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Effect of Filipin on Liposomes Prepared with Different Types of Steroids[1]

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ABSTRACT

The action of the polyene antibiotic filipin on the permeability of liposomes prepared with lecithin and several plant and other steroids was studied. The effect of filipin was found to be dependent upon the type of steroid incorporated into the membrane. The interaction of filipin with steroids was found to be related both to the functional group at the 3-position and the aliphatic chain of the steroid. Complex formation of the steroid with filipin in aqueous solutions, as detected by ultraviolet spectrophotometry, correlated with the ability of filipin to increase the permeability of the liposomes.

From these results it was suggested that the action of filipin could be used as a diagnostic tool to determine the presence of different steroids in plant membranes. Differences in the sensitivity of subcellular organelles to filipin may be due to differences in the steroid composition of their membranes.

Although work has been done concerning identification and biosynthesis of steroids in plants, their function has not yet been defined. Schöenheimer *et al.* (26) have suggested that steroids in plants are merely metabolic waste products. Others (12, 17) have suggested that plant steroids perform functions similar to those in animals, *e.g.*, hormonal and membranal. Recently Galliard (7, 8) reported notably large amounts of steroids in plant tissue: 29.2% of the total lipid in postclimacteric apples and 9.1% of the total lipid in potato tubers are steroid.

The pentaene antibiotic filipin (1, 2) inhibits fungal spore germination and growth (9, 10, 24, 25, 29). *Mycoplasma laidlawii* and *Pythium* spp. are sensitive to filipin only when selected steroids are incorporated into their membranes (25, 29). Gottlieb *et al.* (10) reported that some steroids antagonize the action of filipin while others do not. Filipin has also been shown to cause leakage of cellular constituents from red beets and potato tuber discs (20). Work done with model membrane systems of lipid monolayers and bilayers show that low concentrations of filipin affect these membranes only when cholesterol is incorporated into the membrane, while higher concentrations of filipin affect membranes with no cholesterol (5, 6, 13–15, 27–30). It now appears that the effect at high concentrations is due to the fact that filipin is a complex of at least four components and only the major component shows specificity for sterols (1, 28).

If the specificity of filipin with respect to steroids could be defined, it would be possible to use filipin as a test for the presence of certain steroids in cellular membranes. This paper describes the specificity of filipin to several steroids including most of those commonly found in higher plants.

MATERIALS AND METHODS

Filipin complex was a generous gift from Dr. G. B. Whitfield, Jr., Upjohn Company, Kalamazoo, Michigan. Dicetyl phosphate, TPN, hexokinase, glucose-6-P dehydrogenase, ATP, cholesteryl chloride, 5α-cholestane, stigmasterol, 5α-cholestan-3-one, and Δ5-androsten-3β-17β-diol were obtained from Sigma Chemical Company, St. Louis, Missouri. Campesterol, β-sitosterol, cholesterol, cholesteryl oleate, cholesteryl palmitate, and cholesteryl acetate were obtained from Applied Science Laboratories, State College, Pennsylvania. DTNB[2] was obtained from Aldrich Chemical Company, Gardena, California. Ergosterol was obtained from Nutritional Biochemicals, Cleveland, Ohio.

Phosphatidyl choline was isolated from eggs by the method of Pangborn (23) and further purified by passing it through a 1.5 × 30.0 cm silicic acid column. The eluting solvents were 150 ml of $CHCl_3$, 150 ml $CHCl_3$-methyl alcohol (3:1 v/v), and 150 ml of $CHCl_3$-methyl alcohol (1:1 v/v). Fifty-milliliter fractions were collected and monitored by thin layer chromatography. Phosphatidyl choline began eluting in the second fraction of the 3:1 mixture. Only fraction 3 of the 3:1 and fraction 1 of the 1:1 mixture contained pure phosphatidyl choline. The concentration of phosphatidyl choline was determined by the inorganic phosphate method of Clark (3).

