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INTERACTION OF FILIPIN AND DERIVATIVES WITH ERYTHROCYTE MEMBRANES AND LIPID DISPERSIONS: ELECTRON MICROSCOPIC OBSERVATIONS

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SUMMARY

The effect of the polyene antibiotic, filipin, on the morphology of erythrocyte membranes and lipid dispersions has been investigated with the electron microscope using negative staining (phosphotungstate). Filipin induces pit formation in rat and human erythrocytes; similar pits are also produced by the antibiotic in lecithin–cholesterol dispersions. Pit formation requires the presence of cholesterol. Evidence is presented which suggests that the light ring surrounding each pit may consist predominately, if not exclusively, of sterol which had interacted with the antibiotic. Pits are not produced in lecithin–cholesterol dispersions by derivatives of the antibiotic which have little (perhydrofilipin) or no (irradiated filipin) hemolytic activity. Thus, the characteristic lamellar pattern of the dispersions is retained in the presence of the irradiated derivative, whereas perhydrofilipin has an effect which is apparently similar to that produced by lysolecithin. The pits produced by filipin resemble those produced in human erythrocyte membranes by immune lysis in the presence of complement but are quite different from those produced by saponin. The relevance of these observations to current concepts regarding the mode of filipin action is discussed. It is suggested that filipin may interfere with the ability of cholesterol to stabilize the bilayer configuration of phospholipids. Experiments which suggest that the sterol can function as a stabilizer are described.

INTRODUCTION

The possibility that sterols may function in membranes to stabilize a bilayer configuration of lipids has been the subject of much speculation. In support of this hypothesis, previous studies with lipid monolayers have demonstrated that the

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presence of cholesterol can reduce the mean molecular area of certain lecithins, perhaps due in part to a tighter packing of phospholipid in the monolayer (see *e.g.* ref. 1).

The evidence that polyene antibiotics can interact with sterols localized in the cell membrane of sensitive organisms, and thus produce permeability alterations, has been recently reviewed[2-4]. These observations raise the possibility that the bilayer configuration may no longer be the preferred arrangement of lipids in the presence of the antibiotics.

In this paper, we present some evidence which appears to support both of the above hypotheses. We have studied the effect of the polyene antibiotic, filipin, on the morphology of erythrocyte membranes and lipid dispersions by electron microscopy with phosphotungstate as negative stain. Filipin was investigated in detail for the reasons cited in the introduction to the preceding paper[5]. A preliminary account of this work has been presented[2].

MATERIALS AND METHODS

Chemicals

Filipin was generously supplied by Dr. G. WHITFIELD of the Upjohn Company, Kalamazoo, Mich.*. The preparation of perhydrofilipin and irradiated filipin has been described previously[5]. Saponin and cholesterol were obtained from the Sigma Chemical Company, St. Louis, Mo. Egg lecithin was purchased from the Pierce Chemical. Co., Rockford, Ill., and dicetylphosphate from K and K Laboratories, Plainview, N.J. Lipids were dissolved in chloroform and stored under N_2 at $-17°$; they were routinely checked for purity by chromatography on silicic acid-impregnated paper as described by MARINETTI[6]. The concentration of lecithin was determined by organic phosphate analysis according to the procedure of GERLACH AND DEUTICKE[7]; these values were converted to dry weights by assuming a molecular weight for the lecithin of 770.

Treatment of lipid dispersions with filipin

The effect of the antibiotic on lipid dispersions was studied by 2 methods. In Procedure A, a $CHCl_3$ solution (containing 500 μg of lecithin and 250 μg of cholesterol) was added to 10 mm $\times$ 70 mm test tubes and the solvent was removed under a stream of N_2 followed by evacuation. Isotonic saline (0.1 ml of 0.154 M NaCl) was added to the dried lipid residue which was then dislodged from the walls of the test tube with the aid of a glass stirring rod. After further dilution with either 0.1 ml H_2O or 0.1 ml of the salt solution, the lipids were agitated with a Vortex mixer to give a fine milky suspension. The lipid dispersion was then incubated for 20 min at room temperature with 100 μg of filipin (10 μl of a stock solution containing 10 mg antibiotic per ml of dimethylformamide). An equal volume (0.2 ml) of 2% potassium phosphotungstate (pH 5.1–5.4) was added and a drop of the resulting mixture

* It must be emphasized that Filipin is a registered trademark of The Upjohn Company, Kalamazoo, Mich. The generic name for this antibiotic is filimarisin. Since filipin has been almost exclusively employed in the extensive literature on this compound, we prefer to continue use of this name to avoid the unnecessary confusion which would result if the generic name were substituted.

placed on a grid. Excess liquid was removed with lens paper. Usually, 5 grids were prepared from each tube.

