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STUDIES ON THE BIOLOGICAL PROPERTIES OF POLYENE ANTIBIOTICS: COMPARISON OF OTHER POLYENES WITH FILIPIN IN THEIR ABILITY TO INTERACT SPECIFICALLY WITH STEROL

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SUMMARY

1. The interaction of the five polyene antibiotics filipin, etruscomycin, pimaricin, nystatin and amphotericin B with sterol, primarily free, liposomal and membrane bound cholesterol has been examined. Each of these antibiotics has a characteristic ultraviolet absorption spectrum in aqueous or organic solvents with three or four ultraviolet absorption maxima.

Addition of free cholesterol to aqueous solutions of these antibiotics results in a change of the ratio of the ultraviolet absorbance maxima. The order of effectiveness of interaction with cholesterol as judged by this criterion was filipin, amphotericin B, etruscomycin and pimaricin.

2. The same alteration in ultraviolet absorption spectra of filipin, etruscomycin and amphotericin B observed with addition of free cholesterol to aqueous solutions of these antibiotics also occurs upon addition of these antibiotics to liposomal, erythrocyte or *Acholeplasma* membrane bound cholesterol. No spectral change was found in membranes devoid of cholesterol. This spectral alteration of the antibiotic was accompanied by a binding of the antibiotic to the membrane. Both nystatin and pimaricin showed little change in spectrum with sterol in these systems, either the artificial or natural membranes which contained cholesterol.

3. The structural requirements of the sterol for the spectral change with filipin, etruscomycin and amphotericin B include a planar sterol nucleus, an intact side chain at C-17 and a 3β-hydroxyl group. The spectral change was not affected by the pH except in the case of amphotericin B.

4. Measurements by differential scanning calorimetry of the effect of these polyene antibiotics on the phase transition of lecithin and lecithin–cholesterol showed that all polyenes can reduce the lecithin–cholesterol interaction.

INTRODUCTION

Approximately some 40 polyene antibiotics have been described in the literature[1]. They are characterized by a macrolide ring and varying numbers of double

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bonds and hydroxyl functional groups. For the most studied polyenes, filipin[2], etruscomycin[3], pimaricin[4], nystatin[5] and amphotericin B (ref. 6), complete or nearly complete structures have been proposed. As can be seen by comparison of the published structures for the antibiotics (Fig. 1) only filipin is a neutral molecule. The other antibiotics contain a carboxyl function and an amino sugar, mycosamine, which render these antibiotics amphoteric.

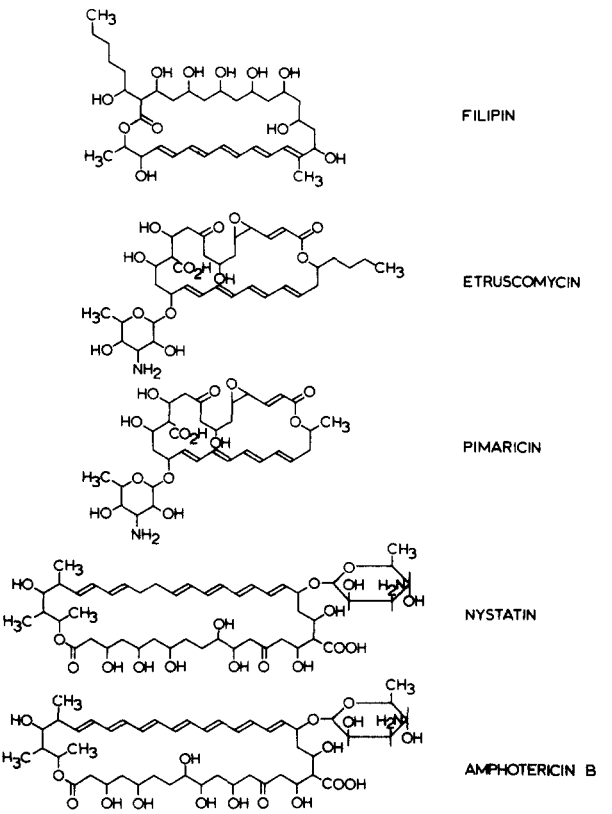

Fig 1 Structures proposed for the different polyene antibiotics.

Polyene antibiotics have been shown to mediate changes in permeability of a variety of cells and model systems, as a consequence of interaction of the polyene with either natural cell membranes or synthetic membranes or bilayers. The above antibiotics have been shown to interact with or cause permeability changes in erythrocytes[7,8], intestinal mucosal cells from vitamin D-deficient chicks[9,10], fungi[11], mycoplasma[12,13] with liposomes[14–16], with bilayers or "black films"[17], and with either pure monolayers or mixed lipid monolayers[18,19]. The concept has been advanced that the presence of sterols in natural membranes is a requirement for polyene sensitivity, particularly filipin sensitivity. This has also proven to be the case in the artificial liposomal, lipid bilayer and monolayer systems. However, an important unresolved question is whether the other polyene antibiotics, amphotericin B, nystatin, etruscomycin and pimaricin, have as "absolute" a requirement for sterol as does filipin.