Steryl glucoside and acyl steryl glucoside were isolated from a commercial preparation of soybean lecithin (Sigma Chemical Co.), since this preparation contains appreciable quantities of these steroids as impurities. Approximately 1 g of the crude lecithin dissolved in hexane was placed on a 1.5 × 30 cm silicic acid column and eluted with a hexane-diethyl ether gradient followed by 300 ml of acetone. The gradient was formed by placing 550 ml of hexane in the column reservoir and running 1 liter of anhydrous diethyl ether into the column reservior which was continually stirred. Eight-milliliter fractions were collected, and every fifth fraction was tested for

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[2] Abbreviation DTNB: 5,5′-dithiobis-(2-nitrobenzoic acid).

sterol by the Liebermann-Burchard reaction (4). Figure 1 shows the elution pattern of sterols from the silicic acid column. The fractions under each peak were combined and aliquots were thin layer chromatographed with chloroform-methanol-acetic acid-water (80:15:3:3, v/v) as the solvent system (21). Sulfuric acid was used to visualize the separated compounds. From R_F values on thin layer chromatograms the fractions were tentatively identified as: fraction 1, steryl esters; fraction 2, free steroids; fraction 3, acyl steryl glycoside; fraction 4, steryl glycoside. The identification of fractions 3 and 4 were confirmed by hydrolyzing the acyl steryl glycoside fraction for 3 hr at 40 C with 0.5 N NaOH. Mild alkaline hydrolysis removes the fatty acid from the acyl steryl glycoside, converting it to steryl glycoside (22). Rechromatographing on a thin layer plate and on a silicic acid column showed that the acyl steryl glycoside fraction 3 was converted to steryl glycoside, fraction 4 (Fig. 1). The acyl steryl glycoside fraction gave a single spot with thin layer chromatography. The steryl glycoside fraction was contaminated with several other components and therefore discarded. To obtain pure steryl glycoside the purified acyl steryl glycoside was hydrolyzed, and the steryl glycoside was extracted with chloroform. The steroid concentration was determined by the Liebermann-Burchard reaction (4). Different steroids have been reported to produce different color intensities in this reaction (4). The glycosides were hydrolyzed to the free steroid, and the color intensities developed in the Liebermann-Burchard reaction with equimolar concentrations of the free steroid and sterol glycosides were compared. The color intensities developed were found to be approximately the same. Since the major steroid found in soybean is β-sitosterol (16, 19), it was used to prepare the standard curve.

Liposomes were prepared by methods described previously (15, 28). Chloroform solutions of phosphatidyl choline (7 μmoles), dicetyl phosphate (2 μmoles), and the respective steroid (0 or 1 μmole) were placed in a screw-cap tube. The solvent was removed under a stream of nitrogen and subsequently under vacuum to assure complete removal of all solvent. One milliliter of 0.3 M glucose solution or 0.1 M DTNB suspension prepared in 0.1 M tris buffer, pH 8.0, was added. The lipids were dispersed into liposomes by vigorous shaking for three 15-min intervals on a Vortex mixer and allowing 10 min equilibration between each shaking period. The liposomes containing glucose or DTNB were placed on a 1.5 × 20 cm Sephadex G-25 column to remove the unsequestered glucose or DTNB. The liposomes were eluted with a solution of 75 mM KCl, 75 mM NaCl in 0.1 M tris buffer, pH 8.0, in the void volume, while the small molecular weight materials were retained.

