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Fluorometric Investigations of the Interaction of Polyene Antibiotics with Sterols†

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ABSTRACT: Changes in specific fluorescent properties, partial quantum efficiency (PQ), and corrected fluorescence (CO) can be used to monitor the interaction of various polyenes with sterols. The changes in PQ and CO, caused by addition of sterols, are different for each polyene antibiotic. Addition of cholesterol decreases the PQ and CO of filipin in aqueous solution 36 and 62%, respectively, but cholesterol increases the PQ and CO of pimaricin more than 80-fold. Addition of cholesterol to nystatin or amphotericin B has little effect on the fluorescence emission. The filipin complex as well as pimaricin strongly binds sterols that contain both a 3β-hydroxyl group and a long alkyl side chain that is attached to the D ring of sterols. The filipin complex and pimaricin interact weakly or not at all with cholesterol palmitate and 3-keto or 3α-hydroxy sterols. The methanol degradation product of filipin (a tetraene epoxide) does not interact with cholesterol. The stoichiometry of the filipin:cholesterol interaction is, within experimental error, 1:1. The interaction of filipin with cholesterol is independent of pH from pH 4.5 to 8, but is dependent on the method of isolation and preparation of the filipin complex. The filipin complex isolated from organic solvents does not interact with sterols—using fluorescence as a criteria of interaction—but it will interact with sterols after prolonged standing in aqueous solution or heating to 50° for 2 hr. These studies strongly indicate that filipins can exist in conformational states or bonded conditions such as dimers that do not bind sterols in a specific manner and that such forms undergo changes in aqueous solutions to a form or forms that can interact specifically with cholesterol and other 3β-hydroxy sterols.

Many polyene antibiotics can produce lethal permeability alterations in microorganisms containing sterols in their membranes (Whitfield *et al.*, 1955; Perritt *et al.*, 1960; Gottlieb *et al.*, 1960; Lampen and Arnow, 1961; Johnson *et al.*, 1962; Ghosh, 1963; Weber and Kinsky, 1965; Child *et al.*, 1969). These polyenes can also lyse erythrocytes (Kinsky *et al.*, 1962, 1967; Kinsky, 1963), and alter the structure and permeability of liposomes (Weissmann and Sessa, 1967; Sessa and Weissmann, 1968), and model membranes (Demel *et al.*, 1965; Van Zutphen *et al.*, 1966, 1971; Demel, 1968; Kinsky, 1970). Effects *in vitro* include stimulation of glucose utilization in mammalian fat cells (Kuo, 1968), decreased DNA synthesis and cellular respiration in Ehrlich ascites and Novikoff hepatoma tumor cells (Mondovi *et al.*, 1971), increased phospholipid turnover in beef thyroid slices (Larsen and Wolff, 1967), and rupture of beef spermatozoa (Morton and Lardy, 1967). Some polyenes, especially filipin, decrease serum cholesterol levels in dogs and chicks (Schaffner and Gordon, 1968; Fisher *et al.*, 1969), reduce canine prostatic hyperplasia (Gordon and Schaffner, 1968), mimic vitamin D mediated calcium transport in chick ileum (Adams *et al.*, 1970; Wong *et al.*, 1970), act as larvicides and chemosterilants in some insects (Sweeley *et al.*, 1970; Schroeder and Bieber, 1971), and are toxic to snails (Seneca and Bergendahl, 1955). The mechanism of polyene action that is suggested by these studies is the binding to sterols, free or membrane bound, by polyene macrolides. Spectrophotometric evidence for complex formation has been presented (Lampen *et al.*, 1960; Gottlieb *et al.*, 1961; Norman *et al.*, 1971, 1972) but interpretation of such data is difficult because reduction of the absorption peaks by sterols does not correlate with the ability of the sterol to alter the fungicidal activity of filipin (Gottlieb *et al.*, 1961). No spectral shifts were noted. Decreases in absorbance of filipin could indicate lowered solubility in aqueous solution (Kinsky, 1967), and, as shown herein, can be due to small differences in experi-

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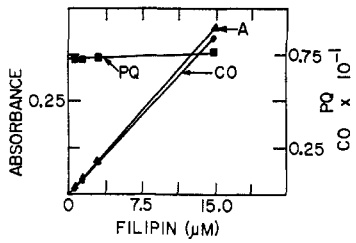

FIGURE 1: Affect of dilution on A, CO, and PQ of filipin. Filipin was prepared from a 14.8 μM stock solution in distilled water. Each filipin solution was excited at 338 nm and fluorescence emission was monitored at 496 nm. (Δ) A (absorbance), (○) CO (corrected fluorescence), and (□) PQ (partial quantum efficiency).

mental conditions. Fluorescence investigations, however, provide definitive evidence for the binding of cholesterol to the filipin complex (Schroeder *et al.*, 1971). Herein, the interaction of filipin and pimaricin with several sterols is demonstrated by simultaneous measurement of absorbance (A), corrected fluorescence (CO), and partial quantum efficiency (PQ) by means of a unique computer-centered fluorimeter.

