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FURTHER STUDIES ON THE HEMOLYTIC ACTION OF FILIPIN AND DERIVATIVES

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SUMMARY

The interaction of filipin with model membrane systems, such as lipid monomolecular layers and bilayer films, has been described previously. To find additional criteria by which to evaluate various membrane models, several methods for producing derivatives of filipin, which have little or no biological activity, have been examined. Two methods have proved particularly useful: (a) reduction of the conjugated double bond sequence and (b) irradiation with visible light in the presence of FMN. Perhydrofilipin has approx. 1/100 the hemolytic activity of the parent antibiotic, whereas irradiated filipin has no biological activity (either hemolytic or antifungal). The effect of these derivatives on model membrane systems is described in the following papers.

We have extended our observations that also the antibiotic/erythrocyte ratio, and not only the absolute concentration of antibiotic, determines the rate and extent of hemolysis. This finding provides an additional criterion which must be met by an appropriate membrane model.

INTRODUCTION

The availability of agents (mainly, antibiotics) which interfere specifically with the biosynthesis or structure of macromolecules has played an important role in the elucidation of the structure of nucleic acids, proteins, and bacterial cell walls. For the past several years, we have been studying the mode of polyene antibiotic action on the assumption that these compounds might similarly prove useful tools for investigating the lipid architecture of plasma and cytoplasmic membranes. Considerable evidence now indicates that the polyene antibiotics cause permeability alterations in sensitive organisms resulting in the leakage of essential cytoplasmic components from the cell (reviewed in refs. 1–3). Only organisms (*e.g.* fungi, protozoa, erythrocytes) which contain sterols are affected by the polyenes. It has, in fact, been possible to convert some normally insensitive organisms which lack sterols (*Myco-*

plasma laidlawii, Pythium ultimum) to polyene sensitivity by cultivating them in media supplemented with cholesterol[4–6]. These observations have led to the contention that the selective toxicity of the polyene antibiotics is due to interaction with a unique component (sterol) found only in the cell membrane of sensitive organisms.

The exact mechanism by which the polyenes affect cell membrane structure, after combining with sterol, is not yet known. Our approach to this problem has been to study interaction of the polyenes with different membrane models such as lipid monomolecular layers and bilayer films[7,8]. Obviously, the most suitable membrane model is the one that can account for all the effects which these antibiotics have on natural cell membrane systems. For example, an appropriate membrane model must not only be consistent with the observation that only cells with sterols are apparently affected by the polyenes; it should also explain why (as indicated by numerous studies with yeast cells, fungal protoplasts, or mammalian erythrocytes) filipin causes the most extensive cell membrane damage with etruscomycin, amphotericin B, pimaricin, and nystatin following in that order[2,3].

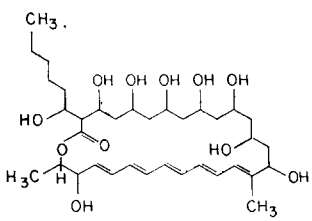


Fig. 1. Structure of filipin proposed by CEDER AND RYHAGE.

Filipin has been extensively investigated with both natural and model membrane systems for 2 reasons: (a) as noted above, it causes the greatest cell membrane damage of all the polyenes thus far examined and (b) it is one of the few polyenes whose structure has been determined (Fig. 1)[9]. To find some additional criteria by which to evaluate the various membrane models, we have investigated several methods for producing derivatives of filipin which have little or no biological activity. As demonstrated in this paper, 2 methods have proved particularly useful: (a) reduction of the conjugated double bond sequence and (b) irradiation with visible light in the presence of flavin nucleotides. The effect of these derivatives on various model membranes is described in subsequent papers[10–12].

We have also extended our earlier observations[13] that the extent of hemolysis induced by the polyenes is also dependent on the antibiotic/cell ratio and not only on the absolute concentration of the antibiotic. This finding provides an additional criterion which must be met by an appropriate membrane model.

MATERIALS AND METHODS

Chemicals

The filipin used in this investigation was generously provided by Dr. G. B.