The liposomes containing different steroids were tested for filipin sensitivity by following the release of sequestered DTNB spectrophotometrically. Reaction mixtures were based on those described by Kinsky *et al.* (15). The following reagents were added to the cuvette: 1 ml of salt solution (0.15 M KCl and 0.15 M NaCl, prepared in 0.1 M tris buffer, pH 8.0); 0.1 ml of GSH (20 mM in 0.1 M tris buffer, pH 8.0); liposomes to give the initial absorbance 0.05; and tris buffer (0.1 M, pH 8.0) to make the final volume 2.0 ml. When the sequestered DTNB is released from the liposome, it reacts with GSH producing a strong chromophore at 412 nm which was measured spectrophotometrically. Various amounts of filipin were added to the cuvette from a 5 mg/ml methanolic solution prepared fresh daily. The total amount of DTNB trapped was determined by complete disruption of the liposomes with 0.2 ml 10% (w/v) Triton X-100 prepared in tris buffer (0.1 M, pH 8.0).

Several liposome preparations in which glucose was sequestered were also checked for filipin sensitivity. The amount of glucose released was measured by the enzymic method of Kinsky *et al.* (15).

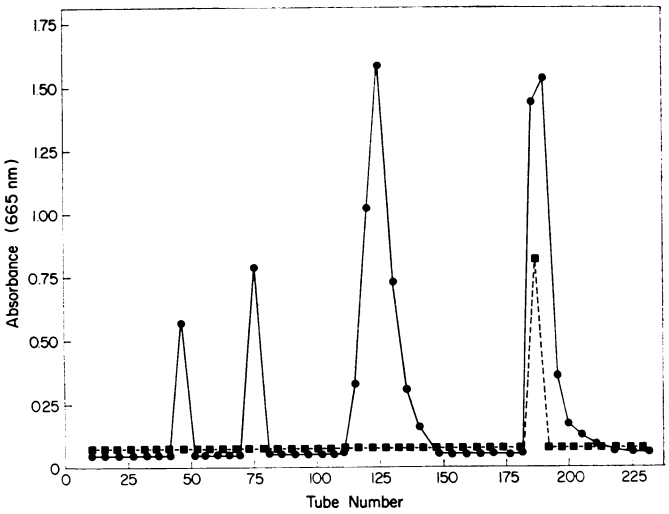

FIG. 1. Steroids from a commercial preparation of soybean lecithin were eluted from a silicic acid column with a diethyl ether-hexane gradient followed by acetone. Fractions are numbered in order of elution: 1: steroid ester; 2: steroid; 3: acyl steryl glycoside; 4: steryl glycoside. The broken line represents fraction 3, acyl steryl glycoside, rechromatographed after hydrolysis with 0.5 N NaOH at 40 C for 3 hr. Absorbance is developed color in the Liebermann-Burchard reaction (4).

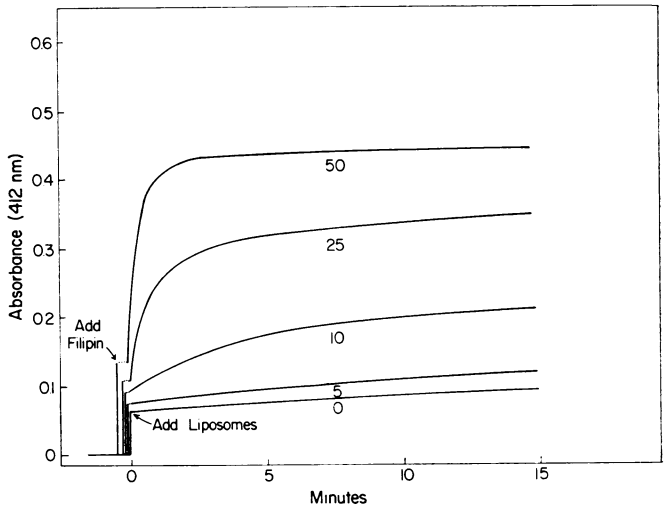

FIG. 2. Effect of 0, 5, 10, and 25 μg/ml filipin on DTNB permeability of liposomes prepared with campesterol. The increase in absorbance at 412 nm is due to reaction of the leaked DTNB with GSH in the incubation mixture. Details are described in "Materials and Methods."