We have recently reported spectrophotometric and calorimetric data[19] which have permitted a direct study of the structural properties of the filipin–sterol complex. Further, we have found that some physical properties of artificial bilayers and natural membranes change as a result of the formation of the filipin–cholesterol complex, thus validating the "sterol hypothesis" for filipin. It is the purpose of this report to extend the spectrophotometric and differential calorimetric scanning assay to a study of the interaction of the other polyene antibiotics with sterol.

METHODS

Polyene antibiotics

The following polyene antibiotics were employed in this study: filipin Lot U-5956 supplied by Dr G. B. Whitfield of the Upjohn Co., Kalamazoo, Mich.; amphotericin B and nystatin, supplied by the Squibb Institute for Medical Research, New Brunswick, N. J.; pimaricin supplied by Mycofarm-Delft, Division of Royal Netherlands Fermentation Industries, Delft, Holland; etruscomycin, supplied by Farmitalia, Milan, Italy.

Separate stock solutions of antibiotics, except amphotericin B, were prepared by dissolving 1–5 mg in 1.0 ml of dimethylformamide. Stock solutions of amphotericin B were made in dimethylsulfoxide.

Lipids and steroids

Egg lecithin was isolated and purified according to established procedures[20], 1,2-dielaidoyl-*sn*-glycero-3-phosphorylcholine (18:1t/18:1t/lecithin) was synthesized as described before[28]. The purity was ascertained by thin-layer chromatography employing solvent systems of chloroform–methanol–water (65:35:4, v/v/v).

The steroids were obtained from the following sources: cholest-5-en-3β-ol (cholesterol), 5α-cholestan-3β-ol (cholestanol) (Fluka, Buchs, Switzerland); cholest-5-en-3-one, 5α-cholestan-3-one, (Koch-Light Laboratories, Colnbrook, Bucks, England); cholest-5-en-3α-ol(epi-cholesterol), cholesterol acetate, (Mann Biochemicals, New York); 5β-cholestan-3β-ol (coprostanol), and cetyl alcohol (K and K Laboratories, Hollywood, Calif.); and androstan-3β-ol (Ikapharm, Ramat-Gan, Israel). The sterols were checked for purity by silica gel thin-layer chromatography using a solvent system of chloroform–acetone (98:2, v/v) and if necessary purified by recrystallization or preparative thin-layer chromatography.

All other chemicals and solvents were analytical reagent grade or equivalent.

Preparation of liposomes

Chloroform solutions of lecithin and/or sterol were transferred to a round-bottomed flask and taken to dryness under vacuum. The dried lipid film was dispersed in 10 mM Tris–acetate (pH 2–9) on a Vortex mixer for 1 min or subjected to ultrasonic radiation for 1 min (Branson Sonic Power instrument, Model S-125, Position 4).

Membrane preparations

Red cell ghosts were prepared from human blood by a minor modification[21] of the procedure of Parpart[22]. They were suspended at 1 mg/ml in 20 mM sucrose, 10 mM

Tris–acetate (pH 7.0) and sonicated for 1 min prior to use. Such preparations normally have approx. 0.20 mg of cholesterol per mg of protein.

Acholeplasma laidlawii B (previously denoted as *Mycoplasma laidlawii* strain B) cells were grown in 0.35–1.0 l quantities of lipid-poor media according to the general techniques of McElhaney and Tourtelotte[23]. Membranes were obtained as described by van Golde *et al.*[29]. Fatty acids and cholesterol, when desired, were added to the growth media according to the techniques specified by de Kruyff *et al.*[24]. The cholesterol content of the various membrane preparations was determined by the Lieberman–Burchard technique on an aliquot of the total lipid extract of the membranes.

Spectrophotometric measurements

A Perkin–Elmer two-wavelength, double-beam spectrophotometer, Model 356, was used for all spectrophotometric analysis. With clear solutions it was utilized in the double-beam mode, with opaque solutions or suspensions of liposomes or membranes it was used in the split-beam mode. In this latter instance appropriate base line corrections were applied. The light path of the sample was usually 1.0 cm, but with concentrations of polyene above $1.6 \cdot 10^{-5}$ M, cuvettes with a 0.1-cm light path were employed.

Differential scanning calorimetry

A Perkin–Elmer differential scanning calorimeter, DSC-1B, operating at a scan rate of 8 °C/min (sensitivity setting 1), was used for all calorimetric experiments. A chloroform solution, containing 1.66 mg 1,2-dielaidoyl-*sn*-glycero-3-phosphorylcholine (18:1t/18:1t/lecithin) and in some cases additional 0.398 mg cholesterol (molar ratio lecithin–cholesterol 2:1), was evaporated under vacuum in a conical test tube to complete dryness. After the addition of 40 μl water–glycol (1·1, v/v) the lipid film was dispersed on a Vortex mixer for 15 min at 37 °C. 5 μl of a polyene antibiotic solution (50 mg/ml dimethylformamide) was added to the lipid dispersion. The tube was vortexed at 37 °C for 15 min. In control experiments it was shown that the glycol added to reduce the freezing point of the water, and the 5 μl of dimethylformamide, had no significant effect upon the heat content of the phase transition of the lecithin. 35 μl of the lipid dispersion was sealed in specially constructed aluminum sample pans. The samples were scanned at least 2 times to show that the transitions were completely reversible. The sample head of the calorimeter was cooled with liquid nitrogen, sample pans were flushed with dried nitrogen gas. The calorimeter was calibrated using benzoic acid as a standard. After the scans the exact amount of lecithin in the sample pan was determined by a phosphorus determination. The ultraviolet spectra of the polyene containing lipid dispersions before and after the scans were identical. Only in the case where cholesterol was present in the 18:1t/18:1t/lecithin liposomes, was the typical shift in the ultraviolet spectrum of the polyene indicating the specific interaction between the polyene antibiotic and cholesterol observed.

