovirus), influenza virus (myxovirus) and the Sindbis virus (togavirus or alphavirus). These viruses have many common structural features. They each are composed of a nucleocapsid (a symmetrical structure composed of a protein shell surrounding a

central core of nucleic acid) surrounded by a lipid membrane containing from 1 to 3 glycoproteins which are anchored in the membrane and protrude externally as spikes (79,80). These glycoproteins play important roles in viral infectivity. Lipids typically comprise 20-30% of the total weight of these viruses. Sendai, vesicular stomatitis, and influenza viruses all contain a non-glycosylated matrix (M) protein which lines the inner surface of the viral membrane (79).

The use of enveloped viruses as models of eukaryotic membranes has been the subject of increasing attention due to the unique advantages these viruses have in membrane research (37,79,80). The most important advantage is the relative simplicity of the viral membrane in which lipids and proteins are organized to form a membrane resembling in many ways the fluid mosaic model of Singer and Nicolson (36). Extracellular viruses (virions) can be easily and quickly prepared in large quantities and purified from contaminating cellular membranes by centrifugation to produce homogeneous and well-defined populations of virions. For simple enveloped viruses, it is possible to do a complete structural analysis on all the membrane components, both lipid and glycoproteins, a feat not yet possible for other biological membranes (80). Another advantage is that viral infection abolishes the host cell's own protein biosynthesis and directs the cellular biosynthetic machinery to produce a small

number of viral envelope proteins so that it is possible to examine the synthesis and insertion of proteins into the viral membrane, a process which may prove useful in understanding the biogenesis of more complicated membranes (80).

Many of the elegant biophysical techniques employed in studies on protein-free lipid vesicles and liposomes can be used to examine the membranes of enveloped viruses. These techniques include electron spin resonance (ESR), nuclear magnetic resonance (NMR), X-ray diffraction, fluorescence spectroscopy and measurements of water permeability. They have provided important insights on the roles phospholipids and sterols play in the organization and the dynamic characteristics of lipid bilayers. In viral membrane studies, these techniques have been used to study the structure of the viral membrane, the fluidity of the viral envelope compared to that of the host cell plasma membrane and the role glycoproteins play in membrane fluidity (79,80).

It is now known from a variety of biophysical techniques that the lipids in enveloped viruses are organized in a single bilayer enclosing the nucleocapsid. The best evidence comes from the X-ray diffraction studies of Harrison et al. (81) who observed that lipids in Sindbis virions existed as a bilayer with a hydrocarbon core similar to that observed for other lipid bilayers. ESR spectra of nitroxide probes located at different depths in the membranes of

influenza, Sindbis and vesicular stomatitis (VS) virions revealed the presence of a fluidity gradient of the type known to exist in synthetic lipid bilayers. Electron microscopic investigations also suggest that the lipids are arranged in a bilayer (37a,79,80).

The membrane of several classes of enveloped viruses have been shown to be more rigid than that of the host cell's. Landsberger and Compans (82) showed that the lipid environment surrounding spin-labeled fatty acid probes was more constrained in VS viral membranes than in the host cell. Barenholz et al. (83) have confirmed this (using the fluorescence depolarization of a fluorescent probe as a measure of membrane fluidity) in both VS viral membranes and in lipids extracted from the virions. Sindbis viral membranes were also observed to be more rigid than the host cells in which they were grown (84). The contribution of glycoproteins to membrane rigidity appears to vary according to the particular virus under study. Proteolytic removal of the external portions of membrane glycoproteins to produce spikeless virions resulted in an increase in membrane fluidity as shown by ^{31}P-NMR and ESR in VS and Sindbis virions (84,85). In contrast to VS and Sindbis viruses, the ESR spectra of intact and spikeless influenza virions were identical (86). The M protein which lies in close juxtaposition to the inner side of the VS membrane may contribute to membrane rigidity, as suggested by Landsberger and Compans (82).

In this study, I used my experience in working with filipin and in measuring the permeability properties of liposomes to investigate lipid-protein interactions and lipid-dependent functions of the VS viral membrane. This was accomplished by examining the permeability properties of intact and glycoprotein-free virions and the membrane-perturbing effects of filipin on several membrane-dependent functions, such as permeability and infectivity. My experimental approach was determined by the two obvious functions of the viral membrane. One function, as Patzer et al. (37b) point out, is to serve as the permeability barrier guarding the nucleocapsid; the second function is to serve as an anchor for glycoproteins, which are essential for the entry of the virus into the host cell. Lipid-dependent functions can be examined by altering phospholipids or cholesterol (as in this study) in viral membranes and then testing the modified virions for changes in biological and physical properties, such as infectivity, membrane fluidity and permeability. In this study, I used the binding of filipin to cholesterol to alter the state of cholesterol in the viral membrane and then tested for changes in membrane fluidity, infectivity, and osmotic properties of modified virions. The importance of cholesterol in the viral membrane is underscored by the fact that cholesterol represents approximately 40% of total VS virion lipids (87). The ESR, electron microscopy and infectivity experiments were out of necessity done in collaboration with Drs. Landsberger and Compans of the Rockefeller University using their instruments and

facilities.

The permeability properties of VS virions were studied by stopped-flow light scattering measurements on virus particles that underwent changes in volume when exposed to hypo- and hyper- osmotic solutions. Under these conditions, the initial rates of water movement into and out of the virions can be measured in a stopped-flow spectrophotometer, as Bittman and Blau were able to show for liposomes (88). The involvement of viral glycoproteins in the lipid permeability barrier was examined by comparing the permeability properties of intact and glycoprotein-free VS virions. The membrane-perturbing properties of filipin were used to show the involvement of cholesterol in the permeability and, also, that the virions exhibited permeability properties similar to those of liposomes and various natural membranes.

The stoichiometry and the equilibrium dissociation constant for the interaction of filipin with cholesterol in the viral membrane was determined. Using filipin as a probe for the state of cholesterol, initial rates of association of filipin with cholesterol in the viral membrane were measured to determine the accessibility of cholesterol in intact and spikeless VS virions. The morphological changes in the viral membrane that accompanied filipin-induced alterations in the permeability and infectivity behavior were examined by electron microscopy and compared to the alterations caused by filipin in other viruses. In addition, the effect of filipin on the rigidity of viral membranes was

examined by ESR spectroscopy using a spin label probe.

VS virions (Indiana serotype) were used in these studies because a great deal of information concerning the involvement of glycoproteins in membrane fluidity of this virus has been accumulated by Patzer et al. (37c). It is among the simplest of enveloped viruses as it contains only one membrane glycoprotein, furthermore it is easily prepared and purified in the large quantities needed for physical studies, such as in permeability measurements.

Materials and Methods

Lipids: The sources of egg yolk lecithin, ergosterol, cholesterol and dicetyl phosphate (DCP) were described earlier. Cholesterol concentrations were determined by the modified Liebermann-Burchard method of Huang et al. (62). Phosphorus was measured by the method of Fiske and SubbaRow (89), and protein by the method of Lowry et al. (90) using bovine serum albumin as the standard.

Antibiotic. Filipin (lot no. 8393-DEG-11-8) was generously supplied by Dr. G.B. Whitfield of the Upjohn Co., Kalamazoo, Michigan. Stock solutions of filipin were prepared in dimethylformamide (DMF) and aliquots were added to virus and liposome preparations to give the desired concentration of filipin and DMF. The concentrations of filipin were determined using the extinction coefficients of filipin in water (5.0×10^4 $M^{-1}cm^{-1}$) or in DMF (5.4×10^4 $M^{-1}cm^{-1}$). Amphotericin

B was prepared as described earlier in Chapter 1 of this dissertation. Fresh stock solutions were prepared daily.

Chemicals. The stearic acid spin labels were purchased from Syva Corp., Palo Alto, Calif., and were used without further purification. Phosphate-buffered salt solution (PBS) was prepared from analytical grade reagents following the directions of Dulbecco and Vogt (90). Bovine serum albumin (less than 0.01% fatty acid) was purchased from Sigma.

Preparation of liposomes and membranes. Liposomes were prepared by dispersing a dried lipid film containing egg yolk lecithin, dicetyl phosphate and cholesterol or ergosterol in PBS solution. Cholesterol- and ergosterol- containing liposomes were prepared at a lecithin: sterol molar ratio of 2:1. All liposomes contained 4 mole per cent dicetyl phosphate. The total lipid concentrations cited in the figure captions and tables were obtained after mixing in the stopped-flow apparatus. Ciliary membranes from Tetrahymena pyriformis W were kindly supplied by Dr. Robert L. Conner and his colleagues of Bryn Mawr College, Bryn Mawr, Pa. The ergosterol and tetrahymanol concentrations of the ciliary membranes were analyzed by Prof. Conner using gas-liquid chromatography (91). Freshly prepared ciliary membranes were centrifuged at 14,000xg for 20 minutes at 2° C and then washed and resuspended in 0.0.M KCl-0.01M Tris buffer (pH 7.2) by

drawing the membranes up and down several times in a Pasteur pipette. In the osmotic shrinking experiments ciliary membranes vesicles were mixed rapidly with an equal volume of buffered hyper- or hypotonic KCl solution. Since the ergosterol and tetrahymanol concentrations of the ciliary membranes were not known at the time of the filipin experiments, approximate values were obtained by comparing the absorbance (light scattering) at 310 nm to that obtained for a membrane preparation known to contain 0.35 mM ergosterol. These concentrations were changed when the actual concentrations were received from Dr. Conner.

