STOICHIOMETRY OF INTERACTION OF THE POLYENE ANTIBIOTIC, FILIPIN, WITH FREE AND LIPOSOMAL-BOUND CHOLESTEROL+‡

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The polyene antibiotic, filipin, is known to interact with cholesterol present in aqueous solutions, artificial membranes and sterol-containing biological membranes. The stoichiometry of interaction between filipin and free or liposomal-bound cholesterol present in an aqueous system was determined by quantitating the ultraviolet spectral change of filipin which occurs in the presence of cholesterol. A preliminary analysis of the data obtained from aqueous dispersions of cholesterol + filipin alone suggested that the stoichiometry was 1:1. According to a more refined analysis of this same data by the SCATCHARD technique, there are 1.11 ± 0.18 molecules of cholesterol associated with filipin in this system. Additionally it was found in a liposome solution consisting of lecithin: dicetylphosphate: [4-^{14}C]-cholesterol that the maximum spectral change of filipin only occurred where a 1:1 ratio of filipin: sterol was present. Thus the likely stoichiometry of the filipin-cholesterol complex in both these systems is 1:1.

A procedure is described wherein the sterol-mediated spectral-change of filipin can be utilized as the basis of a sensitive assay for quantitating the amounts of sterol in membrane fractions.

Filipin belongs to a class of compounds known as polyene antibiotics[1]. Polyenes have attracted the attention of many investigators because, as antibiotics, they have the unusual property of being toxic to fungi while having no effect on bacteria[2]. The concept has emerged from a wide variety of studies that the biological actions of filipin are dependent upon the direct interaction of the polyene with a sterol, usually cholesterol.

LAMPEN[3] and GOTTLIEB[4] showed originally that the addition of sterols to aqueous solutions of filipin produced changes in the ultraviolet absorption spectrum of filipin. It was further shown by these workers that the spectral changes were reversible. Addition of water-miscible organic solvents to the solutions of cholesterol + filipin resulted in a U.V. spectra typical of filipin alone.

These findings were subsequently confirmed and extended by NORMAN *et al.*[5] and KLEINSCHMIDT *et al.*[6] via utilizing ultraviolet spectroscopy and by SCHROEDER *et al.*[7] who employed fluorescence spectroscopy to study the formation of a filipin-cholesterol complex. NORMAN *et al.*[5] found that addition of filipin to cholesterol present in aqueous solutions, artificial membranes such as liposomes, and in membranes from erythrocytes or *Acholeplasma*

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laidlawii all produced identical spectral changes of the polyene which were consistent with the formation of a noncovalent complex with the sterol component. Additionally the formation of this complex was found to be dependent upon certain specific structural requirements of the steroid, particularly a 3β-OH and a cholestane ring structure. It was suggested by NORMAN *et al.*[5] that this ultraviolet spectral change of filipin, which occurs upon addition of sterol, might be utilized for the determination of the stoichiometry of the filipin-cholesterol interaction. The results of the present studies indicate that the stoichiometry for the formation of a complex between filipin and either free or liposomal-bound cholesterol is 1:1.

Methods

Spectrophotometric measurements:

A Beckman DB-GT double beam spectrophotometer was employed in all experimental determinations. Samples were scanned with ultraviolet light from 400 to 260 nm to obtain a characteristic spectrum.

Sample preparation:

The filipin used in this investigation was generously supplied by Dr. G.B. WHITFIELD of the Upjohn Company, Kalamazoo, Michigan. Three different batches of filipin were employed: U-5956, 9098-BBC-94A and 8393-DEG-11-8, which ranged in purity from 93% to 96%. All filipin preparations gave similar experimental results.

Standard solutions of filipin were made up in dimethylformamide (DMF, 0.25 mg/ml). Each day a filipin-buffer stock solution was made up in 0.01 M tris-acetate, pH 7. This solution consisted of 0.5~3.8 ml of the filipin-standard solution diluted to a total volume of 30 ml with the tris-acetate buffer. This was designated solution A.

Weighed portions of cholesterol (Cal Biochem) were dissolved in absolute ethanol (usually 6.22×10^{-4} M). A cholesterol-buffer stock solution was made up in 0.01 M tris-acetate buffer, pH 7. One ml of the cholesterol-ethanol standard solution was diluted to a total volume of 50 ml with tris-acetate buffer. This was designated solution B. Solution C refers to the 0.01 M tris-acetate buffer alone.