RESULTS

Previous studies have shown[19,25] that addition of cholesterol or other related sterols to aqueous solutions of filipin brings about a striking alteration in the ultraviolet absorbance spectrum of the polyene antibiotic. This spectral alteration is

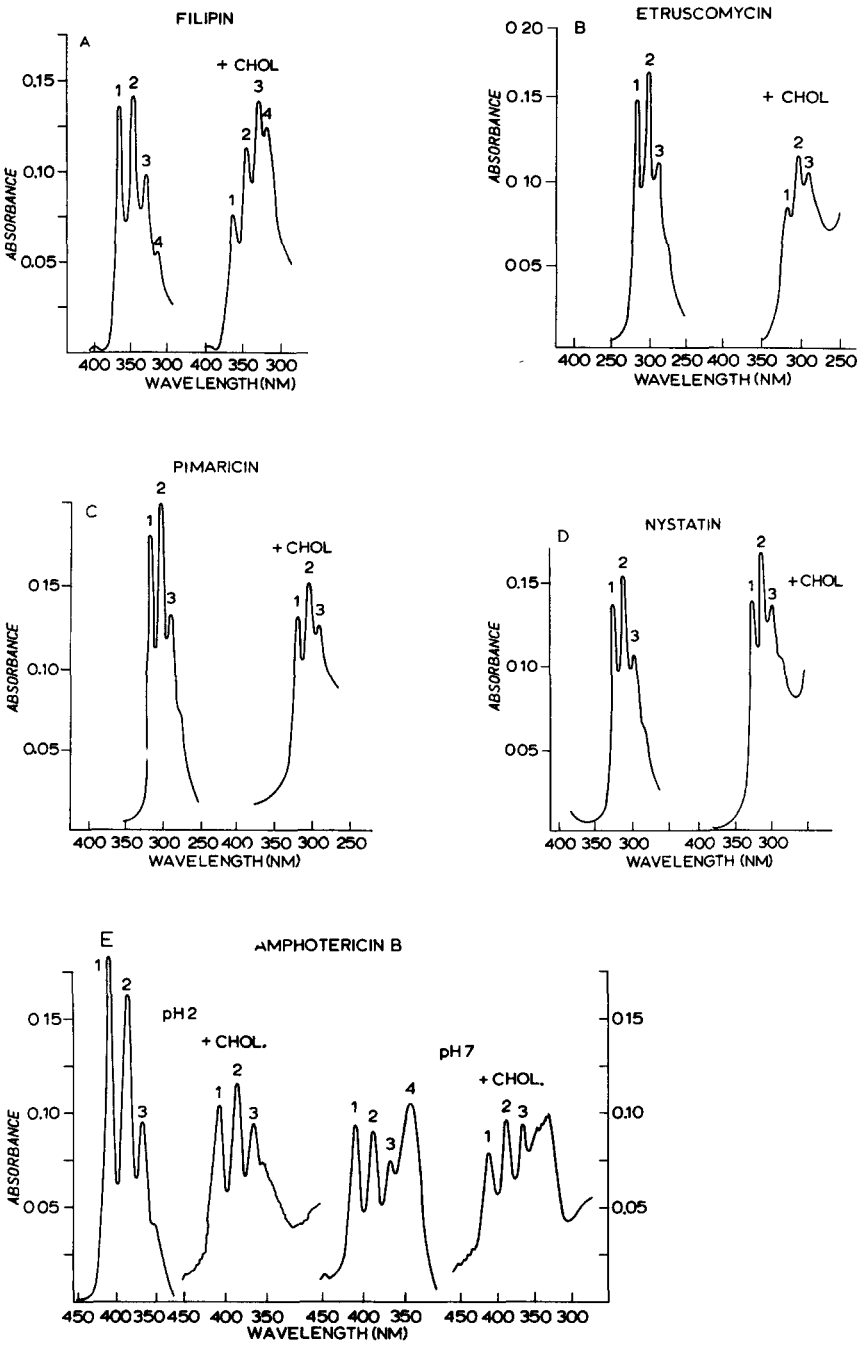

Fig 2 Ultraviolet absorption spectra of the polyene antibiotics in the absence and presence of cholesterol A, filipin, B, etruscomycin; C, pimaricin; D, nystatin; E, amphotericin B. In A–D the polyene antibiotic, approx. $5 \cdot 10^{-6}$ M, was dissolved in 10 mM Tris–acetate buffer (pH 7.0). Cholesterol was added in 25 μl of ethanol to give a sterol concentration of 20 μg/ml For amphotericin B (E) the same protocol was employed at pH 2 0 and 7.0

believed to be a reflection of the formation of a filipin–sterol complex with both a defined stoichiometric and stereochemical relationship. Accordingly, it was of interest to carry out similar studies on other related polyene antibiotics, *e g.* etruscomycin, pimaricin, nystatin and amphotericin B. In Fig. 2 is shown the spectrum of each antibiotic in Tris-acetate buffer (pH 7) and the effect of adding cholesterol to the aqueous solution. The ultraviolet spectra of amphotericin B are strongly influenced by changes in pH and are given at pH 2 and 7 (Fig. 2E).

