Reversible Interconversions of Sterol-Binding and Sterol-Nonbinding Forms of Filipin as Determined by Fluorimetric and Light Scattering Properties†

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ABSTRACT: Combined fluorimetric and Tyndall light scattering measurements have confirmed the existence of different forms of filipin; filipin herein refers to the filipin complex. In these studies, the ratio of two of the absorbance peaks of the filipin molecule (A_{338}/A_{305}) is a consistent indicator of the presence of active and inactive forms. The partial quantum efficiency (PQ) was used to measure the interaction between filipin and sterols. Light scattering (R_{90}) was used to monitor changes in the aggregate size of the polyene. These analyses have shown that filipin can occur in "active forms"—those that interact with cholesterol—and "inactive forms"—those that do not interact with cholesterol—in aqueous solutions (Schroeder, F., Holland, J. F., and Bieber, L. L. (1972), *Biochemistry 11*, 3105). Inactive filipin is converted to the active form by incubation at 50° for 2 hr. The conversion of the nonbinding to the binding form in aqueous systems follows a first-order rate law and appears to involve conformational changes that are time and temperature dependent. The conversion of active to inactive forms of filipin is reversible and independent of the presence or absence of large micelles of filipin. Methods using nonaqueous solvents to isolate and purify filipin consistently produce this nonbinding form. Short-chain alcohols disrupt aqueous filipin micelles in the order of effectiveness: propanol > ethanol > methanol. Light scattering of filipin systems is decreased approximately by an order of magnitude with addition of excess cholesterol. Many of the apparent discrepancies in the literature concerning the interaction of filipin with sterols and sterol-containing membranes may result from the use of the sterol-nonbinding form of filipin and the use of concentrations so high that the observations are independent of sterol-binding properties.

Several groups of investigators using filipin at a concentration of 10^{-5}–10^{-6} M concluded that the antibiotic had no significant effect on subcellular organelles such as mitochondria isolated from fungal (Gottlieb *et al.*, 1961; Kinsky *et al.*, 1965) or mammalian sources (Lardy *et al.*, 1958; Morton and Lardy, 1967). At 10^{-2}–10^{-4} M, filipin bound extensively to mitochondria and inhibited mitochondrial respiration, ruptured lysosomes, and also inhibited cancer cell growth (Balcavage *et al.*, 1968; Haksar and Peron, 1972; Mondovi *et al.*, 1971). Experiments on phospholipid spherules (liposomes) indicated that, at concentrations of 10^{-3}–10^{-5} M, the filipin complex interacted equally well with liposomes whether or not cholesterol was present (Weissmann and Sessa 1967; Sessa and Weissmann, 1968). In contrast, other investigators who used filipin at 10^{-5}–10^{-6} M (Kinsky *et al.*, 1968; Kleinschmidt *et al.*, 1972; Norman *et al.*, 1972b) found a requirement for the presence of cholesterol. At 10^{-8}–10^{-5} M concentrations, filipin interacts preferentially with monolayers and bilayers containing cholesterol (Demel *et al.*, 1965; Van Zutphen *et al.*, 1971), but at 10^{-2}–10^{-4} M filipin concentrations, the antibiotic is able to interact with monolayers of pure lipid in the absence of cholesterol (Demel *et al.*, 1968; Kinsky *et al.*, 1968; Van Zutphen *et al.*, 1971).

It has been suggested that filipin and other polyenes may exist as micelles or aggregates in aqueous solution (Lampen *et al.*, 1959; Kinsky, 1967; Norman *et al.*, 1972a; Schroeder *et al.*, 1972). When the concentration of a micelle-forming molecule is much greater than the critical micelle concentration (CMC), the molecule could act as a detergent (Fendler and Fendler, 1970). The ability of filipin, a neutral amphipathic compound, to act as a detergent at concentrations much higher than the CMC could explain some of the paradoxical results obtained by previous workers who have used extreme differences in concentrations.