RESULTS

Liposomes containing sequestered DTNB were used to evaluate the effect of filipin on permeability of membranes containing different types of steroids. The effect of different concentrations of filipin on the rate of DTNB released from liposomes containing campesterol is shown in Figure 2. The initial absorbance was due to light scattering by the liposomes. After addition of filipin, a slight increase in absorbance due to filipin was observed. The initial rate of release of DTNB was rapid and then slowly tapered off. The total amount of DTNB trapped was determined by complete disruption of the lipo-

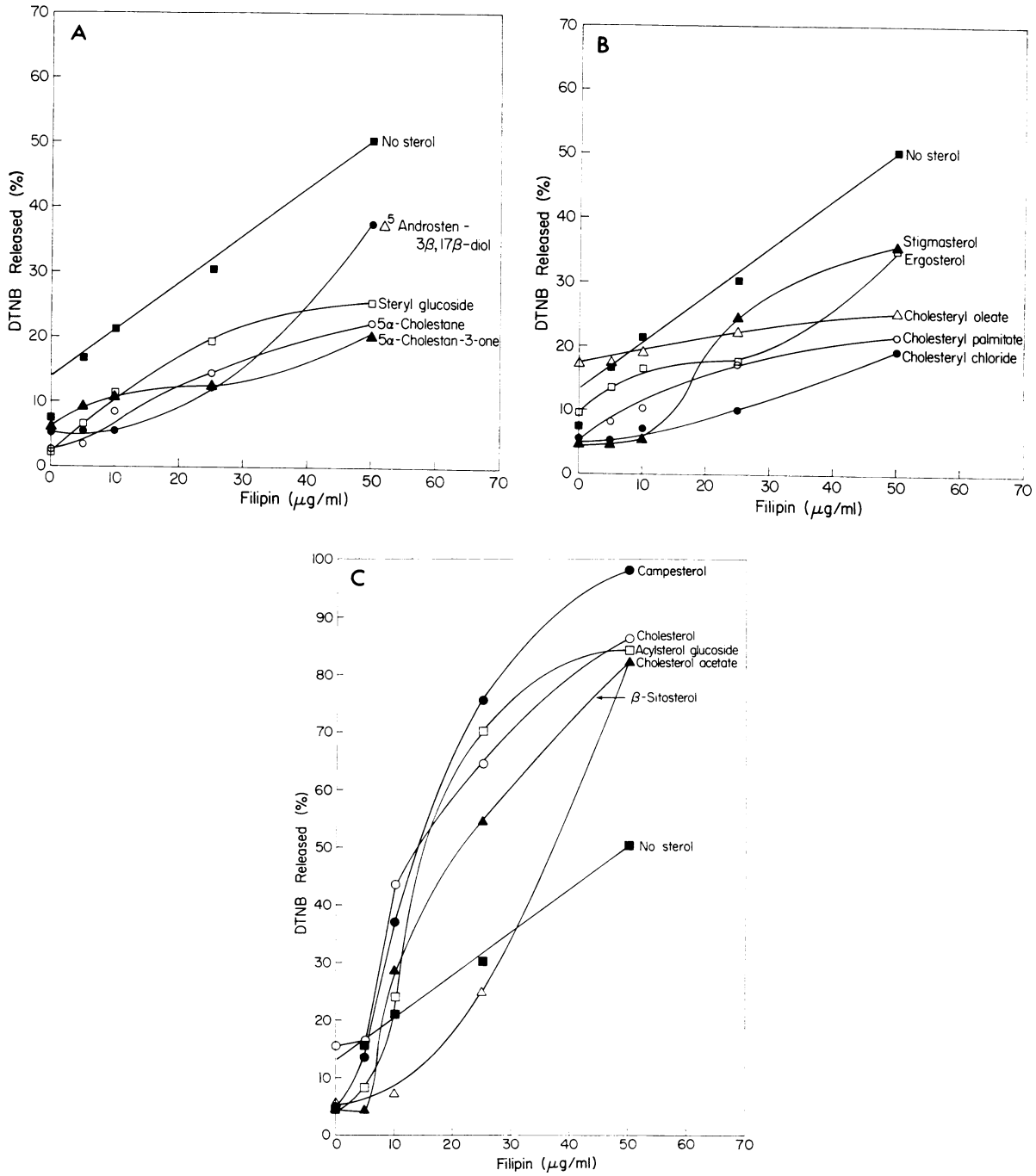

FIG. 3. Effect of filipin concentration on the percentage of DTNB released after 15 min from liposomes prepared with different steroids. A: Those which make the liposomes less susceptible to filipin action; B: those which make the liposomes less susceptible to filipin; C: those which make the liposomes more susceptible to filipin.

somes with Triton X-100. The initial rate of DTNB loss is proportional to filipin concentration. In addition, the amount of DTNB loss is also proportional to filipin concentration if determined after leakage has essentially stopped (*e.g.*, after 15 min). The amount of DTNB released during the first 15 min after the addition of filipin, expressed as percentage of the total DTNB released by Triton X-100, is used as a measure of liposome sensitivity to filipin. Figure 3,A, B, and C, shows the percentage of DTNB released as a function of (*a*) filipin concentration and (*b*) the type of steroid used in the liposome preparation. The liposomes prepared with the various steroids tested generally fell into two categories: (*a*) those which released less DTNB and (*b*) those which released more DTNB than the liposomes prepared without steroid. The one exception was cholesteryl acetate, which consistently showed less DTNB released at filipin concentrations below 25 μg/ml but greater DTNB released at higher concentrations. Previous investigators (15, 28, 30) have shown that filipin has a high specificity for some steroids at low concentration, and this specificity is less at higher concentrations.

After passing the liposome preparation through the Sephadex column, the concentration of the eluted material was not the same for all preparations. The amount of liposomes was kept constant for each experiment by adding an

