FLUOROMETRIC EVIDENCE FOR THE BINDING OF CHOLESTEROL TO THE FILIPIN COMPLEX*

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The data show that the filipin complex in water has a characteristic fluorescence spectrum with a broad emission maxima at 497 nm. Addition of cholesterol to the aqueous solution decreases the absorbance, the corrected fluorescence, and the partial quantum efficiency. The reduction in partial quantum efficiency, which is independent of the concentration of filipin, is definitive evidence that the filipin complex interacts with sterols in aqueous solution. The data indicate that changes in fluorescence may be a sensitive tool for monitoring the interaction of filipins with sterols.

The filipin complex (1) has been used to probe properties and functions of sterols in natural and artificial membranes[1~8]; (2) has antifungal activity[9~13]; (3) has larvicidal and chemosterilant activity towards houseflies[14,15]; (4) reduces serum cholesterol levels in dogs[16,17] chicks[18] and also (5) mimics vitamin D in ileal segments from vitamin D-deficient chicks[19,20]. The above-mentioned effects have been attributed to the apparent affinity of the filipin complex for certain sterols. Herein definitive evidence is presented that filipins interact with cholesterol and that this interaction can be monitored fluorometrically.

Materials and Methods

Filipin, 86 % pure, was kindly provided by the Upjohn Company, Kalamazoo, Michigan. The complex was further purified by washing twice with chloroform and twice with petroleum ether. After drying, the filipin complex was dissolved in tertiary butyl alcohol, lyophilized, and stored in the dark at −20°C. The standard filipin solution was prepared by dissolving 1 mg in 100 ml distilled water. Partial quantum efficiency (PQ), correct fluorescence (CO), and absorption spectra were determined as decribed by HOLLAND and TIMNICK[21].

Results

The fluorescence emission spectrum of the filipin complex in water is shown in Fig. 1. Filipin also has a characteristic absorption spectrum (see Fig. 2A and ref. 9~11) which is decreased by addition of cholesterol. Cholesterol caused a 60 % reduction of the absorption maxima of the filipin complex at 356 and 338 nm and a smaller decrease of the absorption maxima at 305 and 321 nm as shown in Fig. 2B. Cholesterol also decreased the corrected fluroescence (compare Fig. 2D to 2E) and the

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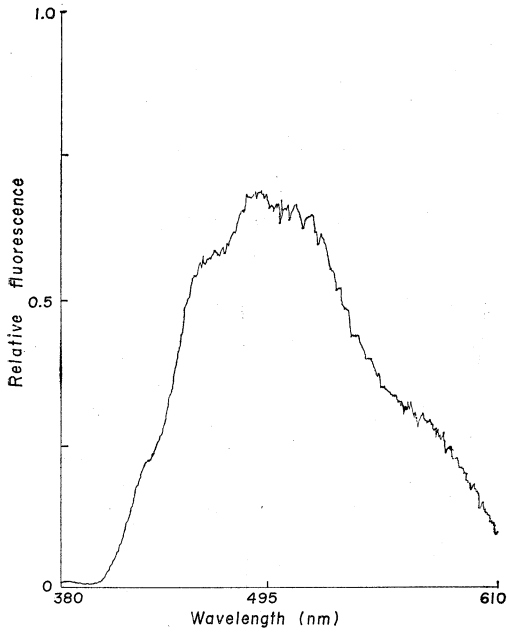
Fig. 1. Fluorescence emission spectrum of the filipin complex excited at 338 nm. The curve was obtained with 2 μg filipin complex per ml distilled water.



partial quantum efficiency (compare Fig. 2G to 2H). In contrast, the absorption spectrum, the corrected fluorescence, and the partial quantum efficiency of filipin plus cholesterol palmitate was very similar, if not identical, to that of filipin; compare Fig. 2A to 2C, 2D to 2F, and 2G to 2I. Thus, cholesterol, but not cholesterol palmitate, alters the absorption and fluorescence properties of the filipin complex in aqueous solution. None of the sterols tested fluoresced when excited at 338 nm.

Addition of methanol, final concentration 50 %, to the cuvette for the experiment of Fig. 2B, 2E, and 2H restored the absorbance, corrected fluorescence and partial quantum efficiency to that shown in Fig. 2A, 2D, and 2G. Thus, methanol prevents and reverses the interaction of filipin with cholesterol.

Other sterols, such as β-sitosterol, stigmasterol, and β-cholestanol, also quench the fluorescence of filipin and reduce the partial quantum efficiency. These sterols and cholesterol, but not cholesterol palmitate, prevent the antifungal effects of the filipin complex[9,13].