Fluorescence changes have been used to demonstrate the occurrence of sterol-binding (herein referred to as the active form) and sterol-nonbinding (inactive form) forms of filipin (Schroeder *et al.*, 1971, 1972) and to monitor the interaction of filipin with sterols (Schroeder *et al.*, 1971, 1972). This technique has recently been used to study the interaction of filipin with membranes (Drabikowski *et al.*, 1973) and to monitor the interaction of other polyenes with sterols (Schroeder *et al.*, 1972; Bittman and Fischkoff, 1972; Strom *et al.*, 1972).

It is apparent that the capacity of filipin to interact with sterols is dependent in a complex manner on temperature, age of solution, and other variables (Sessa and Weissmann, 1968; Norman *et al.*, 1972a,b; Schroeder *et al.*, 1972). Herein, we show that the ability of the filipin complex to interact with cholesterol is independent of the presence of large filipin micelles, and that the conversion of the sterol-nonbinding form to the sterol-binding form is a first-order process which may be indicative of a conformational change.

Materials and Methods

The filipin complex was a gift of the Upjohn Co., Kalamazoo, Mich. This material was 86% pure; therefore, the antibiotic was further purified as previously described (Whitfield *et al.*, 1955; Schroeder *et al.*, 1971, 1972). The filipin used herein is a complex of several components (Bergy and Eble, 1968). The three principal components (96% of the filipin) all interact with cholesterol (Schroeder *et al.*, 1972).

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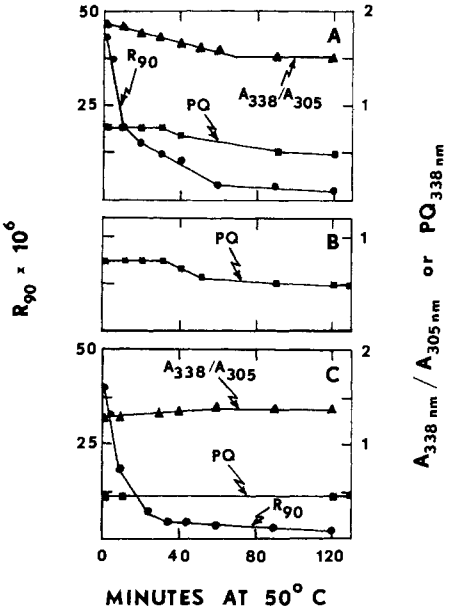


FIGURE 1: Time course for conversion of inactive filipin to active filipin. In A, filipin (2.95 μM) was freshly prepared. Three-milliliter aliquots containing 9 nmol of filipin were incubated at 50° in a heated cuvet. At the times indicated by each point, the solution was mixed with 180 nmol of cholesterol for 2 min and values were determined. A_{338}/A_{305}, R_{90}, and PQ were determined at 50° before and after addition of cholesterol, as described under Materials and Methods. In B, filipin (2.95 μM) was freshly prepared as in A and incubated continuously at 50° with cholesterol. PQ was determined 2 min after the times indicated by each point. In C, filipin (2.95 μM) was prepared from a 60-day-old aqueous filipin solution stored at 4° in the dark. A_{338}/A_{305}, R_{90}, and PQ were determined before and after addition of cholesterol as in A: (●) R_{90} at 338 nm; (▲) A_{338nm}/A_{305nm}; (■) PQ_{338nm}.

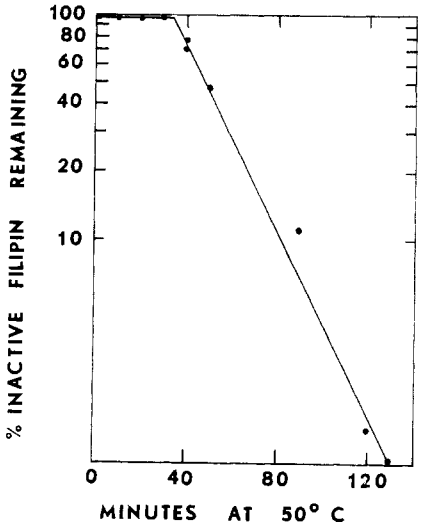


FIGURE 2: First-order rate of conversion of inactive to active filipin. The per cent inactive filipin remaining at the indicated times was calculated from the PQ data of Figures 1A and 1B, as follows: per cent inactive filipin remaining = 100[(maximal decrease in PQ of filipin interacting with cholesterol) − (decrease in PQ of filipin interacting with cholesterol at indicated time)]/[maximal decrease in PQ of filipin interacting with cholesterol].

