EFFECTS OF EXPERIMENTAL CONDITIONS ON THE INTERACTION
OF FILIPIN AND PIMARICIN WITH CHOLESTEROL

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The effect of salts, small neutral molecules, detergents, and organic solvents on the interaction of filipin and pimaricin with cholesterol was investigated using fluorometric techniques. The salts and small molecules, at concentrations of 0.5 mm ~ 100 mm, showed no effect on the interaction of filipin with cholesterol. The interaction of pimaricin with cholesterol was slightly decreased in the presence of 100 mm $MgCl_2$ or $CaCl_2$. The detergents sodium deoxycholate and lauryl sulfate altered the fluorescence properties of the polyenes and precluded the interaction of the polyenes with cholesterol. The organic solvents dimethylformamide, ethanol, acetone, and iso-propanol when present at 0.1 ~ 1 % (v/v) did not affect the fluorescence properties of pimaricin or the pimaricin-cholesterol complex. However, these solvents changed the fluorescence properties of filipin as well as its capacity to interact with cholesterol. These data indicate that low concentrations of some organic solvents and detergents can affect the fluorescence properties of these polyenes in a manner not directly related to the interaction of the polyene with sterol. Light scattering experiments indicate that the different solvents used to solubilize cholesterol produce suspensions of varying size when injected into the aqueous solutions. The extent of the subsequent interaction with the polyene antibiotic correlates with the light scattering properties of these suspensions.

Polyene antibiotics can cause lethal changes in membrane permeability when administered to organisms that contain membrane-associated sterols[1]. Evidence that membrane sterols are the primary target for these antibiotics has come largely from biological[2,3], spectrophotometric[2,4~10] and fluorometric[5,6,9~13] studies. The mechanism of the polyene-sterol interaction and the nature of the resulting complex is not clearly understood and interpretations of experimental results have been complicated by conflicting data reported by different laboratories on similar aspects of the polyene-sterol system. It has been observed that in a single experiment, biological, spectrophotometric, and fluorometric measurements may provide different indices of the extent of polyene-sterol interaction[2,10].

Many of the observed discrepancies undoubtedly result from the low solubilities in aqueous systems of the polyene and sterol components and their complexes. The inherent tendency for aggregation, precipitation, and micelle formation complicates many experiments. It would be expected that such properties would make studies on these systems particularly sensitive to conditions of temperature, pH, ionic strength, method and vehicle of reagent introduction, incubation, time, *etc.* Indeed, certain polyene-sterol systems have been shown to be sensitive to pH[5,7,9], and time and temperature parameters are known to dramatically affect experimental observations[6,9]. Certain organic molecules are known to disrupt the polyene-sterol complexes[5,7,9]. Also, Mg^{++} and Ca^{++} were found to inhibit the effect of pimaricin on acetate oxidation by *Saccharomyces cerevisiae*[19].

The effects of commonly used salts, solubilizing agents, and organic solvents on the interaction of filipin and pimaricin with cholesterol are reported herein. These antibiotics were chosen as subjects

of this study because of the wide range of biological activity, chemical characteristics, and spectroscopic properties which they represent[14].

Materials and Methods

Special chemicals were obtained as follows: Cholesterol and sodium dodecyl sulfate from Sigma Chemical Company, St. Louis, MO., silicic acid (Bio-Sil A, 100~200 mesh) from Bio Rad Laboratories, sodium deoxycholic acid from Pfaltz and Bauer, Stanford, Conn., and pimaricin was a generous gift from the American Cyanamid Company, Princeton, N.J. Other chemicals were of reagent grade.

Preparation of Filipin

Streptomyces filipinensis was kindly provided by P. G. PRIDHAM, ARS, USDA, Peoria, I.L. The organism was grown as referenced[15]. The filipin complex was isolated from the culture filtrate as described by WHITFIELD et al.[16] Further purification was achieved by a modification of the chromatographic procedure described by BERGY and EBLE[17]. Silicic acid, 30 g, was equilibrated in a 97: 3 (v/v) mixture of methylene chloride - methanol. The slurry was poured into a 2.3-cm diameter column and allowed to settle. Thirty mg of the filipin complex, purified as described above, was dissolved in the methylene chloride - methanol mixture [97: 3 (v/v)] and added to the column followed by washing with 125 ml of the same solvent. The filipin complex was eluted from the column with approximately 300 mls of a 90: 10 mixture of methylene chloride: methanol with UV monitoring at 365 nm. The fractions containing polyene were evaporated *in vacuo*, and the polyene antibiotic was resuspended in *t*-butyl alcohol and lyophilized.