Virus and Cells. The different strains of viruses were kindly supplied by Dr. Frank Landsberger of the Rockefeller University and by Dr. Richard Compans, who was at the Rockefeller University during the early part of the experiments and then moved to the University of Alabama Medical Center at Birmingham. Viruses for the initial permeability studies were supplied by Dr. John Lenard of the Rutgers Medical School, Piscataway, N.J., to Dr. Dan Honig working in Dr. Bittman's laboratory. The Indiana strain of VS virus was grown and assayed in BHK-21-F monolayer cell cultures as described previously (93). The WSN strain of influenza virus was grown in MDBK cells (94). Viruses were purified by polyethylene glycol precipitation and equilibrium zonal sedimentation in a 5 to 40% potassium tartrate gradient (86) and dialyzed against a phosphate-buffered salt solution (92).

Preparation of spikeless VS virions. VS virions were treated with either protease type VI from Streptomyces griseus (Sigma Chemical Co., St. Louis, Mo.) or with trypsin (L-(tosyl-amido-2-phenyl) ethyl chloromethyl ketone-treated) from Worthington Biochemicals Corp., Freehold, N.J.) as previously described (93,95). Both these treatments which were performed by Dr. Landsberger, removed the surface glycoprotein spikes from the virions as shown by electron microscopy. After I obtained the virion preparation, I made sure to remove any viral aggregates in the equilibrium binding and kinetic studies by centrifuging both intact and spikeless VS virions at 1,500 rpm in a Sorvall SS34 rotor for 20 min at 4° C and then passing the virions through Pasteur pipettes filled with glass wool.

Spin labeling of virions. VS virions were labeled by Dr. Landsberger with 5- and 16-doxylstearic acid (81,86), and unincorporated label was removed by repurification of the virions on a potassium tartrate gradient. Samples were dialyzed against phosphate-buffered salt solution before electron spin resonance (ESR) spectra were taken. For the filipin experiments, I measured the cholesterol concentrations of the viral suspensions in order to calculate the amount of filipin and DMF to add in order to obtain maximum changes in membrane fluidity with minimal damage.

Electron microscopy. Dr. Compans performed all of the electron microscopic determinations using a Philips EM 300 microscope. For negative staining, virions were stained with sodium phosphotungstate (pH 7.2), while thin sections were obtained from virus pellets that had been fixed with glutaraldehyde and osmium tetroxide and embedded in an epoxy resin mixture as described by Compans et al. (96), and then stained with uranyl acetate and lead citrate.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis scans and radioactivity determinations were also made by Dr. Compans as described previously (97,98).

Permeability measurements. Permeability experiments were performed on a Durrum-Gibson stopped-flow spectrophotometer to which a Tektronix storage oscilloscope was attached for recording initial changes in transmittance. Equal volumes of liposomes, VS virions, or Tetrahymena ciliary membranes were mixed rapidly (less than 3 msec) with osmotic shocking solutions (shrinking or swelling). For VS virions that were dialyzed against phosphate-buffered salt solutions (osmolarity equal to 0.311), the osmotic shocking solutions resembled the buffered salt solution except for the concentration of NaCl which was varied. Liposomes and ciliary membranes were prepared in buffered KCl solutions and exposed to buffered hyper- and hypotonic KCl solutions,

as cited in the table captions. In some experiments, glycerol was used as the pulsing agent instead of KCl or NaCl. In the osmotic permeability studies on VS virions, relative concentrations of spikeless and intact virions were calculated from the absorbance at 280 nm in a 5% sodium dodecyl sulfate solution (obtained by a five-fold dilution of the viral suspension), which was converted to protein content as determined by the procedure of Lowry et al. (90). Initial rates of osmotically induced volume changes, both swelling and shrinking, can be conveniently measured as changes in absorbance (light scattering), as has been demonstrated for liposomes by Bittman and Blau (87). The initial rate of absorbance change, $(dA/dt)t=0$, and the total change in absorbance, ΔA, were determined from the initial rate of change in transmittance dT/dt, and total change in transmittance, ΔT, which was measured within 4 min of mixing. For all of the different membrane suspensions used, the initial rates of transmittance change were measured from the slope of the linear portion of the relaxation curve that followed the region of disturbance in the first 150 msec. The oscilloscope traces for the shrinking and swelling of virions were similar to those reported by Bittman and Blau (87) for liposomes, except that the transmittance changes was in the opposite direction. The rates for all membrane suspensions were examined between 200 and 1000 msec after mixing. For virions

undergoing swelling (decrease in transmittance), the initial change in absorbance was calculated from the initial change in transmittance as follows,

$$dA/dt = \log \frac{S_o + \Delta T}{S_o + \Delta T - dT/dt} \quad \text{and} \Delta A = \log \frac{S_o}{S_o + \Delta T}$$

For virions that underwent shrinking (increase in transmittance), dA/dt was calculated as follows,

$$dA/dt = \log \frac{S_o - \Delta T + dT/dt}{S_o - \Delta T} \quad \text{and} \Delta A = \log \frac{S_o}{S_o - \Delta T}$$

The signal on the oscilloscope, S_o, was generally adjusted to 800 mv and occasionally to 1600 mv by varying the photomultiplier voltage when osmotically shocked membrane suspensions were present in the stopped-flow cuvette. Calculations of the initial rates of absorbance changes for liposomes and ciliary membranes differ from that for virions. Because the initial rate of volume change for liposomes and ciliary membranes is inversely proportional to the absorbance change, the initial rate of absorbance change, d(1/A)/dt, was calculated from the product of dA/dt and $-1/A_i A_f$, where A_f and A_i are the initial and final absorbances of the membrane suspensions. A_i was measured on a Cary 14 spectrophotometer. A_f was calculated from A_i and the change in transmittance. The data in the tables represent the average of between 8 to 15 measurements of virions exposed to a certain osmotic shock.

Results

Permeability studies. To establish that VS virions are osmotically active and that light scattering changes are due to volume changes caused by water movement through the viral membrane, the osmotic properties of the virions were examined in a manner analogous to the way liposomes and other biological membranes have been studied (87,99). It has already been established that liposomes behave as ideal osmometers, that is, they obey the Boylevan't Hoff law, shrinking and swelling in response to osmotic pressure gradients (10). Volumetric and gravimetric determinations have shown that the volume changes are caused by the movement of water in and out of liposomes, and that volume changes are inversely proportional to the changes in absorbance (light scattering) of the lipid dispersions (10,100). When exposed to hyper- and hypoosmolar solutions, the volume of liposomes is inversely proportional to the osmolarity of the resulting solution. Bittman and Blau showed that is possible to measure the initial rates of water movement in liposomes by stopped-flow scattering measurements and that water permeability measurements could be used to probe the fluidity of the lipid bilayer (87).

Preliminary experiments by Dr. Dan Honig, working in Prof. Robert Bittman's laboratory, showed that influenza, parainfluenza SV5 virions and VS virions underwent light scattering changes when

exposed to hyper- and hypo-osmolar solutions. Together with Prof. Bittman, he showed that a linear relationship existed between the absorbance at 480 nm and the reciprocal of the final osmolarity of the viral suspension, indicating that the volume at equilibrium was directly proportional to the absorbance ($V \propto A$) and not to the reciprocal of the absorbance as in the case of liposomes, mitochondria and microsomes. Initial rates of absorbance change of VS virions showed a linear dependence on the change in osmolarity. Dr. Honig also measured the initial rates of absorbance change on the stopped-flow apparatus and found that there was a linear relationship between the initial rates and the change in osmolarity. By combining several of my own initial rates and those previously obtained by Dr. Honig before his departure, I was able to construct a plot of the initial rates of absorbance change as a function of the changes in osmolarity (Fig. 2A, ref. 77). When exposed to swelling solutions (more negative Δosm), the initial rates increased linearly, and conversely, when the virions were exposed to shrinking solutions (more positive Δosm), the rates decreased linearly. The difference in the slopes is probably due to the inability of the virions to shrink beyond a certain point because of the nucleocapsid core which is enclosed by the membrane. Since for an ideal osmometer, the initial rate of volume change is proportional to the change in osmolarity (100a),

the linear relationship between initial rate and change in osmolarity in Fig. 2A of reference 77 is further proof that the absorbance changes are related to movement of water across the viral membrane. In addition, there are other observations to support this conclusion. When virions were exposed to hyperosmotic solutions of glycerol, ethylene glycol, or xylose dissolved in phosphate-buffered saline solution, an initial shrinking phase (decrease in absorbance) was observed. This is followed by a swelling phase (increase in absorbance). The first phase corresponds to movement of water out of the virions in response to the hyperosmotic shock. The second phase corresponds to movement of the non-electrolytes into the shrunken virions, together with the re-entry of water. We also observed a similar pattern of shrinking and swelling when cholesterol-containing lecithin liposomes prepared in phosphate-buffered salt solution were exposed to hyperosmolar solutions of these same non-electrolytes dissolved in the salt solution. When liposomes and virions are exposed to isoosmolar solutions of 0.20 M glycerol in 0.11 osM phosphate-buffered salt solution, they both underwent swelling as water and glycerol moved into the liposomes and virions. Erythrocytes and yeast protoplasts also undergo such biphasic volume changes when exposed to permeant non-electrolytes (11b). In addition, we also observed that the absorbance changes of virions exposed to osmotic pressure gradients were reversible and that gravimetric measurements of swollen virions

showed an increase in the amount of intraparticulate water and an increase in absorbance.