Cholesterol was purchased from Sigma Chemical Co., St. Louis, Mo. Purity of the sterol was checked by thin-layer chromatography and gas chromatography.

Filipin solutions were stored at 4° in the dark for the times indicated in the figure legends. The cholesterol-binding ability of the antibiotic was measured as described earlier (Schroeder *et al.*, 1971, 1972). Unless otherwise stated, absorbance, corrected fluorescence (CO), partial quantum efficiency (PQ), and Tyndall light scattering (R_{90}) were determined at 50° on 3 ml of the filipin solution (2.95 μM) before and after mixing with 20-fold molar excess cholesterol as previously described (Schroeder *et al.*, 1972). After 2 hr at 24° in the dark (Jones and Gordon, 1972), the absorbance, CO, PQ, and R_{90} were determined at 24° on all filipin solutions containing methanol, ethanol, propanol, *p*-dioxane, or dimethylformamide. These solutions were also used for determination of CMC. Light scattering due to solvent was corrected (Kresheck *et al.*, 1966). The dimensionless light scattering factor (R_{90}), absorbance ratios, CO, and PQ were measured on the computer-centered spectrofluorimeter of Holland *et al.* (1973a,b). The absorbances were measured at 305 and 338 nm, the light scattering at 338 nm, and the CO and PQ were measured at an emission wavelength of 496 nm with the excitation wavelength at 338 nm. This instrument treats scattered radiation in an analogous manner to fluorescence. The routine used for obtaining R_{90} values was the corrected fluorescence routine, F_{CO}, the only difference being that the two monochromators were scanning in wavelength synchrony. Hence, R_{90} was determined and is equivalent to R_θ, where $\theta = 90°$ (Tinker and Saunders, 1968). R_{90} is corrected for the inner filter effect, and all intensities are measured in terms of quanta. The instrument was calibrated against water and benzene.

Results

Light Scattering and PQ Measurements of Sterol-Binding and Sterol-Nonbinding Forms of Filipin. A freshly prepared aqueous solution of filipin, as shown in Figure 1A, has an absorbance ratio (A_{338}/A_{305}) of 1.96 and light scattering at 338 nm is 41 $\times$ 10^{-6}. The interaction of this freshly prepared filipin—isolated using organic solvents (Whitfield *et al.*, 1955)—with cholesterol in aqueous solution is delayed until the filipin is converted into an active form, as shown by the PQ curve of Figure 1A. PQ, which is altered by interaction of filipin with cholesterol, does not decrease until the antibiotic has been incubated for 35 min at 50°. Maximal interaction, as measured by per cent decrease in PQ, requires 2 hr. Longer incubation of filipin with cholesterol does not further decrease PQ. In Figure 1A, filipin was incubated at 50° for the times indicated by each point. Cholesterol was then added and values were determined after a 2-min delay. A similar PQ curve is obtained when cholesterol is added to filipin prior to the preincubation, as shown in Figure 1B. The conversion of inactive to active filipin follows first-order kinetics, as indicated in Figure 2. The data in Figure 2 were obtained from the PQ data of Figures 1A and 1B. The conversion of the inactive to the active form is temperature dependent. As shown in Table I, at 24°, only 7% of the filipin is converted to the active form in 2 hr, while at 50° the conversion is completed during this interval.