Experimental Section

Materials. Filipin, 86% pure, was donated by the Upjohn Co., Kalamazoo, Mich. The antibiotic was purified as described previously (Schroeder *et al.*, 1971) and dissolved in distilled water (2.95 μM). Pimaricin (1.37 μmoles/mg (91%)) was kindly provided by the American Cyanamid Co., Princeton, N. J. Nystatin (4300 μ/mg (78%)) and amphotericin B (1.06 μmoles/mg (97%)) were generous gifts of Squibb Laboratories, New Brunswick, N. J. Pimaricin and amphotericin B solutions were prepared by dissolving 1.37 and 1.06 μmoles, respectively, per 100 ml of distilled water. Nystatin was prepared by dissolving 0.839 μmole (1 mg) in 100 ml of pH 4 citrate–phosphate buffer (0.05 M) as described by Lampen *et al.* (1960). The methanol degradation product of filipin was prepared and purified as per Rickards *et al.* (1970). It was dissolved in distilled water (0.36 μmole/100 ml). Filipin isomers were separated by partition chromatography (Bergy and Eble, 1968), dissolved in distilled water, and treated identically with the filipin complex.

Steroids were purchased as follows: 5-cholesten-3β-ol (cholesterol), 5-cholesten-3β-ol 3-palmitate (cholesterol palmitate), ergosta-5,7,22-trien-3-ol (ergosterol), and 17-hydroxyl-11-dehydrocorticosterone (cortisone) from Sigma Chemical Co., St. Louis, Mo.; 5α-cholestan-3β-ol (cholestanol) from Chemed Inc., Odenton, Md.; androstan-3β-ol-17-one, 24-ethyl-5,22-cholestadien-β-ol (stigmasterol), and 24-ethyl-5-cholesten-3β-ol (β-sitosterol) from California Corp. for Biochemical Research, Los Angeles, Calif.; 5α-cholestan-3α-ol (epicholestanol) and 5-cholesten-3α-ol (epicholesterol) from Schwarz-Mann Co., Orangeburg, N. Y.

The following buffers were prepared using reagent grade chemicals 0.10 M: pH 2.6–7.0, citrate–phosphate; pH 8 and 11, phosphate; pH 9, Tris; and pH 10, glycine.

Methods. All polyene antibiotic solutions were stored in the dark at 4°. Nystatin, pimaricin, and amphotericin B solutions were used on the day of preparation. Unless otherwise stated, 10 ml of each polyene solution was incubated in the dark with 1 mg of steroid at 50° for 2 hr. Partial quantum efficiency (PQ), corrected fluorescence (CO), and absorption spectra were determined as described by Holland and Timnick (1972).

For the major part of this work, three output quantities from the computer were used. These quantities are defined as follows: (1) A = absorbance of the sample in solution and is equivalent to values obtained with conventional spectrophotometric instruments; (2) CO = corrected fluorescence. This quantity is a linear function of the number of quanta fluoresced per number of quanta in the excitation beam and is dependent upon the specific instrumental parameters used for the measurement; (3) PQ = partial quantum efficiency. This quantity is a linear function of the number of quanta fluoresced per number of quanta absorbed. PQ is obtained from excitation scans only. If the fraction of the total fluorescence detected for a specific fluorophore is known and if the instrumental parameters are accurately determined (Holland, 1971), this quantity can yield the total quantum efficiency. In practice, it has been more convenient to use PQ to determine relative quantum efficiency changes. Relative PQ measurements have been employed for this investigation.[1] Absorbance for filipin is expressed as A_{338}, an absorption maximum, or as a ratio of two absorbance maxima, A_{338}/A_{305} (Lampen *et al.*, 1960). Absorbance values for pimaricin are similarly expressed as A_{319}, or as a ratio of two absorbance maxima, A_{310}/A_{292}. All measurements were made at 23–24°.

