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STUDIES ON THE RELATIVE AFFINITIES OF POLYENE ANTIBIOTICS FOR CHOLESTEROL AND STIGMASTEROL

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STUDIES ON THE RELATIVE AFFINITIES OF POLYENE ANTIBIOTICS FOR CHOLESTEROL AND STIGMASTEROL

By

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ABSTRACT

STUDIES ON THE RELATIVE AFFINITIES OF POLYENE ANTIBIOTICS FOR CHOLESTEROL AND STIGMASTEROL

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By noting the fractional change in the corrected fluorescence of pimaricin or filipin in the presence of a limiting amount of sterol and a competing polyene antibiotic, the relative affinities of amphotericin B, nystatin, filipin, and pimaricin were determined for stigmasterol and cholesterol. The relative affinities of the polyene antibiotics for cholesterol were filipin > amphotericin B > pimaricin > nystatin, while the relative affinities for stigmasterol were filipin > pimaricin > amphotericin B > nystatin. The competition of the polyene antibiotics amphotericin B, pimaricin, and filipin for stigmasterol or cholesterol gave results which indicated the existence of polyene-polyene-sterol ternary complexes. Furthermore, the order of addition of cholesterol or stigmasterol to solutions of pimaricin and filipin affected the degree of binding of these polyenes to the sterol.

The apparent stoichiometries of the interaction of pimaricin or filipin with stigmasterol or cholesterol in dilute aqueous solutions was 1:1.

DEDICATION

I would like to dedicate this thesis to my loving wife and child, Mary Lou and Shanna, my mother, and my grandparents, who have made my life infinitely richer and more meaningful. Their sown seeds of wisdom and love have found fertile fields in the depths of my heart. May their influence continue to radiate throughout creation and enliven the hearts of the Family of Man.

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INTRODUCTION

It has been established that membrane bound sterols are a target for the action of polyene antibiotics. However, the exact mechanism for this interaction has not yet been elucidated. Certain antibiotics, such as filipin, cause gross membrane disruption and consequently have limited clinical value. These effects have been attributed, in part, to the strong affinities of these polyenes for certain sterols. Therefore, a systematic study dealing with the affinities of polyene antibiotics for sterols would be useful.

Aqueous solutions of filipin and pimaricin exhibit characteristic changes in absorbance and fluorescence properties in the presence of sterols. Under certain conditions, the fluorescence of filipin and pimaricin changes in a predictable manner when sterols are added to these polyenes. This predictable change in the fluorescence was utilized to determine the relative affinities of amphotericin B, nystatin, pimaricin and filipin for cholesterol and stigmasterol.

LITERATURE REVIEW

Biological and Model Membrane Evidence for the Interaction of Polyene Antibiotics with Sterols

The proposed structures of several commonly used polyene antibiotics are shown in Figures 1 through 4. Investigations have used these polyenes as well as others to determine their effects on a variety of biological and model systems. These studies include experiments in which the addition of polyene antibiotics to cells resulted in a loss of cytoplasmic constituents (Caltrider and Gottlieb [1]; Sutton et al. [2]; Marini et al. [3]; Kinsky [4,5]), which is indicative of altered membrane permeability. Experiments performed by Lampen and Arnow [6] and Lampen et al. [7] have implicated the cell membrane as the site of nystatin activity. They found it necessary for fungal cells to accumulate nystatin in order to achieve a subsequent inhibition in cell growth. Also, they observed high concentrations of nystatin had little effect on the growth of bacteria, which accumulated only small amounts of the antibiotic. From these experiments, they concluded that fungi, and not bacteria, are affected by polyene antibiotics because bacteria do not contain membrane bound sterols which are necessary for polyene activity.

Data from various workers suggest that the interaction of polyene antibiotics with sterols is responsible for the inhibition of cell growth and other changes in cell viability. Early work by Gottlieb et al. [8,9] and Lampen et al. [10] recognized the addition of sterol

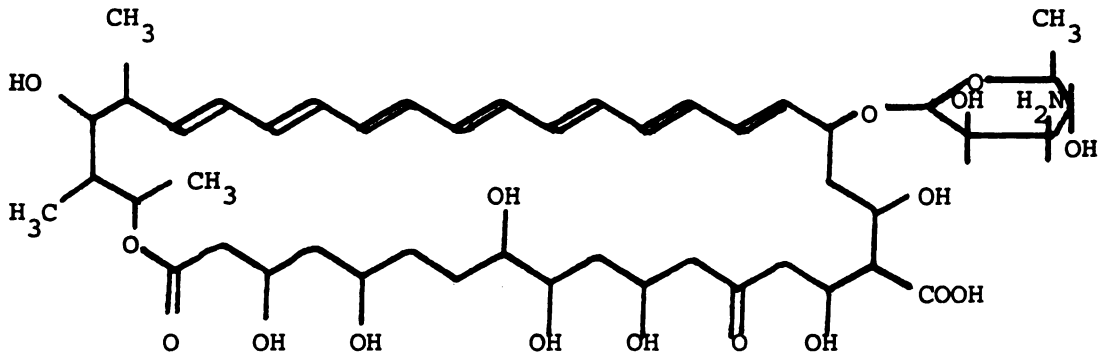

Figure 1. Structure of Amphotericin B.

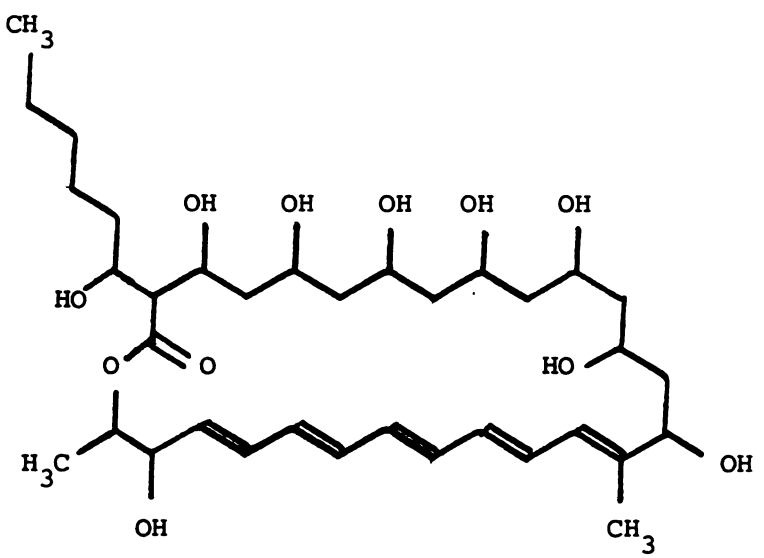

Figure 2. Structure of Filipin III.

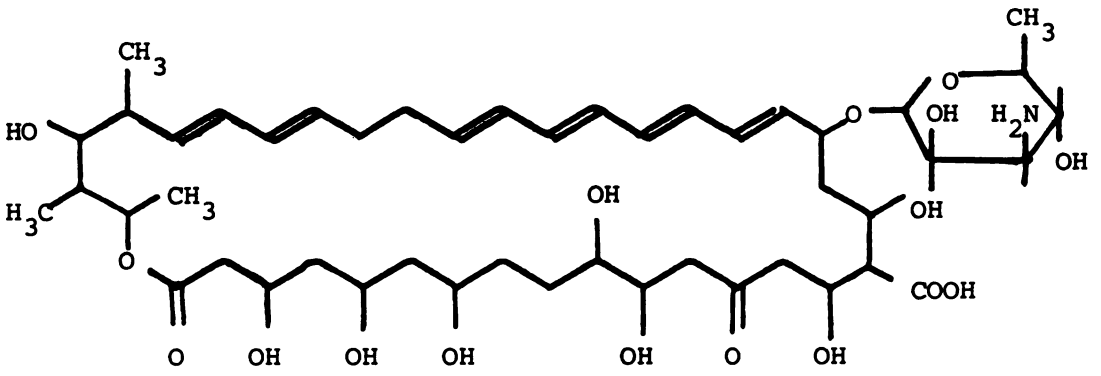

Figure 3. Structure of Nystatin.

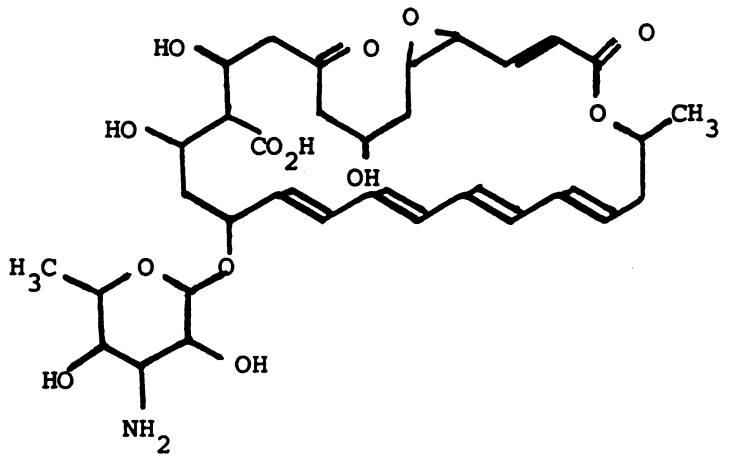

Figure 4. Structure of Pimaricin.

to a medium containing *Saccharomyces cerevisiae* and polyene antibiotics resulted in a decrease in the antifungal activity of the polyenes. They attributed this effect to the interaction of the polyenes with the added sterol which resulted in a lowering of the amount of polyene antibiotic available to inhibit the growth of the fungi. Additional evidence for the interaction of polyene antibiotics with sterol was provided by Feingold [11] and Weber and Kinsky [12]. This involved the use of *Acholeplasma laidlawii*. This organism is unable to synthesize sterols *de novo* but, if grown in the presence of sterol, the sterol is incorporated into the cell membrane. By using this organism, they observed that filipin had no effect upon the growth of these cells in the absence of sterol. However, with cells containing sterol, filipin and amphotericin B severely inhibited their growth. Other work with *Pythium* sp. by Schlosser and Gottlieb [13,14] and Schlosser et al. [15] gave similar results.

A variety of model membrane systems have been used to verify the need of membrane sterols for polyene antibiotic activity. Weissman and Sessa [16] and Sessa and Weissman [17] studied the loss of chromate, glucose, and phosphate from liposomes, with and without incorporated sterol, upon the addition of polyene antibiotics. It was reported that at concentrations of approximately .1 mM to 1 mM, nystatin, amphotericin B, etruscomycin, and filipin could enhance the release of the marker compounds. They also noted, in contrast to the results of other workers, that amphotericin B and nystatin were more potent than filipin and etruscomycin in their ability to release ions from cholesterol containing liposomes. Zutphen et al. [18] noted filipin, nystatin, etruscomycin, and pimaricin at concentrations of

10^{-5} M were able to disrupt lipid films containing lecithin and cholesterol in a 1:1 molar ratio. However, these antibiotics were unable to disrupt lecithin bilayer membrane while using similar concentrations of the polyenes; but at filipin and nystatin concentrations of 10^{-4} M to 10^{-3} M, lecithin bilayers, without cholesterol, were disrupted. These results are consistent with the results seen by Weissman and Sessa at high concentrations of polyenes. Therefore, it is quite likely that at high concentrations of polyene antibiotics, ($>10^{-4}$ M) certain nonspecific detergent effects may arise that do not require the presence of sterol. Experiments performed by Demel et al. [19] with filipin, nystatin, and pimaricin support this idea. These polyenes were able to interact with lecithin monolayers in the absence of cholesterol at high ratios of antibiotic/lipid. However, at a low ratio of polyene antibiotic/lipid, etruscomycin, amphotericin B, and pimaricin interact only with cholesterol monolayers. Additional work by other investigators has implicated membrane bound sterols as the site of polyene activity. DeKruijff et al. [20] observed cholesterol containing lecithin liposomes were necessary to effect permeability changes with filipin, amphotericin B, nystatin, and etruscomycin. Also, Cass et al. [21] have reported sterol is required for the activity of nystatin and amphotericin B on thin lipid membranes.

Spectrophotometric and Fluorimetric Evidence for the Interaction of Polyene Antibiotics with Sterols

Spectrophotometric and fluorimetric techniques are widely used in polyene antibiotic research. The initial spectrophotometric studies with polyene antibiotics were done by Lampen et al. [10], using filipin, nystatin, and antimyocin, and by Gottlieb et al. [22],

who studied filipin. When cholesterol was added to aqueous solutions of these polyenes, the absorbance of these polyenes decreased significantly. This might have been due to a decrease in the solubility of the polyenes. However, it was noted that the absorbance maxima of filipin was also significantly altered. This effect could not be explained in terms of solubility and indicated an interaction between the sterol and the polyene antibiotic. Norman et al. [23] studied the changes in the ultraviolet spectra of polyenes upon the addition of sterols and found when polyenes were added to liposomes which did not contain sterols, no spectral changes were observed, whereas the use of sterol containing liposomes resulted in altered absorbance manifolds for the antibiotics. This same effect was seen for polyenes in the presence of free cholesterol, RBC ghost membranes, and membrane fractions from *Acholeplasma laidlawii* cells grown in the presence of cholesterol.

Schroeder et al. [24,25] have employed the use of fluorescence measurements to investigate the polyene sterol interaction. They noted a 62% decrease in the corrected fluorescence (CO) and a 36% decrease in the relative fluorescence efficiency (RFE) of filipin upon the addition of cholesterol. The quantity RFE is related to the quantum efficiency of the molecule. At wavelengths where only a single fluorophore is absorbing, the changes in RFE are independent of the concentration of the fluorophore and are indicative of changes in the quantum efficiency of the emission process. Therefore, a change in RFE upon the addition of sterol to polyene lends strong evidence for some sort of molecular interaction.

The use of absorbance and fluorescence spectroscopy as an index of polyene sterol interaction is subject to the variability of the experimental conditions employed (Norman et al. [26]. Free polyenes and polyene sterol complexes may exist as clusters in aqueous solution under certain conditions (Norman et al. [23,26]; Schroeder et al. [24]). Such aggregation may complicate spectroscopic data. Also, light scattering caused by the highly insoluble sterol can contribute to errors in the measurements. In some cases the absorption changes from the interaction of polyenes with sterols do not correlate with the ability of the sterol to effect fungicidal activity (Gottlieb et al. [22]), and this may reflect problems of insolubility of the components used. In spite of these drawbacks, fluorescence and absorbance data, in general, correlate well with the effects of polyene antibiotics upon biological and model membrane systems (Kleinschmidt et al. [27]; Bittman and Blau [28]; Bittman and Fischkoff [29]; Bittman et al. [30,31]; Crifo et al. [32]; Strom et al. [33,34,35]). Furthermore, the use of RFE, which is independent of the concentration of the fluorophore under certain conditions, may alleviate many of the problems associated with solubility, resulting in more meaningful spectroscopic data.

The Stoichiometry of the Polyene Sterol Interaction

Various methods have been used to determine the stoichiometry of the polyene sterol complex. Most of the methods used have been indirect methods and have produced a wide range of stoichiometries for the different polyene-sterol interactions.

Norman et al. [36], using differential scanning calorimetry, determined the stoichiometries of the cholesterol/polyene complexes

for filipin, etruscomycin, pimaricin, nystatin, and amphotericin B as 2.3, 1.2, 3.5, 2.4, and 7.9, respectively. These measurements suffer from the fact that they must be carried out at high concentrations of polyene and the resulting aggregation of the highly insoluble polyenes may complicate the results. DeKruijff et al. [20] also attempted to measure the stoichiometry of the polyene-sterol interaction by measuring the K^+ effluxed from *Acholeplasma* cells as a function of membrane sterol/polyene ratios. Using this method, they determined sterol/polyene stoichiometries of 0.7, 3.3, 1.6, and 0.3 for filipin, amphotericin B, nystatin, and etruscomycin, respectively. Other investigators have used different methods and different experimental conditions in arriving at a sterol/polyene stoichiometry. The results from Spielvogel et al. [37] and Gent and Prestegard [38] suggested a sterol/polyene stoichiometry of 1:1. Schroeder et al. [24] have also concluded the stoichiometry of filipin/cholesterol to be 1:1.