Previous studies by SCHROEDER et al.[6] showed that freshly prepared solutions of filipin, did not interact immediately with cholesterol. In aqueous solutions the absorbance spectrum of this preparation underwent a change which apparently coincided with a conversion to a form capable of binding cholesterol. Those investigations were all done with the same preparation of filipin obtained from the Upjohn Company. We have since tested other lots and have isolated several batches of filipin. None of these latter preparations have shown an inactive form of the antibiotic. The basis for this difference is not known. However, recent preparations of filipin are purer than the earlier ones as judged by thin-layer chromatography and the shape of the relative fluorescence efficiency (RFE) curves*. The distortions of the RFE curves for the initial preparation indicated the presence of some absorbing but non-fluorescing species, while subsequent preparations gave a flatter curve, indicating that these preparations contain less non-filipin chromophoric components.

Protocol of Binding Experiments

Stock solutions of filipin were prepared by dissolving 1.0 mg of the antibiotic in 200 ml of doubly distilled water with vigorous stirring for 24 hours at room temperature in the dark. Cholesterol was dissolved in iso-propanol and added to aqueous filipin samples by injecting small volumes with a Hamilton syringe. For the binding experiments involving pimaricin, stock solutions of 15 μM pimaricin were prepared by dissolving 2.0 mg in 100 ml of 0.1 M tris-HCl (pH 7.0) with vigorous stirring for 2 hours at room temperature. Cholesterol preparations were made by injecting an acetone solution of the sterol into boiling 0.01 M Tris-HCl (pH 7.0) to give the appropriate concentration. Boiling was continued for 3 minutes to remove the acetone and aliquots were added, while still hot, to the polyene solution. Mixtures were allowed to stand at room temperature in the dark for 3 hours prior to measurement with pimaricin.

Fluorescence measurements were obtained using a computer-centered spectro-fluorimeter[18]. The analytical quantities, absorbance corrected fluorescence (CO) and relative fluorescence efficiency (RFE), originally termed partial quantum efficiency (PQ), were used in these experiments. Definitions of these quantities and a discussion of the advantages of their use over conventional fluorescence techniques are discussed elsewhere[5,18].

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* The term filipin, as used throughout this paper, refers to preparations of filipin complex, which are known to contain several isomeric components.

Results and Discussion

Effects of Inorganic Salts and Various Organic Compounds on the Interaction
of Filipin and Pimaricin with Cholesterol

As shown in Table 1, the addition of a 1.4 molar excess of cholesterol to 7 μM solutions of filipin resulted in a 33% decrease in RFE, indicating interaction of filipin with cholesterol. The presence of NaCl, KCl, MgCl$_2$, CaCl$_2$, KH$_2$PO$_4$, sucrose, citrate, malate, succinate, or urea at concentrations ranging from 0.5 mM to 100 mM did not appreciably affect the extent of interaction as evidenced by these fluorescence measurements. The interaction of filipin with cholesterol was moderately inhibited by the presence of urea when the concentration of this hydrogen-bond disrupting agent exceeded 3 M. Similarly, the addition of large amounts of urea (5 M final concentration) to solutions containing the filipin-

Table 1. Effect of salts and small molecules on the interaction of filipin with cholesterol.

Compound	RFE				
	(No Salt)	$[5 \times 10^{-4}$ M]	$[5 \times 10^{-3}$ M]	$[5 \times 10^{-2}$ M]	$[10^{-1}$ M]
Control	100±2	—	—	—	—
No cholesterol	150±2	—	—	—	—
NaCl	—	101±2	102±3	101±2	99±2
MgCl$_2$	—	101±2	98±3	98±3	99±3
CaCl$_2$	—	100±2	100±3	99±1	99±3
KCl	—	101±1	99±2	99±1	100±2
KH$_2$PO$_4$; pH 6.5	—	100±2	100±4	99±3	99±3
Sucrose	—	100±1	99±1	99±0	99±1
K Citrate; pH 7.4	—	100±1	100±3	99±4	99±4
K Malate; pH 6.0	—	100±2	102±3	98±2	99±3
Na Succinate; pH 6.0	—	100±3	98±2	97±2	98±4
Urea	—	100±1	99±2	98±1	100±3
Glycerol	—	99±1	100±2	100±1	101±2
(v/v% glycerol only)	(0%)	(5%)	(10%)	(20%)	(30%)

For each measurement, 3 ml sample containing 2.3×10^{-8} moles of filipin was dissolved in the above solutions of salts or small molecules and incubated with 3.22×10^{-8} moles of cholesterol in isopropanol. Measurements were made at 338 nm excitation and 495 nm emission.