Results

Effect of Dilution on Absorbance, Corrected Fluorescence, and Partial Quantum Efficiency of the Filipin Complex. Changes in A, CO, and PQ of the filipin complex in aqueous solution are monitored at 338 nm. This wavelength corresponds to a major absorbance maximum for filipin. Figure 1 shows that both A and CO decrease linearly with dilution through the range 14.8–0.74 μM; however, the PQ of filipin, as predicted by theory (Holland Timnick, 1972), is essentially independent of concentration. PQ decreased approximately 2%. Similar results are obtained when A, CO, and PQ are determined at 356 nm, the other major absorption maximum of filipin.

Effect of Cholesterol on Fluorescence Emission of Filipin, Nystatin, and Pimaricin. In the presence of cholesterol, the fluorescence emission spectrum of some polyenes is altered and these changes are different for each polyene. Cholesterol decreases the fluorescence emission of the filipin complex (Figure 2A), but apparently has little effect on its methanol degradation product which is a very weak fluorophore (Figure 2B). Cholesterol and cholesterol palmitate did not change the A, CO, and PQ of the methanolic degradation product. The fluorescence emission of nystatin decreases slightly in the presence of cholesterol (Figure 2C). The fluorescence emission of pimaricin, a polyene that fluoresces only weakly, is increased over 80-fold when cholesterol is added to the solution (see Figure 2D). The fluorescence emission of amphotericin (data not shown) is less than 1% that of the filipin complex, and fluorescence changes with cholesterol are small—decreases of approximately 10%.

Stoichiometry of the Filipin–Cholesterol Interaction. The purity of the filipin complex used was approximately 96% (Whitfield *et al.*, 1955; Bergy and Eble, 1968). The molecular

[1] Since the relationship between A and PQ is not linear and absorbance may occur by species other than the fluorophore, it should be noted that there exists no convenient mathematical correlation between the two quantities. In similar chemical systems, changes in CO represent the gross effects of changes in concentration, absorptivity, and quantum efficiency of the fluorophore, but changes in PQ represent changes in the ratio of absorbance between the fluorophore and any chromophore that may be present.

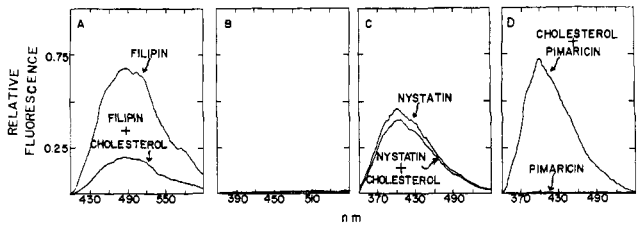

FIGURE 2: Affect of cholesterol on the fluorescence emission spectra of filipin, the methanol degradation product of filipin, nystatin, and pimaricin. Ten-milliliter aliquots of the following polyenes were incubated for 2 hr at 50°, with 2.56 μmoles of cholesterol as described in the Experimental Section: (A) filipin (2.95 μM in distilled water), (B) methanol degradation product of filipin (3.6 μM in distilled water) with cholesterol (lower curve) and without cholesterol (upper curve), (C) nystatin (8.39 μM in distilled water), and (D) pimaricin (13.7 μM in distilled water). Filipin and the methanol degradation product of filipin were excited at 338 nm, nystatin at 323 nm, and pimaricin at 292 nm.

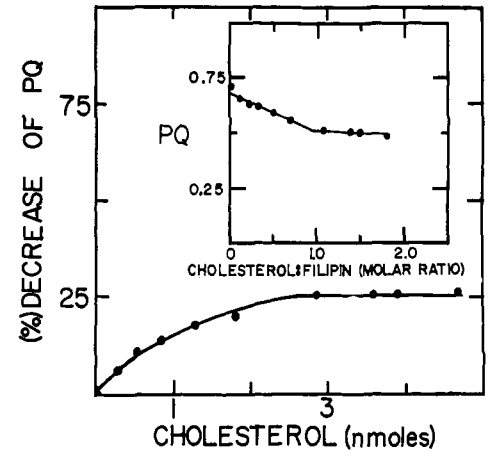

FIGURE 3: Affect of cholesterol concentration on the PQ of filipin. Increasing amounts of cholesterol (2.56 μM in distilled water) were added to 1.8-ml aliquots of a 1.48 μM filipin stock solution and incubated at 37° for 2 hr in the dark. Decreases in PQ and CO were determined at 338 nm. CO was corrected for dilution. Fluorescence emission was monitored at 496 nm. The insert shows the effect of increasing molar ratios of cholesterol:filipin on PQ. A molecular weight of 650.14 for the filipin complex (Schroeder and Bieber, 1971) was used to calculate the molar cholesterol:filipin ratio.