It has been documented that even small changes in the experimental conditions used in polyene work can alter the results (Patterson et al. [39]). It seems likely that the different methods and experimental conditions used in the above experiments have resulted in the wide range of sterol/polyene stoichiometries.

The Structural Requirements of Sterol for the Interaction of Sterol with Polyene Antibiotics

Experiments have suggested that not all sterols have the ability to interact with polyene antibiotics. For example, filipin is unable to interact with epicholestanol (Schroeder et al. [24]). This has led to investigations into the structural requirements of sterol that are necessary for the interaction of polyene antibiotics with sterol.

Norman et al. [23] have determined the presence of a cholestane ring structure containing a Δ22 bond produced the most favorable polyene-sterol interaction in aqueous solution. In lecithin-sterol liposomes, they noted the additional requirements of a 3B-OH on the steroid nucleus. Using RFE measurements, Schroeder et al. [24] have verified the need for a 3B-OH and a 17 alkyl side chain for interaction of filipin with sterol. Other investigators have also observed the requirement of a 3B-OH and an alphatic side chain on the steroid nucleus for favorable polyene-sterol interaction (Kleinschmidt et al. [27]; DeKruijff et al. [20]; Norman et al. [36]; Bittman et al. [30]).

Norman et al. [22,35] have noted the addition of nonionic detergents such as Triton X-100, or organic solvents which are miscible in water, such as dioxane or methanol, resulted in a loss of the filipin-cholesterol interaction. Patterson et al. [39], using filipin and pimaricin, have noted that concentrations of lauryl sulfate and sodium deoxycholate, above their CMC, resulted in the loss of the interaction of filipin and pimaricin with cholesterol. Furthermore, high concentrations of urea can disrupt the filipin-cholesterol complex (Demel et al. [19]; Patterson et al. [39]). These results suggest the interaction of polyenes with sterols is primarily hydrophobic.

The Relative Affinities of Polyene Antibiotics for Sterols

Variations have been observed in the degree of damage caused by polyenes in natural and model membranes (Zutphen et al. [18]; Kinsky et al. [40,41]; Crillo et al. [42]). These differences may be the result of different affinities of polyene antibiotics for membrane bound sterols. Several experimental methods and conditions have been

used to determine these relative affinities. These experimental methods have included the use of thin lipid films (Cass et al. [21]), single lipid bilayer vesicles (Gent and Prestegard [38]), cholesterol monolayers (Demel et al. [19]), *Neurospora* protoplasts (Kinsky [40]), free cholesterol (Norman et al. [36]; Bittman et al. [29]), liposomes (Norman et al. [36]), erythrocyte membranes (Norman et al. [36]), and *Acholeplasma* membranes (Norman et al. [36]). These investigations have given differences in experimental results. For example, Norman et al. [36] have observed an order of relative affinities of filipin > etruscomycin > amphotericin B > nystatin = pimaricin for free cholesterol, while for liposomes they observed an order of filipin > amphotericin B > etruscomycin >> nystatin or pimaricin. These differences in experimental results are likely due to the wide range of experimental conditions used. However, in spite of these differences, most of the results from the above investigations gave an order of affinity of polyene for cholesterol as filipin > amphotericin B > nystatin and pimaricin.

MATERIALS AND METHODS

Special chemicals were obtained as follows: cholesterol, stigmasterol, nystatin, and amphotericin B from Sigma Chemical Co., St. Louis, MO; silicic acid (Bio-Sil A, 100-200 mesh) from Bio-Rad Laboratories; and pimaricin was a generous gift from the American Cyanamid Co., Princeton, NJ. Other chemicals used were of reagent grade.

Preparation of Filipin

Streptomyces filipinensis was kindly provided by P. G. Pridham, ARS, USDA, Peoria, IL. The organism was grown for 3 days, at 30°C with shaking, in 3 ml of media containing .4 g palmitate, .4 g yeast extract (Difco), and 1.0 g malt extract (Difco) per 100 ml of distilled water. After 3 days the entire sample was transferred to 500 ml of the same media and allowed to incubate for an additional 5 days. The media and cells were centrifuged at 500 x g for 10 minutes and the precipitate discarded. To the supernatant was added an equal volume of ethyl acetate and, after stirring for 10 minutes, the solution was centrifuged as above. The organic layer was then concentrated *in vacuo* to a volume of 15 ml and mixed with 30 ml of ligroine. After 15 minutes at 4°C, a yellow precipitate formed which was collected after centrifugation on an analytical centrifuge.

Further purification was achieved by a modification of the chromatographic procedure described by Bergy and Eble [45]. Silicic

acid (30 g) was equilibrated in a 97/3 (v/v) solution of methylene chloride/methanol. The slurry was poured into a 2.3 cm diameter column and allowed to settle. Thirty milligrams of filipin, purified as described above, was dissolved in the methylene chloride/methanol solution and added to the column, followed by washing with 125 ml of the same solvent. Elution of the filipin complex was achieved with approximately 300 ml of a 90/10 (v/v) mixture of methylene chloride/methanol. The eluate was monitored at 365 nanometers, and those fractions containing the polyene were evaporated *in vacuo*, resuspended in t-butyl alcohol, and lyophilized.

Methods

Stock solutions of filipin and pimaricin were prepared by dissolving 1 mg of the polyenes in 200 ml of distilled water with vigorous stirring at room temperature for 24 hours in the dark. Stock solutions of nystatin and amphotericin B were prepared by dissolving 2 mg of the antibiotics in 283 ml of distilled water with vigorous stirring as above. Solutions of cholesterol and stigmasterol were prepared by dissolving various amounts of the sterols in isopropanol to give the concentrations needed. The sterols were added to the polyenes by injection with a Hamilton syringe.

Fluorescence measurements were obtained using a computer centered spectrofluorimeter. The analytical quantity, absorbance corrected fluorescence (CO), was used in these experiments. This quantity is discussed in detail elsewhere (Schroeder et al. [24]; Holland et al. [43]).

For all experiments, filipin was monitored at an excitation wavelength of 338 nanometers and an emission wavelength of 495 nanometers.

Also, pimaricin was monitored at an excitation wavelength of 308 nanometers and an emission wavelength of 405 nanometers. All experimental points presented in the figures and tables of this thesis are the average of 5 separate determinations.

Previous studies by Schroeder et al. [24,25], performed with a batch of filipin received as a gift from the Upjohn Company, showed that freshly prepared solutions of filipin in distilled water do not interact immediately with cholesterol. The absorbance ratio, 338 nanometers/305 nanometers (see Figure 5), of a freshly prepared aqueous solution of filipin was approximately 2.0, and it was observed that this preparation showed little or no decrease in RFE upon the addition of cholesterol, thus indicating a lack of interaction between the sterol and the polyene. However, if allowed to remain for 24 hours at room temperature, or if heated at 50°C for 2 hours, this aqueous solution of filipin underwent a change in the 338 nanometer/305 nanometer absorbance ratio to a value of 1.5. With the subsequent addition of cholesterol, a decrease in RFE of 32-38% was achieved, indicating an interaction of the sterol with the polyene.

Numerous batches of filipin have since been isolated by the procedure described in the Methods. Table I gives the results of the interaction of cholesterol with 12 batches of filipin isolated by the present procedure. From Table I, it can be seen that the 338 nanometer/305 nanometer absorbance ratio varies from a value of 2.90 to a value of 1.58. However, regardless of the 338 nanometer/305 nanometer ratio, filipin retained the ability to interact immediately with cholesterol, as shown by the 32-40% decrease in RFE. The filipin solutions used in these experiments were prepared by stirring at room temperature for

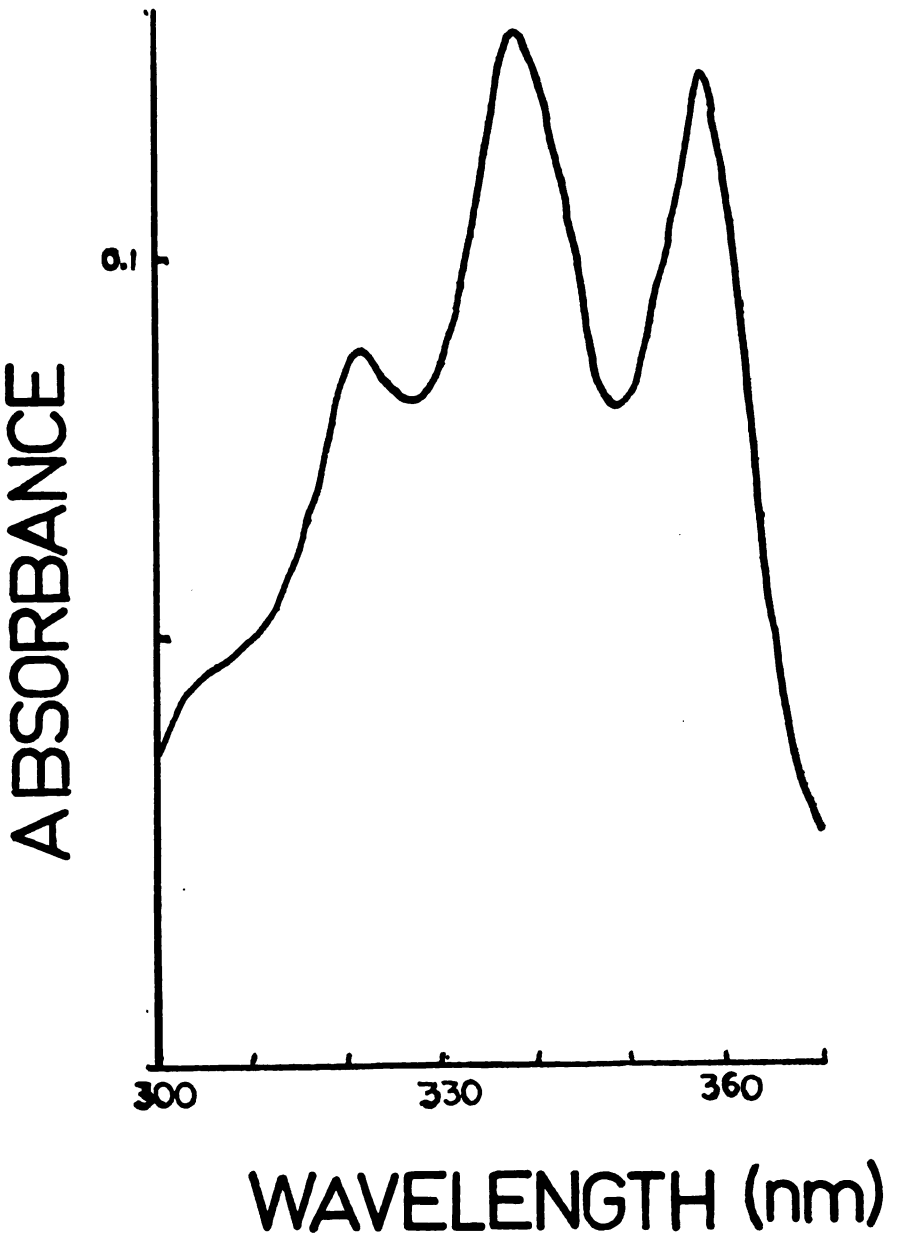

Figure 5. The Absorbance Spectrum of 3.83 μM Filipin in Distilled Water.

TABLE I

The Variation in the Absorbance and Fluorescence Properties of Different Batches of Filipin

Date	Absorbance Ratio[a] 338 nm/305 nm	% Decrease in RFE Upon the Addition of Cholesterol[b]
6/7/76	1.95	40
7/8/76	1.82	34
7/12/76	2.05	36
9/3/76	1.85	40
9/18/76	2.30	32
10/28/76	2.00	32
4/1/77	1.95	38
4/7/77	2.25	35
4/25/77	1.87	33
7/16/77	1.58	33
7/25/77	1.65	36
7/28/77	2.90	35

Filipin was isolated as described in the Methods. Cholesterol (19.8 nanomoles) was added by injection with 10 µl of isopropanol to 11.4 nanomoles of filipin in 3 ml of distilled water. Samples were incubated for 2 hours at 37°C and allowed to cool to room temperature before measurements were made.

[a]The 338 nm/305 nm ratio is the ratio of the absorbance values at those wavelengths.

[b]The measurements for RFE were made at an excitation wavelength of 338 nanometers and an emission wavelength of 495 nanometers.

24 hours in the dark. Also, the experimental techniques used in these experiments allowed cholesterol to incubate for 2 hours at room temperature with filipin. However, solutions of filipin stirred in distilled water for only 15 minutes had the ability to interact with cholesterol and the addition of cholesterol to filipin with an incubation time of only 5 minutes allowed for an essentially complete interaction of the polyene with sterol.

Recent filipin preparations have given evidence for a partially inactive form of filipin that gives a decrease in RFE of only 20-22%. These preparations become more active with time and, after 4 days in distilled water, the observed decrease in RFE was 29%. Some changes in the absorbance ratio were noted with this increase in activity. However, they were not the same changes that were noted by Schroeder et al. [25].

The basis for these differences in absorbance ratios and inactive and active forms of filipin is not known. It is known that filipin exists as a series of isomers and that the various isomers have different affinities for sterols. Therefore, different mixtures of isomers could explain some of the above results; however, this has yet to be proved. Experiments were also performed that ruled out the isolation procedure used by Upjohn and described by Whitfield et al. [44] as causing the differences discussed above.

RESULTS

The Stoichiometry of the Interaction of Filipin and Pimaricin with Cholesterol and Stigmasterol

Figure 6 represents the change in the fluorescence of filipin upon the addition of increasing amounts of cholesterol. The CO decreases from 100 to 37 at a cholsterol/filipin ratio of 1.13. With the further addition of cholesterol, to a cholesterol/filipin ratio of 5.65, there is a small additional decrease in the CO. From this figure, the apparent stoichiometry of cholesterol/filipin is 1.1. Figure 7 gives the change in the fluorescence of filipin upon the addition of increasing amounts of stigmasterol and presents essentially the same type of results as Figure 6. The stoichiometry of stigmasterol/filipin from this figure is .97.

Figure 8 indicates the change in the fluorescence of pimaricin with the addition of increasing amounts of cholesterol. The CO increases sharply from 0 to 64 where the cholesterol/pimaricin ratio is 1.13. With the further addition of cholsterol, the CO increase is less per mole of cholesterol. The observed stoichiometry of cholesterol/pimaricin is 1.15. Figure 9 exhibits the change in the fluorescence of pimaricin upon the addition of increasing amounts of stigmasterol. The results are similar to Figure 8 and give an apparent stoichiometry of 1.1 for stigmasterol/pimaricin.

Figure 6. Titration of Filipin with Cholesterol.

Cholesterol (0, 1.6, 3.2, 5.4, 6.5, 8.0, 13.0, 21, 32, and 65 nanomoles) was added by injection with 10 µl of isopropanol to samples containing 11.4 nanomoles of filipin in 3 ml of distilled water. Samples were incubated for 2 hours at 37°C and allowed to cool to room temperature before measurements were made. Samples were measured at an excitation wavelength of 338 nanometers and an emission wavelength of 495 nanometers.