The ultraviolet spectra of these five polyene antibiotics dissolved in water are highly similar. This of course reflects the ultraviolet-absorbing chromophore of four double bonds for nystatin, pimaricin and etruscomycin, five double bonds for filipin and seven double bonds for amphotericin B. Each spectrum has three major absorption maxima, and for all antibiotics except amphotericin B, Peak 2 has the highest extinction. The wavelength of the absorption maxima for all antibiotics are tabulated in Table I. Qualitatively similar spectra are obtained when each antibiotic is dissolved in methanol except that the extinction coefficient of each peak is increased 20–30 %. We previously noted similar results for filipin[19]. The absorbance of filipin in methanol, but not in water, followed the Beer–Lambert law. A similar behaviour has been noted for all other antibiotics, except amphotericin B. It is likely that each of the polyene antibiotics tends to aggregate or form micelles in water, in the concentration range $1 \cdot 10^{-7}$–$1 \cdot 10^{-4}$ M and that this process has some effect upon the molar extinction coefficient. The ultraviolet absorption spectrum of amphotericin B is dependent on the concentration used. With all antibiotics, except amphotericin B, it was possible to use the apparent molar extinction coefficient of Peak 2 in methanol to determine the concentration of the antibiotic.

Also shown in Fig. 2 is the effect of addition of excess cholesterol, 20 μg/ml, to the aqueous solution of the polyene antibiotics. In each instance there is evidence of some kind of interaction between cholesterol and the respective antibiotic which has an effect upon the ultraviolet absorbance spectrum. This can be characterized by both an overall lowering of the several extinction coefficients and a change in the ratio of absorbance of Peaks 3/1. These results are summarized in Table I

If the extent of the spectral alteration is a reflection of interaction between

TABLE I

WAVELENGTH OF ABSORPTION MAXIMA OF THE VARIOUS POLYENE ANTIBIOTICS

These data are compiled from spectra like those shown in Fig. 2 A–E The antibiotics were all dissolved in 10 mM Tris–acetate buffer (pH 7 0) at approx $5 \cdot 10^{-6}$ M The ratio of ultraviolet absorbance of Peaks 3/1 in the presence of cholesterol should be considered only qualitatively, since the absolute value varies slightly from experiment to experiment.

Antibiotic	*Wavelength of peak (nm)*				*Effect after cholesterol addition*	
	1	*2*	*3*	*4*	*− Cholesterol (peak ratio 3/1)*	*+ Cholesterol (peak ratio 3/1)*
Filipin	356	337	321	311	0.73	1 82
Etruscomycin	319	304	292	282	0 74	1 29
Pimaricin	319	304	292	282	0 73	0 97
Nystatin	320	306	293	281	0 77	0 98
Amphotericin B, pH 2	408	385	363	350	0 52	0 92
Amphotericin B, pH 7	408	385	363	338	0 78	1.19

cholesterol and the antibiotic, as shown for filipin–cholesterol[19] then filipin, etruscomycin, pimaricin and nystatin interact with cholesterol in this order. The closely related polyenes, etruscomycin and pimaricin, which have nearly identical ultraviolet spectra, show a difference in the extent of the spectra change which is in accordance with their different potencies[8]. The behaviour of amphotericin B is more complex. While an increase in the absorbance ratio of Peaks 3/1 occurred at both pH 2 and 7, after cholesterol addition the absorbance of Peak 2 behaved oppositely at these two pH values. Addition of organic solvents like methanol, ethanol or dioxane to 50 vol. percent to aqueous solutions of the antibiotic–cholesterol complex results in complete reversion of the spectra to that characteristic of the free antibiotic. This likely means that such organic solvents are capable of breaking the complex and suggests that no covalent linkages are involved in its formation.

Fig. 3 summarizes the pH-dependent behaviour of the absorbance ratio of Peaks 3/1 after the addition of free cholesterol to aqueous solutions of the polyene antibiotics. No significant pH dependence of the spectral change was found for any of the polyenes except for amphotericin B. The order of effectiveness of interaction of the antibiotic with free cholesterol as evaluated by the magnitude of the ratio change is filipin > etruscomycin > amphotericin B > nystatin = pimaricin.

Fig. 4 summarizes the pH-dependent behaviour of the absorbance ratio of Peaks 3/1 after the addition of lecithin or lecithin–cholesterol liposomes to aqueous solutions of the antibiotics. The order of effectiveness of interaction of the antibiotic with liposomally bound cholesterol as evaluated by the magnitude of the ratio change was principally the same as for crystalline cholesterol. The attainable effect for filipin is even more pronounced with liposomal than with free cholesterol. However, for etruscomycin, pimaricin and nystatin the ability to interact with cholesterol is decreased by the presence of lecithin. As shown in Fig. 3, each of the latter antibiotics gave a definite spectral evidence of interaction with cholesterol, whereas as shown in Fig. 4, these same antibiotics gave little or no spectral evidence of interaction with liposomally bound cholesterol. The heptaene, amphotericin B, on the other hand, has an increased peak ratio 3/1 in the presence of pure lecithin at low pH values. The effects of lecithin–cholesterol liposomes were also very much dependent on the pH. Interestingly, incorporation of 33 or 50 mole % of cholesterol in the