In the above procedure, the lipid dispersion contained equimolar quantities of egg lecithin and cholesterol (0.625 μmole). This standard assay was modified in some experiments but, in all cases, the final volume of lipid dispersion and filipin was 0.2 ml before addition of phosphotungstate. Thus, in experiments to determine the effect of cholesterol, the amount of phospholipid was kept constant (*i.e.*, 500 μg) and varying quantities of sterol were added to make dispersions with the desired lecithin/cholesterol ratio. When the amount of filipin was varied, appropriate amounts of dimethylformamide were added to control tubes. In some experiments, the pH of the phosphotungstic acid was adjusted to 7.1 with KOH but this had no apparent effect.

In Procedure B, egg lecithin and/or cholesterol in the desired molar ratio were dissolved in 0.2 ml of a CH_3OH–$CHCl_3$ solution (2:1, v/v). 10 μl of antibiotic solution (see above) were added followed by 54 μl of 2% potassium phosphotungstate (pH 5.1–5.4). Under these conditions, the solvents form a single phase in which the antibiotic and lipids are completely soluble and the negative stain is initially present as a slightly opalescent suspension. Immediately after addition of phosphotungstate a drop of the mixture was placed on several grids and rapidly drained of excess liquid as described for Procedure A. The organic solvents will dissolve the collodion support but, if sufficient carbon is present, enough material is retained on the grids.

Preparation and treatment of erythrocytes with filipin

Fresh human and rat blood was obtained by venapuncture and decapitation, respectively. Approx. 4 ml of whole blood were diluted with 80 ml of Alsever's solution and the erythrocytes isolated by centrifugation for 10 min at 4° at 3000 × *g*. The erythrocytes were extensively washed with 0.154 M NaCl (total vol. approx. 100 ml) by alternate resuspension and centrifugation as above. The washed packed erythrocytes (0.3–0.5 ml) were lysed by the addition of 5–10 vol. of isotonic saline containing the amounts of filipin indicated below. Control erythrocytes were hemolyzed by the addition of 10–20 vol. of distilled water containing the appropriate amounts of dimethylformamide. Following incubation at room temperature for 20 min with occasional mixing by inversion, the erythrocyte ghosts were isolated by centrifugation for 20 min at 30 000 × *g*. The ghosts were washed once with 10 ml of distilled water or, in some experiments, 0.01 M potassium phosphate buffer (pH 7.1). The membranes obtained by filipin lysis were resuspended in 1–2 ml of distilled water; because the yield of membranes obtained by hypotonic dilution was consistently higher, these were resuspended in a larger volume of water (3–5 ml). To the membrane suspension, an equal volume of 2% potassium phosphotungstate (pH 5.1–5.4) was added. This mixture was sprayed onto grids with an ordinary nebulizer.

In studies of the type described, the membranes were usually prepared by lysis with a total of 500–1500 μg of filipin. This amount of antibiotic was chosen on the basis of the following preliminary experiments. First, the minimum quantity of filipin necessary to cause rapid complete hemolysis of a dilute suspension of erythrocytes was determined. An amount of erythrocytes was chosen which, upon complete hemolysis in a final volume of 1 ml, would produce a supernatant solution with an absorbance of approx. 0.6 at 550 mμ. In the case of both rat and human red

blood cells, 100% hemolysis was obtained with 2.5 μg/ml of the antibiotic (*cf.* ref.[5]). Next, a packed erythrocyte sample (0.3–0.5 ml), similar to the one to be used for isolation of the membranes, was lysed with an excess of water (20 ml) and the absorbance of the supernatant was measured. From these data, the 'theoretical' absorbance, which should result if the packed erythrocytes could be lysed in a final volume of 1 ml, was estimated. For example, in a representative experiment, this value was 110. Since the experiments described in the preceding paper[5] have shown that it is the antibiotic/cell ratio which is the critical factor in determining the extent of hemolysis, the amount of filipin required to lyse the packed erythrocytes was calculated to be approx. 460 μg (*i.e.*, 2.5 × 110/0.6).