weight of the filipin complex used for these calculations in these experiments was an average of the molecular weights of the isomers based on their per cent composition in the complex; see Pandey and Rinehart (1970) for the per cent composition and Schroeder and Bieber (1971) for the calculation of average molecular weight. A known amount of filipin complex in water was titrated with cholesterol, and the per cent change in PQ and CO at 338 nm was determined. As shown in Figure 3, PQ decreased until 0.95 nmole of cholesterol was added per nmole of filipin. Similar results were obtained when the per cent change in CO *vs.* cholesterol concentration was determined. The stoichiometry, nmole of cholesterol to nmole of filipin, as shown in the insert of Figure 3, was 0.95. In three experiments, using 1.5 μM filipin, the stoichiometry varied between 0.8 and 0.95. When 3.0 and 6.0 μM filipin were used, the stoichiometry was 1.0 and 0.8, respectively.

Interaction of the Individual Filipin Components with Cholesterol. Filipin is a mixture of four components, two of which are conformational isomers (isomers III and IV), and two of which differ by one and two hydroxyl groups, respectively (isomers II and I) (see Pandey and Rinehart, 1970). The four major filipin components separated by the column chromatography system of Bergy and Eble (1968) interact with cholesterol, but components II, III, and IV, which comprise 96% of the filipin complex, decrease CO 53, 43, and 65%, respectively, and also decrease PQ 35, 48, and 49%, respectively, when incubated with cholesterol (see Table I). Component I apparently interacts slightly with cholesterol as shown by decreases in CO and PQ of 3 and 11%, respectively. An average of the PQ values of the four filipin components (normalized for per cent composition of each component in the filipin

TABLE I: Interaction of Filipin Components with Cholesterol.[a]

Expt	% Decrease in CO (at 338 nm)	% Decrease in PQ (at 338 nm)
Filipin component I + cholesterol	3	11
Filipin component II + cholesterol	53	35
Filipin component III + cholesterol	43	48
Filipin component IV + cholesterol	65	49

[a] Filipin components were separated by partition chromatography, as described in the Experimental Section. Components I, II, III, and IV were dissolved in distilled water 0.5, 1.4, 1.4, and 0.85 μM, respectively. Aliquots (10 ml) of each solution were incubated with and without 2.59 μmoles of cholesterol at 50° for 2 hr. Changes in CO and PQ were measured at 338 nm. Fluorescence emission was monitored at 496 nm.

TABLE II: Effect of the Length of Storage in Aqueous Solution and Temperature on PQ Changes of Filipin.[c]

Filipin Age	Absorbance Ratio of Filipin	% Decrease in PQ with Cholesterol[a]	% Decrease in PQ with Cholesterol[b]
2 hr	1.96	4	24
12 hr	1.82	2	35
2 days	1.55	40	38
7 days	1.40	38	39
4 months	0.73	8	9

[a] 10-ml aliquots of each solution were treated with 2.59 μmoles of cholesterol for 2 hr in the dark at 24°. [b] 10-ml aliquots of each solution were treated with 2.59 μmoles of cholesterol for 2 hr in the dark at 50°. The per cent decrease in PQ of filipin treated with cholesterol was determined at 338 nm. Fluorescence emission was monitored at 496 nm. [c] Filipin solutions (2.95 μM in distilled water) were kept in the dark at 4° for the times indicated. The absorbance ratio (A at 338 nm/A at 305 nm) of filipin was measured as described in the Experimental Section.

TABLE III: Effect of Temperature on A, CO, and PQ of Filipin and Filipin Plus Cholesterol in Aqueous Solutions.[a]

		Temperature of the Principal Incubation											
		4°, 72 hr			24°, 72 hr			4°, 64 hr; Then 37°, 8 hr			4°, 70 hr; Then 50°, 2 hr		
Expt	Expt Conditions	A	CO	PQ	A	CO	PQ	A	CO	PQ	A	CO	PQ
1	Filipin	0.138	1.07	0.78	0.144	1.26	0.79	0.117	1.03	0.76	0.102	0.79	0.72
2	Filipin: 2 hr at 50° (dark)	0.103	0.84	0.72	0.098	0.85	0.72	0.103	0.87	0.72	0.102	0.85	0.72
3	Filipin + cholesterol: 2 hr at 50° (dark)	0.055	0.46	0.38	0.059	0.46	0.38	0.054	0.46	0.37	0.059	0.46	0.38