WHITFIELD of The Upjohn Company, Kalamazoo, Mich.*. This preparation was approx. 95% pure on the basis of ultraviolet absorption and 80% pure as determined by bioassay. Perhydrofilipin was prepared essentially as described by WHITFIELD *et al.*[14]. The antibiotic was suspended in a mixture of dimethylformamide–methanol (1:3, v/v) with either palladium on charcoal or platinum oxide as catalyst. Incubation was carried out under a H_2 atmosphere at room temperature in the dark for periods as long as 18 h. H_2 uptake was usually complete, however, after 3 h at which time 5 moles per mole of filipin had been consumed. After removal of the catalyst by filtration or centrifugation, the reaction mixture was used directly or the perhydrofilipin was further purified by thin-layer chromatography on silica-gel plates with $CHCl_3$–CH_3OH–H_2O (65:35:4, v/v/v) as solvent. The majority of the experiments described below were performed with a crystalline sample of perhydrofilipin that had been kindly prepared for us by Dr. J. DUTCHER of the Squibb Institute for Medical Research, New Brunswick, N.J.

Saponified filipin was prepared by dissolving the antibiotic in a small volume of dimethylformamide (approx. 1 ml) followed by dilution with 15 ml of 0.1 M NaOH. After incubation at 70–80° for 3 h under N_2 in the dark, unreacted antibiotic was removed by filtration. The filtrate was acidified with 60 ml of 0.05 M H_2SO_4 and the resulting precipitate was washed successively with water and $CHCl_3$. The dried material was dissolved in dimethylformamide before use.

The preparation of irradiated filipin is described in the following section; illumination was carried out in the apparatus previously employed[15]. Absorbance changes and spectra were determined in cuvettes with a 1-cm light path.

Measurement of hemolysis

Rat or rabbit blood was defibrinated by shaking with glass beads and the erythrocytes extensively washed with isotonic saline (0.154 M NaCl), before the lysis experiments. Hemolysis was determined by either of 2 methods. In the first method, tubes containing the indicated amounts of antibiotic, isotonic saline, and erythrocytes were incubated at 37°. At various times, the tubes were centrifuged at 3000 × *g* for 10 min to remove any intact cells and the hemoglobin released was determined by measuring the absorbance of the supernatant solution at 550 mμ. The reaction was started by addition of the erythrocytes; an amount of erythrocytes was employed which, upon complete hemolysis in 1 ml of water, would produce an absorbance between 0.6 to 0.9. Control tubes containing the appropriate quantity of dimethylformamide were always included in each experiment. The percent hemolysis, relative to water, was calculated after correction for this control value.

The preceding method, however, could not be used conveniently to follow the kinetics of hemolysis because, under some conditions, the reaction was complete in less than 2 min. For this purpose, hemolysis was determined spectrophotometrically by measuring the increase in light transmission at 625 mμ at 20°. In these experiments absorbance (actually, light scatter) was not a linear function of the number of re-

* It must be emphasized that Filipin is a registered trade mark of the Upjohn Company, Kalamazoo, Mich. The generic name for this antibiotic is filimarisin. Because filipin has been employed almost exclusively in the extensive literature on this antibiotic, we prefer to continue use of this name in order to avoid the unnecessary confusion which would result if the generic name were substituted. The trade names for nystatin and amphotericin B (Squibb and Sons, New York, N.Y.) are Mycostatin and Fungizone, respectively.

maining intact cells and a calibration curve, prepared by mixing appropriate amounts of non-lysed with lysed cells, was used to calculate the percent hemolysis from the transmission data. Additional experimental details are provided below.

For the determination of an approximate value of the molar ratio, filipin added to membrane lipid, a lipid content of 5.15 mg/ml of packed erythrocytes was assumed (see also PRANKERD[17]). For this calculation a molecular weight of 654 was used for the antibiotic and an average value of 580 for the lipid based on the lipid composition of rabbit erythrocytes[18].