amount which would give an initial absorbance of 0.05, usually about 0.1 ml. The relative amount of liposomes did not appear to be critical at the levels used, since a 2-fold change in the amount of liposomes added gave approximately the same percentage of DTNB released by 25 μg/ml filipin. However, Kinsky *et al.* (13) have reported that the filipin/cell ratio was important in the rate of erythrocyte hemolysis.

No inhibition of the action of filipin on liposomes could be observed at a concentration of 5 mM GSH, five times higher than that used for the DTNB assay. Gottlieb *et al.* (10) reported that high concentrations of GSH could vitiate the action of filipin on fungi.

Previous workers (10, 18) have shown that filipin forms a loosely bound complex with cholesterol in aqueous solutions, and this complex can be broken by addition of equal volumes of methanol. In order to determine if there was a correlation between filipin-steroid complex formation and sensitivity of steroid-containing liposomes, filipin-steroid complex formation was determined. Figure 4 is an ultraviolet spectrum of filipin showing peak maxima at 322 nm, 338 nm, and 355 nm. When an equal amount of campesterol was added to the aqueous solution of filipin and allowed to sit for 2 hr in the dark, the intensities of the 338 nm and 355 nm peaks decreased, while the 322 nm peak remained approximately constant. The decrease in the ratio of 338 and 355 to 322 nm peaks gives an indication of complex formation between steroid and filipin. According to Gottlieb *et al.* (10) this decrease is not stoichiometric and, therefore, may not give an absolute value for amount of complex formed. But, it does indicate if a complex is formed and whether the sensitivity of liposomes to filipin can be correlated with the complex forming capabilities of filipin and the steroid. Table I shows the complex formation with filipin and the steroids tested. Generally, the large change in 338/322 and 355/322 nm peaks was observed in those steroids which allow the release of more DTNB from liposomes by filipin, filipin-sensitive steroids, and a smaller change in these ratios was observed with the steroids which allow the release of less DTNB, filipin-insensitive steroids. However, stigmasterol, which is a filipin-sensitive steroid, showed a large change in these ratios, and its behavior is therefore anomalous. If stigmasterol failed to be incorporated into the membrane during preparation of the liposomes, this would explain why the liposomes were not sensitive to filipin. The amount of stigmasterol recovered from the liposome fraction after elution on the Sephadex column was determined by chloroform extraction and the Liebermann-Burchard reaction (4). More than 65% of the added stigmasterol was recovered in the liposome fraction.