Discussion

The data show that cholsterol alters the fluorescence properies of the filipin complex and indicate that fluorescence could be a useful tool for probing the interactions of polyene antibiotics with sterols. The fluorescence properties of each polyene antibiotic may be different. This conclusion is supported by unpublished data which show that cholesterol, stigmasterol, sitosterol, and β-cholestanol all alter the fluorescence properties of pimaricin, but unlike the effect obtained with filipin, the partial quantum efficiency is greatly increased rather than decreased.

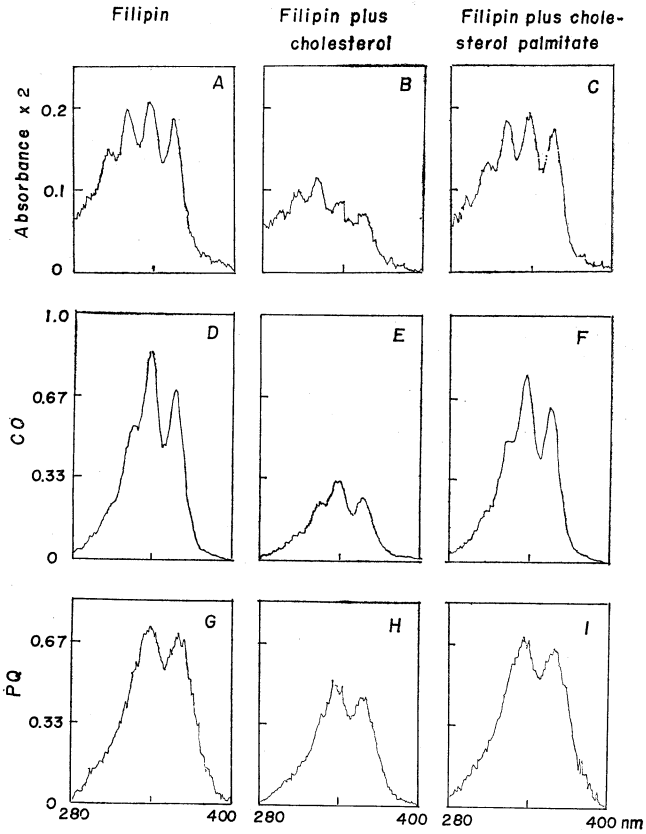
Our results correlate well with the results of GOTTLIEB and others[9,13] on the prevention of filipin toxicity to fungi by certain sterols. Sterols that prevent the fungicidal effects of filipin alter the fluorescence properties while compounds such as cholesterol palmitate, cortisone, and androstane-3β-ol-17-one which do not prevent the fungicidal effects of filipin also do not affect the corrected fluorescence or the partial quantum efficiency.

The reduction in the absorption intensity of filipin by addition of cholesterol agrees with the results of others[9,11]. A reduction of the absorption intensity of filipin by addition of sterols in aqueous solution might be due to a decrease of the concentration of the polyene in solution. Since partial quantum efficiency is independent of concentration, the change in partial quantum efficiency of the fluorescence of filipin caused by sterols is very strong evidence that filipins interact with certain sterols. The absorbance spectrum of filipin in the presence of cholesterol shows a large decrease in absorbance with no

detectable change in the energy of the bands. This suggests a close approximation of the cholesterol molecule to filipin such that the charge distribution of the excited state orbitals of the filipin could be altered sufficiently to produce the observed loss in transition moment. It is of interest to note that the two bands, at 338 and 356 nm, are preferentially diminished when compared with the total absorbance spectrum. This could indicate close proximity of the cholesterol to the conjugated double bond system of the filipin. The observed decrease in the partial quantum efficiency strongly suggests pi*–pi interaction between the excited states of the fluorophore and the orbitals of the cholesterol. Failure to observe any shift in the energy of the excitation or emission spectra indicates that any strong interactions between the two molecules are not located in the vicinity of the chromophores or fluorophores.

Fig. 2. Effect of cholesterol and cholesterol palmitate on the corrected fluorescence, partial quantum efficiency, and absorption spectrum of the filipin complex in water.

The partial quantum efficiency, PQ, and the corrected fluorescence, CO, are identical to that defined by HOLLAND and TIMNICK[21]. Fluorescence, λ-emission, was monitored at 497 nm. The filipin complex, 2 μg/ml, in distilled water was incubated at 50℃ for two hours: 1) without added sterol in A, D and G: 2) with 50 μg/ml chlolesterol in B, E, and H, and 3) with 50 μg/ml cholesterol palmitate in C, F, and I. Then absorbance, CO, and PQ were determined.