Effect of filipin and derivatives on growth of Neurospora

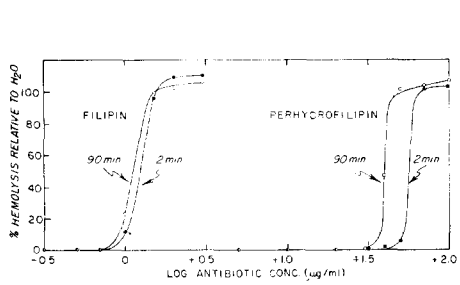
This was determined by methods previously described[16].

RESULTS AND DISCUSSION

Perhydrofilipin

Fig. 2 compares the ability of filipin and perhydrofilipin to lyse rat erythrocytes. Reduction of the conjugated double bond sequence produces a derivative which is far less potent than the parent antibiotic. Thus, with the amount of erythrocytes employed in this experiment, the critical threshold concentration (*i.e.* the lowest concentration of antibiotic necessary to induce hemolysis) was approx. 0.7 μg/ml for filipin and 70 μg/ml for perhydrofilipin. It is important to note that the critical threshold concentration does not change appreciably by increasing the incubation time. This phenomenon is in accord with the view that a definite ratio of antibiotic per cell must be attained before hemolysis can occur (see below).

The above experiment was performed with a sample of perhydrofilipin purified by recrystallization from dioxane (J. DUTCHER, personal communication). The spectrum of this material and filipin in methanol is shown in Fig. 3. There was no evidence for the presence of the conjugated chromophore (peaks at 320, 337, and 355 mμ) which is characteristic of filipin. It should be emphasized that these spectra were obtained with solutions containing perhydrofilipin at a concentration approx.



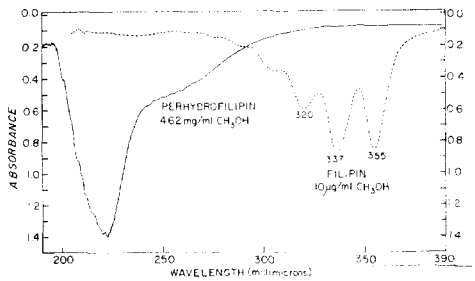


Fig. 2. Comparative potencies of filipin and perhydrofilipin. Tubes contained 0.9 ml of isotonic saline and the indicated amounts of antibiotic or derivative dissolved in 0.05 ml of dimethylformamide. The reaction was started by addition of 0.05 ml of a rat erythrocyte suspension. One series of tubes was incubated for 2 min, the other for 90 min, before removal of non-lysed cells by centrifugation. In this experiment, complete hemolysis of the erythrocytes in 1.0 ml of water gave a supernatant solution with an absorbance (550 mμ) of 0.770, whereas the corresponding value in control tubes, containing dimethylformamide (no antibiotic), was 0.030.

Fig. 3. Spectra of filipin and perhydrofilipin preparation used in experiment described in Fig. 2.

450 times greater than the concentration of filipin. We therefore conclude that the hemolysis observed with the reduced derivative is a property of this compound *per se* and not due to contamination by traces of unreacted filipin.

Saponified filipin

Preliminary experiments with the alkali-treated filipin also indicated that this material was hemolytic under the conditions of the experiment shown in Fig. 2. This material was, like perhydrofilipin, far less potent than filipin; the critical threshold concentration was approx. 100 μg/ml. Saponified filipin has essentially the same spectrum as the parent antibiotic. Attempts to detect and, if present, remove any unreacted filipin from the saponified material by thin-layer chromatography were unsuccessful. Only 0.7% contamination of the saponified preparation by filipin would, however, account for the observed hemolysis. This amount could not have been detected by the techniques which were employed. Therefore, the possibility that the hemolytic activity of the saponified filipin was due to contamination by the parent antibiotic cannot be excluded and further experiments with this material were not attempted.