Several liposome preparations were made in which glucose was the trapped molecule. The rate of glucose released was measured with hexokinase, glucose-6-P dehydrogenase, ATP, and TPN (15). Filipin has an absorption maximum at 338 nm and consequently strongly absorbs at 340 nm, which interferes with the glucose determination. Exposure to ultraviolet light causes the 338 nm absorption maximum of filipin to be reduced (10, 13, 32). The change in filipin absorbance during the reaction was also taken into consideration when final calculations were made. The steroids tested by this method generally showed results similar to those in which DTNB was used.

DISCUSSION

The action of filipin on liposomes is dependent upon the type of steroid incorporated into the membrane. The specific structural requirements of the steroid necessary to provide filipin sensitivity are not clear. A double bond in the aliphatic chain as in stigmasterol and ergosterol reduced the amount of DTNB permeability caused by filipin. Changing the functional group at the 3 position to a chloride, a carbonyl, or adding a long chain fatty acid reduced the effect of filipin on the liposomes. 5α-Cholestane, which differs from cholesterol by lacking the 3-hydroxy group and Δ^5-double bond, decreased the amount of DTNB released from liposomes by filipin. Therefore, it appears that the specificity of filipin to steroids lies in part with the type of functional group at the 3-position and the type of aliphatic chain present.

In order for filipin to be effective in changing membrane permeability, it appears that filipin must be capable of forming a complex with the steroid present in the membrane. How-

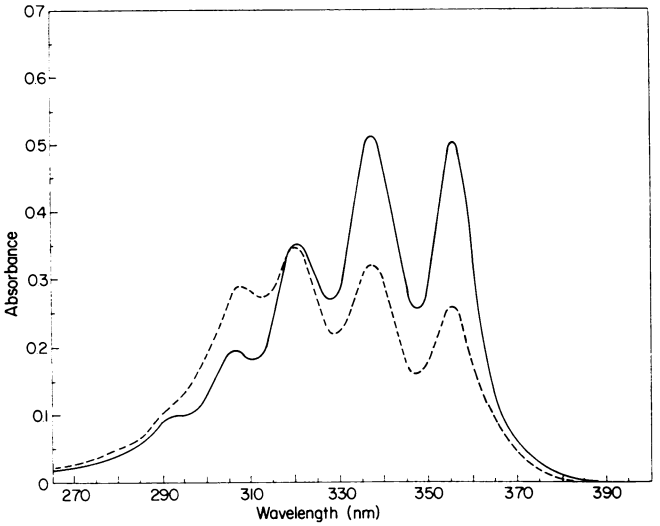

FIG. 4. Ultraviolet spectra showing complex formation between campesterol and filipin. Solid line represents 5 μg/ml filipin. Broken line is spectrum after addition of 5 μg/ml campesterol and incubated 2 hr in the dark.

Table I. *Filipin Complex Formation with Steroids as Measured by the Ratios of 338/322 and 355/322 nm Absorption Maxima*

The filipin absorbance spectrum is changed when a complex with steroid is formed. Absorbance at 322 nm is essentially unchanged, but absorbance at 338 nm and 355 nm is less (see Fig. 4). Lowering of the 338/322 and 355/322 nm ratios therefore is an index of complex formation.