Electron microscopy

The grids used were slotted and had been previously coated with collodion and stabilized with carbon. The grids were examined on the day of preparation in either an RCA EMU-3F electron microscope, equipped with a double condenser lens, or an RCA EMU-3H. Both electron microscopes were equipped with high magnification accessories. Instrumental magnifications were 20 000–50 000 fold with photographic enlargement as desired.

Preparation and assay of liposomes

Liposomes were prepared by minor modification of the procedure described by WEISSMANN, SESSA AND WEISMANN[8]. A $CHCl_3$ solution, containing egg lecithin, cholesterol, and dicetylphosphate in the desired molar ratio, was taken to dryness on a rotary evaporator. For every 10 μmoles of lecithin present in the lipid residue, 1 ml of swelling solution (either 0.154 M KH_2PO_4 or 0.3 M glucose) was added. The lipids were dispersed by agitation with a Vortex mixer under N_2 and kept at room temperature for 2–5 h before being dialyzed to reduce the level of untrapped marker. Approx. 6 ml of the liposome preparation were dialyzed overnight against 5 l of a solution containing KCl and NaCl each at a final concn. of 0.075 M.

The experiment described in Fig. 13 was performed in the following manner. A series of tubes containing 0.32 ml of the appropriate liposomes and 0.68 ml of isotonic saline were incubated at various temperatures for 30 min. An aliquot (0.7 ml) of the mixture was removed and dialyzed at room temperature against 15 ml of the KCl/NaCl salt solution. Equilibrium was obtained in 1 h at which time the dialysate was assayed for either inorganic phosphate[7] or glucose[9]. We are indebted to Dr. J. V. PASSONNEAU for her generous assistance with the fluorimetric determination of glucose.

RESULTS

Effect of filipin on mammalian erythrocytes

Fig. 1 is a micrograph of a rat erythrocyte membrane preparation obtained by lysis of cells with filipin; for comparison, a picture of a membrane preparation obtained by lysis with water is shown in Fig. 2. It is apparent that the antibiotic causes pit formation in the cell membrane, *i.e.*, a dark area, where the phosphotungstate has accumulated, surrounded by a light band. Although some asymmetry is apparent, the pits are nearly circular; the diameter is, however, somewhat variable (80–

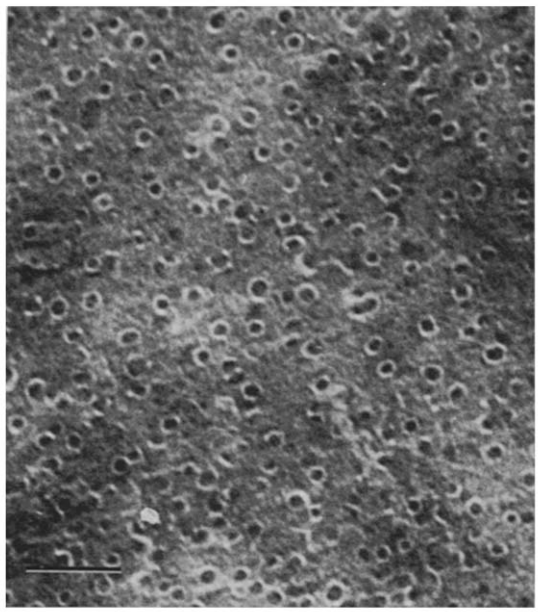

Fig. 1. Rat erythrocyte membrane preparation obtained by lysis of cells with filipin. See text for experimental details. Marker indicates 1000 Å.