Irradiated filipin

ZONDAG, POSTHUMA, AND BERENDS[19] have reported that illumination of the polyene antibiotic, pimaricin, with visible light in the presence of flavins results in destruction of the conjugated chromophore. This phenomenon has been used as the basis for an assay of nystatin in the presence of compounds which would otherwise interfere with the spectrophotometric determination of this antibiotic[15]. The following experiments demonstrate that filipin exhibits a similar behavior and that, as might be anticipated, treatment of a polyene antibiotic in this manner abolishes biological activity completely.

In aqueous media the absorption peaks for filipin are shifted towards slightly longer wavelengths: 357, 339, and 322 mμ (*cf.* Fig. 3). Fig. 4 (Curve A) shows that illumination in the presence of FMN causes a rapid decrease in absorbance at 339 mμ; although not indicated a similar absorbance decline was also observed at 357 and 322 mμ. This decrease was appreciably slower when the flavin nucleotide was omitted (Curve B) and did not occur at all in the dark even in the presence of FMN (Curve C).

Illumination of filipin in organic solvents had much less effect, in agreement with the original observations of ZONDAG, POSTHUMA AND BERENDS[19]. Thus, as shown by Curve D, the absorbance of filipin in methanol declined by only 16% of the initial value after 20-min illumination whereas, under identical conditions, the absorbance of filipin in phosphate buffer had decreased by 80% (Curve A). It should be noted that the absorbance of filipin in organic solvents, such as methanol or dimethylformamide, is significantly greater than the absorbance of the antibiotic in water (*cf.* Curves A and D, 'zero time'). This observation has some experimental significance as discussed below. LAMPEN *et al.*[20] first described a similar phenomenon with nystatin and have also reported that this antibiotic is only slowly dialyzable, if at all. On the basis of these observations, they have suggested that the polyenes may be present to some extent as micelles in aqueous media. This conclusion is also supported by the observation of LAMPEN, ARNOW AND SAFFERMAN[21] that filipin is sedimentable at low speeds in the centrifuge. This latter finding, which we have

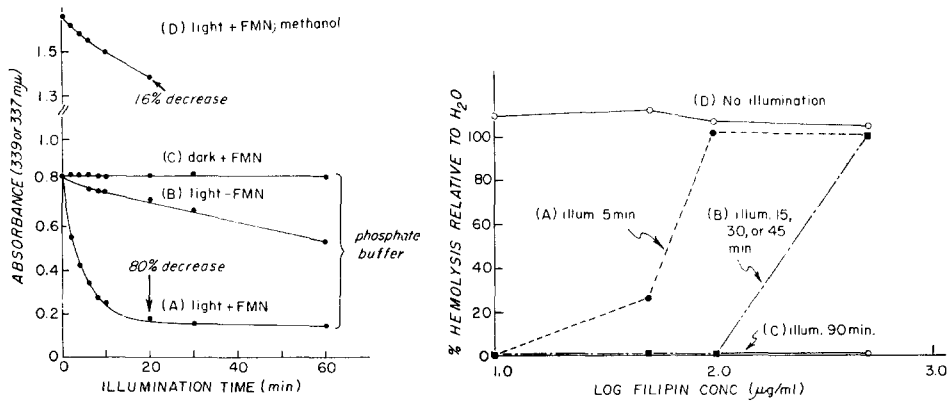


Fig. 4. Requirements for photoinactivation of filipin. Cuvettes contained the following: 19.6 μg of filipin and 0.01 μmole of FMN per ml of 0.05 M potassium phosphate (pH 6.9; A, C); 19.6 μg of filipin per ml of phosphate buffer (B); 19.3 μg of filipin and 0.01 μmole of FMN per ml of methanol (D). A, B, and D were illuminated as described previously; C was kept dark. At the times indicated, the absorbance was measured at 339 mμ (A, B, C) or 337 mμ (D).