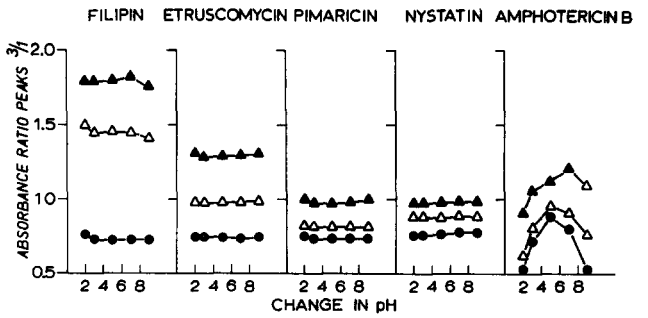

Fig. 3 Summary of interaction of polyene antibiotics with free cholesterol. Each polyene antibiotic, approx $5 \cdot 10^{-6}$ M, was placed in 10 mM Tris–acetate at the indicated pH. Cholesterol was first added to 10 μg/ml, and then 20 μg/ml. After each addition the sample was mixed and allowed to stand 30 min before the spectrum was measured No sterol (●); 10 μg/ml cholesterol (△); 20 μg/ml cholesterol (▲)

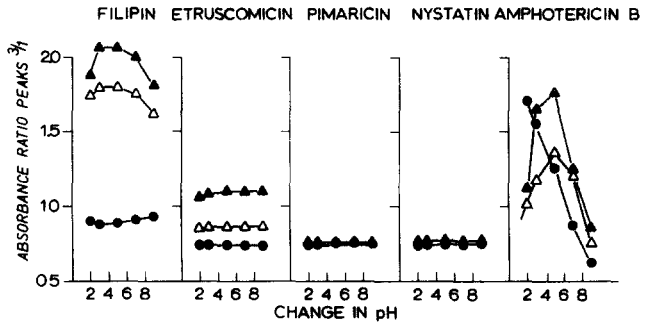

Fig 4 Summary of interaction of polyene antibiotics with cholesterol in liposomes Each polyene antibiotic, approx $5 \cdot 10^{-6}$ M, was placed in 10 mM Tris–acetate buffer at the indicated pH in the presence of either egg lecithin, 100 mole% (●), or liposomes of lecithin–cholesterol, 67 33 (△) or 50:50 mole% (▲). The final concentration of the cholesterol was 20 and 40 μg/ml, respectively

lecithin liposomes suppressed the interaction of amphotericin B with these liposomes at pH 2–3.

Shown in Table II are the results of a comparative study of some of the structural requirements of liposomally bound sterol for interaction with the five different polyene antibiotics. Previous more detailed studies involving only filipin[19] have emphasized that with free sterol the interaction is primarily hydrophobic in nature. When the sterol was incorporated into liposomes additional structural requirements were apparent. Sterols with a 3β-hydroxyl group were much more effective than those with either 3α-hydroxyl or a 3-keto functional group. Also the planar ring structure and the side chain of the sterol are involved in the interaction. Cholesterol, cholestanol and stigmasterol proved to be the most effective sterols. Similar results are shown in Table II for etruscomycin and amphotericin B. Of the sterols tested, cholesterol followed by cholestanol were the most effective sterols. The 3α-hydroxyl sterol, epicholesterol, and the 3-keto steroids, were much less effective in their ability to mediate any spectral alteration in these antibiotics. Thus similarities exist between the filipin–sterol structural requirements for interaction and the etruscomycin or amphotericin B sterol structural requirements for modification of the ultraviolet

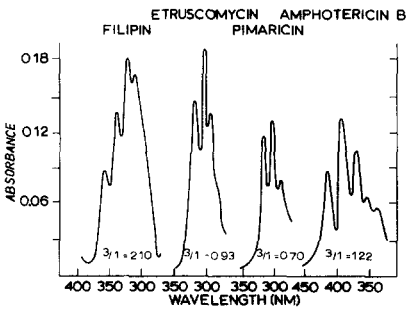

Fig. 5 Interaction of polyene antibiotics with erythrocyte ghost membranes. Sonicated erythrocyte ghost membranes, prepared as described under Methods were suspended in 10 mM Tris–acetate buffer (pH 7.0), 1.0 ml, and the indicated polyene antibiotic added in 10 μl of dimethylformamide to give approx $5 \cdot 10^{-6}$ M The ratio of absorbance of Peaks 3/1 is indicated on the figure

TABLE II

INTERACTION OF VARIOUS POLYENE ANTIBIOTICS WITH EGG LECITHIN:STEROL LIPOSOMES

The interaction of the various polyene antibiotics with egg lecithin liposomes containing 16 mole% of steroid was determined by measuring the absorbance ratio of Peaks 3/1 In each instance the liposomes were suspended in 10 mM Tris–acetate buffer (pH 7 0) so that the steroid concentration was 7 7 μg/ml The final concentration of the antibiotic was $5 \cdot 10^{-6}$ M The liposomes and antibiotic were mixed and allowed to stand 30 min before measurement