Steroid	338/322 nm Ratio			355/322 nm Ratio		
	Before complex formation	After complex formation	Net change	Before complex formation	After complex formation	Net change
Campesterol	1.45	0.92	0.53	1.42	0.74	0.68
β-Sitosterol	1.44	0.61	0.83	1.40	0.33	1.07
Cholesterol	1.45	0.66	0.79	1.43	0.40	1.03
Acyl steryl glycoside	1.32	0.79	0.53	1.26	0.56	0.70
Cholesteryl acetate	1.43	1.18	0.36	1.39	0.93	0.46
Δ^5Androsten-3β-17β-diol	1.42	1.13	0.29	1.39	1.03	0.36
Stigmasterol	1.46	0.67	0.79	1.43	0.40	1.03
Ergosterol	1.42	1.21	0.21	1.40	1.20	0.20
Cholesteryl palmitate	1.44	1.35	0.09	1.40	1.02	0.38
Cholesteryl oleate	1.43	1.12	0.31	1.39	1.36	0.03
5α-Cholestane	1.40	1.35	0.05	1.39	1.32	0.07
5α-Cholestan-3-one	1.41	1.30	0.11	1.37	0.89	0.48
Cholesteryl chloride	1.41	1.35	0.06	1.37	1.30	0.07
Steryl glycoside	1.31	1.24	0.07	1.23	1.09	0.14

ever, filipin forms a complex with stigmasterol which did not give sensitivity to the liposomes. Norman, Demel, de Kruyf, and van Deenen (private communication) have recently made a study of the interaction of filipin with steroids by a spectrophotometric method. Their results are in agreement with those reported in this paper, but they have found that interaction of filipin with steroids in solution is not precisely the same as the interaction with steroids incorporated into liposomes. In all cases except stigmasterol, the interaction is less when the steroid is incorporated into the liposome. Thus, in this case also, the behavior of stigmasterol is anomalous.

The sigmoidicity of the response to filipin in some cases (Fig. 3C) suggests cooperative effects of increased amounts of filipin. This may be interpreted as a requirement for a minimum number of filipin molecules to change the permeability of the liposome. One might suggest that the distribution of the steroid in the liposome is reorganized in the presence of filipin. Log plots of the data from Figure 3C indicate a cooperativity number of 2. However, we would prefer not to speculate further at this point.

Demel *et al.* (6) reported that filipin interacted with cholesterol monolayers more than with ergosterol monolayers. These findings with artificial membrane preparations contrast with the results of the *in vivo* experiments of Gottlieb *et al.* (9, 10) and Schlosser and Gottlieb (25). They reported that there was no correlation between complex formation and biological activity and that filipin caused similar leakage of phosphate from cells of *Pythium irregulare* whether cholesterol, sitosterol, ergosterol, or stigmasterol was incorporated into their membranes. With the exception of stigmasterol our experiments show complex formation correlates well with liposome sensitivity to filipin.

Our results indicate that filipin may be used to test for the presence of certain types of steroids in membranes. If filipin affects permeability of one type of membrane and not another, this difference could be due to differences in the steroids of the membranes. Preliminary studies in this laboratory indicate that although potato discs treated with filipin release large amounts of ultraviolet-absorbing material, isolated mitochondria did not release significant amounts, although the mitochondria may contain low amounts of releasable ultraviolet-absorbing material. It appears that the steroid compositions of the plasmalemma and mitochondria may be quite different. Weissman *et al.* (31) found that filipin released lysosomal enzymes but did not have any measurable effect on the mitochondria. It is likely that the differences observed by Weissman *et al.* (31) are due to differences in the steroids of the lysosomal and mitochondrial membranes.

Grunwald (11) has shown large differences between the steroid composition of whole tobacco leaf, the nuclei-cell debris fraction, the chloroplast fraction, the mitochondrial fraction, and the microsomal fraction. The mitochondrial fraction contained 62% of the steroid as esters, and of the 31% free steroid, over 50% was stigmasterol. Therefore, one might predict that filipin would not affect tobacco leaf mitochondria.

Evidence strongly suggests that plant membranes contain steroids, and the steroid composition of various subcellular membranes is different. This difference in steroid composition may have interesting consequences as far as physiological properties are concerned.

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