Fig. 5. Effect of illumination on hemolytic activity of filipin. Tubes contained initially the indicated amounts of filipin (10, 50, 100 or 500 μg) and 0.1 μmole of FMN in 0.95 ml of isotonic saline. After illumination for the times shown, 0.05 ml of a rat erythrocyte suspension was added. Following incubation for 90 min at 37°, intact cells were removed by centrifugation. In Curve D, filipin was added after illumination and hemolysis was determined after 2 min incubation with the erythrocytes. In this experiment, complete hemolysis of the erythrocytes in 1.0 ml of water gave a supernatant solution with an absorbance (550 mμ) of 0.864, whereas the corresponding value in control tubes, containing dimethylformamide (no antibiotic) was 0.066.

been able to confirm, has precluded the use of centrifugation (approx. at 10 000 $\times$ g for 10 min) as an experimental method by which to measure the amount of filipin which is bound to the erythrocyte membrane.

Fig. 5 shows that irradiation of filipin in the presence of FMN results in complete destruction of hemolytic activity. Thus, under the conditions employed, 5 min illumination destroys 10 μg filipin (Curve A), 15 min inactivates 100 μg (Curve B), whereas 90 min is required for the inactivation of 500 μg of the antibiotic (Curve C). It should be emphasized that these values represent the time necessary to reduce the amount of active antibiotic below the minimum threshold level. With the amount of erythrocytes used in this experiment, filipin induces hemolysis at a concentration of approx. 1 μg/ml (Fig. 2). Thus the possibility that 499 μg of the antibiotic may have been inactivated in less than 90 min would not have been detected in the above experiment. Filipin, at concentrations of approx. 50 μg/ml water or greater, is present as a visible suspension. This may account for the fact that relatively long periods of irradiation are required for inactivation. It would probably be better to carry out the reaction under conditions in which the antibiotic is completely soluble (see above) but organic solvents cannot be employed since they inhibit (Fig. 4). Unfortunately, the structure of the irradiated product(s) is not known. POSTHUMA AND BERENDS[22] have suggested, on the basis of experiments with model compounds, that a *trans–cis* rearrangement may be involved. We have observed that the antibiotic becomes more water soluble as illumination proceeds. Nevertheless, the fact that irradiation results in complete loss of hemolytic activity provides an important tool.

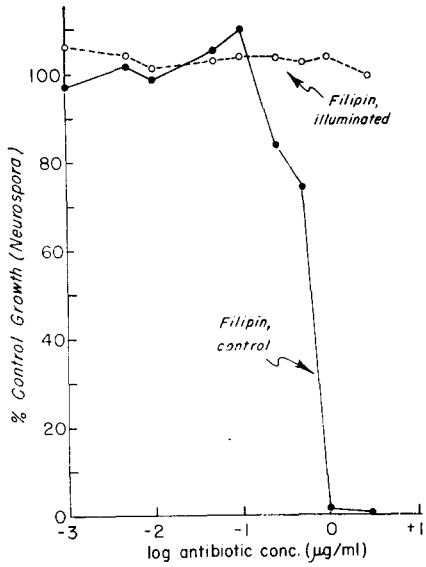


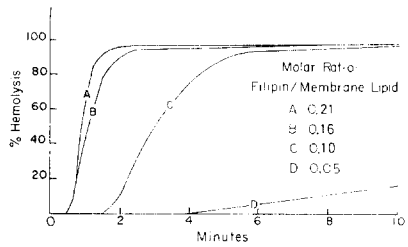
Fig. 6. Effect of illumination on antifungal activity of filipin. An aqueous suspension, containing 125 μg/ml of filipin and 0.05 μmole/ml of FMN, was illuminated for 90 min. Appropriate aliquots were then added to 125-ml erlenmeyer flasks, containing 25 ml of Fries minimal medium, to give the final antibiotic concentrations indicated on the abscissa. Flasks were inoculated with approx. 0.2 ml of a dense spore suspension of *Neurospora crassa*, strain 5297a. After 44 h growth at 30° in the dark, mycelial mats were harvested and dried over P_2O_5. Results are expressed as percent of control growth (dry wt.) obtained in flasks containing no antibiotic.