Each experiment consisted of eleven tubes containing different proportions of solutions A, B, and C in a total volume of 10 ml. In every experiment there was also 11 tubes containing 10 ml of only solutions B and C; these served as blanks and were used in the reference cuvette to correct for the turbidity caused by cholesterol in the aqueous media when taking spectrophotometric measurements. All samples were vortexed and allowed to stand three hours prior to analysis.

The concentration of filipin per sample was determined spectrophotometrically. The molar extinction coefficient of Peak 2 in methanol of the U.V. spectra (see Fig. 1) is 6.2×10^4.[5] Experimentally it was found that this extinction coefficient was also valid for a 1:1 mixture of solution C and methanol.

Liposomes consisting of egg lecithin alone or lecithin : dicetylphosphate: [4-^{14}C]-cholesterol at the molar ratio of 40:10:50+1.0 μCi of ^{1}carbon-14 were prepared exactly as described by PAPAHADJOPOULOS[19]. This method involved homogenization of the lipid mixture in 50 mM tris-HCl, pH 8.0, 1 mM NaCl, 10 mM KCl with 6~8 strokes of a Potter-Elvehjem homogenizer. Next the lipid dispersion was sonnicated under nitrogen (20~40 min.) until it became clear to slightly opalescent. Visible aggregates were removed by centrifugation. These liposomes were chromatographed in the same buffer over a 35×1.5 cm column of Sephadex G-25 to separate free cholesterol from liposome-bound [4-^{14}C]-cholesterol. The liposome peak was detected by absorbance measurements at 300 nm. After chromatography the cholesterol concentration in the liposome solution was determined by assessing the radioactivity content.

Next the liposomes were diluted to give $5\sim15\times10^{-6}$ M cholesterol and were incubated with filipin, 3×10^{-5} M, for 3 hours at room temperature under nitrogen. An aliquot was applied to a column of Sephadex 6-25 and the fractions containing lipid vesicles were used for determination of the filipin spectrum in aqueous solution, the filipin concentration, and the cholesterol concentration.

Brush borders from the intestinal mucosa of 100 g chicks were prepared exactly as described previously[8]. Briefly the procedure consists of the dissociation of the brush border fraction from the epithelial cells by homogenizing scraped mucosal cells with 30 volumes of 5 mM EDTA. The brush borders are repeatedly pelleted at low speed centrifugation ($450\times g$). The brush border pellet was diluted to appropriate protein concentrations with buffer containing 50 mM tris-HCl, pH 8.0, 1 mM NaCl, 1 mM KCl, and 1 mM $MgCl_2$.

The cholesterol content of the brush border fraction was determined on lipid extracts by the procedure of LIEBERMAN-BURCHARD.

Theory of Treatment:

Adding cholesterol to a filipin solution induces a spectral change which may best be quantitated by calculation of the ratio of ultraviolet absorbance of Peak 3 : Peak 1. As will be shown later in this report, the changes in this ratio in the presence and absence of cholesterol make possible the determination of the amount of filipin-cholesterol complex present.

The type of treatment that was employed was the method of SCATCHARD[9,10]. If it is assumed that the filipin-cholesterol complex follows from simple binding parameters, an expression can be developed to establish the binding stoichiometry. The two binding species are filipin and its ligand, cholesterol. If it is assumed that the filipin molecule has n groups capable of binding one ligand, the simplest association would occur when $n=1$. Taking this case where n=1 and setting the activities of the two components equal to their concentrations, the only reaction is

$$F+C' \rightarrow FC \tag{1}$$

Definition of all the parameters used in this treatment are as follows:

F=free filipin
C'=free cholesterol $(C_o-FC)-(C_o-nFC_n)$
F_o=initial filipin before interaction with cholesterol
C_o=initial cholesterol before interaction with filipin
FC=filipin-cholesterol complex
n=number of cholesterols associated with filipin
K=association constant
K_d^a=dissociation constant
[ ]=concentration of the indicated species

$$K_a=\frac{[FC]}{[F]\,[C']}=\frac{1}{K_d} \tag{2}$$

The fraction of binding sites that are unoccupied on filipin is defined as

$$\alpha=\frac{[F]}{[FC]+[F]}=\frac{1}{1+K_a[C']}=\frac{K_d}{[C']+K_d} \tag{3}$$

$\bar{V}$ is defined as the average number of moles of cholesterol bound by one mole of filipin.