[a] In expt 1 for the principal incubation, each of four 10-ml aliquots of filipin (2.95 μM in distilled water) was incubated in the dark at one of the following conditions: 4°, 72 hr; 24°, 72 hr; 4°, 64 hr, followed by 37°, 8 hr; 4°, 70 hr, followed by 50°, 2 hr. A, CO, and PQ were determined at 338 nm. Fluorescence emission was monitored at 496 nm. For expt 2, the four solutions treated from expt 1 were incubated an additional 2 hr in the dark at 50°. A, CO, and PQ were determined as above. In expt 3, the four solutions treated from expt 1 were incubated with 2.59 μmoles of cholesterol at 50°, 2 hr in the dark. A, CO, and PQ were determined as above.

complex) differs from the measured PQ of the total mixture by only 5%, indicating that there is little, if any, interactions between the individual filipin components in solution which affect fluorescence efficiency.

Relation of the Absorbance Ratio at 338–305 nm to the Cholesterol Binding Capacity of Filipin. The capacity of an aqueous solution of filipin to elicit changes in PQ due to a specific interaction of filipin with cholesterol can be monitored by measuring the absorbance ratio. Table II shows that the absorbance ratio (ratio of absorbance at 338 nm to that at 305 nm) of filipin in aqueous solutions decreases from 1.96 for newly prepared aqueous solutions to 0.73 after storage for four months at 4°. Cholesterol binding ability of filipin—determined by changes in PQ—increases to a maximum in 2 days, remains constant for several weeks, and then decreases slowly, as shown in Table II. Thus, the potential binding ability of filipin can be estimated from the absorbance ratio. Incubation of the apparent noncholesterol binding form of filipin at 50° for 2 hr converts filipin to a form that readily binds cholesterol (see Table II, last column). It is important to note that the filipin used in these experiments was a standard preparation that had been isolated and purified using organic solvents.

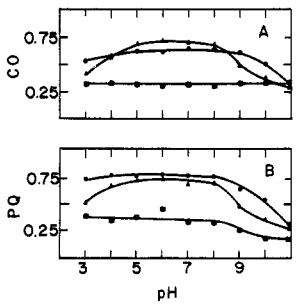

FIGURE 4: Effect of pH on the cholesterol-induced PQ changes of filipin. Filipin (2.95 μM) at pH 3–11 was prepared by diluting filipin (14.8 μM in distilled water) with buffer to a final buffer concentration of 0.05 M. Buffers were prepared as described in the Experimental Section. The solutions were then incubated for 2 hr at 50° with and without cholesterol as described in the Experimental Section A, CO, and PQ were determined at 338 nm. Fluorescence emission was monitored at 496 nm. (○) Unincubated filipin, (△) incubated filipin, and (□) filipin incubated with cholesterol.

Effect of Temperature on A, CO, and PQ of Filipin and Mixtures of Filipin Plus Cholesterol in Aqueous Solutions. The absorbance and corrected fluorescence values of aqueous filipin solutions vary as much as 30 and 26%, respectively. The variations are dependent on temperature and other experimental conditions. For example, filipin incubated in water for the time intervals given in Table III, expt I at 4, 24, 37, and 50° has CO values of 1.07, 1.26, 1.03, and 0.79, respectively. Similar responses to temperature in PQ were obtained (see Table III, expt I). Incubation at 50° for 2 hr of filipin solutions used for expt I gives a form of filipin with almost identical A, CO, and PQ values (see Table III, expt 2). Thus, aqueous filipin solutions that have different spectral properties—hence, different forms—can be converted to a form that has reproducible spectral properties. This form of filipin is the form that interacts with cholesterol as shown by equal decreases in A, CO, and PQ (Table III, expt 3).

Effect of pH on Filipin–Cholesterol Interaction. Corrected fluorescence and partial quantum efficiency changes of filipin interacting with cholesterol are independent of pH over the range pH 4.5–8.0, as shown in Figure 4. At high pH, the CO of the filipin complex is decreased until no interaction with cholesterol can be detected by CO changes (Figure 4A); however, PQ shows that even at pH 11, cholesterol interacts with filipin (Figure 4B). At pH's lower than 4.5, CO and PQ values of filipin, but not of cholesterol-bound filipin, decrease.