Irradiation of filipin not only destroys hemolytic activity but also abolishes the ability of the antibiotic to inhibit growth of Neurospora. As shown in Fig. 6, 1 μg/ml of filipin inhibits growth completely; under identical conditions, no effect is obtained with 10 μg/ml of the irradiated derivative. In other experiments, even 50 μg/ml of the irradiated derivative did not inhibit growth. These observations are significant because they illustrate again that antifungal and hemolytic activities are properties of the same molecule.

Effect of filipin and erythrocyte concentration

In an earlier investigation, it was demonstrated with amphotericin B, pimaricin and nystatin that both the rate and extent of hemolysis was dependent on antibiotic/cell ratio[13]. These antibiotics, however, are relatively weak compared to filipin thus necessitating the use of higher amounts and longer incubation times to achieve the same degree of hemolysis. It was therefore of some interest to determine whether a similar phenomenon occurs with filipin. Fig. 7 indicates that the rate of hemolysis was markedly influenced by the initial antibiotic concentration. There was a pronounced lag phase before the onset of hemolysis with low levels of filipin. Similar results with amphotericin B suggest that binding and/or penetration of the polyene antibiotics to the erythrocyte membrane may be a rate-limiting step in hemolysis[13].

In the preceding experiment, a constant number of erythrocytes was employed and the amount of filipin was varied. Results of the converse experiment, using a constant amount of antibiotic and varying amounts of erythrocytes, are shown in



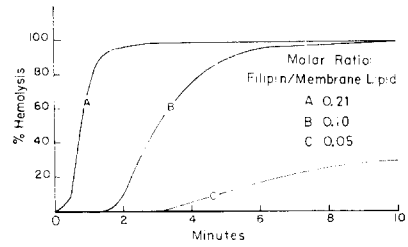


Fig. 7. Effect of filipin concentration on hemolysis of rabbit erythrocytes. Hemolysis was followed spectrophotometrically. Cuvettes contained initially the following concentrations of filipin in 5 ml of isotonic saline: 6.2 μM (A); 4.6 μM (B); 3.1 μM (C); 1.5 μM (D). The reaction was initiated by addition of 50 μl of stock erythrocyte suspension (33%, by vol. as determined by hematocrit measurements). The molar ratios of filipin to membrane lipid were calculated as described in the text.

Fig. 8. Effect of rabbit erythrocyte concentration on hemolysis by filipin. Hemolysis was folowed spectrophotometrically. Cuvettes contained initially 5 ml of isotonic saline and 3.1 μM filipin. The reaction was initiated by addition of the following amounts of stock erythrocyte suspension (33%, by vol. as determined by hematocrit measurements); 25 μl (A); 50 μl (B); 100 μl (C). The molar ratios of filipin to membrane lipid were calculated as described in the text.

Fig. 8. Hemolysis was least extensive, and occurred at the slowest rate, when the number of erythrocytes was relatively high. These observations demonstrate that with filipin, as with the other polyenes, the antibiotic/cell ratio and not only the absolute concentration of antibiotic is a critical factor.

In Figs. 7 and 8, we have also indicated the molar ratios of filipin to erythrocyte lipid. Thus, complete hemolysis was obtained when the number of filipin molecules added per molecule of lipid in the erythrocyte membrane was approx. 0.10–0.21, at least under the conditions used. These values are significant because experiments to be described in a subsequent paper[11] have shown that at these molar ratios, filipin preferentially interacts with lipid monolayers containing cholesterol.

NOTE ADDED IN PROOF (Received September 25th, 1967)

We have been informed by Drs. M. E. BERGY and T. E. EBLE (The Upjohn Company, Kalamazoo, Mich.) that they have recently been able to separate crystalline filipin, which has up to now been regarded as a single chemical entity, into 3 major pentaene components. These components are closely related chemically as indicated by elemental analysis, ultraviolet, infrared, and NMR spectra. Although these components show some variation in biological potency, it would indeed be surprising if they did not share the same basis for selective toxicity (*i.e.* interaction with membrane sterols) since they all induce erythrocyte hemolysis, are all growth inhibitory to a variety of fungi, and, like the original filipin complex, have no antibacterial activity.

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