The initial value of the light scattering in Figure 1A indicates that the filipin exists in an aggregated form. Incubation at 50° for 35 min decreases light scattering by 77%, showing partial disruption of the large aggregates. This incubation also reduces the absorbance ratio from 1.96 to 1.7; however, PQ does not decrease upon the addition of cholesterol; hence the antibiotic is not binding cholesterol at this point. After 1.5 to 2.0 hr, light scattering of filipin is decreased by more than 90%, the absorbance ratio is decreased to 1.5, indicating the

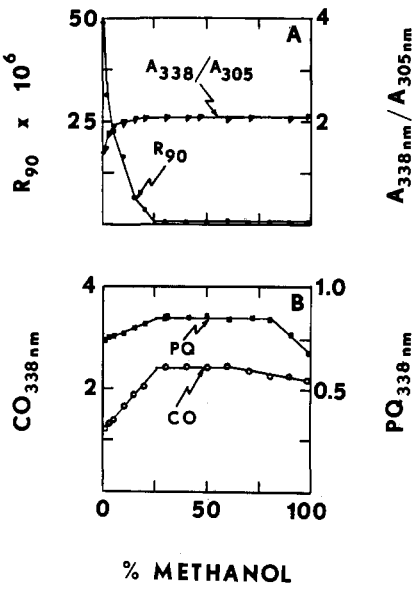


FIGURE 3: Effect of methanol on R_{90}, A_{338}/A_{305}, CO, and PQ of filipin. A_{338}/A_{305}, R_{90}, CO, and PQ were determined at 24°. Filipin (2.95 μM) was prepared from a 2-day-old stock solution, as described under Materials and Methods: (●) R_{90} at 338 nm; (▲) $A_{338\,nm}/A_{305\,nm}$; (○) $CO_{338\,nm}$; (■) $PQ_{338\,nm}$.

presence of the active form, and the interaction with cholesterol is maximized as demonstrated by a 34% decrease in PQ.

As shown in Figure 1C, filipin stored for 60 days at 4° in the dark immediately interacts with cholesterol, indicated by the 32% decrease in PQ. The low value of the absorbance ratio signifies that the active form is present and the high initial value of light scattering indicates that active filipin as well as inactive filipin can exist in micellar or aggregated form.

Effect of Temperature on the Activity and Light Scattering of Freshly Prepared Filipin Solutions. Incubation at 24° instead of 50° increases the time necessary for formation of the active form of filipin. This decrease in incubation temperature of inactive filipin results in a 12-fold increase in time required to produce the active form (see Table I).

Effect of Dimethylformamide on Activity and Light Scattering of Filipin. Dimethylformamide is frequently used as a solvent for filipin, but as shown in Table II, filipin dissolved in dimethylformamide is initially inactive as is indicated by the absorbance ratio of 2.3. However, since the light scattering at 338 nm is only 1×10^{-6}, the filipin freshly prepared in dimethylformamide is not in the micellar form. If the dimethylformamide stock solution is stored for 2.5 days at 4° in the dark, the absorbance ratio indicates that the antibiotic is now active, as confirmed by PQ. The increase in the light scattering indicates that the filipin has aggregated.

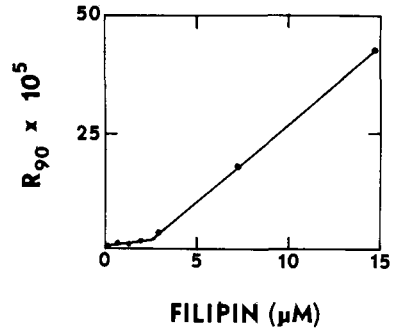


FIGURE 4: Determination of critical micelle concentration of filipin in water. Two-day-old filipin (14.7 μM) was diluted with distilled water to the concentrations indicated. After mixing and incubation at 24° for 2 hr, R_{90} was determined as described under Materials and Methods.

Effect of Alcohols on the Activity and Light Scattering of Filipin. Methanol solubilizes filipin and also inactivates the antibiotic (Gottlieb *et al.*, 1961; Norman *et al.*, 1972a,b). As the methanol concentration is increased, the absorbance ratio increases from 1.4 to 2.1, indicating that filipin is being transformed from an active to an inactive form. Light scattering at 338 nm decreases from 50×10^{-6} to almost zero (see Figure 3A). As shown in Figure 3B, CO (corrected fluorescence) and PQ also increase as the methanol concentration is changed from 0 to 25%.