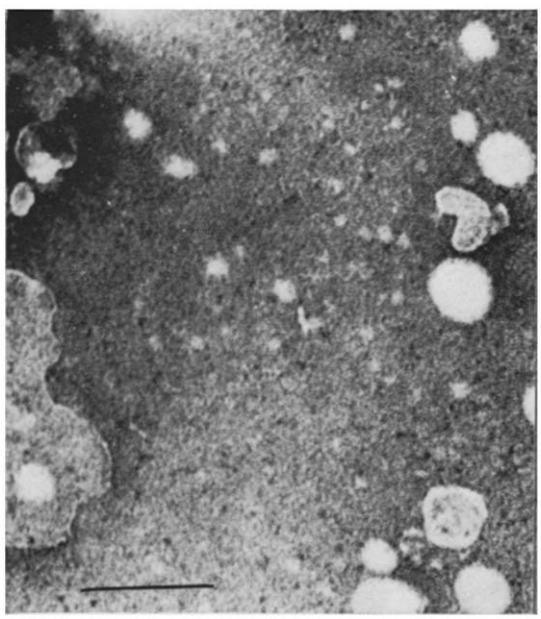

Fig. 2. Rat erythrocyte membrane preparation obtained by lysis of cells with water. See text for experimental details. Marker indicates 1000 Å.

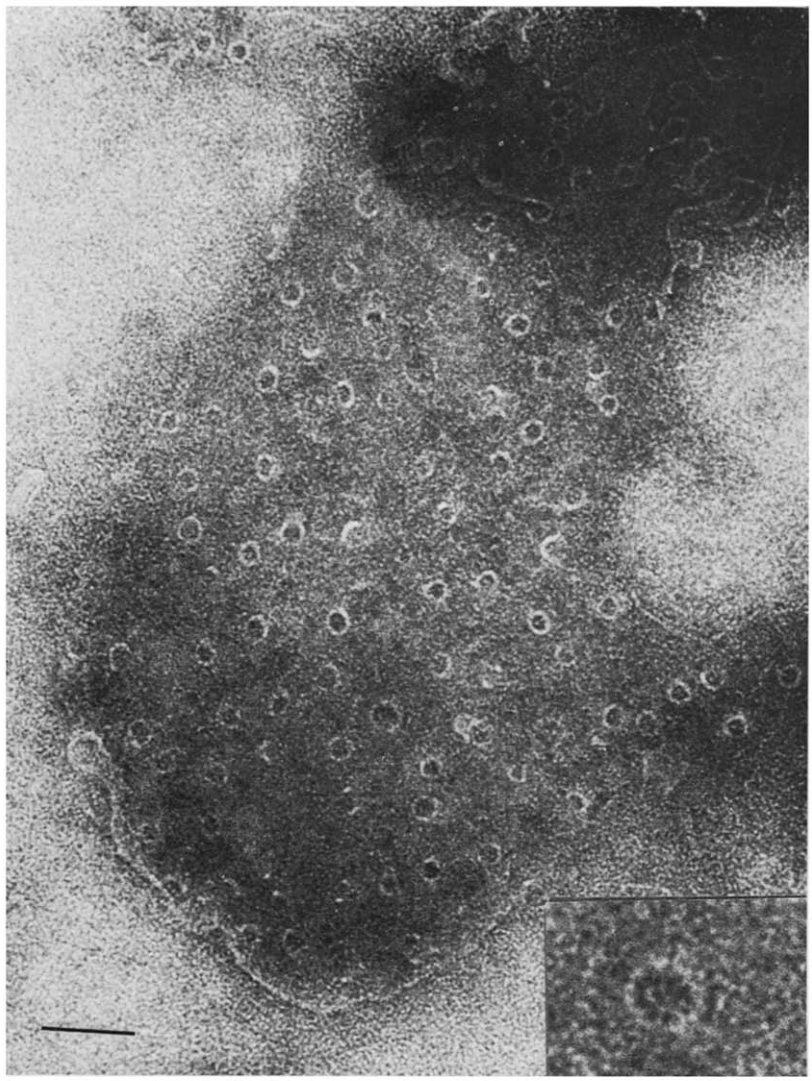

Fig. 3. Human erythrocyte membrane preparation obtained by lysis of cells with filipin. See text for experimental details. Marker indicates 1000 Å. Inset shows a single pit enlarged 270 000 fold.

120 Å). Filipin also induces pit formation in human erythrocytes (Fig. 3). We have generally observed that the pits in the latter are significantly larger (200 – 250 Å in diameter) than those found in rat erythrocyte ghosts.