$$\bar{V}=\frac{\text{moles cholesterol complexed}}{\text{total moles filipin}}=\frac{[FC]}{[F]+[FC]}\,\frac{[FC]}{[F_0]} \tag{4}$$

$\bar{V}$ may be considered as the probability that any one site chosen at random from any filipin molecule in the solution is occupied by a cholesterol molecule. If filipin, instead of containing one site capable of binding cholesterol, contains n such sites which are equivalent and

independent it follows that

$$\frac{\bar{V}}{n}=1-\alpha=\frac{K_a[C']}{1+K_a[C']} \tag{5}$$

Rearranging equation (5) we get

$$\bar{V}=\frac{nK_a[C']}{1+K_a[C']} \tag{6}$$

Scatchard plots utilize equation[6] in the following form

$$\frac{\bar{V}}{[C']}=K_a(n-\bar{V}) \tag{7}$$

A linear plot is obtained when $\bar{V}/[C']$ is plotted *versus* $\bar{V}$. The intercept on the abscissa is n, the number of cholesterols bound to one filipin and the intercept on the ordinate is $K_a n$.

Results

Filipin has the characteristic ultraviolet absorption spectrum shown in Fig. 1. Both in methanol and aqueous solutions the three maxima occur at 356, 338 and 322 nm; however, in the latter the molar extinction coefficient decreases. As shown in Fig. 2, a definite spectral change results when cholesterol is added to the aqueous solution of filipin. The major peak of a filipin spectrum is Peak 2, while the major peak of a spectrum of the steroid-antibiotic mixture is Peak 3. The alteration in absorbance intensity for Peaks 1 through 3 was obtained consistently throughout a wide range of filipin and cholesterol concentrations. This reinforced previous suggestions that the stoichiometry could be determined by mathematically relating this change in peak height to the concentrations of the reacting components.

One of the means of quantitating the spectral change was to calculate the ratio of ultra-

Fig. 1. Ultraviolet spectrum of filipin in methanol and tris-acetate buffer.

Spectra of identical concentrations of filipin (*ca.* 10^{-5} M) were taken. In both methanol and water the maxima occur at 356, 338 and 322 nm. The molar extinction coefficients were less for filipin in water than for filipin in methanol. The major peak is designated Peak 2 and the minor peak as Peak 3.

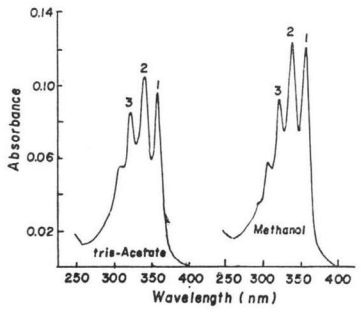
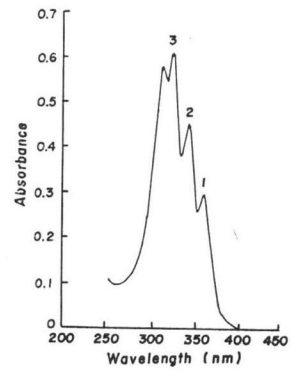


Fig. 2. Spectrum of filipin-cholesterol complex.

Aqueous solutions of cholesterol were added to aqueous solutions of filipin. The final concentrations of cholesterol was 7.0×10^{-5} M and filipin was 2.39×10^{-5} M respectively. The maxima of Peaks 1, 2 and 3 occur in order at 356, 338 and 322 nm, but the relative magnitude of the molar extinction coefficients has changed markedly. The major peak is Peak 3 and the minor peak is Peak 1.



violet absorbance of Peak 3 : Peak 1. In the absence of any sterol this ratio for aqueous solutions of filipin was approximately 0.8; as sterol was added in increasing amounts, the ratio of ultraviolet absorbance of Peak 3 : Peak 1 approached a limiting value of 2.0. The first attempt at determining the stoichiometry from spectral data was to plot the ratio of Peak 3 : Peak 1 *versus* the cholesterol concentration. This resulted in a rectangular hyperbolic curve (see Fig. 3). If two lines are drawn through the asymptotes they intersect at a cholesterol concentration of 9.15×10^{-6} M. When this is compared with the filipin concentration of 9.04×10^{-6} M for this particular sample run it can be implied that there may be a 1:1 stoichiometry of the interaction which results in the spectral change.