CMC values for filipin in 25% methanol were calculated from the data given in Figure 3 as well as by a determination of R_{90} *vs.* increasing concentration of filipin in 25% methanol. The values obtained are 3.1 and 3.0 μM, respectively. The CMC of filipin in aqueous solution is 2.7 μM as shown in Figure 4.

Other experiments (data not shown) show that the minimum per cent alcohol required for disruption of both the active and inactive filipin micelle is linearly related to the number of methylene groups: propanol < ethanol < methanol. Increasing hydrophobicity of the alcohol increases its ability to solubilize filipin micelles. These results agree with those predicted by others (Emerson and Holtzer, 1967) that the micelle is held together by hydrophobic forces and its dis-

TABLE I: Effect of Temperature on the Conversion of Filipin[a] from Inactive to Active Forms.

Incubation		$10^6 R_{90}$ at	$A_{338\,nm}/A_{305\,nm}$	% Decrease in $PQ_{338\,nm}$ with
Temp (°C)	Time (hr)	338 nm		Cholesterol
24	0.33	41	1.96	0
24	2.0	23	1.78	2
24	24.0	2	1.56	33
50	2.0	2	1.50	36

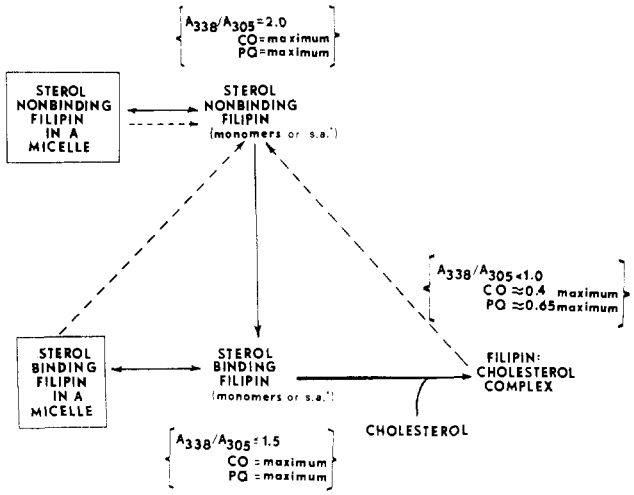
[a] Filipin (2.95 μM) was freshly prepared and incubated at 24 or 50°, as described under Materials and Methods. R_{90}, A_{338}/A_{305}, and PQ were determined as stated under Materials and Methods.

TABLE II: Effect of Dimethylformamide on R_{90} and Activity of Filipin.[a]

Time at 4° (Days)	$10^6 R_{90}$ at 338 nm	$A_{338\,nm}/A_{305\,nm}$	% Decrease in $PQ_{338\,nm}$ with Cholesterol
0	1	2.3	3
2.5	40	1.0	25

[a] Filipin (2.9 μM) solutions were prepared from fresh and 2.5-day-old stock solutions of filipin in dimethylformamide, as described under Materials and Methods. R_{90}, A_{337}/A_{305}, and PQ were determined at 24° as stated under Materials and Methods.

SCHEME I: Characteristics and Proposed Model for Some States of the Sterol-Binding and the Inactive Forms of Filipin.[a]



[a] Solid arrows refer to processes occurring in aqueous solutions. Broken arrows refer to effects of organic solvents such as methanol, ethanol, and propanol added to aqueous solutions: A_{ratio} = absorbance at 338 nm/absorbance at 305 nm; CO = corrected fluorescence; PQ = partial quantum efficiency; s.a.* = small aggregates.

ruption is dependent on the solvent's power to solvate the hydrophobic monomeric species. The dielectric constant of the solvent appears to have little effect on the ability of the solvent to break up filipin micelles since the minimum per cent solvent required to break up the micelles is 29% w/w for *p*-dioxane (dielectric constant $\epsilon = 2.2$) while that of methanol is 25% w/w ($\epsilon = 32.6$).