The effect of filipin on the permeability properties of VS virions was compared with those for liposomes and Tetrahymena ciliary membranes. The binding of filipin to sterol-containing membranes is known to cause rearrangements of lipids within the lipid bilayer and to make the membrane leaky to solutes, thus abolishing the osmotic gradient to varying degrees (87). Such rearrangements have been observed in Tetrahymena ciliary membranes containing either ergosterol or tetrahymanol (72). First the effect of filipin on the initial rate of swelling and the volume change in liposomes and ciliary membranes was examined. Table 1 shows that filipin is able to decrease significantly both the rate and extent of volume change in both liposomes and ciliary vesicles, presumably by reducing the ability of the membrane to act as an effective osmotic barrier to electrolytes. Filipin was less effective in reducing the magnitude of the osmotically induced absorbance changes in liposomes prepared from egg lecithin alone and in reducing the initial rate of volume change in ciliary vesicles containing tetrahymanol compared with ergosterol-containing membranes. This is not surprising since the affinity of filipin for sterol-containing liposomes exceeds that for lecithin liposomes, and filipin produces more pronounced membrane perturbations in ergosterol-containing ciliary membranes than in those containing tetrahymanol (72).

The effect of filipin on the permeability properties of intact VS virions and cholesterol-containing liposomes is shown in Table 2. Liposomes were prepared with a cholesterol/lecithin molar ratio (0.50) comparable to that in virions grown on BHK cells (0.65). When present at a comparable ratio of cholesterol to filipin, the antibiotic reduced the extent and initial rate of volume change in both virions and liposomes. When I examined the spent polyene-treated virions recovered from the stopped-flow apparatus, I found that filipin had undergone spectral changes indicative of a cholesterol-filipin interaction. The permeability results suggest that filipin interacted with cholesterol-containing VS virions to produce rearrangement of membrane components, making the virions leaky to solutes and reducing the osmotic gradient across the viral membrane. The ability of filipin to reduce the absorbance changes that accompany swelling of VS virions is still another indication that the virions are osmotically active.

In order to examine the involvement of VS viral glycoproteins in the lipid permeability barrier, the initial rates of swelling and equilibrium volume changes in intact and spikeless VS virions were measured. Virions were rendered glycoprotein-free by treatment with protease VI or trypsin. Table 3 shows that in experiments where hypotonic NaCl gradients were used to swell virions, both equilibrium volume changes and the initial rates of swelling were greater for

intact virions than for spikeless virions. In a separate experiment (data not shown here), virions exposed to hyperosmolar solutions of glycerol in phosphate-buffered saline underwent an initial rapid shrinking phase (due to water loss) followed by a swelling phase due to glycerol permeation and water re-entry. The initial rates of swelling and extent of volume change in this second phase were measured and the results (not shown) indicated that intact virions exhibited both greater initial rates of swelling and extent of swelling. Since treatment with protease VI or trypsin are known not to affect the internal proteins of VS virions (93,95), the permeability results indicate that the viral glycoproteins are involved in the permeability barrier of VS virions. Because the initial rates of water movement in spikeless virions were too small to be measured accurately, the effect of the glycoproteins on the accessibility of cholesterol to filipin could not be determined.

Filipin-induced modification of viral membrane morphology.

The morphological alterations in the viral membrane that accompany filipin-induced modification of VS viral permeability properties were examined by electron microscopy and are shown in Figure 1. Treatment with filipin results in a striated or corrugated appearance in the viral membrane and a redistribution of membrane glycoprotein spikes. Along the membrane edge, a series of depressions and ridges appears, repeating themselves every 100-200 A (Fig. 1B). The glyco-

protein spikes are no longer evenly distributed in the membrane but are attached only to the ridges as revealed by negative staining. A similar striated appearance is also observed in thin-sectioned virions (Fig. 1D), demonstrating that both preparative procedures reveal similar morphological alterations. In those VS virions that were sometimes disrupted by the negative-staining procedures, 200-300 A circular depressions or pits were observed, giving the virions a cratered appearance (Fig. 1E). Similar morphological alterations have been observed in filipin-treated erythrocytes (7), sterol-containing liposomes (102) and ergosterol-containing fungal cells (33). These pits are believed to be areas in which filipin-cholesterol complexes are congregated. Saponins, which are known to specifically bind to cholesterol and extract the sterol from membranes, interestingly enough also produces evenly distributed pits (6). As shown in Fig. 1C, similar striations occur in spikeless VS virions.

Filipin-induced morphological alterations in bullet-shaped VS virions were compared with those in the roughly-spherically shaped influenza and Rauscher leukemia virions, both of which are lipid enveloped virions containing glycoprotein spikes. Rauscher leukemia virions treated with filipin were dramatically altered, exhibiting numerous pores or pits ranging in size from 100 to 250 A in diameter (Fig. 2). In some instances, the circular pores appear to go all the way through the membrane. Pits that go through the membrane have not

been observed in liposomes, erythrocytes or Acholeplasma laidlawii cells, either by freeze-etch or negative-staining techniques (7,33,103). The striking morphological changes in Rauscher leukemia virions resemble the alterations in partially disrupted VS virions (Fig. 1E), both in the general shape of the virion and the cratered surface morphology (Fig. 1E). Influenza virions treated with filipin under similar conditions showed no dramatic membrane alterations as reported by Prof. Compans.

VS virions were treated with amphotericin B in order to determine if it too would cause morphological alterations similar to those induced by filipin. As shown in Fig. 3, amphotericin B-treated virions exhibited long tail-like out-foldings of the viral membrane. The glycoprotein spikes are redistributed on the main body of the virion, as well as on the outfolding. No pits or depressions are visible in amphotericin B-treated virions. It is clear that this antibiotic causes a different redistribution of viral membrane components (lipids and glycoproteins) compared to that observed with filipin. The differences between the mode of action of filipin and amphotericin B that have been proposed by deKruijff and Demel (8) are apparently reflected in the morphological alterations induced by these two polyenes. In liposomes and natural membranes filipin causes widespread and severe damage to membranes, while amphotericin B produces a less dramatic rearrangement of membrane components,

resulting in the formation of hypothetical pores, too small to be visible (8). However, in fungal cells containing ergosterol, amphotericin B has been observed to induce severe damage to the membrane. Small pits and protrusions have been observed in the freeze fractured membranes (6).

To determine if filipin-induced morphological changes in VS virions involved loss of protein or lipid material, viral proteins and lipids were labeled, as suggested by Prof. Bittman, with 4,5-^{3}H-leucine and ^{14}C-glycerol, respectively. As shown in Fig. 4, the protein profile obtained by Prof. Compans on polyacrylamide gels reveals that the amino acid label is incorporated into all of the polypeptides, while the glycerol labels the polypeptides, as well as a major peak at the dye front which also includes viral lipids. With incorporation of label assured, labeled virions were treated with filipin and then analyzed by density gradient centrifugation. An examination of Fig. 5 shows no release of either ^{3}H- or ^{14}C-labeled molecules. Similar results were obtained with a 5-fold higher concentration of filipin. When the distribution of filipin in the gradient is monitored by scanning the gradient at 338 nm (one of the absorption maxima of filipin), it can be seen that filipin is almost entirely incorporated into the virus (Fig. 5B). Control viral particles do not possess any significant absorbance at this wavelength (Fig. 5A). Other investigators have observed no significant change

in the composition of filipin-treated Neurospora and other fungal cells (4), and in amphotericin B-treated VS virions (107).

Effect of filipin on biological activity of virions. To answer the question whether or not filipin-induced rearrangements of viral membrane lipids had an effect on virus-host cell interactions, the effect of filipin on the infectivity of VS and influenza virions was examined. The results in Table 4 show that a cholesterol/filipin ratio of 0.21, there is approximately a 500-fold reduction in the infectivity of VS virions. Under similar conditions, there was no significant reduction in the infectivity of influenza virions. This may be related to the minor morphological alterations caused by filipin on the membranes of influenza virions that Dr. Compans observed.

Stoichiometry of filipin-cholesterol interaction in viral membranes. To determine the stoichiometry of the filipin-cholesterol interaction in viral membranes, binding curves were obtained for both intact and spikeless virions. For a given concentration of cholesterol, saturating concentrations of filipin gave rise to a linear portion of the binding curve where there is a constant ratio of cholesterol to filipin (Fig. 6). For intact and spikeless VS virions, the values for the number of moles of cholesterol bound per mole of filipin

were 1.25 ± 0.15 and 1.35 ± 0.33, respectively, at the 90% confidence level. Thus, the stoichiometry of the filipin-cholesterol interaction was similar for intact and spikeless VS virions, indicating that glycoproteins do not inhibit the binding of filipin to virions.

Equilibrium dissociation constant. The values of the equilibrium dissociation constant for the binding of filipin to intact and spikeless virions were determined using the same procedure described in Chapter 2 of this dissertation. The values for the dissociation constants were determined by least squares analysis of the data presented in Fig. 7. The values, obtained by dividing the slope of the line by the value of the intercept, were 34 ± 2.0 μM and 0.46 ± 0.24 μM at the 90% confidence level for intact and spikeless virions respectively. As expected, the y-intercepts were approximately 1.0 which validates the assumption used in these calculations that there is approximately a 1:1 mole ratio of filipin to cholesterol in the sterol-antibiotic complex. The results indicate that the binding of filipin to spikeless VS virions is much stronger than to intact virions.

Initial rates of association. The results of kinetic experiments are presented in Fig. 8. They show that the initial rate of association of filipin with cholesterol is faster in spikeless than in intact

virions. The rates are linearly dependent on the cholesterol concentration of the viral preparation at low sterol concentrations.