Effect of filipin on lipid dispersions

Pits, similar to those that filipin induces in erythrocytes, were readily demonstrable in dispersions prepared from lecithin and cholesterol (1:1 molar ratio) which had been treated with the antibiotic (Fig. 4). No pits were found after incubation

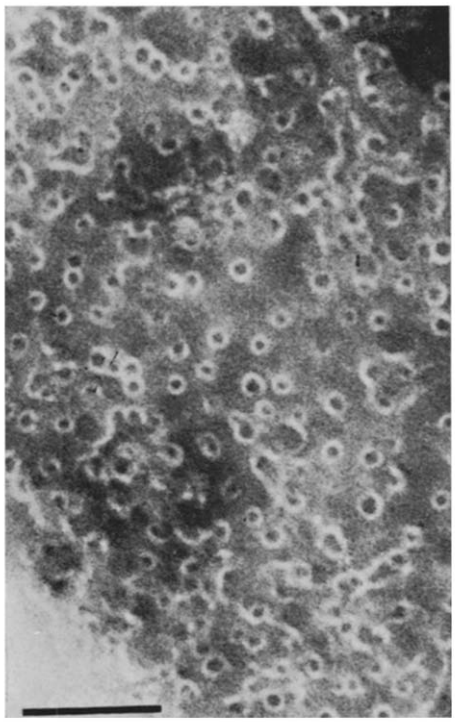

Fig. 4. Lecithin–cholesterol dispersion (1:1 molar ratio) treated with 100 μg of filipin (Procedure A). Marker indicates 1000 Å.

with dimethylformamide, the solvent for filipin (Fig. 5). It should be noted that, with both erythrocytes and the dispersions, the pits produced by filipin were distributed 'randomly' and appeared isolated from each other; this aspect is discussed in greater detail below.

Effect of cholesterol and filipin concentration

The experiments described above were performed with lipid dispersions containing equimolar quantities of lecithin and sterol because analysis in several laboratories has shown that rat and human erythrocyte membranes are composed of nearly identical amounts of phospholipid and cholesterol[10]. Dispersions with lecithin: cholesterol ratios of 1.25, 2.5, and 5.0 also gave pits when treated with filipin. We did not obtain any clear indication that the size or distribution of the pits was influenced by the phospholipid:sterol ratio in this range. The least amount of filipin which gave evidence of pits in the standard assay (Procedure A) was 25 μg; this minimum threshold level was also not affected by the lipid ratio. At this low level of filipin, the number of pits was markedly reduced; however, between 50 and 300 μg, there was no consistent and reproducible effect of antibiotic concentration on the number, size, and distribution of pits.

When cholesterol was omitted from the lipid dispersion, pits were not observed with the highest level of antibiotic tested (300 μg) and the characteristic lamellar