Discussion

The results are consistent with our previous observations that filipin can occur in forms that bind and others that do not bind 3-β-hydroxy sterols and that these forms are interconvertible depending on the experimental conditions (Schroeder *et al.*, 1971, 1972).

The sterol-binding activity of filipin, as measured by its capacity to immediately alter PQ when exposed to cholesterol, appears to be independent of the presence or absence of micelles. Nonmicellar, active filipin—either monomers or small aggregates—interacts with cholesterol; however, since micelles are not static, but exist in equilibrium with monomers (Kresheck *et al.*, 1966; Jaycock and Ottewill, 1967; Bennion *et al.*, 1969), with rate constants yielding half-lives in the millisecond range (Bennion *et al.*, 1969), any possible interaction of micelles of active filipin with sterols would not be detected with the present methodology.

The results of our investigations can be summarized as indicated in Scheme I. It is logical to assume that inactive monomers or small molecular aggregates that are not detectable by light scattering are in equilibrium with micelles of inactive filipin. The decrease in absorbance ratio during incubation or long periods of standing suggests that filipin undergoes a time- and temperature-dependent change, most likely conformational, to active filipin form(s) which can also form micelles. The finding that the conversion of the sterol-nonbinding to the sterol-binding form follows first-order kinetics is consistent with a conformational change. Conformational changes of antibiotics dissolved in organic or aqueous solvents have been previously demonstrated with the cyclic peptides valinomycin and polymyxin B (Patel, 1973; Patel and Tonelli, 1973; Chapman and Golden, 1972). As is indicated by the dashed arrows in Scheme I, alcohols disrupt the filipin–cholesterol complex and break up both types of micelles, always resulting in sterol-nonbonding forms of filipin, possibly monomers. Conversion to the inactive form is shown by the increase in absorbance ratio from less than 1.5 to about 2.1.

Although the data presented herein and previously by Schroeder *et al.* (1971, 1972) and by others (Bittman and Fischkoff, 1972; Norman *et al.*, 1972a,b; Drabikowski *et al.*, 1973) are generally consistent with the model shown in Scheme I, some contradictions do exist and, thus, it seems appropriate to mention some potential problems that may occur with the sensitive fluorescence techniques.

Changes in Concentration of the Polyene. Any process that changes the concentration of the polyene in solution, such as precipitation or absorption to a solid surface or to a particle, will alter the intensity of fluorescence and absorption, thereby producing apparent, but not necessarily real, changes in these parameters, making these parameters of questionable value for studying molecular interactions unless the exact nature of the concentration changes are known. It is of importance to note that the parameter, PQ, is dependent upon the quantum efficiency of the fluorophore and independent of its concentration. In a study of this type where micelle formation, precipitation, and denaturation processes are prevalent, such a parameter has unique capability in the detection of binding or conformational changes within the vicinity of the fluorophore.

Use of High Concentrations of the Polyene. (a) DETERGENT EFFECTS. Amphipathic compounds have a low CMC, which is 2.7 μM for filipin. At concentrations two to three orders of magnitude above the CMC, 10^{-4} M and greater, these compounds can be detergents. Detergent effects would make the interaction of the polyene nonspecific rather than specific for sterols.

(b) ABSORPTION ARTIFACTS. At high concentrations of the polyene, the absorption of the primary excitation beam is so great that the observed fluorescence would actually *increase with dilution*, a classical example of the inner filter effect (Holland *et al.*, 1973a). With such conditions, it is easy to obtain apparent, but artifactual, increases in fluorescence by simple dilution or any other process which results in a lower total absorption in the sample cell. The instrument used for our studies corrects for this artifact (Holland *et al.*, 1973a,b), always presenting a more accurate measure of the actual fluorescence emission.