Factors Influencing the Filipin–Cholesterol Complex. Other experiments have shown that organic solvents and extraction of filipin from the filipin–cholesterol complex affect the absorbance ratio, CO, and PQ of filipin. Methanol (50% final concentration) disrupts the filipin–cholesterol complex and CO increases to 80% of the value for unbound filipin, and PQ is the same as that of unbound filipin. The PQ of filipin, without cholesterol, in 50% methanol increases only 2%. This indicates that methanol disrupts the complex and some of the filipin was lost from solution without binding to cholesterol. Extraction of the filipin–cholesterol complex with 1-butanol, followed by methanol extraction of the residue after removal of 12-butanol gives a product that has, in distilled water, the same absorbance ratio, CO, and PQ as unbound filipin. Thus,

TABLE IV: Interaction of Filipin with Sterols.[a]

Steroid Added	% Decrease			
	Absorbance Ratio	In A	In CO	In PQ
None	1.38			
Cholesterol (3β-OH)	0.92	55	62	32
Epicholesterol (3α-OH)	1.32	24	36	13
Cholestanol (3β-OH)	0.96	33	57	36
Epicholestanol (3α-OH)	1.40	31	27	2
β-Sitosterol (3β-OH)	0.58	41	85	52
Stigmasterol (3β-OH)	0.79	39	71	62
Ergosterol (3β-OH)	1.02	35	28	5
Cholesterol palmitate	1.50	9	5	4
Cortisone	1.70	6	2	3
Androstan-3β-ol-17-one	1.34	3	14	4

[a] Ten-milliliter aliquots of filipin (2.95 μM in distilled water) were incubated with and without 1 mg of sterol at 50° for 2 hr. Absorbance ratio, A, CO, and PQ were determined at 338 nm. Fluorescence emission was monitored at 496 nm.

TABLE V: Interaction of Pimaricin with Sterols.[a]

Steroid Added	Absorbance Ratio	% of Pimaricin + Cholesterol CO	% of Pimaricin + Cholesterol PQ
None (unincubated)	2.85	1	1
None	3.02	1	1
Cholesterol (3β-OH)	1.39	100	100
Stigmasterol (3β-OH)	1.34	81	80
β-Sitosterol (3β-OH)	1.14	69	76
Cholesterol palmitate	2.76	2	1
Cortisone	2.76	2	1
Epicholesterol (3α-OH)	1.55	10	12
Epicholestanol (3α-OH)	1.53	11	7

[a] Ten-milliliter aliquots of pimaricin (13.7 μM in distilled water) were incubated with and without 1 mg of sterol at 50° for 2 hr. Pimaricin interacting with cholesterol fluoresces strongly and is arbitrarily assigned CO and PQ values of 100%. Absorbance ratio, CO, and PQ were determined at 319 nm as described in the Experimental Section. Fluorescence emission was monitored at 402 nm.

the filipin–cholesterol complex can be disrupted and the reisolated filipin exhibits properties of the original filipin. If filipin is extracted from aqueous solutions, reduced to dryness *in vacuo*, and dissolved in *tert*-butyl alcohol, lyophilized, and then dissolved in distilled water, the absorbance ratio, CO, PQ are the same as unbound, unreactive filipin; but the absorbance ratio differs by 28% from that of the extracted filipin. Thus, the absorbance ratio of filipin is affected by organic solvents and by the treatment after extraction from the filipin–cholesterol complex, but PQ is affected only by whether filipin is in the bound or unbound form.

Interaction of Filipin with Sterols. Filipin interacts strongly with 3β-hydroxy sterols as shown in Table IV. Cholesterol, cholestanol, β-sitosterol, and stigmasterol decrease the absorbance ratio, A, CO, and PQ of filipin by more than 30%. β-Sitosterol and stigmasterol, which contain one more double bond than cholesterol, interact more strongly with filipin (52 and 62% decreases in PQ, respectively) than any of the 3β-hydroxy sterols tested. Both sterols also contain C-24 ethyl groups. Ergosterol interacts only weakly as shown by the 5% decrease in PQ. This may be due to cleavage of the ring structure of ergosterol by ultraviolet irradiation, which could prevent interaction with filipin. Epicholesterol (3α-hydroxy) has much less of an effect on PQ. Cholesterol palmitate (the 3β-ester of cholesterol and palmitic acid), cortisone (3-keto), and androstane (3β-ol-17-one) do not, or only weakly, interact with filipin as shown by the small decreases in A, CO, and PQ. Sterols such as α-cholestanol do not decrease PQ, but reduce A and CO 31 and 27%, respectively.

Binding of Sterols by Pimaricin. Addition of 3β-hydroxy sterols such as cholesterol, stigmasterol, sitosterol, and cholestanol to pimaricin enhances its fluorescence 80- to 100-fold, as shown in Table V. β-Cholestanol increases CO and PQ more than 10 times as much as cholesterol (data not shown). Epicholesterol (3α-hydroxy), epicholestanol (3α-hydroxy), cholesterol palmitate, and cortisone interact only weakly (12, 7, 1, and 1% increases in PQ, respectively). It should be noted that sterols which interact with pimaricin also interact with filipin.