In order to test the general validity of the 1:1 stoichiometry for the filipin-cholesterol complex, many repetitive experiments were conducted where the cholesterol concentration was varied for a range of filipin concentrations of $6.2\sim90.0\times10^{-7}$ M. For each separate experiment, a plot of the ratio of Peak 3 : Peak 1 was prepared as a function of the cholesterol concentration (see Fig. 3 for example). Then the asymptotes were drawn and the concentration of cholesterol where the intersection occurred was determined. The cholesterol : filipin ratio obtained over this series of eleven experiments was 1.31 ± 0.34 (SD).

As a consequence of this preliminary analysis of the data which suggested a 1:1 stoichiometry for the complex, it was felt appropriate to carry out a more precise calculation of the stoichiometry using the techniques of SCATCHARD[9,10] and JOB, as described by HAMMES *et al*[11]. Both methods have been used by investigators to study the stoichiometry of binding of one molecule to another. The JOB method of determining the stoichiometry of binding involves taking a difference

Fig. 3. Cholesterol concentration dependency of the ratio of ultraviolet absorbance of Peak 3: Peak 1.

A plot of Peak 3: Peak 1 *versus* cholesterol concentration resembles a rectangular hyperbola. The asymptotic intersection is at a cholesterol concentration of 9.15×10^{-6} M. [F]$=9.04\times10^{-6}$ M.

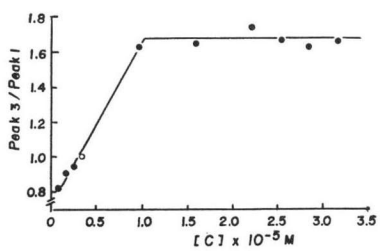


Fig. 4. SCATCHARD analysis of cholesterol mediated changes in the u.v. spectra of aqueous solutions of filipin.

$\overline{V}/C'$ is plotted against $\overline{V}$. The abscissa intercept gives the number of cholesterols associated per filipin molecule. The equation of the line is $Y=1.24\times10^{6}\sim1.20\times10^{6}\times$. From the data given the number of cholesterols per filipin is 1.037. [F]$=3.22\times10^{-6}$ M

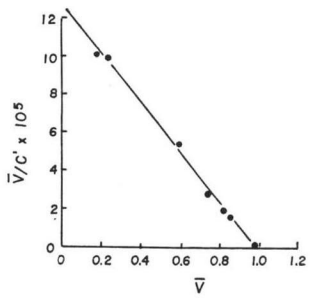
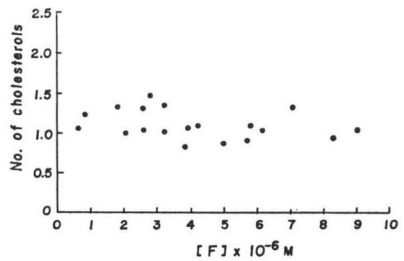


Fig. 5. Apparent stoichiometry of the cholesterol-filipin interaction as determined by the SCATCHARD method.

When a linear regression analysis of all the data is carried out, the ordinate intercept, which is the number of cholesterols interacting with filipin, has a value of 1.11 ± 0.18 (S.D.).



spectrum and plotting mole fraction *versus* the change in absorbance. Although theoretically attractive, this method proved unsuccessful, because when a difference spectrum is taken there is no way to blank out the turbidity caused by the high cholesterol concentrations necessitated by the technique.

An extrapolation of the number of cholesterols interacting with filipin was obtained from a SCATCHARD analysis of the available data. This technique required a calculation of the free cholesterol left after the putative filipin-cholesterol complex is formed, *i.e.* [C']. But this was experimentally impossible since there was no way to directly measure the free cholesterol in the presence of the cholesterol associated with the complex. However, the parameter [C'] could be determined if a stoichiometry of the complex was assumed. Thus the measure of the concentration of the filipin-cholesterol complex, [FC_n] (a measurable parameter) could be utilized to calculate the free cholesterol concentration, [C'[, when differing values of n were utilized. It became apparent that the data only successfully followed a stoichiometry of 1:1.

A linear result was obtained when $\bar{V}/[C']$ was plotted *versus* $\bar{V}$ as shown in Fig. 4. The intercept on the abscissa indicates the number of moles of cholesterol interacting with one mole of filipin. With the SCATCHARD technique, n is 1.037 for the data in Fig. 4.