Compound	*Absorbance ratio, Peaks 3/1*				
	Filipin	*Etruscomycin*	*Pimaricin*	*Nystatin*	*Amphotericin B*
None	0.73	0.74	0 73	0 77	0 78
Egg lecithin	0 88	0 74	0.73	0 77	0 87
Cholesterol	1.40	0 98	0 78	0 85	2 00
Cholestanol	1.35	0 93	0 77	0 83	1.85
Androstane-3β-ol	0 94	0 86	0 78	0 80	1 14
Coprostanol	0 90	0.77	0.78	0.78	1.11
Epicholesterol	0 85	0 82	0 78	0.79	1 08
5α-Cholestan-3-one	0 89	0 84	0 79	0 82	1 11
Cholestan-3-one	0 90	0 80	0 77	0 80	1 28
Cholesterolacetate	0.88	0 81	0 77	0 79	1 17
Cetylalcohol	0.78	0 82	0 78	0 79	1 31

TABLE III

SUMMARY OF INTERACTION OF VARIOUS POLYENE ANTIBIOTICS WITH MEMBRANES FROM *A laidlawii* CONTAINING OR LACKING CHOLESTEROL

Membranes were prepared as described under Materials and Methods from *A laidlawii* grown either in the presence (+C) or absence (−C) of cholesterol They were suspended in 10 mM Tris–acetate buffer (pH 7 0 at 3 0 mg/protein per ml) Then the indicated antibiotic was added in 25 μl of dimethylformamide to 2 5 ml of membrane suspension to give a concentration of $5 \cdot 10^{-6}$ M After 30 min the spectra were recorded using an untreated membrane suspension as blank Then all membrane suspensions (blanks and antibiotic treated) were centrifuged at 50000 rev/min in a Spinco rotor 50 for 60 min and the spectra and protein determinations were made on the supernatant solution

	Antibiotic									
	Filipin		*Etruscomycin*		*Pimaricin*		*Nystatin*		*Amphotericin B*	
	−C	+C	−C	+C	−C	+C	−C	+C	−C	+C
Peak ratio 3/1:	*0 81*	*2 25*	*0 73*	*1.25*	*0 67*	*0 75*	*0 75*	*0 72*	*1 00*	*1 55*
After centrifugation										
% antibiotic in supernatant	84	36	69	25	95	75	96	84	60	45
% protein in supernatant	36	37	35	39	38	32	39	45	34	37

absorbance spectra. Again nystatin and pimaricin showed very little evidence of interaction with any sterols, in agreement with the results in Fig. 3.

Having established that several of the polyene antibiotics could interact with either free or liposomally bound sterol, it was of interest to further examine this interaction in sterol-containing natural membranes. In Fig. 5 are shown the effect of addition of sonicated erythrocyte ghost membranes to aqueous solutions of filipin, etruscomycin, pimaricin and amphotericin B. Again, the order of effectiveness of interaction, as evaluated by the change in ratio of ultraviolet absorbance of Peaks 3/1 is filipin $\gg$ amphotericin B $>$ etruscomycin $>$ pimaricin, filipin being significantly more effective than amphotericin B It should be noted that erythrocyte ghost membranes normally have approximately 50 mole% of their total lipid present as cholesterol which is similar to the liposomes formed from lecithin–sterol (50:50) shown in Fig. 4.

In Fig. 6 a further examination of the interaction of the five polyene antibiotics with cholesterol-free and cholesterol-containing membranes isolated from *A. laidlawii* is presented. Again, the largest change in spectra occurs only with the cholesterol-containing membranes in the presence of either filipin, etruscomycin or amphotericin B. Essentially no alteration of the spectra of these antibiotics occurred in the cholesterol-free membranes. No change in spectra occurred with nystatin or pimaricin with either the cholesterol-free or cholesterol-containing membranes.

In Table III the results are shown of an additional experiment with these same membrane preparations and antibiotics. The spectra of the five antibiotics in the presence of sterol-free and cholesterol-containing membranes, were measured before and after centrifugation at 50000 rev./min for 1 h.

The actual polyene antibiotic concentration was determined by diluting the solution 1.1 with methanol. As can be noticed from the high percent of protein in the supernatant, only part of the membrane fragments could be sedimented. Thus the ratio change due to the presence of sterol was measured, and also the binding of the antibiotic to the membrane fractions was assessed. With the cholesterol-free membranes only amphotericin B had an increased ratio of ultraviolet absorbance of Peaks 3/1 and only amphotericin B and etruscomycin did bind significantly to the cholesterol-lacking membrane fractions. With the cholesterol-containing membrane fraction, filipin, amphotericin B and etruscomycin all had a significant increase in ultraviolet absorbance ratio of Peaks 3/1. Also, for these same three antibiotics there was a good correlation between percent polyene sedimented and the percent of the membrane protein sedimented. The order of binding of these antibiotics with cholesterol-containing membranes is filipin $>$ etruscomycin $>$ amphotericin B $\gg$ nystatin $=$ pimaricin.