CO = corrected fluorescence

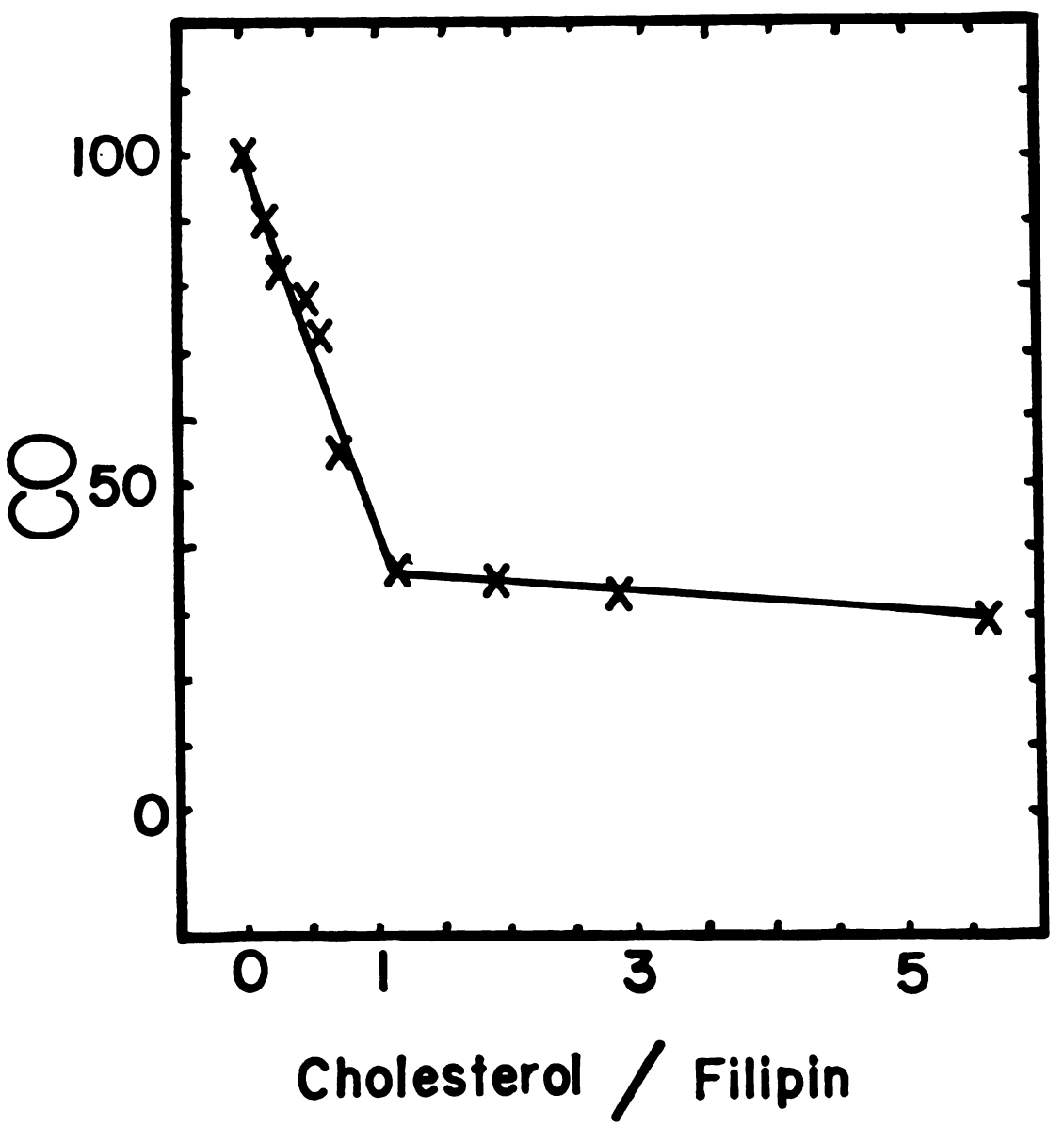

Figure 6

Figure 7. Titration of Filipin with Stigmasterol.

Stigmasterol (0, 1.6, 3.1, 5.2, 6.3, 7, 8, 12, 21, 31, and 63 nanomoles) was added by injection with 10 µl of isopropanol to samples containing 11.4 nanomoles in 3 ml of distilled water. Samples were incubated for 2 hours at 37°C and allowed to cool to room temperature before measurements were made. Samples were measured at an excitation wavelength of 338 nanometers and an emission wavelength of 495 nanometers.

CO = corrected fluorescence

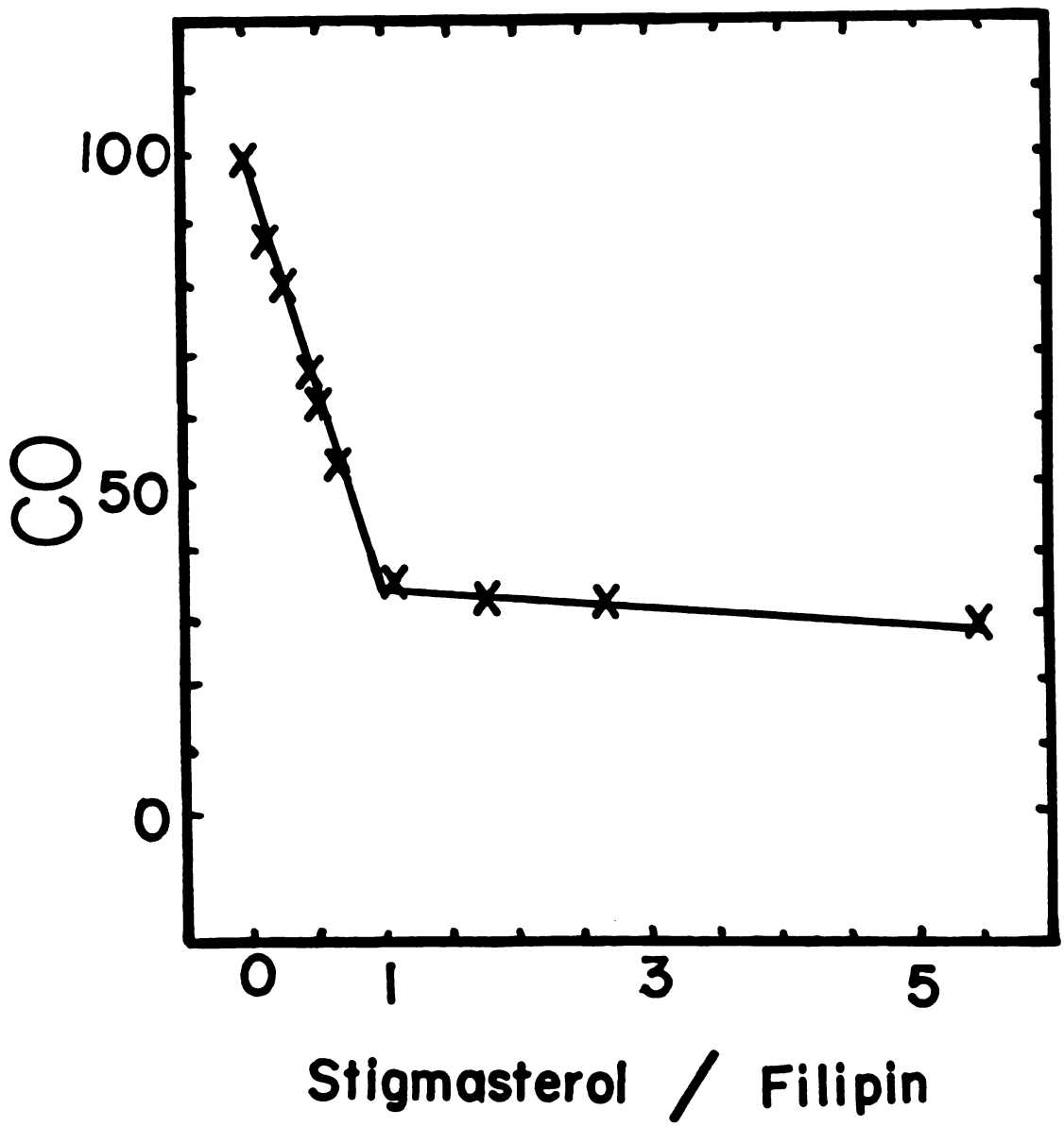

Figure 7

Figure 8. Titration of Pimaricin with Cholesterol.

Cholesterol (0, 1.6, 3.2, 5.4, 6.5, 8.0, 18, 21, 32, and 65 nanomoles) was added by injection with 10 µl of isopropanol to samples containing 11.4 nanomoles of pimaricin in 3 ml of distilled water. Samples were incubated for 2 hours at 37°C and allowed to cool to room temperature before measurements were made. Samples were measured at an excitation wavelength of 308 nanometers and an emission wavelength of 405 nanometers.

CO = corrected fluorescence

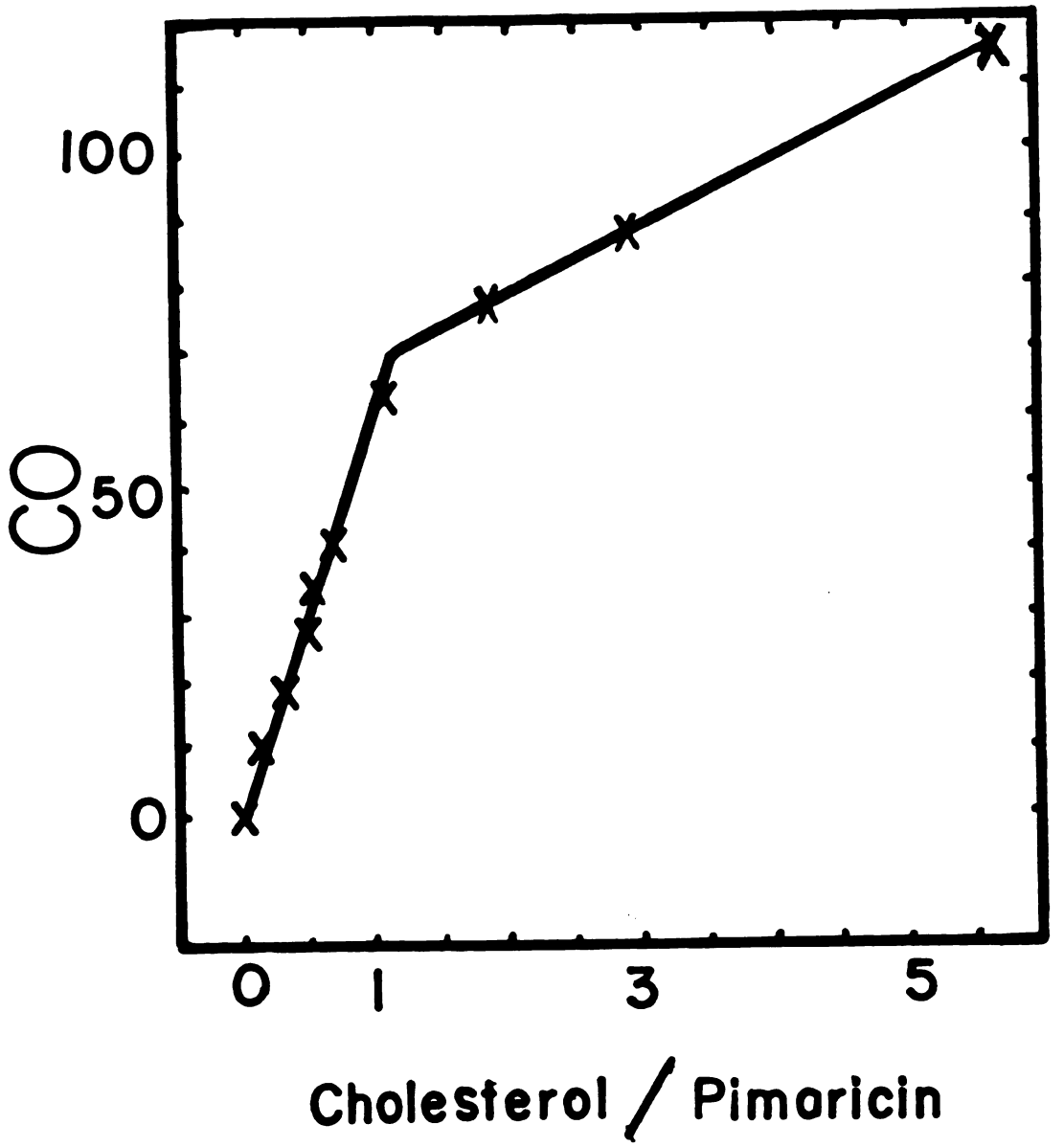

Figure 8

Figure 9. Titration of Pimaricin with Stigmasterol.

Stigmasterol (0, 1.6, 3.1, 5.2, 6.3, 7, 8, 12, 21, 31, and 63 nanomoles) was added by injection with 10 µl of isopropanol to samples containing 11.4 nanomoles of pimaricin in 3 ml of distilled water. Samples were incubated for 2 hours at 37°C and allowed to cool to room temperature before measurements were made. Samples were measured at an excitation wavelength of 308 nanometers and an emission wavelength of 405 nanometers.

CO = corrected fluorescence

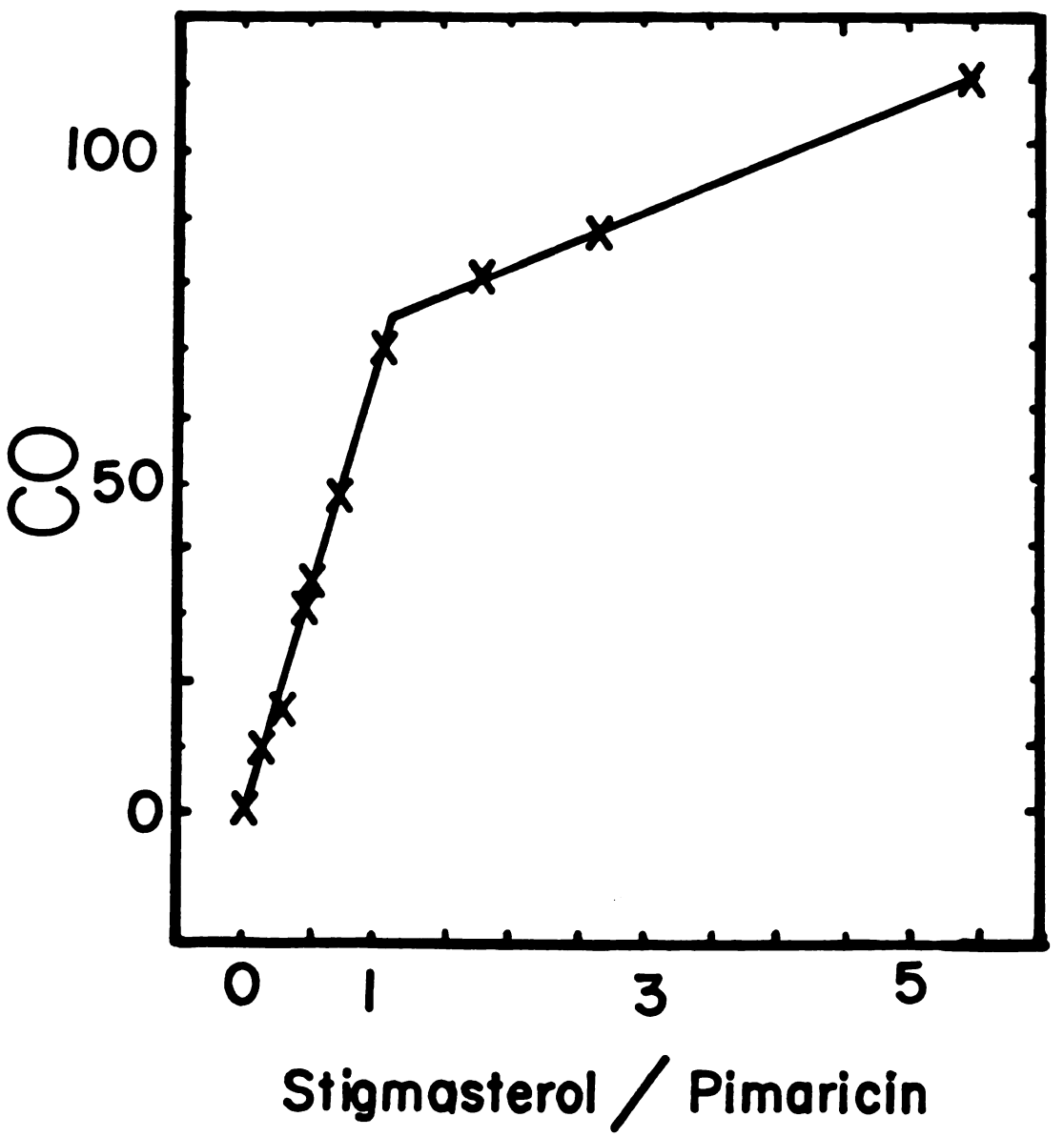

Figure 9

The Effect of Nystatin or Amphotericin B on the Fluorescence Properties of Pimaricin

Figure 10 presents the change in the fluorescence of pimaricin upon the addition of increasing amounts of amphotericin B or nystatin in the presence or absence of cholesterol. In the absence of cholesterol, the CO of pimaricin is unaffected by the addition of amphotericin B or nystatin. In the presence of cholesterol, the CO of pimaricin is 100 and remains unchanged upon the addition of increasing amounts of nystatin. However, as increasing amounts of amphotericin B are added to pimaricin, in the presence of cholesterol, the CO of pimaricin decreases sharply from 100 to 55 at an amphotericin B/pimaricin molar ratio of 0.1. As additional amphotericin B is added, the CO of pimaricin decreases less per mole of added amphotericin B until the CO is 24 at an amphotericin B/pimaricin molar ratio of 1.0.