ESR spectroscopy. To determine the effect of filipin on the fluidity of the viral membrane, the ESR spectrum of a nitroxide labeled stearic acid probe exogeneously incorporated into the virions was recorded by Prof. Landsberger. Figure 9 shows the ESR spectrum of VS virions labeled with the C5 (the nitroxide reported group is positioned on the fifth carbon from the carboxyl end of the fatty acid, as shown in the insert in Fig. 9). The distance between the vertical lines drawn through the outermost high and low magnetic field extrema can be used to report on the motion of the spin label located within the lipid membrane because in general, the splitting (distance) increases as the motion of the spin label is restricted by its environment (104,105). The observed increase in the splitting between the spectral extrema, the "broad-line" spectrum, upon filipin treatment is indicative of an increase in the rigidity of the viral membrane in the region probed by the spin label. Exposure of influenza and spikeless VS virions to filipin also resulted in an increase in the rigidity of the membrane (spectra not shown). The appearance of the "liquid-line" in the ESR spectrum of filipin treated virions indicates that a significant amount of spin label is in the buffer (104,105). This is consistent with the

diffusion of spin label out of the viral membrane as a result of the rigidity of the viral membrane. In other spectra also recorded by Dr. Landsberger, the region near the middle of the bilayer was also found to be more fluid in both filipin-treated and untreated virions than the region near the polar surface probed by the C5 spin label.

Discussion

Permeability studies. In order to use measurements of the initial rates of water permeability as a non-perturbing means of studying the involvement of cholesterol in the VS viral membrane's permeability barrier, the relationship between absorbance and volume changes has to be known. An examination of the absorbance changes occurring in VS virions in response to osmotic shocks indicates that VS virions behave as osmometers, swelling and shrinking in response to changing osmotic conditions, and that absorbance changes represent osmotically driven water movement across the viral membrane, as Bittman et al. have shown (77). These investigators showed that the osmotically induced volume changes in VS virions are directly proportional to changes in absorbance ($\Delta V \propto \Delta A$). In the case of liposomes and other biological membranes, volume changes are inversely proportional to changes in absorbance. Differences among various osmometers in their relationship between ΔV and ΔA are not uncommon, as Bittman et al. (77) have pointed out; experiments and theory both reveal that light scattering is a complex function of several factors, including particle size and scattering angle. As Bryant et al. (106) have shown, it should not be automatically assumed that increased light scattering (absorbance) means particle shrinkage, as indicated by certain theoretical considerations.

They point out that shrinkage of yeast cells is accompanied by a decrease in light scattering, while the reverse is true for other types of membrane systems (106).

The permeability properties of VS virions were modified by treating the virus particles with the lipophilic antibiotic filipin. The drug is known to form complexes with cholesterol in the hydrophobic portion of the membrane that produce severe membrane disruption (4). The results in Table 2 indicate that filipin treatment results in a decrease in the viral membrane's ability to behave as an effective barrier to electrolytes so that both the extent and the initial rate of volume change are decreaed. The degree of filipin-induced damage appears to be greater in sterol-containing liposomes and ciliary membrane vesicles (Table 1) than in VS virions (Table 2) and tetrahymanol-containing ciliary vesicles (Table 1). Glycoproteins may limit the access of filipin to cholesterol located within the membrane or they may hinder the ability of filipin to form stable-cholesterol-filipin complexes within the membrane. Tetrahymena ciliary membrane proteins apparently do not interfere with the cholesterol-filipin interaction, at least not to the extent glycoproteins do in virions. The ability of filipin to perturb the viral permeability barrier in the same way as it does in liposomes and ciliary membranes indicates that the viral membrane exhibits permeability properties similar to those of other biological membranes and reveals the involvement of cholesterol in the viral permeability barrier.

Experiments in which I measured the initial rate of absorbance change versus the change in osmolarity of intact VS virions and those in which I exposed viral particles to different non-electrolytes (for example, glycerol and xylose) are further evidence that VS virus is enclosed by an uninterrupted semipermeable lipid membrane capable of functioning as a barrier, impermeable to water and non-electrolytes much in the same manner as other membranes, both natural and artificial.

In addition to cholesterol, the involvement of glycoproteins in the permeability barrier of VS virions was also examined. This was done by comparing the permeability properties of intact and spikeless virions. (The effect of filipin on the already slow rates of volume change was to lower the rates to a point where they could not be measured precisely, indicating damage to the permeability barrier.) As shown in Table 3, intact VS virions underwent faster initial rates of volume change and exhibited greater equilibrium volume changes than did spikeless virions. This suggests that the overall fluidity of spikeless virions is less than that of intact virus particles. Initial rates of water movement in liposomes have been shown to be sensitive indicators of bilayer fluidity, as Bittman and Blau have demonstrated (87). However, the decreased rates of water movement in spikeless virions is unexpected since it has been shown by ESR and NMR techniques (82,85) that removal of spikes leads to an increase

in the fluidity of the VS viral membrane, with the largest increase occurring in the polar head group region of the bilayer. It is conceivable that removal of the spikes leads to local rearrangements of the lipids so as to hinder passive transport of water molecules. In connection with this, it would be of interest to compare the interaction of glycoproteins with membrane lipids in influenza virions by removing the glycoprotein spikes and measuring the effect on the initial rates of water permeability.

Filipin-induced morphological changes in viral membranes.

The permeability changes in VS virions that accompany filipin treatment are also accompanied by profound morphological alterations in the viral membrane. Filipin treatment leads to the formation of striations and to the segregation of membrane glycoprotein spikes. These alterations occur without any discernible dissociation of lipids or proteins from the viral membrane and do not appear to be the result of artifacts resulting from a particular staining procedure. The observation that no membrane components are released upon filipin treatment is consistent with published observations that filipin, as well as other polyenes, does not cause the release of membrane cholesterol (4) and with more recent studies indicating that the polyene amphotericin B does not induce release of membrane constituents from VS virions (107). The fact that similar ridges

and depressions were observed in both intact and spikeless VS virions indicates that these alterations are independent of the presence of glycoproteins. The alterations in disrupted VS, influenza and Rauscher leukemia virions differ from those observed for VS virions, possibly because of the different geometries of the virions, as will be shown next.

The appearance of the striations on the VS membrane cannot be satisfactorily explained by two current models of the filipin-cholesterol interaction, unless an important modification based on the curvature of the viral membrane is included. As I mentioned in Chapter 1 of this dissertation, deKruijff and Demel summarized much available evidence concerning the behavior of individual polyenes, and in the case of filipin, they proposed a filipin-cholesterol complex in the shape of a planar aggregate (diameter 150-250 A) lying in the hydrophobic core of the membrane parallel to the bilayer surface (8). Cholesterol and filipin molecules are aligned so that their long axes are parallel to each other and to the membrane surface. The presence of these aggregates is thought to lead to the appearance of pits and depressions, although they don't show any pits in their filipin-cholesterol complex model. For amphotericin B and nystatin, deKruijff and Demel proposed a circular array of polyene and cholesterol molecules aligned so that their long axes are parallel to the

fatty acid chains of the phospholipids, forming a half pore with an 8 A diameter. Two such pores must come together, one on each side of the membrane, to form a pore extending through the membrane. In the Seeman model (6), cholesterol and filipin molecules are interdigitated with their long axes parallel to the fatty acid chains of membrane phospholipids, forming a ring and depressing the membrane region enclosed by the ring to form a pit 150 A in diameter. Possibly, as Prof. Bittman has suggested, the striations observed in filipin-treated VS virions are due to the formation of planar filipin-cholesterol complexes 150 A to 250 A in diameter, similar to those proposed by deKruijff and Demel for amphotericin B and nystatin, which because of the curvature of the VS viral membrane were unable to form pits (diameter 150 A-250 A) and instead took on an elongated structure of the same diameter. The same can be said of the Seeman model of the filipin-cholesterol complex where there is already a circular array of sterol and polyene molecules lying at the bilayer surface. The circular-shaped complex can also be squeezed because of the viral membrane curvature until it too takes on an elongated shape. In both these models, the cholesterol-filipin complex would take on the shape of a pit in those cases where the membrane curvature is not so great, as in disrupted VS and Rauscher leukemia virions. There is some recent evidence to favor the original planar model of deKruijff

and Demel in which the long axes of filipin and cholesterol are parallel to each other and perpendicular to the phospholipid fatty acyl chains. Flick and Gelerinter have shown (108) that steriod spin label probes incorporated into lecithin planar multibilayers free of any curvature constraints altered their orientation from parallel to perpendicular to the fatty acyl chains in filipin-treated bilayers, just as in the deKruijff and Demel model. Further studies on the morphological alterations induced by filipin in membranes of other geometries and degrees of curvature, such as the spherically shaped Toga virus (diameter 700 A), would be of interest in determining whether or not the formation of pits or striations depends on membrane geometry.

Effect of filipin on membrane fluidity. Perturbation of viral membranes by filipin results in an increase in the rigidity of the region near the glycerol backbone probed by the spin label in the membranes of influenza, as well as intact and spikeless VS virions. An increase in the rigidity of this region is consistent with the formation of filipin-cholesterol complexes close to the membrane surface. In agreement with these present results, Ohki et al. (109) have recently shown with both steroid and phospholipid spin label probes that filipin decreased the fluidity of cholesterol-containing phospholipid vesicles near the glycerol backbone region of the bilayer, while at the same time increasing the fluidity in the hydrophobic core of the vesicles. They interpreted this to mean that filipin

swept cholesterol from the middle of the bilayer to the region near the carbonyl group region of the bilayer. They also showed that filipin interacted with and reduced the mobility of the steroid probes incorporated into the vesicles and that filipin increased the rigidity of erythrocyte membranes near the glycerol backbone of the bilayer, as revealed by the ESR spectrum of the same stearic acid spin label probe used in this present study. The increase in the rigidity of the VS membrane by filipin treatment of both intact and spikeless virions may result from the formation of cholesterol-filipin complexes that squeeze both lipids and glycoproteins into those regions between the complexes. These regions would correspond to the ridges we observe in the electron micrographs. In these same regions, the packing of membrane components would be tighter, thus accounting for the increased rigidity of filipin-treated membranes. This picture is also consistent with the observed increase in the liquid-line spectrum which can be thought to result from an increase in the diffusion of the spin label out of the compressed regions because of the increased rigidity. Changes in the rigidity of the VS viral membrane have been shown to result from changes in the lipid phase such as depletion of cholesterol and cleavage of membrane glycoproteins (82,110).