Interaction of Other Polyenes with Cholesterol. Table VI indicates that nystatin and amphotericin B also interact with cholesterol. PQ decreases 33 and 22%, respectively. Cholesterol palmitate decreases the PQ of amphotericin B by 5% while the PQ of nystatin is lowered 1%.

Discussion

Since much of this research embodies measurements of absorbance, corrected fluorescence, and a unique quantity, partial quantum efficiency, it would be appropriate to discuss the nature of these measurements and their significance. These parameters have been obtained from a single instru-

TABLE VI: Interaction of Nystatin and Amphotericin B with Cholesterol.[a]

Polyene	Sterol Added	% Decrease in PQ	
		At 323 nm	At 355 nm
Nystatin	Cholesterol	33	
Nystatin	Cholesterol palmitate	1	
Amphotericin B	Cholesterol		22
Amphotericin B	Cholesterol palmitate		5

[a] Ten-milliliter aliquots of nystatin (8.39 μM in pH 4 citrate–phosphate buffer, 0.05 M) were incubated with and without sterol for 2 hr at 30°. Each solution contained 0.3% dimethyl sulfoxide. PQ was determined at 323 nm. Fluorescence emission was monitored at 402 nm. Ten-milliliter aliquots of amphotericin B (10.6 μM in distilled water) were incubated with and without 1 mg of sterol for 2 hr at 50°. PQ was determined at 355 nm. Fluorescence emission was monitored at 475 nm.

ment system. This system is a computer-centered combination spectrophoto–spectrofluorimeter that is capable of simultaneous absorption and fluorescence measurements (see Holland (1971) and Holland and Timnick (1972)). PQ, when monitored as a function of excitation wavelength, produces a value that is linearly related to the total quantum efficiency. For a single fluorophore, this value will be constant across an excitation band and independent of concentration within the measuring capability of the instrument system. This concentration independence presents a powerful new approach to detection of conformational, bonding, or solvation changes.

For fluorophores in the presence of nonfluorescing chromophores, the PQ will deviate from the theoretical linear shape and tend to reveal the absorption bands of the fluorophore (Holland, 1971). This is precisely the case encountered in the filipin complex investigation (Schroeder *et al.*, 1971).

In mixtures of fluorophores that absorb and fluoresce within the same wavelength windows, the total PQ of the system will be the sum of the concentration normalized components of the mixture provided no interaction occurs between the various components, as illustrated by the components of the filipin complex (Table I).

The data show that changes in PQ, the partial quantum efficiency, of filipins and pimaricin on addition of sterols can be used to study an interaction of these polyene antibiotics with sterols. *Interaction in this discussion refers to the specific interaction between polyenes and sterols that alters fluorescence.* Since PQ is independent of concentration, this eliminates many of the problems inherent in quantitating changes in fluorescence or absorption of compounds that are sparingly soluble in water.

The specific interaction of filipin with cholesterol, that is detected fluorometrically, depends on how filipin was treated prior to exposure to sterols. Freshly prepared aqueous solutions of the filipin complex that were isolated using organic solvents or dissolved in solvents such as methanol do not show changes in PQ immediately. Thus, these solutions of filipin apparently do not interact with sterols. If such solutions are allowed to stand for long periods of time or are heated for 2 hr at 50°, the ratio of the absorption maxima at 338–305 nm changes from 1.96 to 1.55. Such solutions readily interact with sterols as shown by changes in PQ (see Table II). Thus, the absorbance ratio is a sensitive measure of the capability of filipin to bind sterols in a specific manner that affects fluorescence properties. Interactions between filipin and sterols, specific or nonspecific, that do not alter fluorescence properties of the system would not be detected.

A likely explanation of these results is that filipin, isolated using organic solvents, dissolved in alcohols or lyophilized from *tert*-butyl alcohol, acquires a conformation or bonding that is unfavorable for complexing with sterols in a specific manner.

The above-mentioned results appear to be entirely consistent with the results of Amman *et al.* (1955) and Lampen *et al.* (1960) and provide a possible explanation for their findings. These investigators showed greater than an 18-hr lag in the fungicidal activity of filipin toward *Candida albicans* and *Saccharomyces cerevisiae*. Weber and Kinsky, (1965) also observed a 36-hr lag in the inhibition of growth of *Mycoplasma laidlawii;* however, no growth inhibition of *Mycoplasma* was observed in previous studies (Kinsky (1963, 1964)). Our results indicate that such a lag could be partly, if not entirely, caused by starting with a form of filipin that does not bind sterols. In the aqueous media, this could be slowly converted to a sterol-binding form.