The experiments shown in Figure 11 are identical to those shown in Figure 10, except that stigmasterol is used instead of cholesterol. As in Figure 10, in the absence of sterol, the CO of pimaricin remains unchanged with the addition of increasing amounts of amphotericin B or nystatin. In the presence of stigmasterol, the CO of pimaricin is 100. Upon the addition of increasing amounts of nystatin, the CO of pimaricin decreases from 100 to 78 at a nystatin/pimaricin molar ratio of 1.0. When increasing amounts of amphotericin B are added to pimaricin, in the presence of stigmasterol, the CO decreases from 100 to 66 at an amphotericin B/pimaricin molar ratio of 0.1. With the addition of more amphotericin B, the CO continues to decrease to 46 at an amphotericin B/pimaricin molar ratio of 1.0.

Controls, not shown here, were run to determine the CO of amphotericin B and nystatin in the presence and absence of cholesterol and

Figure 10. The Effect of Nystatin and Amphotericin B on the Fluorescence Properties of Pimaricin in the Presence and Absence of Cholesterol.

Nystatin or amphotericin B (0, 1.1, 2.3, 3.3, 4.5, 5.7, 7.5, and 11.4 nanomoles) were added to samples containing 11.4 nanomoles of pimaricin. The final volume was 3 ml in distilled water. The samples were incubated for 2 hours at 37°C and allowed to cool to room temperature before measurements were made. Samples were measured at an excitation wavelength of 308 nanometers and an emission wavelength of 405 nanometers. The above experiment was repeated with the addition of 6.5 nanomoles of cholesterol by injection with 10 µl of isopropanol.

X-X-X-X = pimaricin + nystatin
●-●-●-● = pimaricin + amphotericin B
nys = nystatin
amp B = amphotericin B
chole = cholesterol
CO = corrected fluorescence

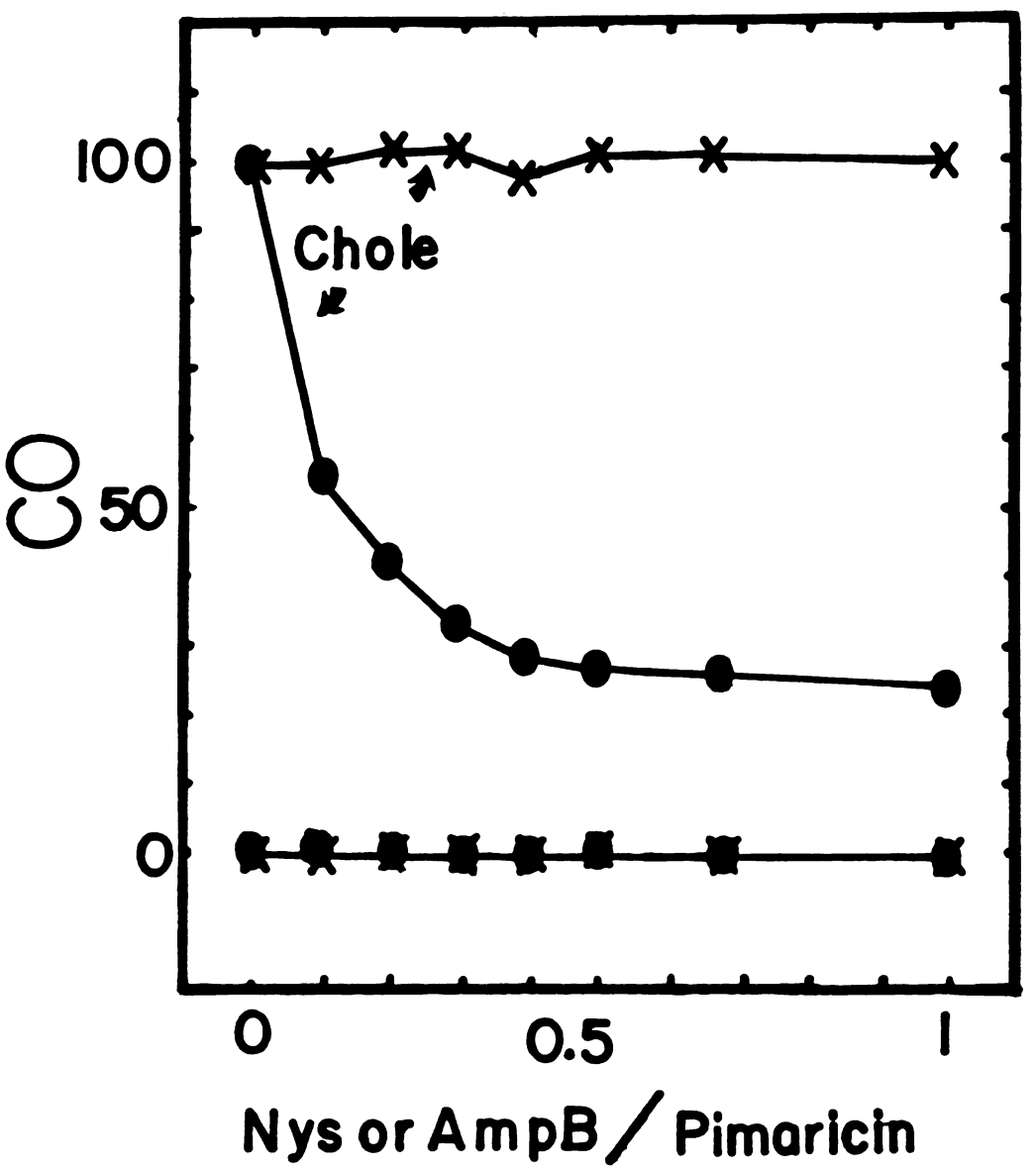

Figure 10

Figure 11. The Effect of Nystatin and Amphotericin B on the Fluorescence Properties of Pimaricin in the Presence and Absence of Stigmasterol.

Nystatin or amphotericin B (0, 1.1, 2.3, 3.3, 4.5, 5.7, 7.5, and 11.4 nanomoles) were added to samples containing 11.4 nanomoles of pimaricin. The final volume was 3 ml in distilled water. The samples were incubated for 2 hours at 37°C and allowed to cool to room temperature before measurements were made. Samples were measured at an excitation wavelength of 308 nanometers and an emission wavelength of 405 nanometers. The entire above experiment was repeated with the addition of 6.5 nanomoles of stigmasterol by injection with 10 µl of isopropanol.

X-X-X-X = pimaricin + nystatin
●-●-●-● = pimaricin + amphotericin B
nys = nystatin
amp B = amphotericin B
stig = stigmasterol
CO = corrected fluorescence

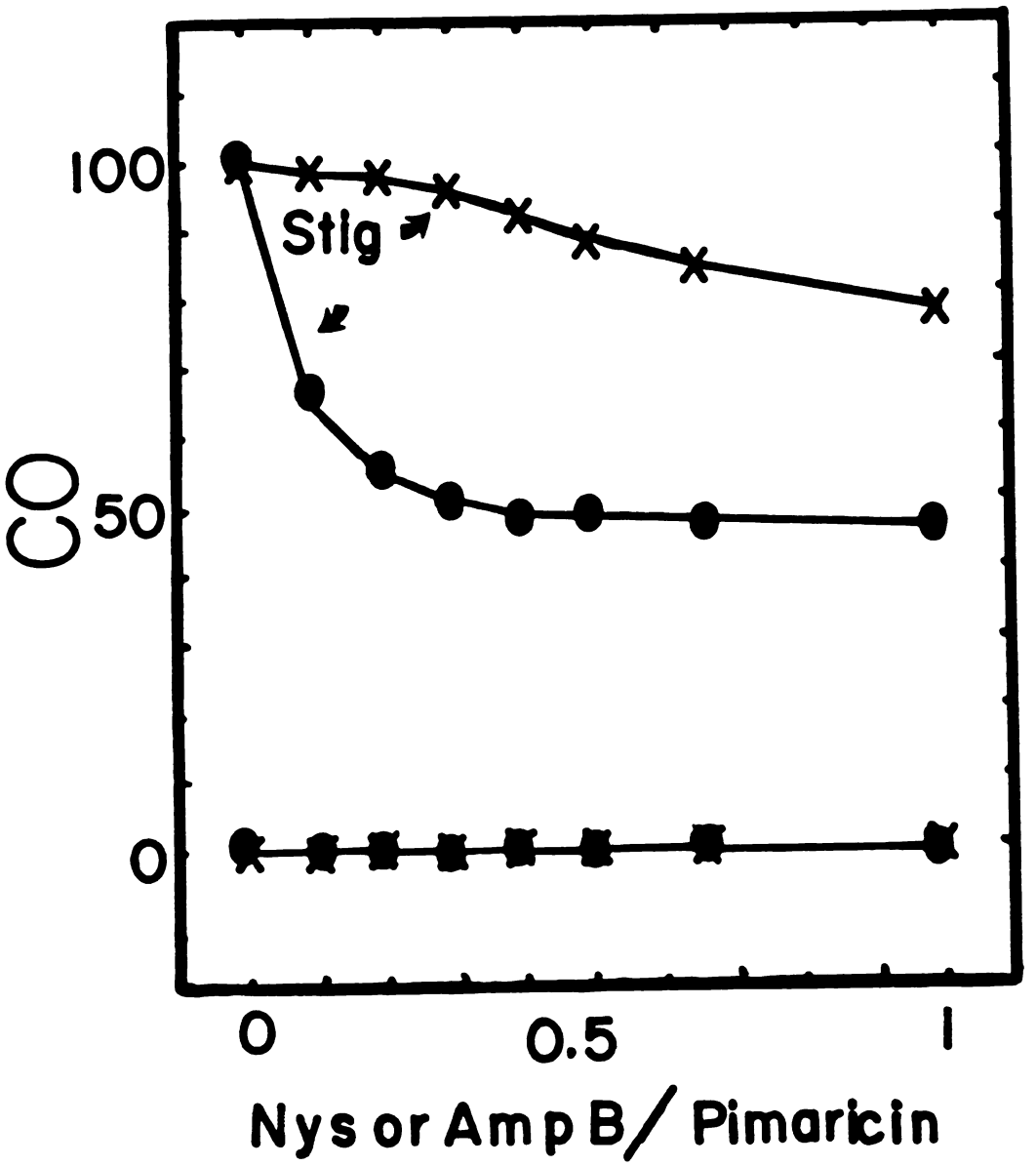

Figure 11

stigmasterol. The CO values were determined at the same wavelengths used in Figures 10 through 13. It was found that amphotericin B and nystatin had CO values of 0 at these wavelengths in the presence and absence of cholesterol and stigmasterol.

The Effect of Nystatin or Amphotericin B on the Fluorescence Properties of Filipin

Figure 12 represents the effect of nystatin or amphotericin B on the fluorescence properties of filipin in the presence and absence of cholesterol. The CO of filipin, in the absence of cholesterol, is 100. The CO of filipin remains at 100, in the absence of cholesterol, as increasing amounts of amphotericin B or nystatin are added up to a nystatin or amphotericin B/filipin molar ratio of 1.0. In the presence of cholesterol, the CO of filipin decreases to a value of 36. With the addition of increasing amounts of nystatin or amphotericin B up to a molar ratio of 1.0, the CO of filipin is unaffected and remains at a value of approximately 36. Figure 13 represents identical experiments as in Figure 12, except that stigmasterol was used instead of cholsterol. The results in Figure 13 are essentially the same as in Figure 12. The CO of filipin remains unchanged with increasing amounts of nystatin or amphotericin B up to a nystatin or amphotericin B/filipin molar ratio of 1.0, in the presence and absence of stigmasterol.

Studies on the Competition of Filipin and Pimaricin for Cholesterol

Figure 14 shows the changes in the fluorescence of pimaricin and filipin with the addition of increasing amounts of pimaricin to a constant amount of filipin, in the presence and absence of cholesterol.

Figure 12. The Effect of Nystatin and Amphotericin B on the Fluorescence Properties of Filipin in the Presence and Absence of Cholesterol.

Nystatin or amphotericin B (0, 1.1, 2.3, 3.3, 4.5, 5.7, 7.5, and 11.4 nanomoles) were added to samples containing 11.4 nanomoles of filipin. The final volume was 3 ml in distilled water. The samples were incubated for 2 hours at 37°C and allowed to cool to room temperature before measurements were made. Samples were measured at an excitation wavelength of 338 nanometers and an emission wavelength of 495 nanometers. The entire above experiment was repeated with the addition of 6.5 nanomoles of cholesterol added by injection with 10 µl of isopropanol.

X-X-X-X = filipin + nystatin
●-●-●-● = filipin + amphotericin B
nys = nystatin
amp B = amphotericin B
chole = cholesterol
CO = corrected fluorescence

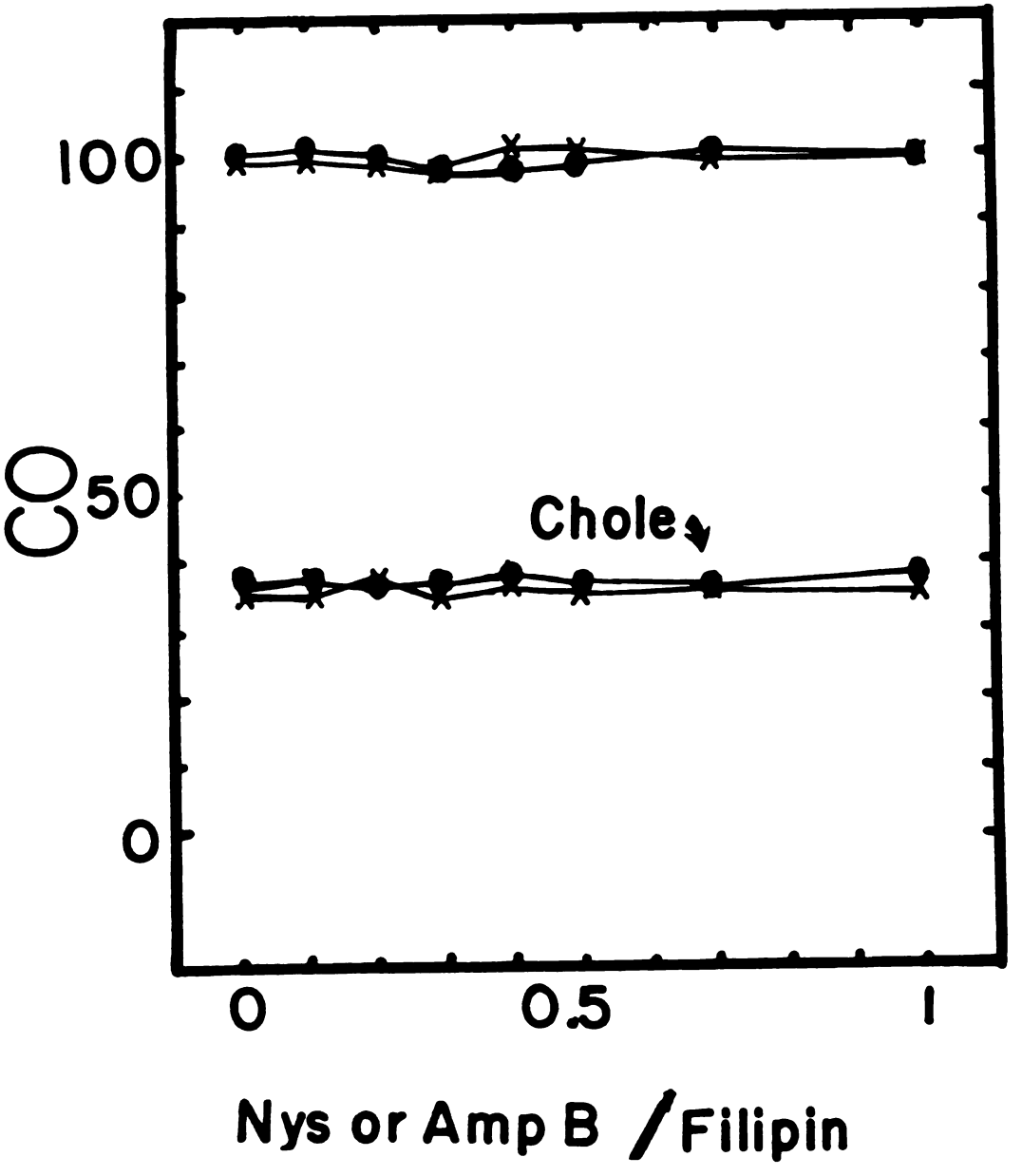

Figure 12

Figure 13. The Effect of Nystatin and Amphotericin B on the Fluorescence Properties of Filipin in the Presence and Absence of Stigmasterol.