State of cholesterol in the VS membrane. Using filipin as a probe for the state of cholesterol within the viral membrane, the initial rates

of association of filipin with cholesterol were measured and it was concluded that cholesterol is more accessible in spikeless than in intact VS virions, possibly because of the presence of surface glycoproteins which may limit the ability of filipin to reach cholesterol, or because the membranes of spikeless VS virions are known to be more fluid (37c). The effect of viral fluidity has to be considered since the accessibility of cholesterol for interaction with filipin has been shown to be dependent on the molecular packing of lipids in liposomal membranes (87). There is some evidence to support the first possibility. The accessibility of cholesterol to filipin has recently been shown to increase when surface components were enzymatically removed from both sealed and unsealed human erythrocyte ghosts (111). Because proteolytic cleavage of influenza surface glycoproteins has no effect on the fluidity of the viral membrane (82), it may be pertinent to determine the extent to which glycoproteins determine the accessibility of cholesterol in viral membranes by measuring the initial rates of association of filipin with cholesterol in intact and spikeless influenza virions.

There appears to be no significant difference between intact and spikeless virions in the stoichiometry of the cholesterol-filipin interaction. The values obtained in this study are similar to the published mole ration of 1.11 ± 0.18 reported by Katzenstein et al. (112) for liposomes and of 1.2 for Acholeplasma laidlawii cells (113), thus indicating that the high curvature of the VS viral membrane does

not affect the stoichiometry of the interaction. It may affect the shape of the complex as our evidence indicates. The fact that the differences in the equilibrium dissociation constants were much larger between spikeless and intact virions than the differences between the rates of association suggests that the rate of dissociation of filipin from the complex must be greater in intact than in spikeless virions. This implies that another difference in the interaction of filipin with VS membranes is the stability of the filipin-cholesterol complex.

Alteration of the state of cholesterol by high concentrations of filipin decreased VS viral infectivity. The mechanism by which filipin reduces infectivity remains to be established. It may result from the severe disruption of the viral membrane that often occurs at high filipin concentrations so that the virions are limited in their ability to invade host cells. Since filipin appeared to be incorporated into all of the virions (Fig. 5B), it was not possible to determine if infectivity was due to undamaged virions. Influenza virions, which exhibited only minor perturbations from filipin, suffered no appreciable loss of infectivity. The possibility that the increased rigidity of the VS viral membrane caused by the formation of filipin-cholesterol complexes somehow inhibits viral penetration may not be tenable in light of recent studies on the involvement of cholesterol in VS viral infectivity. In their studies on the role cholesterol plays in membrane functions, Moore et al. (110) depleted VS

virions of about 90% of their membrane cholesterol by incubation with phosphatidylcholine vesicles and found that viral infectivity was decreased under conditions where the structural integrity of the viral membrane was maintained. In a companion study, they oxidized membrane cholesterol by cholesterol oxidase (after prior treatment with phospholipase to increase the accessibility of the sterol) and observed a decrease in VS viral infectivity and no change in fluidity beyond the increase in fluidity caused by the phospholipase treatment. These experiments involving the manipulation of membrane cholesterol by either complexation with filipin, depletion or oxidation reveal the involvement of cholesterol in viral infectivity by an as yet unexplained mechanism. Studies on the mechanism for the antiviral properties of filipin, as well as that of amphotericin B which has recently also been shown to inactivate VS viral infectivity (107), may prove useful to understanding the properties and functions of the viral membrane, much in the same way that agents that interfere with the structure and biosynthesis of macromolecules have been used to study nucleic acids and proteins (4).

Table 1: Effect of filipin on the initial shrinking rates, d(1/A)/dt, and equilibrium volume changes of liposomes and Tetrahymena ciliary membrane vesicles. Liposomes were prepared in 0.04 M KCl-0.01 M Tris solution (pH 7.2) except for lecithin/cholesterol liposomes (1), which were prepared in 0.06 M KCl-0.01M Tris solution. Ciliary vesicles were suspended in 0.01 M KCl-0.01M Tris solution (pH 7.2). Liposome and membranes suspensions contained 1% dimethylformamide by volume and were incubated with filipin in the dark at room temperature for approximately 1.5 h. Liposomes were mixed with 0.20 M KCl-0.01 M Tris solution containing 1% dimethylformamide by volume (except for lecithin/cholesterol liposomes (1), which were mixed with 0.18 M KCl-0.01 M Tris solution containing 1% dimethylformamide by volume). The final lipid conccentration in the liposomes was 1 mM and the molar ratio of lecithin to sterol was 2:1. The final concentrations of tetrahymanol and ergosterol in the membranes were 0.068 and 1.08 mM, respectively. The absorbance was measured at 600 nm. The ratios shown are molar ratios of lecithin or sterol or tetrahymanol to filipin.

Table 1

	Liposomes				Ciliary Membranes	
	Lecithin	Lecithin/ Ergosterol	Lecithin/ Cholesterol (1)	Lecithin/ Cholesterol (2)	Tetrahymanol	Ergosterol
(Lecithin)/(Filipin)	7.7	-	-	-	-	-
(Alcohol)/(Filipin)	-	2.0	1.3	2.0	0.5	0.6
% Decrease in Shrinking d(1/A)/dt	14	78	73	40	27	88
% Decrease in equilibrium volume (1/A)	5	58	58	45	64	59

Table 2: Effect of filipin on the initial swelling rates and equilibrium volume changes of liposomes and VS virions. Liposomes derived from lecithin and cholesterol in 2:1 ratio and virions were suspended in phosphate-buffered salt solution and mixed in the stopped-flow spectrophotometer with an equal volume of a phosphate-buffered saline solution whose NaCl concentration was lower than that of the phosphate-buffered salt solution. The change in osmolarity was -0.104. Light scattering (absorbance) changes were measured at 500 nm. The final lipid concentrations of the liposomes were 0.5 and 0.2 mM, respectively. The total protein content of the virions was 0.47 mg/ml. Liposomes and virions were treated with filipin as described in Table 1.

Table 2

		Liposomes				Virions			
				% Decrease				% Decrease	
	Cholesterol/Filipin Molar Ratio	Δ^1/A	$d^1/A/dt$	Δ^1/A	$d^1/A/dt$	ΔA	dA/dt	ΔA	dA/dt
Untreated	-	0.073	0.0218 ± 0.0020	-	-	0.085	0.0134 ± 0.0013	-	-
Filipin treated	1.1	0.022	0.0073 ± 0.0010	70	65	0.040	0.0086 ± 0.0017	47	36
	0.56	-	-	-	-	0.043	0.0050 ± 0.0009	50	63

Table 3: Initial swelling rates and equilibrium volume changes of intact and spikeless VS virions. Intact and spikeless virions from the same preparation were suspended in phosphate-buffered salt solution. Each virion suspension was exposed to an equal volume of hypoosmolar phosphate-buffered saline solution (0.137 osM) whose NaCl concentration differed from that present in phosphate-buffered salt solution. The change in osmolarity was (1) -0.087 and (2) -0.104 osM. Changes in light scattering at 375 nm were recorded on the stopped-flow spectrophotometer using a total signal of 800 mV for (1) and 1600 mV for (2). This accounts for the larger changes in signal amplitude observed in (2) compared to (1). The total protein concentrations of the virions were 0.31 mg/ml for preparations (1) and (2) of the intact particles, 0.14 mg/ml for the protease type VI-treated virions (spikeless preparation (1)), and 0.23 mg/ml for the trypsin-treated virions (spikeless preparation (2)).

Table 3

Type of Virions	$dA/dt \times 10^4$ (sec^{-1})	ΔA
Intact (1)	30.8 ± 3.4	0.019
Intact (2)	50.9 ± 2.8	0.050
Spikeless (1)	6.4 ± 1.3	0.012
Spikeless (2)	8.4 ± 2.7	0.032

Figure 1: Effect of filipin on the morphology of intact and protease-treated VS virions. A. Control particle showing projections uniformly distributed on the surface. B. Filipin-treated particle showing depressions and ridges in the membrane, and redistribution of surface projections. C. Trypsin-treated particles that have been exposed to filipin. Despite the absence of surface spikes, depressions in the viral membrane are still detected. D. Filipin-treated VS virion in thin section showing striated appearance. E. Partially disrupted particle exhibiting ring-like pits or pores approximately 200 A in diameter. A, B, C, E: Negative staining with sodium phosphotungstate. Magnification: A and D, x 150,000; B, x 300,000; C, x 180,000; E, x 130,000.