Table IV presents results of differential scanning calorimetric analysis of pure lecithin and lecithin–cholesterol mixtures in the absence and presence of various polyene antibiotics. Pure lecithin with 2 mono-unsaturated *trans*-structured fatty acids exhibits in excess water–glycol (1·1, v/v) a phase transition, with a maximal heat absorption at 13 °C, characteristic of conversion of the lecithin from the L-(β)-crystalline phase to the L-(α)-liquid crystalline phase. The addition of cholesterol reduces the energy content of the phase transition (Table IV and Fig. 7). This can be explained by a liquefying effect of cholesterol on a lecithin bilayer in the crystalline state[27]. The addition of polyene antibiotic to the lecithin–cholesterol phase causes

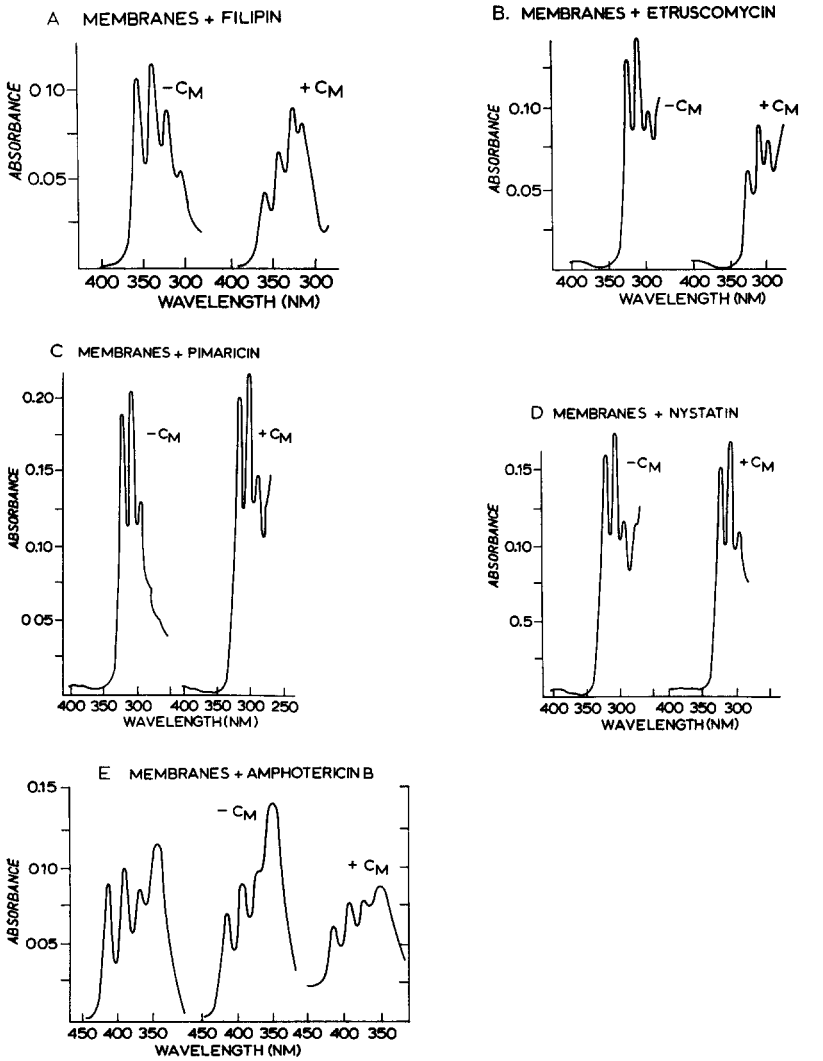

Fig 6 Interaction of polyene antibiotics with membranes from *A laidlawii*. Cholesterol-free ($-C_m$) and cholesterol-containing ($+C_m$) membranes were obtained from *A. laidlawii* as described under Methods They were suspended in 1 0 ml of 10 mM Tris–acetate buffer (pH 7.0), filipin (A), etruscomycin (B), pimaricin (C), nystatin (D) and amphotericin B (E) were added in 10 μl of dimethylformamide to give a concentration of approx $5 \cdot 10^{-6}$ M

a striking reversal of the effect of cholesterol on the phase transition. All the polyene antibiotics show the ability to bind and reorient cholesterol in such a way that cholesterol can no longer interact with the lecithin molecules. No significant effect of the polyenes is observed on pure lecithin preparations. From the data presented in Table IV, and Fig. 7 of ref. 24, it is possible to calculate some approximate stoichiometrics for the polyene antibiotic–sterol interaction. The calculated number of cholesterol molecules that can be complexed by filipin, etruscomycin, pimaricin, nystatin, amphotericin B is, respectively, 2.3, 1,2; 3.5; 2.4; 7.9. It has to be noticed

however, that these experiments had to be carried out in highly concentrated solutions, in which the polyenes can aggregate to a different extent than in the dilute solutions, so that these experiments can only be compared qualitatively with the other experiments. Whether under more physiological conditions the same "stoichiometrics" for the cholesterol–polyene antibiotic interaction can be found has still to be answered.