Bibliography

1) SESSA, G. & G. WEISSMANN: Effects of four components of the polyene antibiotic, filipin, on phospholipid spherules (liposomes) and erythrocytes. J. Biol. Chem. 243: 4364～4371, 1968.
2) KINSKY, S. C.; R. A. DEMEL & L. L. M. VAN DEENEN: Further studies on the hemolytic action of filipin and derivatives. Biochim. Biophys. Acta 135: 835～843, 1967
3) KINSKY, S. C.: Nystatin binding by protoplasts and a particulate fraction of *Neurospora crassa*, and a basis for the selective toxicity of polyene antifungal antibiotics. Proc. Natl. Acad. Sci. U.S.A. 48: 1049～1056, 1962
4) KINSKY, S.C.: Antibiotic interaction with model membranes. Ann. Rev. Pharmacol. 10: 119～142, 1970
5) DEMEL, R. A.; L. L. M. VAN DEENEN & S. C. KINSKY: Penetration of lipid monolayers by polyene antibiotics. Correlation with selective toxicity and mode of action. J. Biol. Chem. 240: 2749～2753, 1965
6) DEMEL, R. A.: Studies on phospholipid mono- and bilayers. J. Am. Oil Chem. Soc. 45: 305～312, 1968
7) VAN ZUTPHEN, H.; L. L. M. VAN DEENEN & S. C. KINSKY: The action of polyene antibiotics on bilayer lipid membranes. Biochem. Biophys. Res. Commun. 22: 393～398, 1966

8) WEISSMANN, G. & G. SESSA : The action of polyene antibiotics on phospholipid-cholesterol structures. J. Biol. Chem. 242 : 616～625, 1967
9) GOTTLIEB, D.; H. E. CRATER, J. H. SLONEKER, L. C. WU & E. GAUDY : Mechanisms of inhibition of fungi by filipin. Phytopathology 51 : 321～330, 1961
10) WHITFIELD, G. B.; T. D. BROCK, A. AMMAN, D. GOTTLIEB & H. E. CARTER : Filipin, an antifungal antibiotic : Isolation and properties. J. Am. Chem. Soc. 77 : 4799～4801, 1955
11) LAMPEN, J. O.; P. M. ARNOW & R. S. SAFFERMAN : Mechanism of protection by sterols against polyene antibiotics. J. Bact. 80 : 200～206, 1960
12) CHILD, J. J.; G. DEFAGO & R. H. HASKINS : The effect of cholesterol and polyene antibiotics on the permeability of the protoplasmic membrane of *Pythium* PRL 2142. Canad. J. Microbiology 15 : 599～603, 1969
13) GOTTLIEB, D.; H. E. CARTER, L. C. WU & J. H. SLONEKER : Inhibition of fungi by filipin and its antagonism by sterols. Phytopathology 50 : 594～603, 1960
14) SWEELEY, C. C.; J. D. O'CONNOR & L. L. BIEBER : Effect of polyene macrolides on growth and reproduction of *Musca domestica* and on the uptake of cholesterol in *Galleria mellonella* larvae. Chem.-Biol. Interactions 2 : 247～253, 1970
15) SCHROEDER, F. & L. L. BIEBER : Effects of filipin and cholesterol on housefly, *Musca domestica* (L.), and wax moth, *Galleria mellonella* (L.). Chem.-Biol. Interactions 4 : 239～249, 1971
16) SCHAFFNER, C. P. & H.W. GORDON : The hypocholesterolemic activity of orally administered polyene macrolides. Proc. Natl. Acad. Sci. (U.S.) 61 : 36～41, 1968
17) GORDON, H. W. & C. P. SCHAFFNER : The effect of polyene macrolides on the prostate gland and canine prostatic hyperplasia. Proc. Natl. Acad. Sci. (U.S.) 60 : 1201～1208, 1968
18) FISHER, H.; P. GRIMINGER & C. P. SCHAFFNER : Effect of polyene macrolides on cholesterol metabolism of the chick. Proc. Exptl. Biol. & Med. 132 : 253～255, 1969
19) WONG, R.G.; T. H. ADAMS, P. A. ROBERTSON & A. W. NORMAN : Studies on the mechanism of action of calciferol. IV. Interaction of the polyene antibiotic, filipin, with intestinal mucosal membranes from vitamin D-treated and vitamin D-deficient chicks. Biochim. Biophys. Acta 219 : 61～72, 1970
20) ADAMS, T. H.; R. G. WONG & A. W. NORMAN : Studies on the mechanism of action of calciferol. II. Effects of the polyene antibiotic, filipin, on vitamin D-mediated calcium transport. J. Biol. Chem. 245 : 4432～4442, 1970
21) HOLLAND, J. F. & A. TIMNICK : Simultaneous absorbance and corrected fluorescence measurement by a versatile computerized spectrofluorimeter. Analytical Chemistry and Applied Spectroscopy, Pittsburgh Conference, Paper # 115, Cleveland, Ohio, 1971