Nystatin or amphotericin B (0, 1.1, 2.3, 3.3, 4.5, 5.7, 7.5, and 11.4 nanomoles) were added to samples containing 11.4 nanomoles of filipin. The final volume was 3 ml in distilled water. The samples were incubated for 2 hours at 37°C and allowed to cool to room temperature before measurements were made. Samples were measured at an excitation wavelength of 338 nanometers and an emission wavelength of 495 nanometers. The entire above experiment was repeated with the addition of 6.5 nanomoles of stigmasterol added by injection with 10 µl of isopropanol.

X-X-X-X = filipin + nystatin
●-●-●-● = filipin + amphotericin B
nys = nystatin
amp B = amphotericin B
stig = stigmasterol
CO = corrected fluorescence

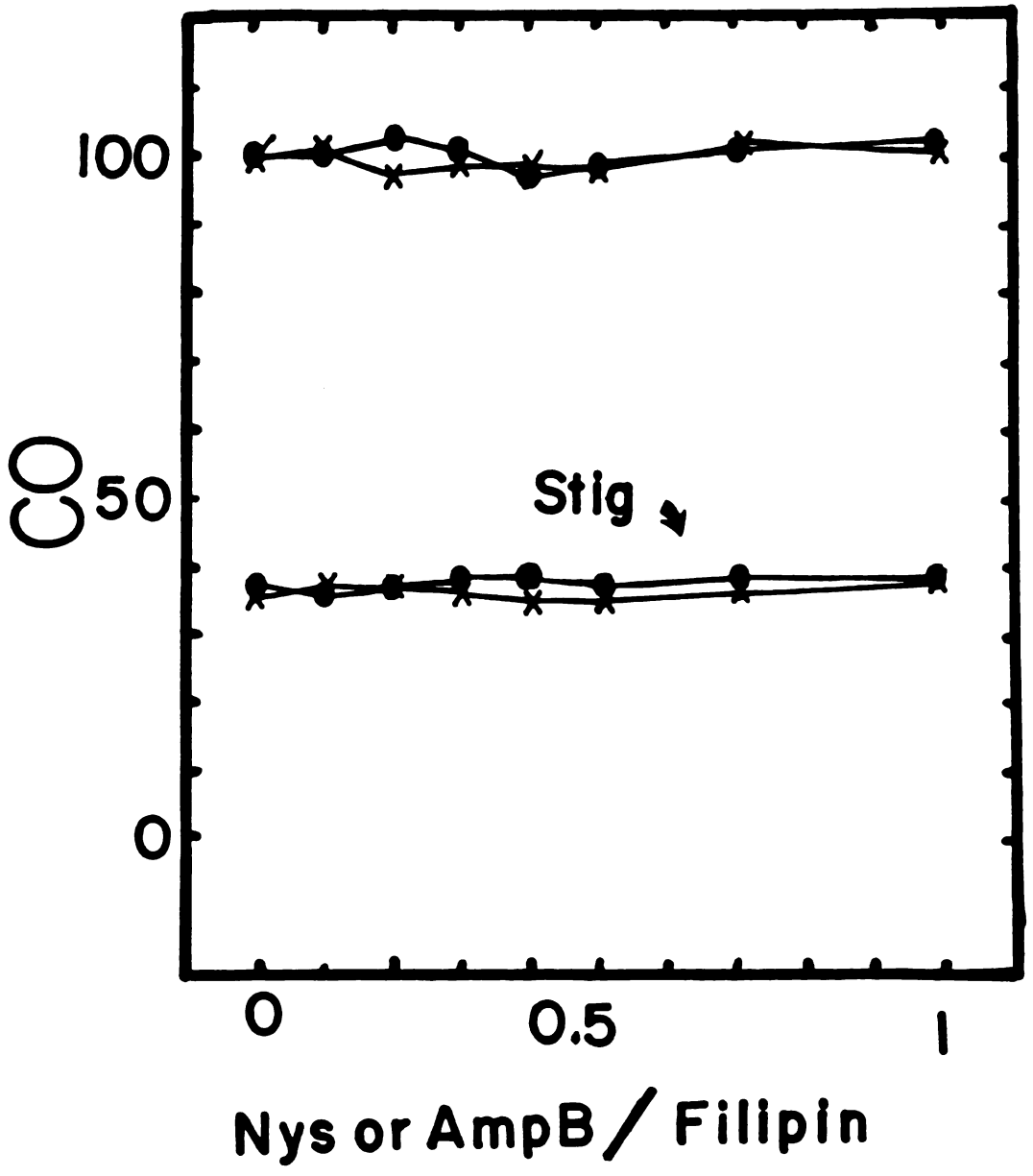

Figure 13

Figure 14. The Effect of Different Amounts of Pimaricin on the Competition of Filipin and Pimaricin for Cholesterol.

Pimaricin (0, 1.1, 2.3, 3.3, 4.5, 5.7, 7.5, and 11.4 nanomoles) was added to samples containing 11.4 nanomoles of filipin. The final volume was 3 ml in distilled water. The samples were incubated for 2 hours at 37°C and allowed to cool to room temperature before measurements were made. Samples were measured at an excitation wavelength of 338 nanometers and an emission wavelength of 495 nanometers. Samples were also measured at an excitation wavelength of 308 nanometers and an emission wavelength of 405 nanometers. The entire above experiment was repeated with the addition of 6.5 nanomoles of cholesterol by injection with 10 µl of isopropanol.

●-●-●-● = measurements made at an excitation wavelength of 308 nanometers and an emission wavelength of 405 nanometers

X-X-X-X = measurements made at an excitation wavelength of 338 nanometers and an emission wavelength of 495 nanometers

O = To 11.4 nanomoles of pimaricin in 3 ml of distilled water, 6.5 nanomoles of cholesterol was added as above. Samples were incubated as above and measurements were made at an excitation wavelength of 308 nanometers and an emission wavelength of 405 nanometers

chole = cholesterol

CO = corrected fluorescence

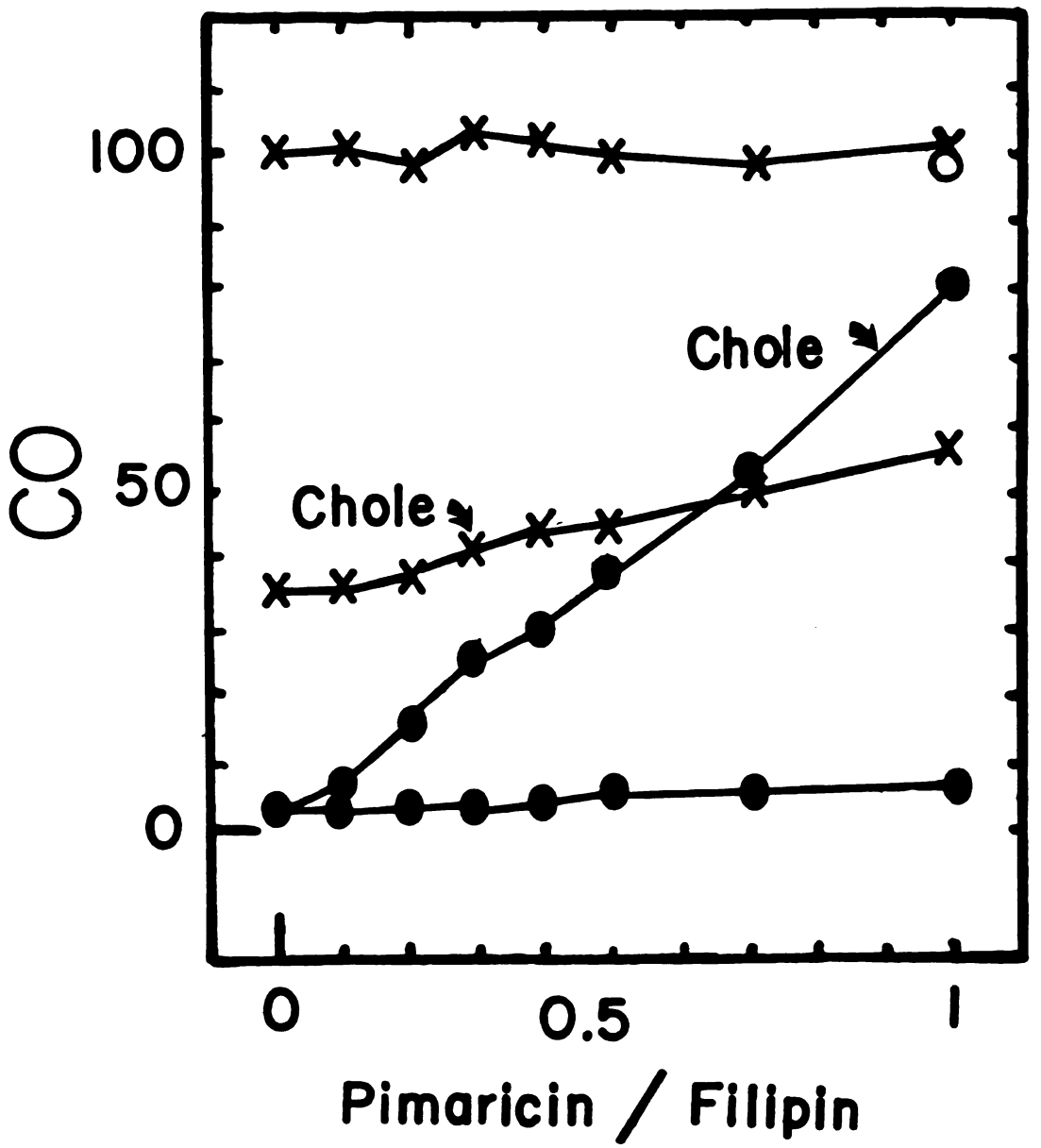

Figure 14

In the absence of cholesterol, the fluorescence of filipin remains unchanged at 100 as increasing amounts of pimaricin are added. However, in the presence of cholesterol and the absence of pimaricin, the CO of filipin is 36 and, as pimaricin is added, the CO increases to 57 at a pimaricin/filipin molar ratio of 1.0. The fluorescence of pimaricin, in the absence of cholesterol, remains essentially unchanged upon the addition of filipin. In the presence of cholesterol, the CO of pimaricin increases to 80 at a pimaricin/filipin molar ratio of 1.0. The CO of pimaricin in the absence of cholesterol and filipin is 1.0. The CO of pimaricin in the presence of cholesterol and the absence of filipin is 99.

The experiments presented in Figure 15 are the same as in Figure 14, except the pimaricin concentration was held constant while the filipin concentration was increased up to a filipin/pimaricin molar ratio of 1.0. The fluorescence of pimaricin, in the absence of cholesterol, remains essentially unchanged upon the addition of filipin. The fluorescence of pimaricin, in the presence of cholesterol, decreases from 100 to 80 as filipin is added to a filipin/pimaricin molar ratio of 1.0, while in the presence of cholesterol, the CO of filipin increases from 10 to 60. The CO of filipin in the absence of cholesterol and pimaricin is 100, while the CO of filipin in the presence of cholesterol and absence of pimaricin is 38.

Studies on the Competition of Filipin and Pimaricin for Stigmasterol

Figure 16 shows the change in the fluorescence of pimaricin and filipin when increasing amounts of pimaricin are added to a constant amount of filipin in the presence and absence of stigmasterol. The

Figure 15. The Effect of Different Amounts of Filipin on the Competition of Filipin and Pimaricin for Cholesterol.

Filipin (0, 1.2, 2.3, 3.3, 4.5, 5.7, 7.5, and 11.4 nanomoles) was added to samples containing 11.4 nanomoles of pimaricin. The final volume was 3 ml in distilled water. The samples were incubated for 2 hours at 37°C and allowed to cool to room temperature before measurements were made. Samples were measured at an excitation wavelength of 308 nanometers and an emission wavelength of 405 nanometers. Samples were also measured at an excitation wavelength of 338 nanometers and an emission wavelength of 495 nanometers. The entire above experiment was repeated with the addition of 6.5 nanomoles of cholesterol added by injection with 10 µl of isopropanol.