Figure 1

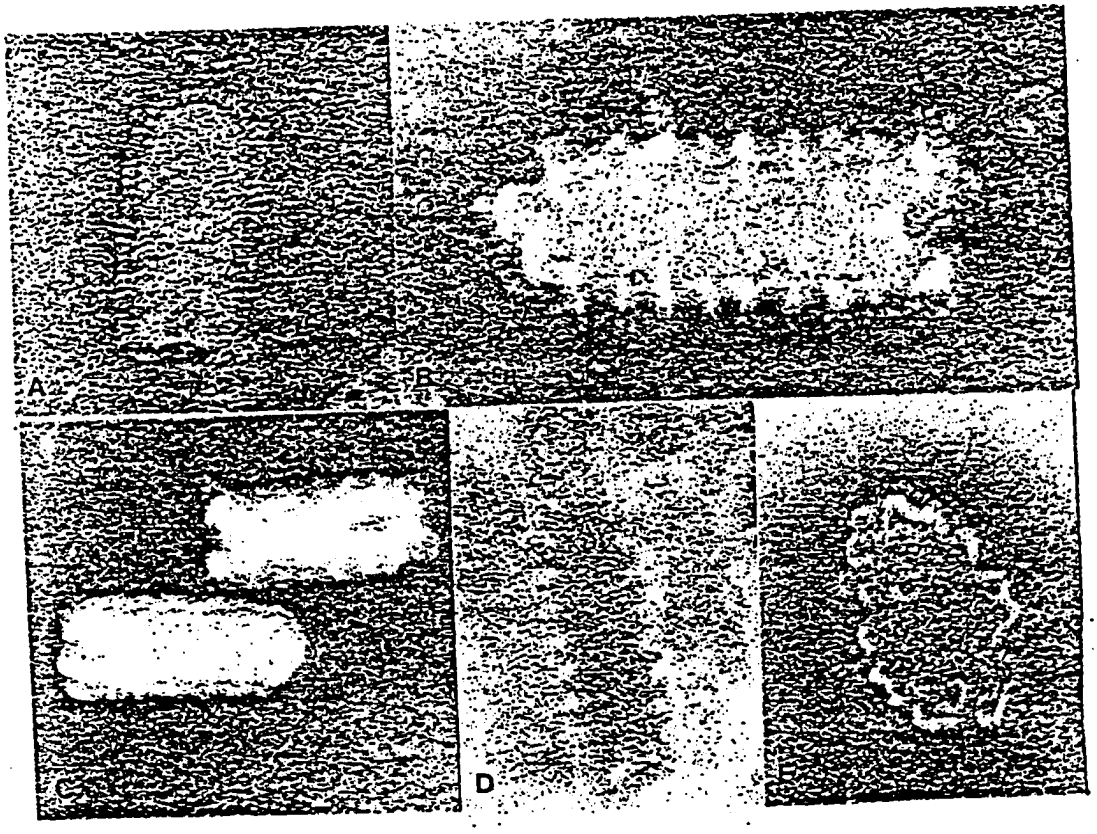


Figure 2: Effect of filipin on Rauscher leukemia virions. The viral membranes have become permeable to phosphotungstate and show numerous pores or pits ranging from 100-250 A in diameter, some of which appear to extend completely through the membrane (arrows). Control particles (insert) show intact envelope and typical head and tail forms in unfixed preparations. Magnification: x 165,000; insert x 150,000.

Figure 2

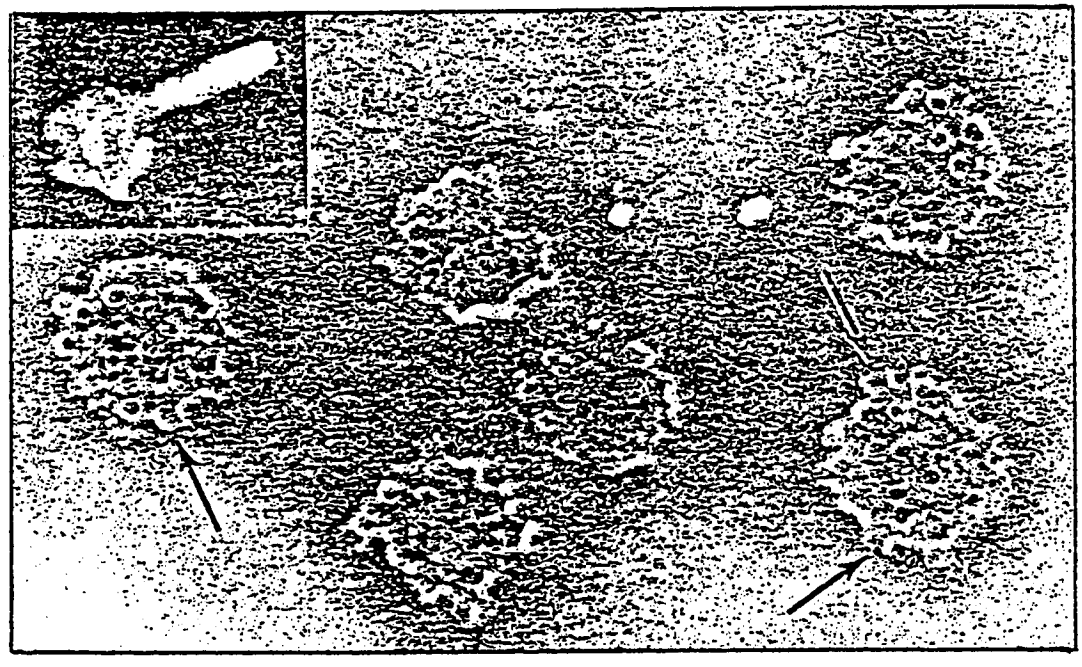


Figure 3: VS virions treated with amphotericin B, showing long tail-like outfoldings of the membrane. Magnification x 120,000.

Figure 3

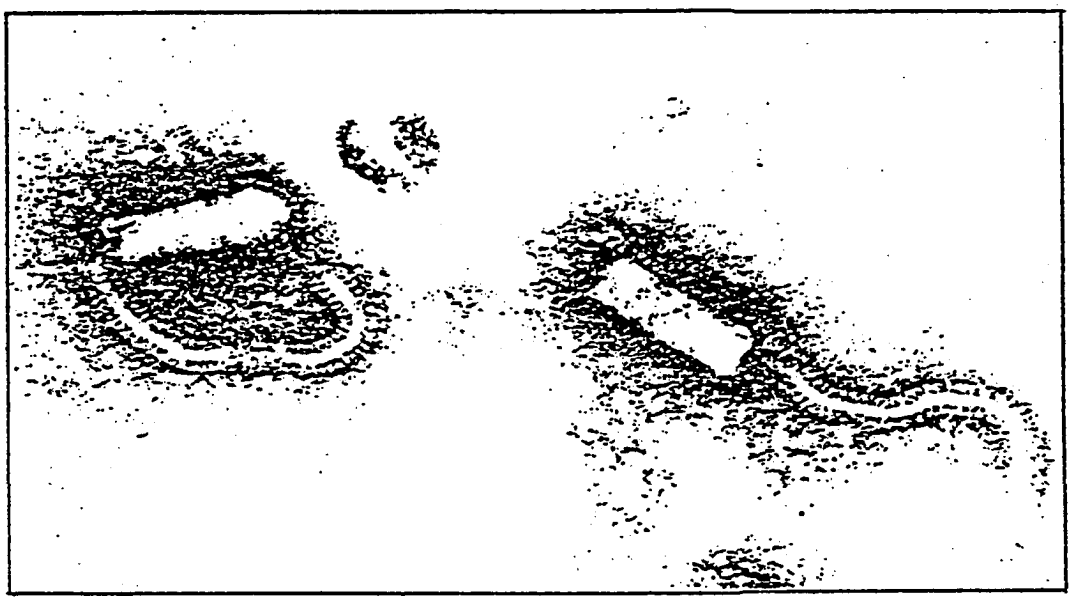


Figure 4: Polyacrylamide gel electrophoresis of SDS-dissociated VS virions labeled with 4,5,^{3}H-leucine (5 μCi/ml) and ^{14}C-glycerol (0.5 μCi/ml). The designations of viral polypeptides (L,G,NS,M) are according to Wagner et al. (114).