A summary of all the results as analyzed by the SCATCHARD technique is given in Fig. 5. Table 1 summarizes these results. As emphasized previously a 1:1 stoichiometric relationship for the complex was assumed. The validity of this assumption was tested by calculating a [C'] by the SCATCHARD method, for the data shown in Fig. 4 by assuming values of 0.50 and 2.00 for n; *i.e.* $[C']=[C_0]-N[FC_n]$. When this was carried out, the number of cholesterols found to be associated with filipin (the abscissa intercept) ranged from 0.41 to 0.80 for $n=0.5$ and from 1.50 to 2.97 for $n=2.0$. Thus the data clearly fit the 1:1 stoichiometry much better than the other tested possibilities. These results provide further support to the original assumption that the stoichiometry of the complex was 1:1. From the data presented in Table 1 it can be concluded that one cholesterol molecule interacts with one filipin molecule to give the observed characteristic spectral change.

Table 1. Number of cholesterols associated with filipin as determined by SCATCHARD analysis.

	n (avg) ±	S.D.	S.E.
SCATCHARD analysis	1.11	0.18	0.04

t-Test*

Experimentally determined n	Theoretical values of n		
	1.00	1.50	2.00
SCATCHARD 1.11	$p>0.5$	$p<0.05$	$p<0.001$

* The values of n obtained by the SCATCHARD analysis were compared with theoretical values of n to establish which value the data followed the best. n is defined as the moles of cholesterol which interact with one mole of filipin to produce a change in the ultraviolet absorption spectrum.

It was also of interest to ascertain whether the experimentally derived values were significantly different from theoretical n's of 1.00, 1.50 and 2.00. This was accomplished by means of STUDENT's *t*-test and the results appear in Table 1. The SCATCHARD data fit an n of only 1.00.

A different approach to the filipin-sterol stoichiometry employed the use of liposomes containing (4-^{14}C)-cholesterol. NORMAN *et al.*[5] have previously shown that liposomal-bound cholesterol gives the same spectral change of filipin as do aqueous dispersions of the sterol.

Fig. 6. Spectral change in filipin on interaction with liposomal cholesterol.

Liposomes consisting of lecithin: dicetylphosphate: [4-^{14}C]-cholesterol, (40:10:50 mole percent) were prepared and incubated with filipin as described under Methods.

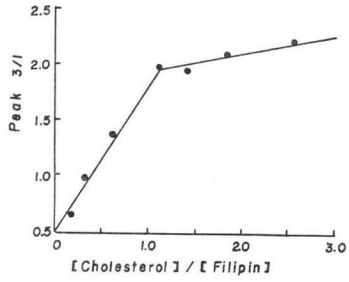
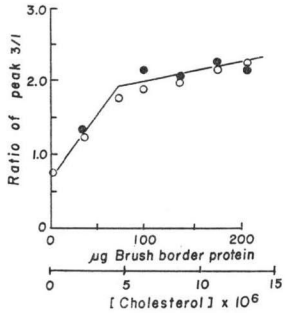


Fig. 7. Stoichiometry of interaction of filipin with brush border cholesterol.

The filipin concentration was 4.8 10^{-6} M. The lower scale represents the cholesterol concentration as determined by LIEBERMANN BURCHARD analysis of the lipid extract of the same brush border preparations.



The results are given in Fig. 6. A maximum change in Peak 3 : Peak 1 ratio only occurs at a stoichiometry of 1:1 of filipin: sterol.

As a consequence of the results described above it was possible to devise a quantitative assay for cholesterol present in biological samples. An example is shown in Fig. 7. When the spectral change of filipin is plotted *versus* the brush border protein or cholesterol a sharp break is evident at a cholesterol concentration of 4.6×10^{-6} M. Since filipin was applied at a final concentration of 4.8×10^{-6} M, again a stoichiometry of 1:1 is evident.

The cholesterol concentration per 100 mg brush border protein was found to be 2.7 mg if the total cholesterol of the lipid extract was assayed by the LIEBERMAN-BURCHARD method or 2.5 mg if determined by the filipin-cholesterol interaction method. Considering that 16 % of the cholesterol present in brush borders is as cholesterol ester and as such unreactive with filipin, the data from the two assays are in very close agreement with one another.

Discussion

The stoichiometry of interaction between filipin and free or liposomal cholesterol present in an aqueous system was determined by quantitating the spectral change of filipin which occurs in the presence of the cholesterol. A preliminary analysis of the data obtained from aqueous dispersions of cholesterol (see Fig. 3) suggested that the stoichiometry which produced this spectral change was 1:1. A more refined analysis of this data was carried out employing the SCATCHARD technique. The SCATCHARD method indicates there are 1.11 ± 0.18 (S.D.) moles of cholesterol associated with one mole of filipin.