It must be emphasized that each family of polyene antibiotics apparently responds differently to sterols. As shown in Figure 2, cholesterol causes a decrease in PQ of filipin, but pimaricin, another polyene, fluoresces very little in aqueous solution. Addition of cholesterol to pimaricin in water causes at least an 80-fold increase in PQ as well as a marked increase in fluorescence. In contrast, changes in PQ and fluorescence of nystatin and amphotericin B were small when sterols were added.

We have concluded that the higher the affinity of the sterol for filipin, the greater will be the decrease in PQ. This conclusion appears to be supported by the biological studies of Gottlieb and coworkers (1960). They found that certain sterols were very effective in overcoming or preventing the fungicidal effects of the filipin on *S. cerevisiae*, but other sterols were less effective and some were ineffective. Our results, as well as the recent results of Norman *et al.* (1972), correlate well with their studies. Sterols such as cholesterol, cholestanol, stigmasterol, and β-sitosterol, which decrease the PQ of filipin greatly were effective in preventing the fungicidal effects of filipin. Sterols such as cortisone and androstan-3β-ol-17-one, did not cause much change in PQ. Gottlieb and coworkers (1960) found that their preparation of androstan-3β-ol-17-one prevented fungicidal effects of filipin. They noted that this finding was not consistent with the remainder of their biological data.

The one exception is ergosterol. It can prevent fungicidal effects of filipin but does not alter PQ. The failure to alter PQ is most likely caused by the well-known rapid photolytic cleavage of the β ring of ergosterol by ultraviolet light (Fieser and Fieser, 1959). Ergosterol should be destroyed in the cuvette during our assay.

Esterification of the β-hydroxyl group of cholesterol with palmitate prevented interaction with filipin. The results strongly indicate that the 3β-hydroxyl group of sterols is of prime importance for interacting with filipin. This agrees with conclusions of others (see Gottlieb *et al.*, 1960; Norman *et al.*, 1972). If the 3-hydroxyl group is α, smaller changes in PQ occur. For example, cholestanol, a 3β-hydroxy sterol, reduced PQ 36% but the α isomer, α-cholestanol, reduced PQ 2%. Similarly, cholesterol reduced PQ 32% and epicholesterol, the α isomer, reduced PQ 13% (see Table IV). The 3β-hydroxyl group also appears to be required for the interaction of pimaricin with sterols. The two α isomers investigated affected PQ only 10% that of the β isomers; see Table V.

The alkyl side chain of sterols appears to be essential for interaction of sterols with filipins. Compounds such as cortisone and androstan-3β-ol-17-one which do not have the long alkyl hydrophobic side chain do not alter PQ significantly. The conjugated pentaene double bonds appear to be essential for binding sterols because the methanol degradation product—a tetraene containing epoxide (see Rickards *et al.*, 1970)—does not show changes in PQ on addition of sterols nor does it have fungicidal activity (see Whitfield *et al.*, 1955). The lactone of filipin is also required for interaction with sterols. Cleavage of the lactone destroys the fungicidal effects of filipin (Kinsky *et al.*, 1967). Filipin loses 74% of its antifungal activity at pH 2.5 and 81% at pH 10.3 after storage for 18 hours (see Amman *et al.*, 1955). Conditions which should cleave the lactone, high and low pH's prevented PQ and fluorescence changes caused by cholesterol (see Figure 4).

The above-mentioned interactions, the reversal of the cholesterol–filipin interaction by methanol, indicate that specific hydrogen and hydrophobic bonds are major forces involved in the interaction of filipin with cholesterol. The fact

that the absorption maxima of filipin are decreased but *not* shifted on interaction with cholesterol indicates that no bond changes are occurring in the vicinity of the fluorophore; rather, the concentration of the fluorophore is reduced or the absorptivity of the molecule has been diminished. The change in the ratio of the absorption peaks indicates that a simple reduction in concentration is not an adequate explanation; however, analysis of these changes does not clearly delineate any interactions that may be involved. Since PQ is independent of concentration, it does present a measure of any interactions between the molecules of filipin and the various sterols occurring in the solution itself. For example, an analysis of the measured PQ for increasing amounts of cholesterol when added to an aqueous solution of filipin reveals a continuing change until a molar ratio of approximately 1:1 is attained.

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