Figure 4

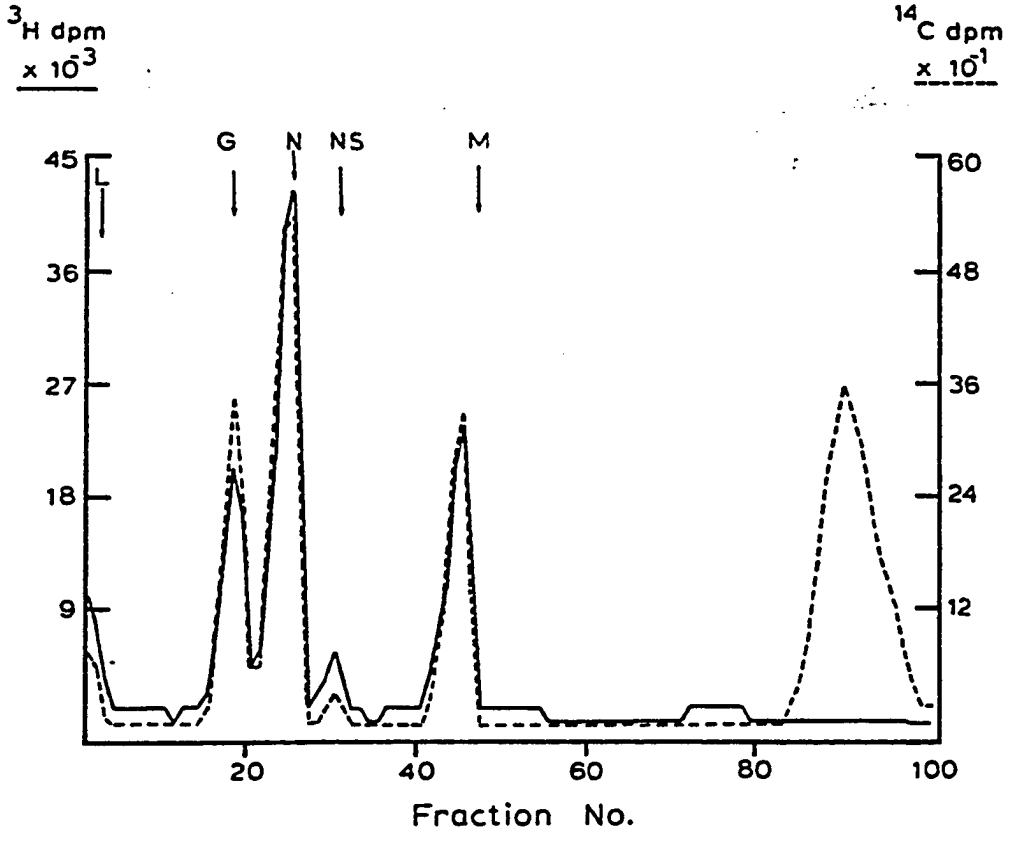


Figure 5: Location of filipin-treated VS virions in a sucrose density gradient. To 0.3 ml of purified virus in phosphate-buffered salt solution, containing 0.5 mg/ml of protein, 1 μl of a 0.29 M solution of filipin in dimethylformamide was added, and the mixture was held at 4°C in the dark for 1 hour. A similar control sample lacked filipin. The samples were layered on 4.5 ml 15-60% linear sucrose gradients in 0.01 M KCl, 0.0015 MgCl, 0.01 M Tris-HCl, pH 7.4, and centrifuged for 1 hr at 35,000 rpm in a Beckman SW56 rotor. Fractions of 0.2 ml were collected, and 25 μl of each was used for radioactivity determinations in a Protosol-toluene-Liquifluor mixture. To the remainder, 0.08 ml of a 1.25% solution of sodium dodecyl sulfate was added, and the optical density at 338 nm was determined on a Zeiss PMQII spectophotometer. (A) Untreated virions; (B) filipin-treated virions.

Figure 5

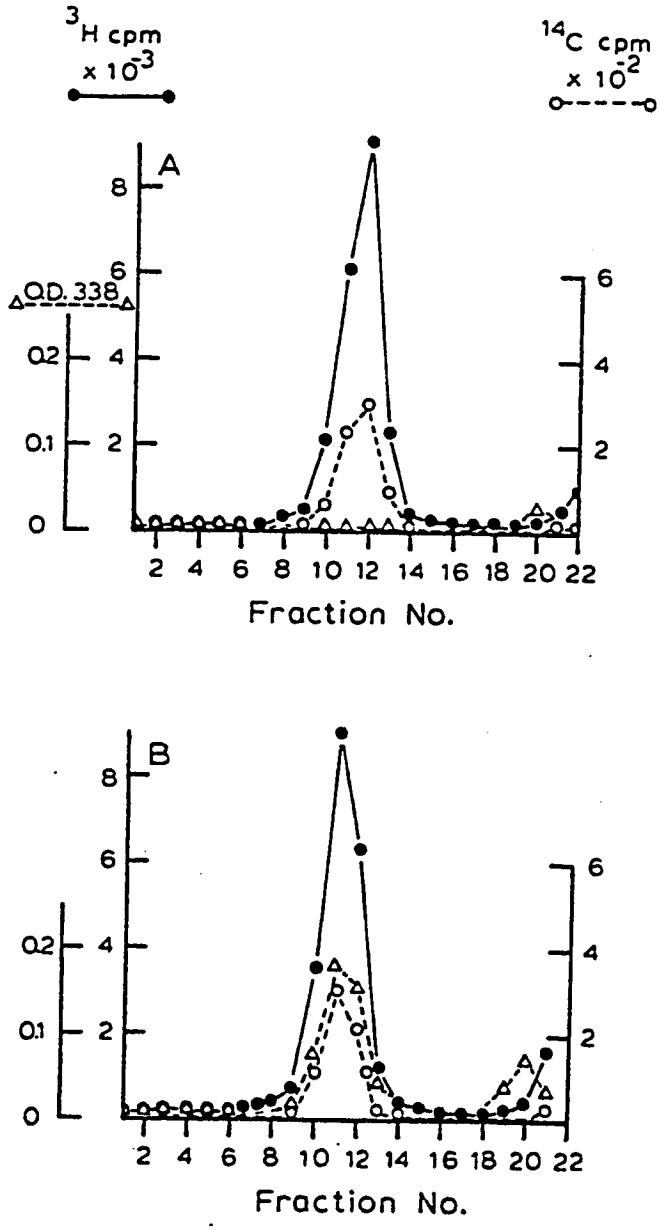


Table 4: Effect of filipin on infectivity of influenza and VS virions. Virions were incubated in the dark with filipin for approximetly 30 min. Cholesterol concentrations varied between 75.3 and 233μM. Control experiments with dimethylformamide showed no inactivation. VS virus plaque assays were carried out on 60 mm BHK-21-F cell monolayers as described previously (115), except that tryptose phosphate broth was not used in the overlay. Influenza virus plaques were as described by Choppin (94) in chicken embryo fibroblasts monolayers.

Table 4

Virus	Molar ratio of cholesterol to filipin	PFU/cell
VSV		
Experiment 1	control	2.5×10^9
	1.0	3.3×10^8
	0.2	5.3×10^6
Experiment 2	control	9.6×10^9
	4.2	2.3×10^9
	1.0	1.1×10^9
	0.4	3.4×10^7
Influenza		
Experiment 1	control	6.8×10^8
	3.7	4.5×10^8
	1.8	3.0×10^8
	0.9	2.3×10^8
Experiment 2	control	7.2×10^9
	0.5	2.6×10^9

Figure 6: Stoichiometry of binding of filipin to intact and spikeless VS virions. To 5 ml of VS virions in phosphate-buffered salt solution, at a concentration of 2.4 and 4.8μM viral cholesterol for intact and spikeless virions, respectively, filipin was added at concentrations that varied from 4.0 to 32.0μM. After the samples were incubated the virions were pelleted and the filipin concentration in the supernatant was measured. Bound filipin concentration was determined as the difference between initial and final concentrations.
A. Spikeless. B. Intact virions.

Figure 6

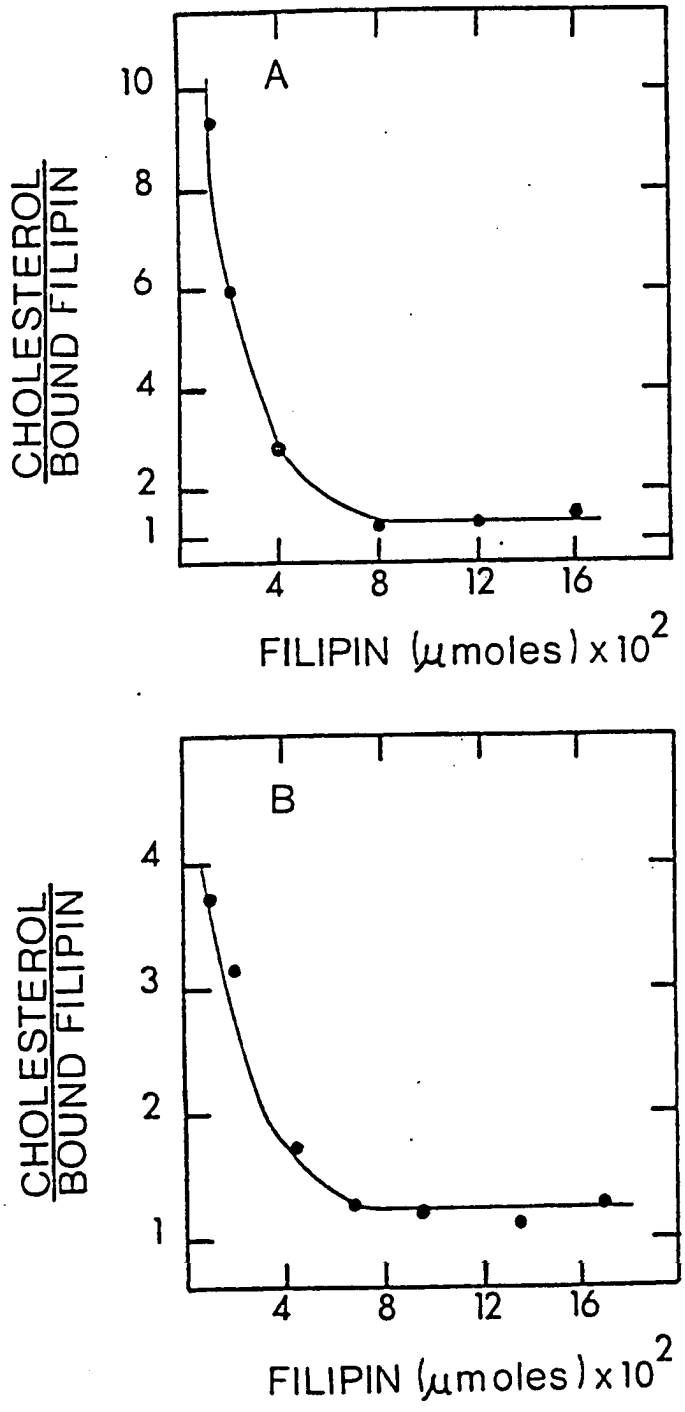


Figure 7: A plot of the ratio of the total filipin to bound filipin (C_T / C_B) versus the reciprocal of the free viral cholesterol concentration in the VS virion suspension. For intact virions (■), the final filipin concentration was held constant at 4.2 μM and the cholesterol concentration was varied from 63.1 to 631 μM. For spikeless particles (●), cholesterol concentrations varied from 6.2 to 84 μM at a constant filipin concentration of 7.1 μM.