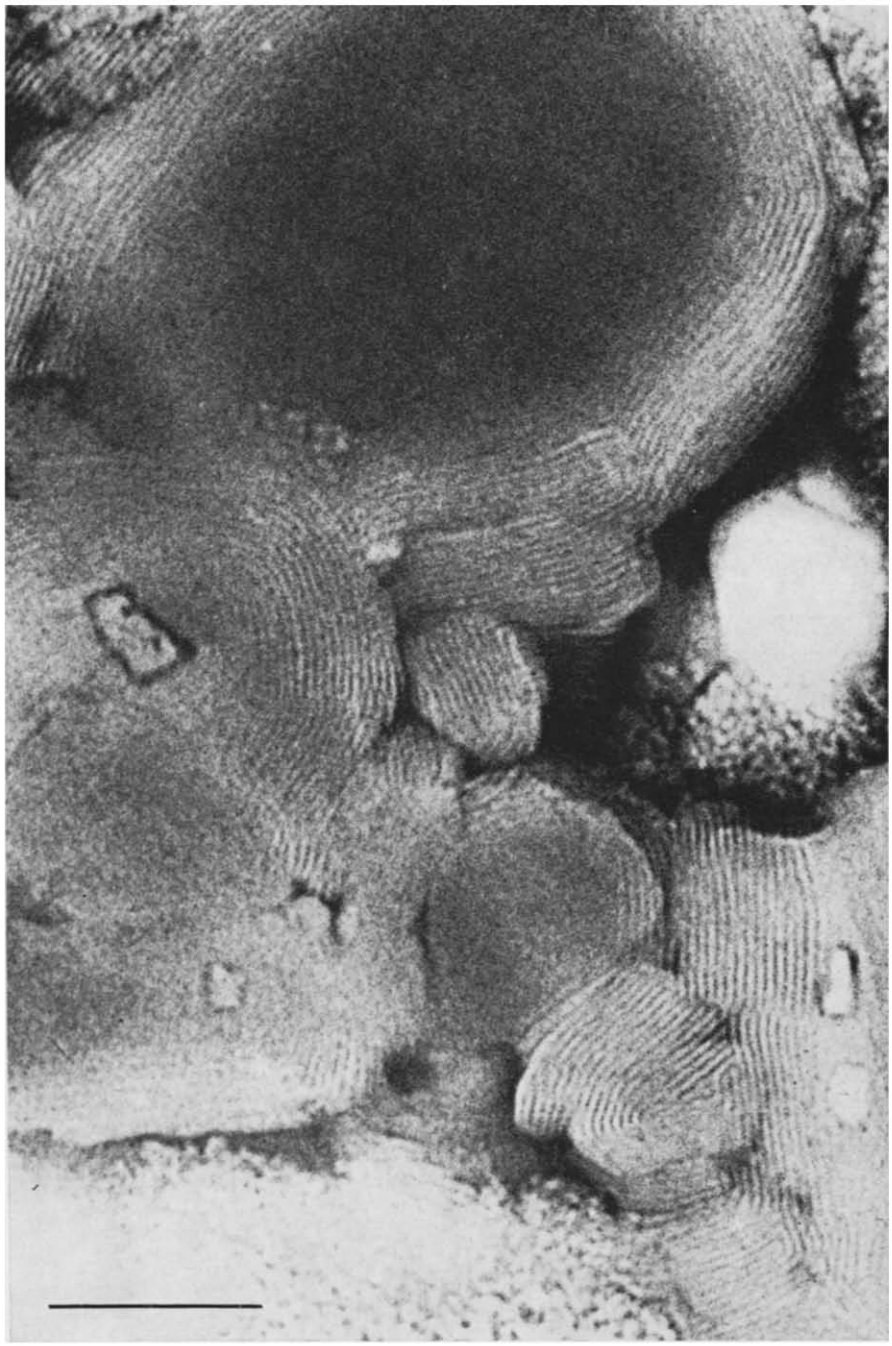

Fig. 5. Lecithin–cholesterol dispersion (1:1 molar ratio) treated with 100 μg of irradiated filipin (Procedure A). See preceding paper[5] for preparation of the filipin derivative. Similar pictures, showing retention of the characteristic lamellar pattern, are obtained when lecithin–cholesterol dispersions are treated with dimethylformamide or when lecithin dispersions (no cholesterol) are treated with filipin. Marker indicates 1000 Å.

pattern of lecithin was retained (Fig. 5). Thus, pit formation shows an absolute requirement for the presence of sterol.

Effect of filipin derivatives

In the preceding paper, it was shown that irradiated filipin (prepared by illumination of the antibiotic with visible light in the presence of FMN) does not possess either hemolytic or antifungal activity[5]. This derivative has no effect on the morphology of lecithin–cholesterol dispersions (Fig. 5).

Perhydrofilipin (prepared by catalytic hydrogenation of the antibiotic), unlike irradiated filipin, has some hemolytic activity although it is approx. 100 times less potent than the parent compound[5]. As indicated in Fig. 6, perhydrofilipin has a visible effect on lecithin–cholesterol dispersions. However, it should be emphasized that the picture obtained with perhydrofilipin is clearly different from that produced by the antibiotic; thus far, we have only observed pit formation with filipin. This aspect is discussed in greater detail below.