The values in parenthesis for glycerol indicate its concentration in distilled water as being 5%, 10%, 20% and 30% (v/v).

Table 2. Effects of salts and small molecules on the interaction of pimaricin with cholesterol.

Sample	RFE							
	No additions	NaCl	KCl	KH$_2$PO$_4$	MgCl$_2$	CaCl$_2$	Sucrose	Succinate
Pimaricin	3	3	3	3	3	3	3	3
Pimaricin & cholesterol	59	60	60	59	56	55	59	60

The RFE values for 2 μM solutions of pimaricin containing the above salts at a concentration of 0.1 M were determined. Solutions were prepared in 0.01 M Tris–HCl buffer and the pH of all samples was adjusted to pH 7 with dilute HCl or NaOH if required. Cholesterol was then added (as described in "Methods and Materials") at a concentration of 3 μM and the samples incubated for 3 hours prior to measurement of RFE. The listed values are the mean of 5 determinations with variations of $\pm < 3$%.

cholesterol complex caused partial disruption of the complex.

The presence of NaCl, KCl, KH₂PO₄, sucrose, or succinate, at concentrations up to 0.1 M had no appreciable effect on the interaction of pimaricin with cholesterol. The addition of cholesterol to solutions of pimaricin caused the RFE to increase from 3 to 60; similar results were obtained when the above-mentioned salts were present (Table 2). The slight inhibition of this interaction caused by 0.1 M CaCl₂ and 0.1 M MgCl₂ was reproducible, although varying the experimental protocol changed the magnitude of the observed effect. These data suggest a direct effect of the divalent ions on the pimaricin-cholesterol interaction and support the biological evidence of interference of this interaction by these ions[19].

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Effects of Deoxycholate and Lauryl Sulfate on the Interaction of Filipin and

Pimaricin with Cholesterol

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Some commerical polyene antibiotic formulations include solubilizing agents which serve to facilitate the dispersion of these sparingly soluble compounds in biological fluids. The effects of bile salts upon the biological activity of polyene antibiotics have been examined[20,21]. While there is evidence supporting a reversible, hydrophobic attraction between polyenes and sterols, the effects on this interaction of relatively strong detergents such as those used in polyene formulations, is not well documented. The effects of additions of deoxycholate on the fluorescence characteristics of aqueous solutions of free (○—○) and sterol-bound (●—●) filipin are shown in Fig. 1. The lower curves show the reduced fluorescence of sterol-bound filipin relative to the unbound species (upper curves). Deoxycholate causes two effects. First, the filipin-cholesterol complex is disrupted by deoxycholate at concentrations ranging from 5 mM to 50 mM. At 50 mM dissociation of the complex is complete. Secondly, the presence of deoxycholate enhances the fluorescence efficiency of the free polyene. Similar results were observed when this experiment was repeated using lauryl sulfate instead of deoxycholate (Fig. 1b). However, being a

Fig. 1. Effects of deoxycholate and lauryl sulfate on the interaction of filipin with cholesterol.