●-●-●-● = measurements made at an excitation wavelength of 308 nanometers and an emission wavelength of 405 nanometers

X-X-X-X = measurements made at an excitation wavelength of 338 nanometers and an emission wavelength of 495 nanometers

◘ = Filipin (11.4 nanomoles) in 3 ml of distilled water was measured at an excitation wavelength of 338 nanometers and an emission wavelength of 495 nanometers after incubation as above

O = To 11.4 nanomoles of filipin in 3 ml of distilled water, 6.5 nanomoles of cholesterol was added as described above. The sample was incubated as above and measurements were made at an excitation wavelength of 338 nanometers and an emission wavelength of 495 nanometers.

chole = cholesterol

CO = corrected fluorescence

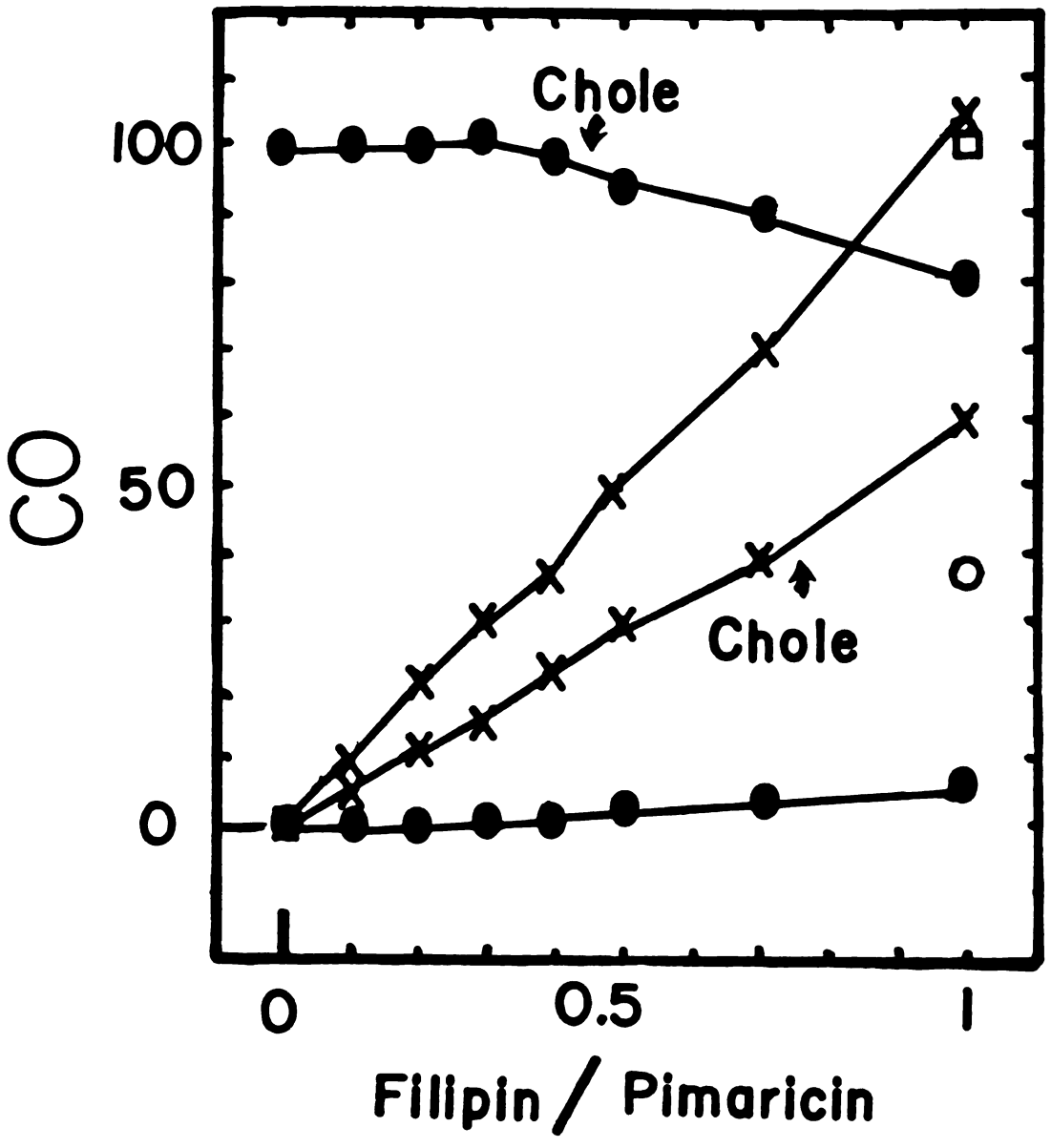

Figure 15

Figure 16. The Effect of Different Amounts of Pimaricin on the Competition of Filipin and Pimaricin for Stigmasterol.

Pimaricin (0, 1.1, 2.3, 3.3, 4.5, 5.7, 7.5, and 11.4 nanomoles) was added to samples containing 11.4 nanomoles of filipin. The final volume was 3 ml in distilled water. The samples were incubated for 2 hours at 37°C and allowed to cool to room temperature before measurements were made. Samples were measured at an excitation wavelength of 338 nanometers and an emission wavelength of 495 nanometers. Samples were also measured at an excitation wavelength of 308 nanometers and an emission wavelength of 405 nanometers. The entire above experiment was repeated with the addition of 6.5 nanomoles of stigmasterol added by injection with 10 µl of isopropanol.

●-●-●-● = measurements made at an excitation wavelength of 308 nanometers and an emission wavelength of 405 nanometers

X-X-X-X = measurements made at an excitation wavelength of 338 nanometers and an emission wavelength of 495 nanometers

O = To 11.4 nanomoles of pimaricin in 3 ml of distilled water, 6.5 nanomoles of stigmasterol was added as above. Samples were incubated as above and measurements were made at an excitation wavelength of 308 nanometers and an emission wavelength of 405 nanometers.

stig = stigmasterol

CO = corrected fluorescence

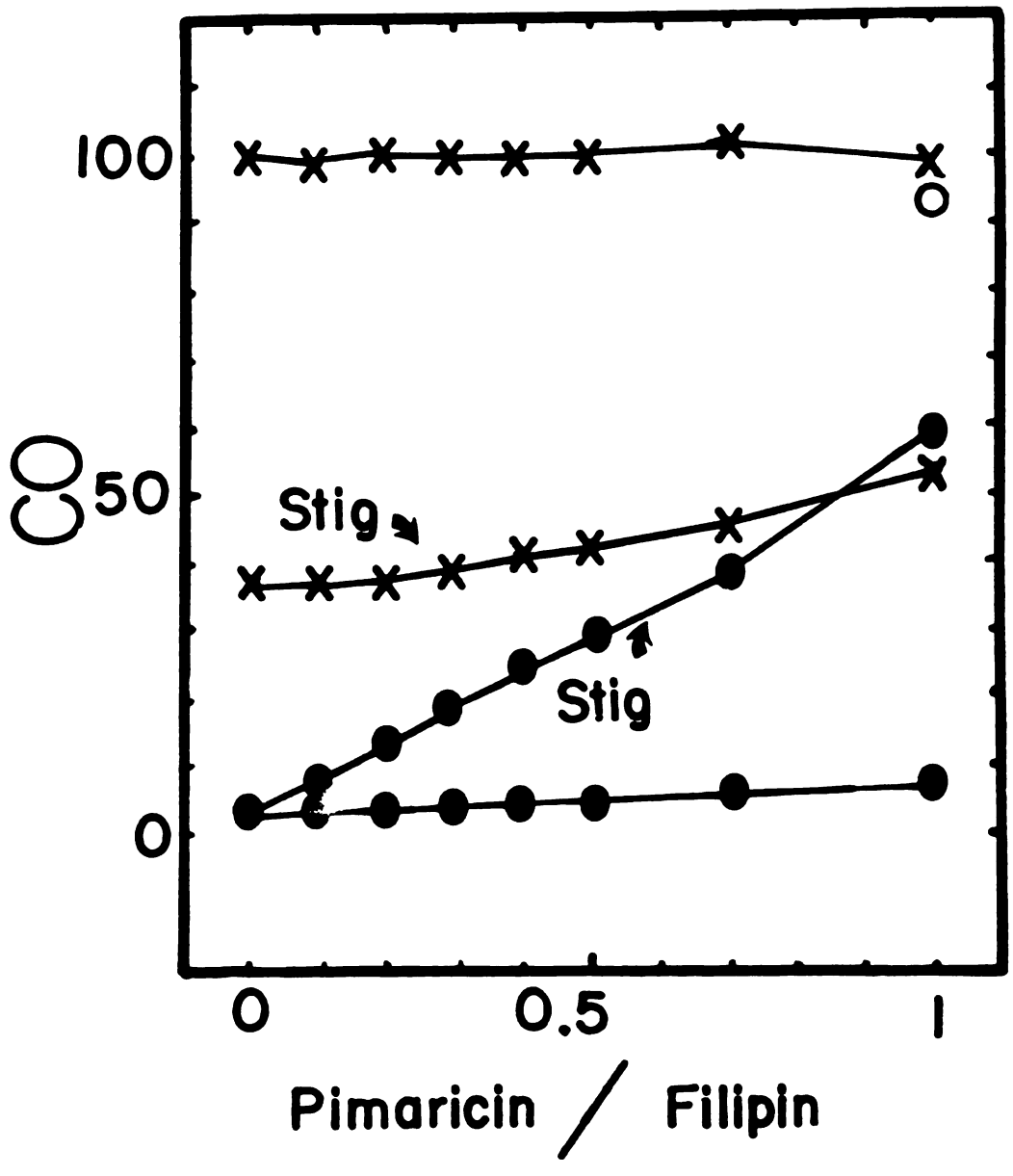

Figure 16

fluorescence of filipin, in the absence of stigmasterol, is unchanged by the addition of pimaricin, while in the presence of stigmasterol the CO of filipin increases from 36 to 53 at a pimaricin/filipin molar ratio of 1.0. In the absence of stigmasterol, the CO of pimaricin increases from 2 to 7, while in the presence of stigmasterol the CO of pimaricin increases from 2 to 59, at a pimaricin/filipin molar ratio of 1.0. The CO of pimaricin in the presence of stigmasterol and absence of filipin is 94.

The experiments presented in Figure 17 are the same as those presented in Figure 16, except the primaricin concentration is held constant while the filipin concentration is increased. In the absence of stigmasterol, the fluorescence of pimaricin remains essentially unchanged; however, in the presence of stigmasterol, the CO of pimaricin decreases from 100 to 67 as filipin is added up to a filipin/pimaricin ratio of 1.0. The CO of filipin, in the absence of stigmasterol, increases from 0 to 103, while in the presence of stigmasterol the CO of filipin increases from 0 to 49, at a filipin/pimaricin molar ratio of 1.0. The CO of filipin in the absence of stigmasterol and pimaricin is 97, while the CO of filipin in the presence of stigmasterol and the absence of pimaricin is 31.

The Effect of the Order of Addition of Cholesterol on the Fluorescence and Light Scattering Properties of Solutions Containing Filipin and/or Pimaricin

Table II shows the effect of the order of addition of cholesterol upon the light scattering and fluorescence properties of solutions containing filipin and/or pimaricin. The data at 338/495 show the CO of filipin and the CO of filipin with pimaricin as approximately the same at 100 and 101, respectively. At 338/495, the addition of

Figure 17. The Effect of Different Amounts of Filipin on the Competition of Filipin and Pimaricin for Stigmasterol.

Filipin (0, 1.1, 2.3, 3.3, 4.5, 5.7, 7.5, and 11.4 nanomoles) was added to samples containing 11.4 nanomoles of pimaricin. The final volume was 3 ml in distilled water. The samples were incubated for 2 hours at 37°C and allowed to cool to room temperature before measurements were made. Samples were measured at an excitation wavelength of 308 nanometers and an emission wavelength of 405 nanometers. Samples were also measured at an excitation wavelength of 338 nanometers and an emission wavelength of 495 nanometers. The entire above experiment was repeated with the addition of 6.5 nanomoles of stigmasterol added by injection with 10 µl of isopropanol.

●-●-●-● = measurements made at an excitation wavelength of 308 nanometers and an emission wavelength of 405 nanometers

X-X-X-X = measurements made at an excitation wavelength of 338 nanometers and an emission wavelength of 495 nanometers

□ = Filipin (11.4 nanomoles) in 3 ml of distilled water was measured at an excitation wavelength of 338 nanometers and an emission wavelength of 495 nanometers after incubation as above

○ = To 11.4 nanomoles of filipin in 3 ml of distilled water, 6.5 nanomoles of stigmasterol was added as described above. The sample was incubated as above and measurements were made at an excitation wavelength of 338 nanometers and an emission wavelength of 495 nanometers.

stig = stigmasterol

CO = corrected fluorescence

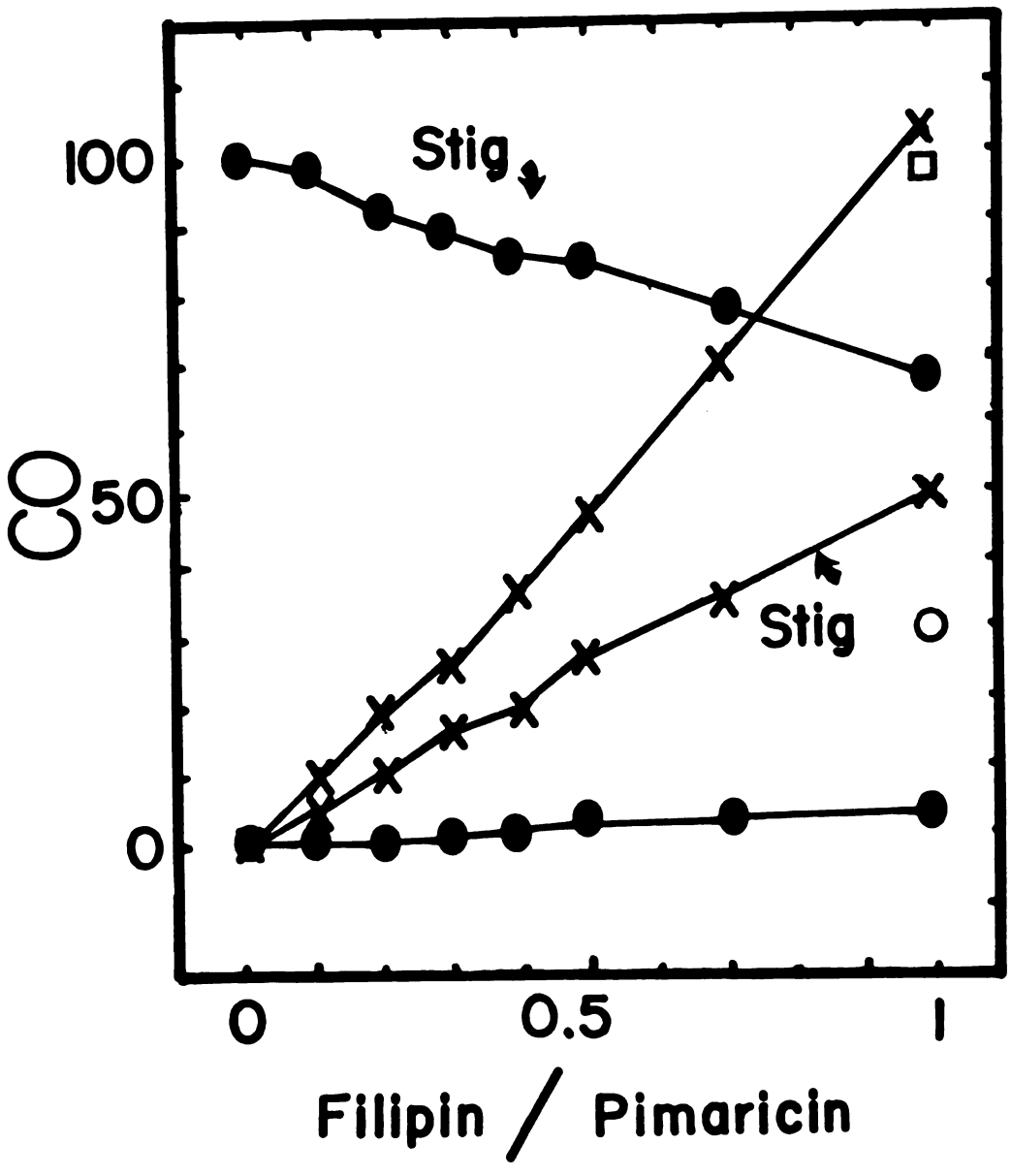

Figure 17

TABLE II

The Effect of the Order of Addition of Cholesterol Upon the Fluorescence and Light Scattering Properties of Solutions Containing Filipin and/or Pimaricin

Solution Components	CO at 338/495[d]	CO at 308/405[e]	R_{90} at 265[f]
Filipin	100	1.8	75
Pimaricin	0.6	0.5	32
Filipin + pimaricin	101	2.2	75
Filipin + cholesterol	38	1.5	84
Pimaricin + cholesterol	0.3	100	43
Filipin + pimaricin + cholesterol[a]	53	80	86
Pimaricin + cholesterol + filipin[b]	65	88	90
Filipin + cholesterol + pimaricin[c]	44	55	85
Cholesterol	---	---	25

Filipin and/or pimaricin (11.4 nanomoles) were used in the above experiments. To solutions containing cholesterol, 6.5 nanomoles of the sterol were added by injection with 10 µl of isopropanol. The final volume in all samples was 3 ml. Samples were incubated for 2 hours at 37°C and allowed to cool to room temperature before measurements were made.

[a]Filipin and pimaricin were mixed together followed after 10 minutes by the addition of cholesterol.

[b]Pimaricin and cholesterol were mixed together followed after 10 minutes by the addition of filipin.

[c]Filipin and cholesterol were mixed together followed after 10 minutes by the addition of pimaricin.

[d]Corrected fluorescence measurements were made at an excitation wavelength of 338 nanometers and an emission wavelength of 495 nanometers.

[e]Corrected fluorescence measurements were made at an excitation wavelength of 308 nanometers and an emission wavelength of 405 nanometers.