TABLE IV

MEASUREMENT, BY DIFFERENTIAL SCANNING CALORIMETRY, OF THE EFFECT OF VARIOUS POLYENE ANTIBIOTICS ON THE PHASE TRANSITION OF LECITHIN AND LECITHIN–CHOLESTEROL

Conditions as described in the legend of Fig 7

	Energy content of the phase transition (kcal/mole)	
	18 1t/18·1t/lecithin	*18 1t/18·1t/lecithin + 33 mole % cholesterol*
No polyene antibiotic	10 0	4 0
Filipin	9 6	6 0
Etruscomycin	9 4	5 4
Pimaricin	9 3	7 8
Nystatin	8 9	5 9
Amphotericin B	10 3	10 0

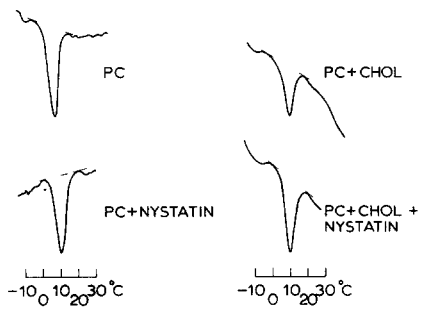

Fig 7 Effect of cholesterol and nystatin on the phase transition of 18:1t/18.1t/lecithin To 1 66 mg 18:1t/18:1t/lecithin or 1 66 mg 18:1t/18:1t/lecithin + 0.398 mg cholesterol in 40 μl water–glycol (1.1, v/v), 0 25 mg nystatin in 5 μl dimethylformamide was added. The effect of cholesterol and nystatin on the phase transition of the 18:1t/18:1t/lecithin was determined by differential scanning calorimetry as described under Material and Methods PC stands for 18.1t/18.1t/lecithin

DISCUSSION

We have previously presented evidence that the polyene antibiotic filipin is capable of interacting in a stereochemically and stoichiometrically defined manner with cholesterol and other closely related sterols to form a filipin–sterol complex[19]. This complex was shown to exist with free or liposomally bound cholesterol and with cholesterol-containing natural membranes. These results were obtained by studying the sterol-mediated alteration in the ultraviolet absorption spectra of aqueous solutions of filipin. In this report we have extended this technique to study the possible interaction of several other polyene antibiotics, *e.g.* etruscomycin, pimaricin, nystatin and amphotericin B with sterol. With free cholesterol the order of effectiveness in

effecting a spectral change is filipin > etruscomycin > amphotericin B > nystatin = pimaricin. However, when the cholesterol was incorporated into liposomes the order of effectiveness became filipin > amphotericin B > etruscomycin while nystatin and pimaricin showed little or no spectral evidence of interaction with liposomally bound cholesterol. Experiments with filipin[19] have established that the spectral alteration is specific for sterol, little or no change in the ultraviolet absorbance ratio occurred with lecithin, cetylalcohol, bovine serum albumin or glucose. For filipin, etruscomycin and amphotericin B it is shown that the interaction with liposomal bound sterol is dependent on: (a) planar sterol nucleus, (b) intact side chain at C_{17}, (c) 3β-hydroxyl group. It is likely that the same mode of interaction with the sterol is applicable to these three antibiotics. In the case of filipin it was previously concluded that this interaction was primarily hydrophobic in nature[19].

Experiments with biological membranes showed that a spectral change can be observed only when cholesterol is present in the membrane. The order of interaction found for erythrocyte membranes and *Acholeplasma* membranes which contain cholesterol is filipin > amphotericin B > etruscomycin > pimaricin = nystatin. Many biological studies have demonstrated that filipin is the most biologically active of these polyene antibiotics[1,8,16-18,25], which is in agreement with the observation that sterols cause the largest spectral change with filipin. Also for the amphoteric polyene, etruscomycin, especially with free cholesterol but also with membrane-bound cholesterol, a significant change in peak ratio is found. However, for the structurally very closely related pimaricin only very weak spectral changes are found. Pimaricin lacks, compared to etruscomycin, a side chain of three carbon atoms. The structures of nystatin and amphotericin B are also very similar. In nystatin, however, the conjugated double bond system is interrupted. The spectral changes of nystatin are weak and comparable with pimaricin. The effects of amphotericin B in the presence of cholesterol are more pronounced but more complicated and influenced by pH and concentration effects.

The experiments on differential scanning calorimetry show that all polyenes tested react with cholesterol in such a way that cholesterol is withdrawn from its interaction with lecithin. In the case of amphotericin B the effect of cholesterol on the phase transition was even completely reversed. All the studied polyenes bind to sterol-containing *Acholeplasma* membranes, although pimaricin and nystatin are bound to the membrane to a smaller extent than the other polyenes. So the absence of spectral changes in the case of pimaricin and nystatin does not indicate that these sterols would not interact with membrane-bound sterol. Possible differences in mode of action between the polyenes has already been suggested from electron microscopic observations[31] and differences in polyene-mediated permeabilities of different solutes through bimolecular lipid films[17,32,33]. The present results establish that filipin, amphotericin B and etruscomycin have the strongest effect on the sterol reorientation which is in good agreement with previous work on model lipid systems[17,18]. Little or no evidence was found that these antibiotics could also interact with sterol-free systems as observed by Weissmann and Sessa[30]. The order of spectral changes as observed in this study does not necessarily mean that other membrane effects induced by polyene antibiotics will emerge in the same order. For the ultimate effect also rate of interaction with the cell membrane and the extent of binding and reorientation is of crucial importance for the physiological effect. The effect of polyene

antibiotics on the permeability of ions from liposomes or *Acholeplasma* cells, having incorporated different sterols, is now under investigation.

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