Similar conclusions concerning the stoichiometry were reached when studies of liposomally bound [4-^{14}C)-cholesterol were carried out. This system has the significant advantage that free filipin can be separated from the filipin-cholesterol complex chromatographically. Thus one can directly quantitate in the liposome fractions the radioactivity of the cholesterol and the spectrum of the filipin of the complex. This independent method also indicates that the stoichiometry of the complex is 1:1.

Earlier DEMEL and coworkers[12,13] found that the penetration of only a few antibiotic molecules through the phase boundary of mixed lipid and pure sterol monolayers caused a surface pressure increase. They suggested one filipin per one hundred molecules of sterol could cause this pressure increase. It was further proposed that the polyene may produce a

reorientation of sterol molecules in the membrane and a consequent alteration in the cell permeability.

In another model system, where the filipin-sterol interaction was studied spectrophotometrically, NORMAN *et al.*[5] have stated that cholesterol, both free and lipsomally bound, interacts with filipin in a reproducible and identical manner in both these systems. The preliminary results of NORMAN *et al.*[5] regarding the stoichiometry, which were obtained from binding ^{14}C-cholesterol to filipin in biphasic systems, suggested that as few as two cholesterol molecules are associated with one filipin molecule.

Measurements by differential scanning calorimetry of the effect of filipin on the phase transition of lecithin and lecithin-cholesterol dispersions indicated that filipin can reduce the lecithin-cholesterol interaction. It has been established by NORMAN *et al.*[5,14] that filipin reacts with cholesterol in such a way that cholesterol is withdrawn from its interaction with lecithin. From this data it was proposed that the number of cholesterol molecules that can be complexed by filipin is 2.3. Highly concentrated solutions of lipid were employed in this work and accordingly the results may be compared only qualitatively with the other experimental systems such as that employed in this study.

BIEBER and coworkers[7] used fluorescence spectroscopy to study the formation of the filipin-cholesterol complex. An analysis of the measured partial quantum efficiency for increasing amounts of cholesterol, when added to an aqueous solution of filipin, revealed a continuing change until a molar ratio of approximately one to one was attained.

It is important to emphasize the fact that in the present studies it is assumed that there exist only two ultraviolet absorbing species, filipin and the filipin-cholesterol complex. Analysis of the spectral data by the SCATCHARD technique requires that the only absorbing species are filipin and the filipin-cholesterol complex. It is possible that additional cholesterol molecules may add to the complex without producing a further ultraviolet spectral change. The validity of this suggestion might be determined when an X-ray crystallographic study is made on the filipin-cholesterol complex. Though assumptions have been made in defining the complex, the close fit of the data with a 1:1 stoichiometry (Table 1) but not 1:2 or 2:1 stoichiometry strongly support the conclusion of a 1:1 value.

The spectral measurement of the filipin-cholesterol complex presents a potentially useful assay for cholesterol. An example of this is demonstrated utilizing intestinal brush border membranes. One difficulty in this application relates to the turbidity of the membrane solutions which are utilized for spectrophotometric assay. With care this can be avoided. The advantage of this assay is the possibility to determine the cholesterol concentration directly in the membrane suspension without tedious and extensive organic solvent extraction. Further, cholesterol esters do not interfere with the assay.

Filipin is a surface-active compound. It has been clearly demonstrated in a number of systems, *e.g.* chick intestinal mucosa[15,8], liposomes[16], black films[17], *Acholeplasma laidlawii*[18] that the presence of filipin results in permeability changes. Very little is known of the events which occur after the antibiotic is bound to the membrane steroid, which ultimately produces the observed permeability changes. The exact mechanism by which polyenes affect the membrane structure after combining with sterol is not yet known. However the differential scanning calorimetry data[5,14] suggest that some reorientation of the membrane lipid may occur. The present data support the concept that this reorientation involves the specific interaction of one polyene molecule with one sterol molecule which in some manner results in changes in the physical properties of the membrane.

The mode of action of polyene antibiotics is being actively studied with the assumption that some of these compounds may prove useful tools for investigating the lipid architecture of plasma and cytoplasmic membranes[15,8]. When the stoichiometry of interaction between several different polyene antibiotics and steroids is obtained, controlled binding and permeability studies of model and natural membranes can be made. Polyenes may also be used as a

diagnostic tool for determining the type and amount of sterols present in various membrane species. Information from such studies may prove of value in understanding the structure and functioning of membranes.

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