Use of the Inactive Form of Filipin. We have shown that, depending on the experimental conditions, filipin can exist in either a sterol-binding or a nonbinding form. Use of the inactive form of filipin has produced anomalous results in the literature. For example, it has recently been reported that filipin interacts about equally well with cholesterol as epicholesterol, but a 50-fold molar excess of cholesterol and epicholesterol was needed to obtain maximum effects (Bittman and Fischkoff, 1972). This result is puzzling and appears to be contrary to previous results which have demonstrated a 1:1 stoichiometry between filipin and cholesterol interaction (Schroeder *et al.*, 1972) and also appears to be inconsistent with results which show that a slight molar excess of cholesterol in the diet can prevent the larvicidal effects of filipin toward *Musca domestica* (Schroeder and Bieber, 1971). We have found that excess cholesterol produces little enhancement of fluorescence when compared to the situation where

cholesterol equals the filipin concentration. Much of this discrepancy could also again be due to use of an inactive form of filipin. Although their samples were incubated for 2.5 hr, the temperature apparently was 15°. Our results indicate that incubating the inactive form of filipin in water for 2.5 hr at 50° is adequate to convert it to the sterol-binding form, but 2.5 hr at 24° is not adequate. At 15°, the rate of conversion to the active form would be much slower. The effect of sterol concentration on potentiating the conversion of inactive filipin to the sterol-binding form is not known, but the sterol appears to assist the conversion when present in large molar excess.

To preclude use of the inactive form, the absorbance ratio $A_{338}/_{308}$ can be used to confirm the presence of predominantly active filipin. In aqueous solution, the ratio should not be greater than 1.6.[1]

Effects by Nonsterol Molecules on Filipin-Produced Fluorescence. It seems likely that in complex systems such as membrane preparations, many factors would be present that could alter the fluorescence properties of filipin. For example, it has been reported that lecithin, in the absence of cholesterol, enhances the fluorescence intensity of filipin (Bittman and Reddy, 1971). This effect of lecithin would be in direct competition with the effect of cholesterol and could lead to paradoxical results if both species were to act on filipin simultaneously.

In another instance, it has been reported that the fluorescence of the tetraene polyene antibiotic, lucensomycin, is greatly altered by various organic solvents in the absence of sterols (Crifo *et al.*, 1971). These and other interactive effects demand great care in the selection of experimental parameters and in the interpretation of the data obtained.

References

Balcavage, W. X., Beale, M., Chasen, B., and Mattoon, J. R. (1968), *Biochim. Biophys. Acta 162*, 525.

Bennion, B. C., Tong, L. K. J., Holmes, L. P., and Eyring, E. M. (1969), *J. Phys. Chem. 73*, 3288.

Bergy, M. E., and Eble, T. E. (1968), *Biochemistry 7*, 653.

Bittman, R., and Fischkoff, S. A. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 3795.

Bittman, R., and Reddy, B. K. (1971), *Proc. Int. Congr. Pure Appl. Chem., XXIIIrd 1*, 407.

Chapman, T. M., and Golden, M. R. (1972), *Biochem. Biophys. Res. Commun. 46*, 2040.

Crifo, S., Strom, R., Santaro, S., and Mondovi, B. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 17*, 121.

Demel, R. A., Crombag, F. J. L., Van Deenen, L. L. M., and Kinsky, S. C. (1968), *Biochim. Biophys. Acta 150*, 1.

Demel, R. A., Van Deenen, L. L. M., and Kinsky, S. C. (1965), *J. Biol. Chem. 240*, 2749.

Drabikowski, W., Lagwinska, E., and Sarzala, M. G. (1973), *Biochim. Biophys. Acta 291*, 61.

Emerson, M. F., and Holtzer, A. (1967), *J. Phys. Chem. 71*, 3320.

Fendler, E. J., and Fendler, H. J. (1970), *Advan. Phys. Org. Chem. 8*, 271.

Gottlieb, D., Carter, H. E., Sloneker, J. H., Wu, L. C., and Gaudy, E. (1961), *Phytopathology 51*, 321.

Haksar, A., and Peron, F. G. (1972), *Biochim. Biophys. Acta 264*, 548.