[f]Light scattering measurements, R_{90}, were monitored at 265 nanometers.

cholesterol to filipin gives a CO of 38. At 338/495, the CO values increase from 44 when pimaricin is added to a solution containing filipin and cholesterol, to 53 when cholesterol is added to a solution of filipin and pimaricin, and finally to 65 when cholesterol is added to pimaricin followed by the addition of filipin. At 308/405, the addition of pimaricin to cholesterol results in a CO of 100. At 308/405, the CO values decrease from 88 when cholesterol is added to pimaricin followed by the addition of filipin, to 80 when filipin and pimaricin are mixed together followed by the addition of cholesterol, and finally to 55 when cholesterol is added to filipin followed by the addition of pimaricin.

Tyndall light scattering measurements (R_{90}) were made at 265 nanometers. The R_{90} values of filipin and filipin mixed with pimaricin are 75. Filipin mixed with cholesterol has an R_{90} of 84, which is approximately the same as seen for filipin mixed with pimaricin followed by the addition of cholesterol and filipin mixed with cholesterol followed by the addition of pimaricin. The addition of pimaricin to cholesterol followed by the addition of filipin has a different R_{90} of 90. The experimental protocol for all of the experiments performed in Table II used a total incubation period of 2 hours. Additional experiments were performed with the samples in Table II at an incubation time of 24 hours and the results were the same.

The Effect of the Order of Addition of Stigmasterol on the Fluorescence and Light Scattering Properties of Solutions Containing Filipin and/or Pimaricin

The experiments shown in Table III are the same as shown in Table II, with the exception that stigmasterol was used as the sterol instead of cholesterol. At 338/495, the CO's of filipin and filipin mixed with

TABLE III

The Effect of the Order of Addition of Stigmasterol Upon the Fluorescence and Light Scattering Properties of Solutions Containing Filipin and/or Pimaricin

Solution Components	CO at 338/495[d]	CO at 308/405[e]	R_{90} at 265[f]
Filipin	100	1.0	74
Pimaricin	0.6	0.6	34
Filipin + pimaricin	101	1.8	75
Filipin + stigmasterol	35	1.2	93
Pimaricin + stigmasterol	0.5	100	57
Filipin + pimaricin + stigmaterol[a]	52	68	96
Pimaricin + stigmasterol + filipin[b]	65	76	105
Filipin + stigmasterol + pimaricin[c]	45	50	95
Stigmasterol	---	---	35

Pimaricin and/or filipin (11.4 nanomoles) were used in the above experiments. To solutions containing stigmasterol, 6.5 nanomoles of the sterol were added by injection with 10 µl of isopropanol. The final volume in all samples was 3 ml. Samples were incubated for 2 hours at 37°C and allowed to cool to room temperature before measurements were made.

[a]Filipin and pimaricin were mixed together followed after 10 minutes by the addition of stigmasterol.

[b]Pimaricin and stigmasterol were mixed together followed after 10 minutes by the addition of filipin.

[c]Filipin and stigmasterol were mixed together followed after 10 minutes by the addition of pimaricin.

[d]Corrected fluorescence measurements were made at an excitation wavelength of 338 nanometers and an emission wavelength of 495 nanometers.

[e]Corrected fluorescence measurements were made at an excitation wavelength of 308 nanometers and an emission wavelength of 405 nanometers.

[f]Light scattering measurements, R_{90}, were monitored at 265 nanometers.

pimaricin are the same. At 338/495, the addition of cholesterol to filipin results in a CO of 35. As the order of addition of stigmasterol changes, the CO's at 338/495 change from 45 when filipin is mixed with stigmasterol followed by the addition of pimaricin, to 52 when pimaricin and filipin are mixed together followed by the addition of stigmasterol, and to 65 when pimaricin is allowed to incubate with stigmasterol before the addition of filipin. At 308/405, the CO of pimaricin with stigmasterol is 100. The addition of filipin to a solution of pimaricin and cholesterol gives a CO of 76. The CO's at 308/405 continue to decrease to 68 when filipin and pimaricin are mixed together followed by the addition of stigmasterol and to 50 when filipin is mixed with stigmasterol followed by the addition of pimaricin.

Tyndall light scattering measurements (R_{90}) in Table III give similar results as those seen in Table II. The R_{90} values of filipin and filipin mixed with pimaricin are 74 and 75, respectively. Filipin mixed with stigmasterol gives a R_{90} of 93, which is, within experimental error, the same as values of 96 and 95 seen for filipin mixed with pimaricin followed by the addition of stigmasterol and filipin mixed with stigmasterol followed by the addition of pimaricin. The R_{90} of pimaricin mixed with stigmasterol, followed by the addition of filipin, is higher at 105. As in Table II, these experiments were performed again with an incubation time of 24 hours and the results were the same as seen at 2 hours.

DISCUSSION

The experiments depicted in Figures 6 through 9 suggest a polyene sterol stoichiometry of 1:1. The results for the stoichiometry of filipin with cholesterol, in Figure 6, are in excellent agreement with the work of Katzenstein [46] and Spielvogel et al. [37], who observed a cholesterol:filipin molar ratio of 1.0 produced a maximum spectral change in filipin. Also, Schroeder et al. [24] have concluded the filipin:cholesterol stoichiometry is 1:1. However, Olinger et al. (unpublished data) have observed that depending upon the concentrations of the reactants, the apparent cholesterol:pimaricin stoichiometry varies from 1:1 to 4:1. Furthermore, it was observed that the addition of pimaricin to cholesterol resulted in sigmoidal titration curves instead of the linear relationship seen in Figures 8 and 9, where the [sterol]≤[pimaricin]. Strom et al. [34], using the polyene antibiotic lucensomycin, which is similar in structure to pimaricin, have observed a stoichiometry of 2.0 for the interaction of lucensomycin with cholesterol and have seen sigmoidal titration curves with erythrocyte membranes but not with colloidal cholesterol. Therefore, the different observed stoichiometries observed for the interaction of pimaricin with cholesterol necessitates the need for standardization of experimental conditions to achieve reproducible experimental results.

Figures 6 through 9 show a linear increase in the CO of pimaricin and a linear decrease in the CO of filipin upon the addition of sterol

where the [sterol]≤]polyene]. Where [sterol]>[polyene], the CO of filipin continues to show small additional decrease. This decrease in CO is attributed to light scattering by sterol aggregates which result in a decrease in the fluorescence intensity.

The CO's of pimaricin, in Figures 8 and 9, show a further increase when sterol is added at a sterol:polyene ratio of >1.0. This increase in CO might be associated with a clustering or aggregation of the pimaricin sterol complex and free cholesterol so that pimaricin is situated in the hydrophobic environment of these clusters, which results in an enhancement of pimaricin fluorescence. Olinger et al. (unpublished data) have observed the fluorescence of pimaricin is markedly increased when pimaricin is added to an environment of high polarizability. Also, there is evidence for aggregates of polyene antibiotics (Schroeder et al. [24]; Norman et al. [36]) and cholesterol (Lucy et al. [47] in aqueous systems. Therefore, as more cholesterol is added at a sterol:polyene ratio of >1.0, this could result in the formation of pimaricin-sterol aggregates and produce an increase in pimaricin fluorescence caused by the association of pimaricin in a hydrophobic environment (or an environment of high polarizability). Future light scattering studies may identify and correlate the size of the pimaricin-sterol aggregates with the fluorescence intensity of pimaricin and elucidate the reason for the increase in pimaricin fluorescence observed in Figures 8 and 9, where the [sterol]>[pimaricin].

The linear relationship between the CO of filipin or pimaricin and the amount of sterol added where the [sterol]≤[polyene], as seen in Figures 6 through 9, was used to determine the relative affinities of filipin, pimaricin, amphotericin B, and nystatin for stigmasterol

and cholesterol. The determination of these relative affinities is based upon several assumptions with regard to the fluorescence properties of filipin and pimaricin. The changes in the CO of filipin or pimaricin, where the [sterol]≤[polyene], are assumed to be a linear measure of the amount of binding between the polyene and the sterol. In the presence of an added polyene antibiotic, the fraction of the total possible change in the fluorescence that was observed for either filipin or pimaricin, in the presence of sterol, was assumed to be a linear measure of the fraction of filipin or pimaricin bound by the sterol. It was assumed that any deviation from the total possible change in fluorescence of filipin or pimaricin, in the presence of sterol, was due to a direct interaction between the added polyene and the sterol. The experimental conditions employed in these relative affinity experiments include the use of 2 polyene antibiotics, with at least one of the polyenes being either filipin or pimaricin, in the presence of a limiting amount of sterol.

In the presence of cholesterol and increasing amounts of nystatin, the results in Figure 10 show the CO of pimaricin remains unchanged. This implies that nystatin is unable to interact with the available cholesterol in the presence of pimaricin and suggests that pimaricin has a stronger affinity for cholesterol than does nystatin. The data in Figure 11 show nystatin is able to reduce the maximal fluorescence of pimaricin by 22% in the presence of stigmasterol. This indicates that nystatin is able to compete with pimaricin for stigmasterol; however, pimaricin has interacted with 78% of the available stigmasterol and this indicates pimaricin has a stronger affinity than nystatin for stigmasterol.

The data in Figures 10 and 11 also show the effect of amphotericin B on the fluorescence of pimaricin. In the presence of cholesterol, the addition of increasing amounts of amphotericin B to pimaricin, as shown in Figure 10, results in a 76% decrease in the fluorescence of pimaricin. This indicates amphotericin B has a stronger affinity for cholesterol than does pimaricin. In the presence of stigmasterol, as shown in Figure 11, the CO of pimaricin decreased by 53%, where the [amphotericin]=[pimaricin]. This indicates that amphotericin B and pimaricin have similar affinities for stigmasterol.

The above results, from Figures 10 and 11, give the relative affinities of amphotericin B, pimaricin, and nystatin for cholesterol as amphotericin > pimaricin > nystatin. For stigmasterol, the relative affinities are amphotericin B = pimaricin > nystatin. The results for the relative affinities of these polyenes for cholesterol are in good agreement with the results of other workers (Demel et al. [19]; Norman et al. [36]).

It is interesting that the fluorescence of pimaricin, in Figures 10 and 11, does not show a linear decrease with the addition of increasing amounts of amphotericin B. Also, above an amphotericin B/pimaricin molar ratio of approximately 0.50-0.55, there is little additional decrease in the pimaricin fluorescence. The small additional decrease that is observed is attributed to the light scattering of the reactants, which results in a decrease in the fluorescence intensity. Another interesting observation in Figures 10 and 11 is that an amphotericin B/pimaricin molar ratio of approximately 0.55 corresponds to 6.5 nanomoles of amphotericin B, which gives an amphotericin B/sterol molar ratio of 1.0. Therefore, a possible explanation for the

lack of any additional decrease in the fluorescence of pimaricin beyond a molar ratio of 0.50-0.55 might be that amphotericin B has interacted with all of the cholesterol. However, if this were true, the predicted CO of pimaricin would be nearly 0.0 and the CO of pimaricin, as seen in Figures 10 and 11, is much greater than 0.0.

There are several possible explanations for the continued fluorescence of pimaricin seen at molar ratio of >0.50. These explanations include the possibility of a ternary complex of amphotericin B, sterol, and pimaricin. This complex would allow for the interaction of pimaricin with sterol and result in the fluorescence of pimaricin. Another possibility is the existence of an amphotericin B sterol aggregate or cluster which interacts with pimaricin so that pimaricin is in an environment of high polarizability, which results in an enhancement of pimaricin fluorescence. Experimental observations by Olinger et al. (unpublished data) have produced results that allow for the spectroscopic differentiation between pimaricin-sterol and pimaricin-micelle interactions and would elucidate whether pimaricin is interacting with sterol or is associated with an amphotericin B-sterol aggregate or cluster. It was observed by Olinger that the excitation spectrum of pimaricin exhibits characteristic changes that are different when pimaricin interacts with sterol, as compared with pimaricin interacting with detergent micelles of deoxycholate or lauryl sulfate. It was found that the interaction of pimaricin with detergent micelles or sterol resulted in an enhancement of pimaricin fluorescence; however, only the interaction of pimaricin with sterol resulted in a dramatic shift in the maxima of the pimaricin excitation spectrum. Furthermore, the absorbance spectrum maxima of pimaricin

changes dramatically only upon interacting with sterol. Therefore, the excitation spectrum of pimaricin was monitored in the presence of 15 mM Na deoxycholate and in the presence of sterol at 3.8 mM pimaricin and amphotericin B. Because pimaricin does not fluoresce in distilled water, it is not possible to examine the excitation spectrum of pimaricin in the absence of sterol. Also, the absorbance spectrum of pimaricin was monitored at an amphotericin B/pimaricin molar ratio of 1.0 in the presence and absence of cholesterol. It was found that the excitation (Figure 1A) and absorbance (Figure 2A) spectra of pimaricin indicated an interaction of the polyene with the sterol as characterized by the significant changes in the excitation and absorption spectra maxima. This would tend to support the existence of a ternary complex. However, this possibility presents some problems in terms of the molecular interactions associated with this complex. Evidence by Schroeder et al. [24], Norman et al. [36] and Olinger et al. (unpublished data) has indicated the interaction of amphotericin B or pimaricin with sterol depends upon a sterol structure that includes a 3BOH, a planar steroid nucleus, and a 17 alkyl side chain. This infers some specific interaction between the polyene and the sterol and makes unlikely the possibility of both polyenes interacting in the same manner with the sterol.

The effect of amphotericin B on the fluorescence of pimaricin, as discussed above, does not seem to have any simple interpretation. It should be pointed out that the quenching of pimaricin fluorescence by amphotericin B was considered as a possible reason for the decrease in pimaricin fluorescence noted in Figures 10 and 11. However, this interpretation seemed unlikely because the CO of pimaricin did not

continue to decrease beyond an amphotericin B/pimaricin molar ratio of 0.50. Also, the absorbance spectrum of amphotericin B and the emission spectrum of pimaricin are such that secondary absorbance is theoretically possible and would result in a decrease in pimaricin fluorescence. In the presence of sterol, the absorption of amphotericin B, at the emission maxima of pimaricin, was monitored in the presence of increasing amounts of pimaricin. It was observed that there was not any secondary absorbance.

The results from Figures 12 and 13 indicate that filipin has a much stronger affinity for stigmasterol and cholesterol than does amphotericin B or nystatin. This is consistent with the observations of others (Cass et al. [2]]; Norman et al. [36]; Bittman et al. [30]).

Figures 14 and 15 show the changes in the fluorescence of filipin and pimaricin as increasing amounts of pimaricin are added, while the filipin concentration is held constant (Figure 14) and as increasing amounts of filipin are added while the pimaricin concentration is held constant (Figure 15). In Figures 14 and 15, the fraction of the total possible change in the fluorescence of filipin is approximately 67% at a filipin/pimaricin molar ratio of 1.0. Therefore, the interpretation of this result is that filipin has interacted with 67% of the available cholesterol and has a stronger affinity for cholesterol than does pimaricin. The fraction of the total possible change in the fluorescence of pimaricin, in Figures 14 and 15, is 80% at a filipin/pimaricin molar ratio of 1.0. Thus, the interpretation of this result is that pimaricin has interacted with 80% of the available cholesterol and therefore has a stronger affinity for cholesterol than does filipin.