Each 3 ml sample contained 2.28×10^{-8} moles of filipin. The samples with cholesterol contained 3.23×10^{-8} mole of the sterol which was added by injecting with iso-propanol. Detergents were added at the concentrations indicated by dissolving the appropriate amount of the sodium salt. Fluorescence of the samples was measured using 338 nm excitation and an emission wavelength of 495 nm.
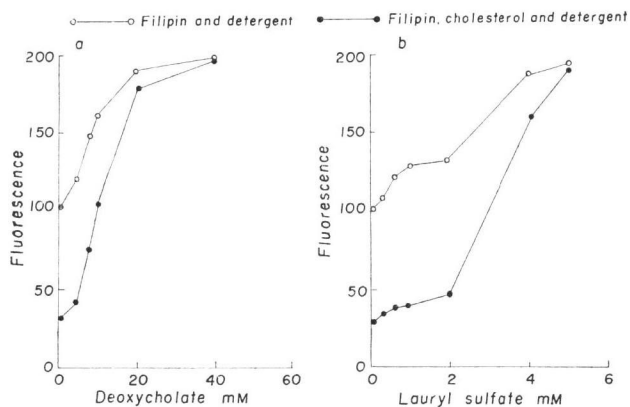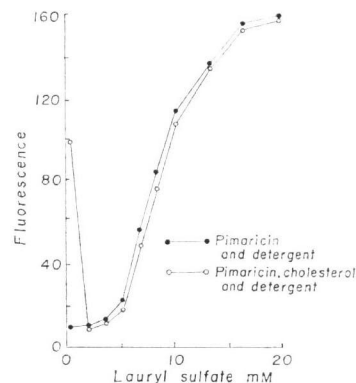
Fig. 2. Effects of lauryl sulfate on the interaction of pimaricin with cholesterol.

Each 4 ml sample contained 2×10^{-8} mole pimaricin and lauryl sulfate at the indicated concentrations. In those samples containing cholesterol 3×10^{-8} moles were used. Fluorescence of the samples was measured using excitation (320 nm) and emission (415 nm) wavelengths.

stronger detergent, lauryl sulfate caused complete disruption of the polyene at much lower concentrations.

The presence of lauryl sulfate in aqueous solutions of pimaricin caused a large increase in the fluorescence efficiency of the free polyene at concentrations above 6 mM (Fig. 2). At lower concentrations, however, this detergent showed a dramatic effect upon the fluorescence of pimaricin-cholesterol mixtures. At a concentration of 2 mM lauryl sulfate, the development of a strongly fluorescing species, characteristic of cholesterol-bound pimaricin, was inhibited, and a level of fluorescence was observed which indicated the presence of free polyene. Higher concentrations of this detergent caused an increase in fluorescence to a level much higher than that of the original pimaricin-sterol complex. Similar results were obtained when deoxycholate was present in solutions of bound and unbound pimaricin. Again, the weaker detergent properties of deoxycholate resulted in less inhibition of complex formation when compared to that observed for lauryl sulfate at a similar concentration.

These experiments indicate that polyene-sterol interaction is prevented by the presence of these detergents at low concentrations, and if pre-formed, such complexes can be disrupted by similar concentrations of detergent. The increased fluorescence efficiency of free filipin and pimaricin in the presence of these detergents may result from the sequestration of the polyene into micelles. In this situation the increased polarizability of the fluorophore's environment would be predicted to cause enhanced fluorescence emission[22].

## Effects of Organic Solvents on the Interactions of Filipin and Pimaricin with Cholesterol

The extremely low water solubilities of sterols such as cholesterol has led to the use of organic solvents as vehicles for the introduction of these compounds into aqueous mixtures. Many polyene antibiotics exhibit low water solubility and are commonly introduced into aqueous solutions in a similar fashion. In studies of polyene-sterol interactions, any effects on the experimental observations that may be produced by the solvent used for these additions have generally been ignored. The following experiments were conducted to test whether this is a valid assumption. The results of additions of various organic solvents (1% v/v) to solutions containing pimaricin and cholesterol are shown in Table 3. As evidenced by fluorescence measurements, these solvents had only minor effects upon the pimaricin cholesterol complex.

As shown in the top curves of Figs. 3a ~ 3d the RFE of filipin in water was not affected by the addition 0.05 ~ 1% (v/v) of ethanol, isopropanol, acetone, or dimethylformamide. However, pronounced changes in corrected fluorescence were found. This indicates that there are variations in the degree of solubilization of the antibiotic in the presence of certain solvents.

In order to test the effect of these solvents on the filipin-cholesterol interaction, a constant amount of cholesterol was injected into aqueous solutions of the polyene using varying amounts of organic solvent. The results are shown in the bottom curves of Figs. 3a ~ 3d. In the experiment using DMF, it was observed that the degree of polyene-sterol interaction, as evidenced by the decrease in RFE, was quite dependent upon the amount of solvent used for the introduction of the sterol. However, after 3 hours the initial interaction was complete, and further additions of solvent had no affect on RFE. This result suggests that low concentrations of these organic solvents have little effect upon the polyene-sterol complex after it has been formed. When used as a vehicle for adding the sterol into aqueous solutions, the concentration and volume parameters of these organic solutions may determine the characteristics of the resulting cholesterol suspension.