Figure 7

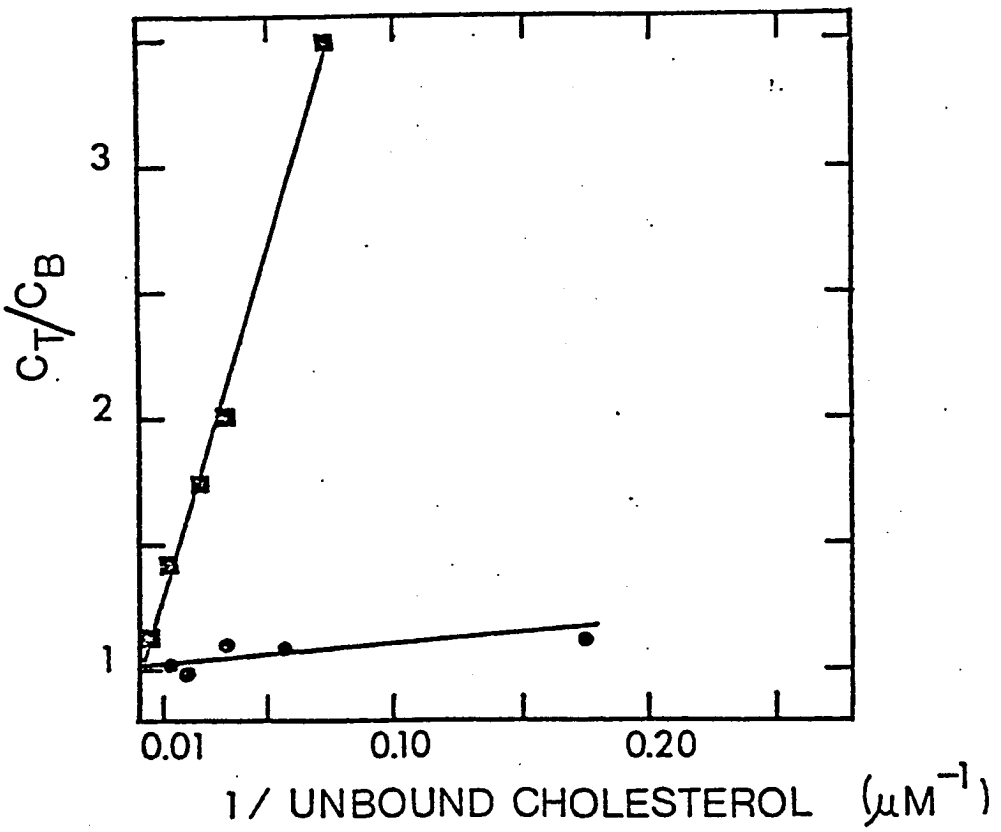


Figure 8: Effect of viral cholesterol concentration on the initial rate of association of filipin with intact (○) and spikeless virions (●). The filipin concentration was 2.4 μM.

Figure 8

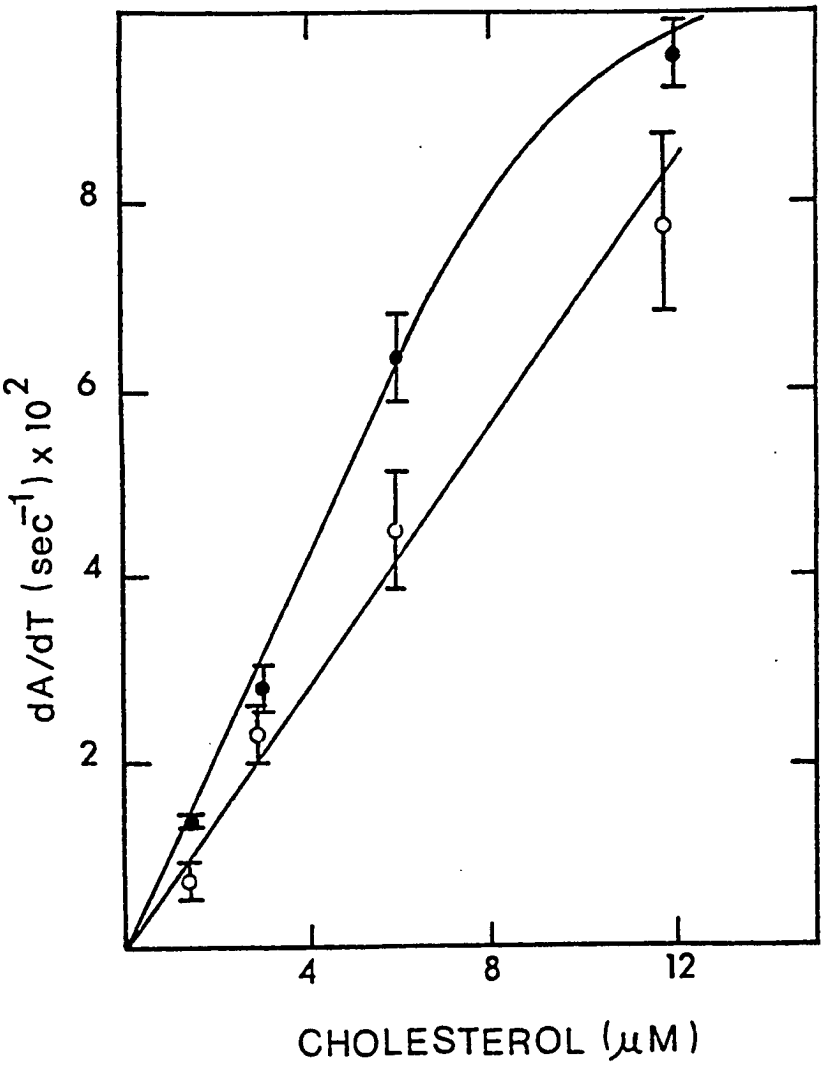
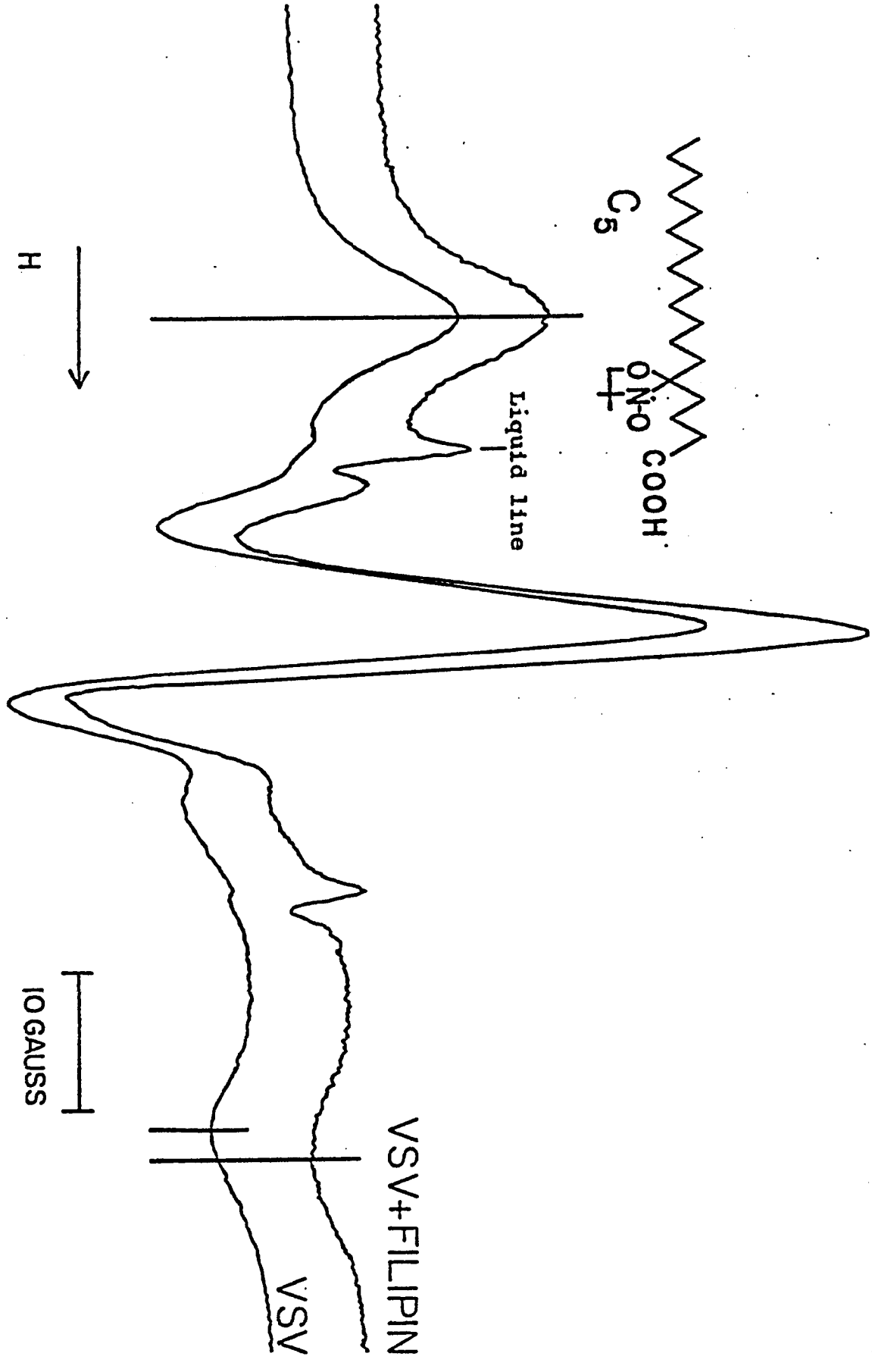


Figure 9: Comparison of the ESR spectra of C_5 spin-labeled untreated and filipin-treated VS virions. A line has been drawn through the high and low magnetic field peaks to demonstrate the greater splitting with filipin-treated virions. The cholesterol/filipin molar ratio was 0.89 and the concentration of dimethylformamide was 9.1 % (v/v).

Figure 9



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