The anomalies in the above results may indicate the possible existence of a polyene-polyene-sterol ternary complex. As previously described, the maxima of the excitation spectrum of pimaricin changes as the polyene interacts with sterol. Results not presented in this study have shown a similar marked change in the excitation spectrum of filipin occurs only when filipin interacts with sterol. Such changes were also observed by Bittman et al. [31]. Thus, in the presence and absence of cholesterol, the excitation spectrum of filipin was observed at a pimaricin/filipin molar ratio of 1.0 (Figure 3A). The results from these spectra indicate an interaction of filipin with cholesterol. Also, data not shown here indicated the excitation spectrum of filipin in water is identical to the excitation spectrum of filipin with pimaricin at a 1:1 molar ratio in the absence of cholesterol. Furthermore, it was observed in the presence of cholesterol that the excitation spectrum of pimaricin at a pimaricin/filipin molar ratio of 1.0 indicated an interaction of pimaricin with cholesterol. These results lend strong evidence for the existence of a ternary complex. As discussed previously, pimaricin has certain requirements of sterol structure which are necessary for the interaction of pimaricin with sterol. Experiments by Norman et al. [23] and Schroeder et al. [24] have verified the same requirements exist for filipin which infers a specific interaction between the sterol and filipin and makes unlikely the possibility of both filipin and pimaricin interacting in the same manner with cholesterol.

Figures 16 and 17 show experiments identical to those shown in Figures 14 and 15, except the sterol used is stigmasterol. In Figure 16, the fraction of the total possible change in the fluorescence of

filipin is 73%, which indicates that filipin has a stronger affinity for stigmasterol than does pimaricin. The fraction of the total possible change in the fluorescence of pimaricin in Figure 16 is 61%, which indicates that pimaricin has a stronger affinity for stigmasterol than does filipin. The results from Figure 17 give similar findings in agreement with those in Figure 16. These observations from Figures 16 and 17 indicate the possibility of a polyene-polyene-sterol ternary complex. Experiments involving changes in the excitation manifold of pimaricin and filipin, as described for Figures 14 and 15, were performed here. The results of these experiments were similar to those seen in Figures 14 and 15 and indicate the possible existence of a ternary complex.

Data from Table II show that variations in the order of addition of cholesterol to solutions of pimaricin and filipin result in different amounts of interaction of filipin and pimaricin with cholesterol. The fraction of the total possible change in the fluorescence of filipin, upon the addition of cholesterol, is greatest when cholesterol is first added to filipin followed by the addition of pimaricin, is less when filipin and pimaricin are mixed together followed by the addition of cholesterol, and finally the change is least when pimaricin is added to cholesterol followed by the addition of filipin. The fraction of the total possible change in the fluorescence of pimaricin, upon the addition of cholesterol, is greatest when cholesterol is first added to pimaricin followed by the addition of filipin, is less when filipin and pimaricin are mixed together followed by the addition of cholesterol, and is least when filipin is added to cholesterol followed by the addition of pimaricin. It is observed that for all of the different

orders of addition, the fraction of the total possible changes in the fluorescence of pimaricin and filipin indicate the existence of a ternary complex as described in Figures 14 through 17.

It is possible that the fraction of the total possible change in fluorescence, that is observed for filipin and pimaricin, would remain the same regardless of the order of addition. However, because of the possible existence of polyene-sterol aggregates, the formation of an initial polyene sterol complex might aggregate in such a way as to preclude an interaction of cholesterol with the polyene added second. The R_{90} data, in Table II, indicate the light scattering of filipin and cholesterol is similar to that seen for solutions containing filipin, pimaricin, and cholesterol. This indicates that if aggregates exist, they are not much larger than the filipin-cholesterol complex. Results not presented here have suggested but not proved that the filipin-cholesterol complex may exist as aggregates. It is also known that free polyenes exist as aggregates in water (Norman et al. [36]; Schroeder et al. [24]) and therefore the existence of polyene-sterol aggregates in water is a distinct possibility.

The results from Table III are very similar to those seen in Table II. The order of addition of the various reactants does influence the apparent amount of polyene that has interacted with stigmasterol. The reason given for this observation is the same as given in Table II.

APPENDIX

Figure 1A. The Excitation Spectrum of Pimaricin Under Various Experimental Conditions.

Pimaricin (11.4 nanomoles) was mixed with (solid line) 15 mM Na deoxycholate or (dashed line) 11.4 nanomoles of amphotericin B in the presence of 6.5 nanomoles of cholesterol. The final volume was 3 ml in distilled water. The samples were incubated for 2 hours at 37°C and allowed to cool to room temperature before measurements were made. The excitation spectra of these mixtures were monitored at an emission of 405 nanometers.

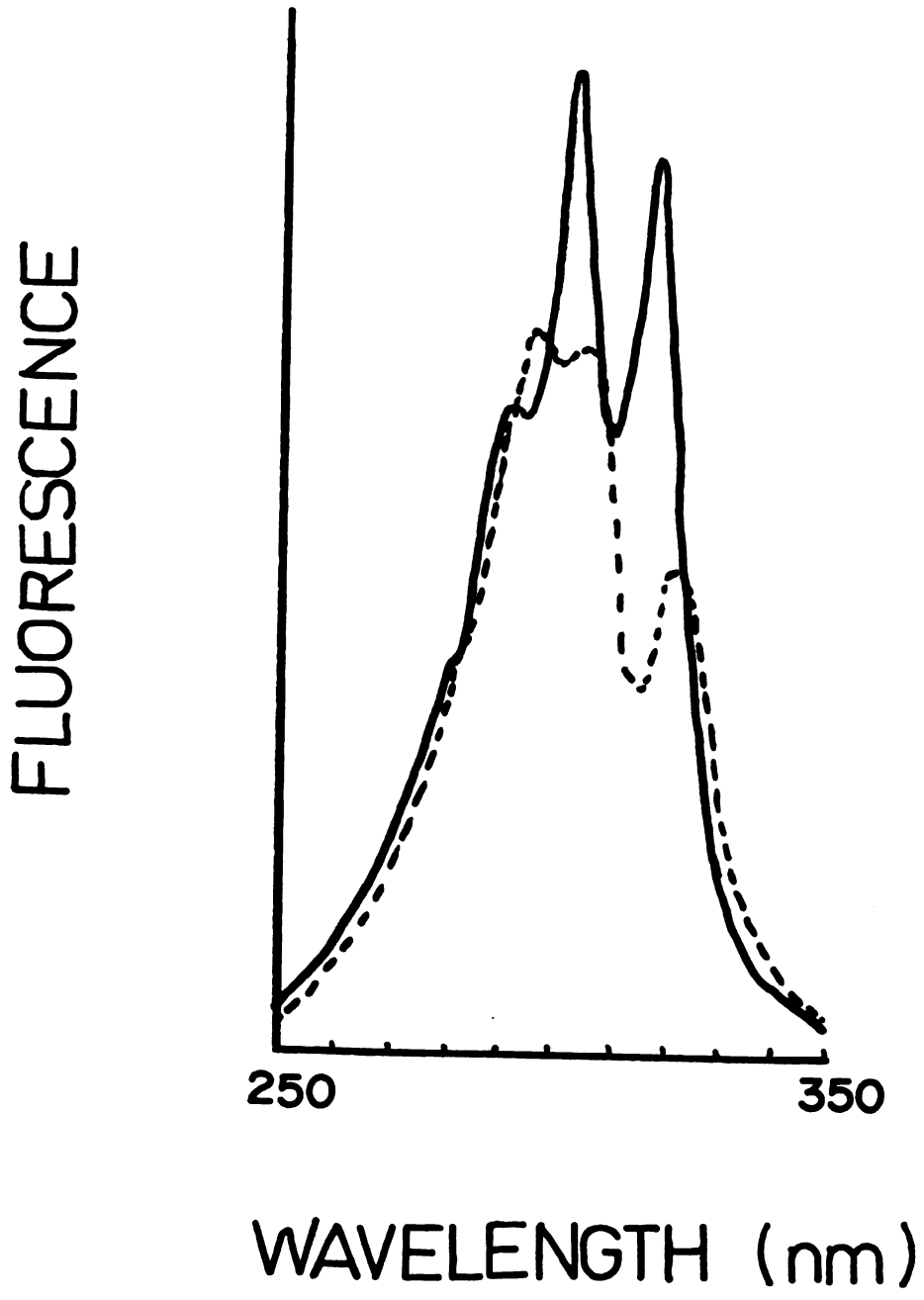

Figure 1A

Figure 2A. The Absorbance Spectrum of Pimaricin Under Various Experimental Conditions.

Pimaricin (11.4 nanomoles) was mixed with (solid line) 11.4 nanomoles of amphotericin B or with (dashed line) 11.4 nanomoles of amphotericin B in the presence of 6.5 nanomoles of cholesterol. The final volume was 3 ml in distilled water. The samples were incubated for 2 hours at 37°C and allowed to cool to room temperature before measurements were made.

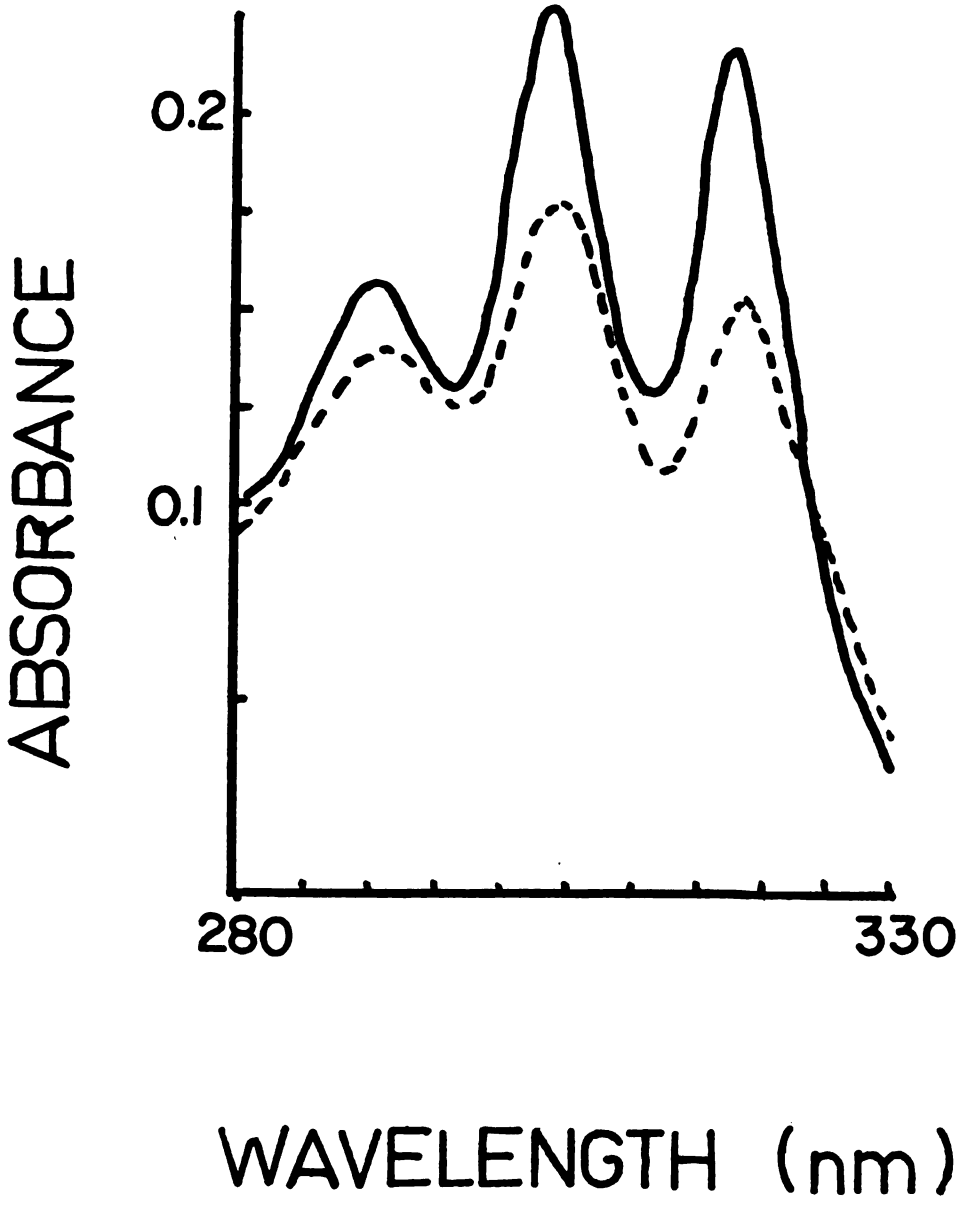

Figure 2A

Figure 3A. The Excitation Spectrum of Filipin Under Various Experimental Conditions.

Filipin (11.4 nanomoles) was mixed with (solid line) 11.4 nanomoles of pimaricin or with (dashed line) 11.4 nanomoles of pimaricin in the presence of 6.5 nanomoles of cholesterol. The final volume was 3 ml in distilled water. The samples were incubated for 2 hours at 37°C and allowed to cool to room temperature before measurements were made. The excitation spectra of filipin were monitored at 495 nanometers.

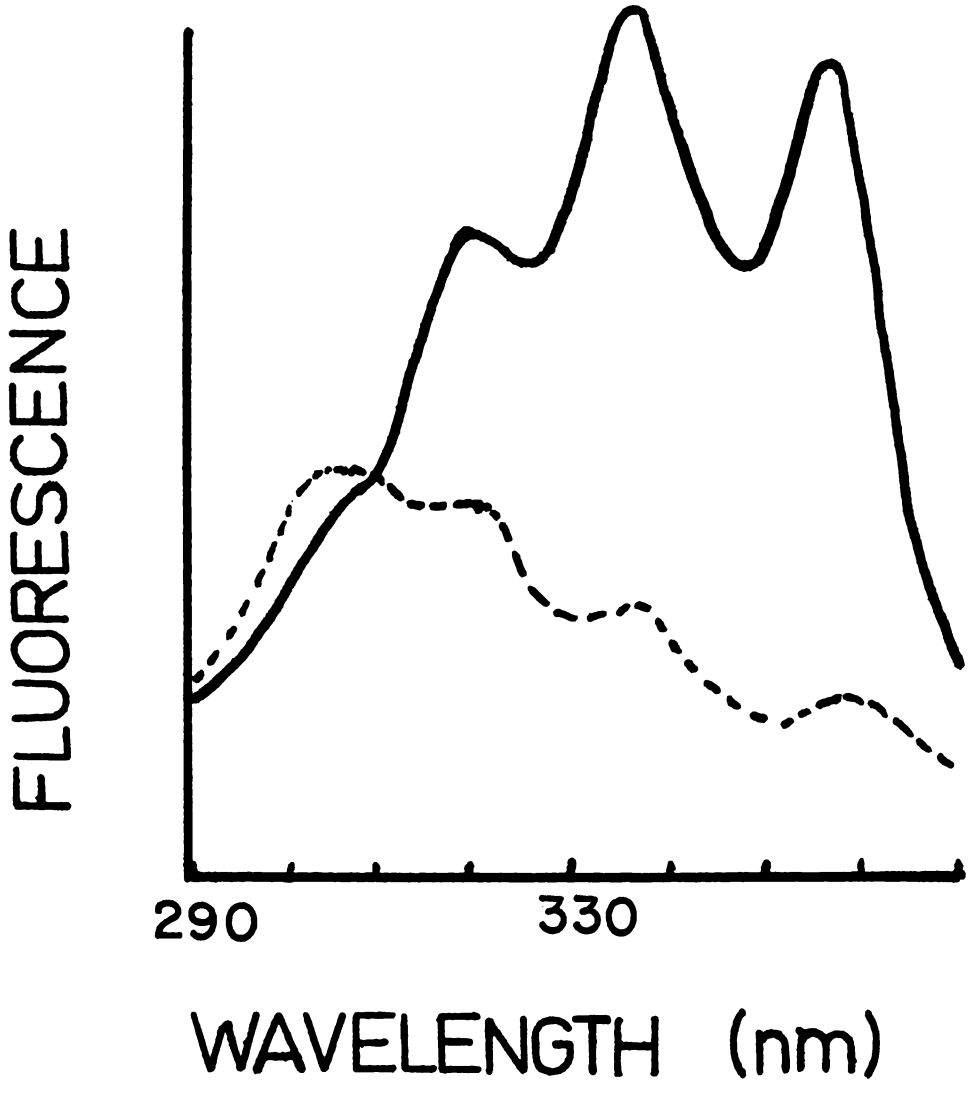

Figure 3A

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