Holland, J. F., Teets, R. E., Sindmack, G., and Timnick, A. (1973a), *Anal. Chem.*, submitted for publication.

Holland, J. F., Teets, R. E., and Timnick, A. (1973b), *Anal. Chem. 45*, 145.

Jaycock, M. J., and Ottewill, R. H. (1967), *in* Chemistry, Physics, and Application of Surface Active Substances, Vol. 2, Overbeek, J. Th. G., Ed., New York, N. Y., Gordon and Breach, p 545.

Jones, J. B., and Gordon, K. D. (1972), *Steroids 20*, 449.

Kinsky, S. C. (1967), *Antibiotics 1*, 122.

Kinsky, S. C., Gronau, G. R., and Weber, M. M. (1965), *Mol. Pharmacol. 1*, 190.

Kinsky, S. C., Haxby, J., Kinsky, C. B., Demel, R. A., and Van Deenen, L. L. M. (1968), *Biochim. Biophys. Acta 152*, 174.

Kleinschmidt, M. G., Chough, K. S., and Mudd, J. B. (1972), *Plant Physiol. 49*, 852.

Kresheck, G. C., Hamori, E., Davenport, G., and Scheraga, H. A. (1966), *J. Amer. Chem. Soc. 88*, 246.

Lampen, J. O., Morgan, E. R., Slocum, A., and Arnow, P. M. (1959), *J. Bacteriol. 78*, 282.

Lardy, H. A., Johnson, D., and McMurray, W. C. (1958), *Arch. Biochem. Biophys. 78*, 587.

Mondovi, B., Strom, R., Agio, A. F., Caiafa, P., Sole, P. D., Bozzi, A., Rotilio, G., and Fanelli, A. R. (1971), *Cancer Res. 31*, 505.

Morton, B. E., and Lardy, H. A. (1967), *Biochemistry 6*, 57.

Norman, A. W., Demel, R. A., de Kruyff, B., Guerts von Kessel, W. S. M., and Van Deenen, L. L. M. (1972a), *Biochim. Biophys. Acta 290*, 1.

Norman, A. W., Demel, R. A., de Kruyff, B., and Van Deenen, L. L. M. (1972b), *J. Biol. Chem. 247*, 1918.

Patel, D. J. (1973), *Biochemistry 12*, 496.

Patel, D. J., and Tonelli, A. E. (1973), *Biochemistry 12*, 486.

Schroeder, F., and Bieber, L. L. (1971), *Chem.-Biol. Interacto 4*, 239.

Schroeder, F., Holland, J. F., and Bieber, L. L. (1971), *J. Antibiot. (Tokyo) 24*, 846.

Schroeder, F., Holland, J. F., and Bieber, L. L. (1972), *Biochemistry 11*, 3105.

Sessa, G., and Weissmann, G. (1968), *J. Biol. Chem. 243*, 4364.

Strom, R., Crifo, C., Faiola, R., Bozzi, A., and Scioscia Santora, A. (1972), *Boll. Soc., Ital. Biol. Sper. 48*, 412.

Tinker, D. O., and Saunders, L. (1968), *Chem. Phys. Lipids 2*, 316.

Van Zutphen, H., Demel, R. A., Norman, A. W., and Van Deenen, L. L. M. (1971), *Biochim. Biophys. Acta 241*, 310.

Weissmann, G., and Sessa, G. (1967), *J. Biol. Chem. 242*, 616.

Whitfield, G. B., Brock, T. D., Amman, A., Gottlieb, D., and Carter, H. E. (1955), *J. Amer. Chem. Soc. 77*, 4799.

[1] This method has been detailed by Schroeder *et al.* (1971, 1972) and was initially presented at the American Society of Biological Chemists Meeting, June, 1971. Copies of the presentation are available from L. L. Bieber or F. Schroeder. Use of this method to confirm the presence of predominantly active or inactive filipin in aqueous systems has been successful in every application that we have tried. Recently, this method apparently has been adopted by others (Bittman and Fischkoff, 1972).