Fig. 3. Effects of organic solvents on the interaction of filipin with cholesterol.

Each 3 ml sample contained 2.28×10^{-8} mole of filipin and the indicated amount of solvent. In those samples containing cholesterol, 3.87×10^{-8} mole of the sterol, dissolved in varying amounts of solvent, was injected into the aqueous sample. Fluorescence of the samples was measured using 338 nm excitation and 495 nm emission wavelengths.
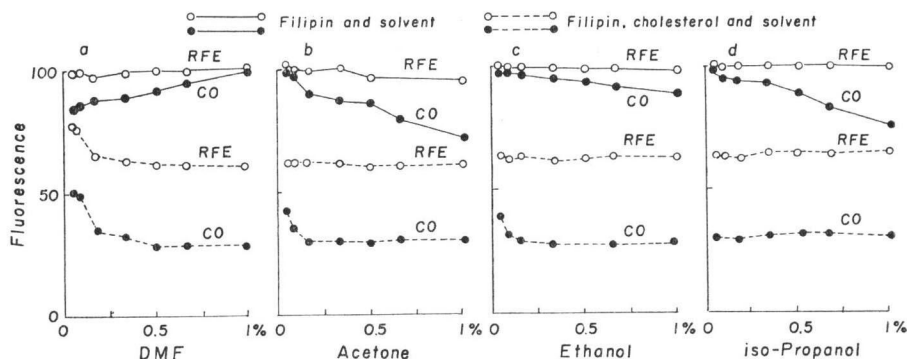
Dispersions of cholesterol prepared by adding ethanolic solutions of the sterol to a large excess of water are known to exist as microcrystals of various sizes[24]. Therefore, the method of addition of sterol into the aqueous solutions of filipin used in these studies may determine the degree of aggregation of the sterol, and consequently, its availability for binding to the polyene. In order to examine this point, cholesterol in DMF was added to samples containing only water using the same protocol as that for the data in Fig. 3. The measurement of 90° light scattering for the resulting dispersions shown in Fig. 4 indicate that the size of the cholesterol aggregates is determined by the volume of solvent used to introduce the sterol into the aqueous phase.

Fig. 4. The affect of solvent volume on the light scattering of cholesterol in water.

Cholesterol, 3.83×10^{-8} moles, in dimethylformamide or iso-propanol was injected to a final volume of 3.0 ml of water. The samples were mixed well and light scattering R_{90} was monitored at 338 nm.
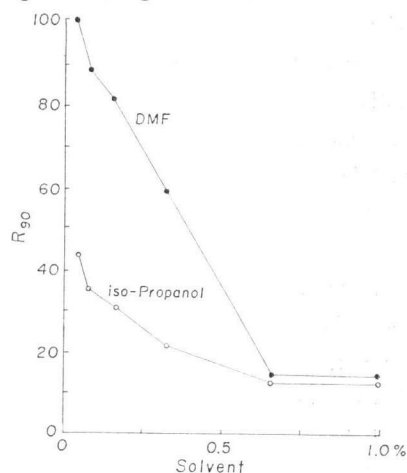

Table 3. Effect of organic solvents on the formation of the pimaricin-cholesterol complex.

Sample	RFE							
	No additions	MeOH	EtOH	i-Propanol	DMF	DMSO	Glycerol	Acetone
Pimaricin	3	3	3	3	3	3	3	3
Pimaricin & cholesterol	58	60	62	55	55	62	62	57

The final concentrations of solvents were 1 % (v/v); 2 μM pimaricin or 2 μM pimaricin and 3 μM cholesterol. After a 3-hour incubation period, RFE was measured. The averages of 5 separate determination are reported in the table and have variations of $\pm < 3\%$.

Abbreviations: DMF = Dimethylformamide, DMSO = dimethylsulfoxide, MeOH = methanol, EtOH = ethanol.

This phenomenon is most pronounced when small volumes of solvent are used. In addition, the rate of injection also alters the nature of the suspension formed. These observations strongly suggest that the mechanism of aggregation is dependent on the rate of exposure of cholesterol to the aqueous environment. Different rates of miscibility with water for the various solvents should produce different exposure rates. When the light scattering results of Fig. 4 are compared with Fig. 3, it becomes apparent that the finer dispersions of cholesterol are most readily accessible to the antibiotic.

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