Observations bearing on the possible mechanism of filipin action

Fig. 7 is another micrograph of a lecithin–cholesterol dispersion treated with

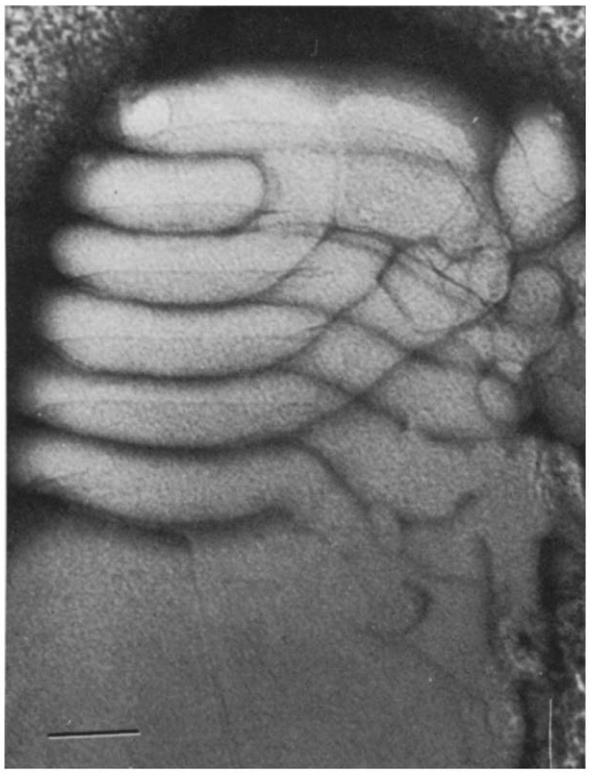

Fig. 6. Lecithin–cholesterol dispersion (1:1 molar ratio) treated with 100 μg of perhydrofilipin (Procedure A). See preceding paper[5] for preparation of the filipin derivative. Marker indicates 1000 Å.

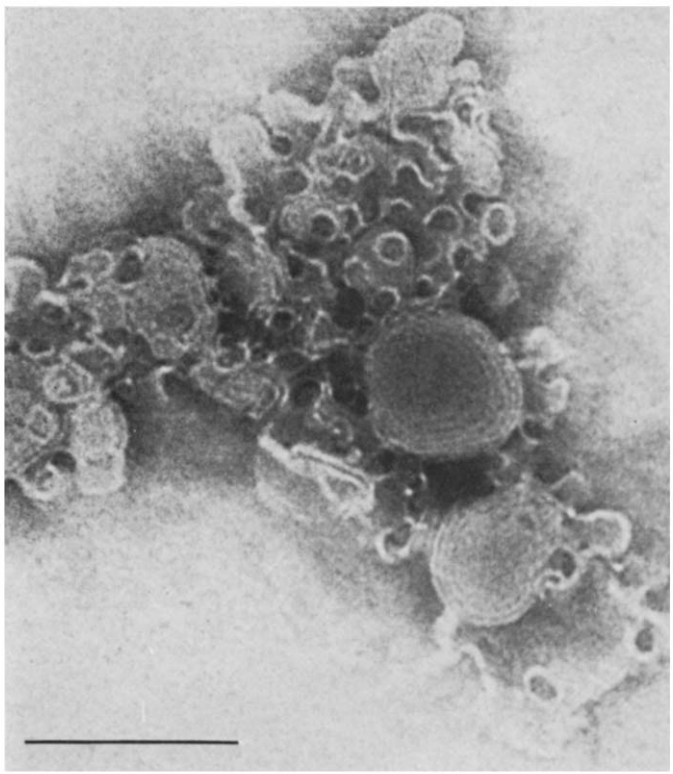

Fig. 7. Lecithin–cholesterol dispersion (1:1 molar ratio) treated with 100 μg of filipin. Procedure A was modified by adding the phosphotungstate after 2-min incubation with the antibiotic. Marker indicates 1000 Å.

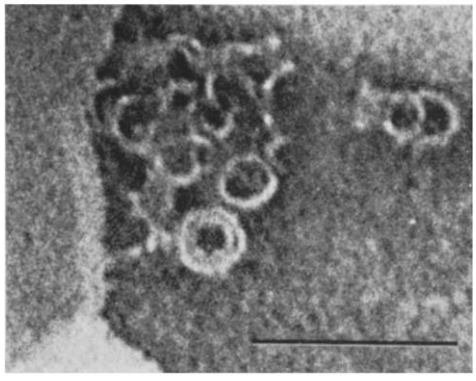

Fig. 8. Lecithin–cholesterol dispersion (5:1 molar ratio) treated with 100 μg of filipin (Procedure A) showing 'substructure' of the light rings. Similar results were also obtained using equimolar lecithin–cholesterol dispersions. Marker indicates 1000 Å.

filipin. In this particular experiment, Procedure A was modified and the lipid dispersion was incubated with the antibiotic for approx. 2 min (instead of 20 min) before addition of phosphotungstate. This picture illustrates the disruption of the characteristic lipid lamellar pattern by filipin with the subsequent production of pits. These observations are consistent with the hypothesis that the antibiotic acts by inducing a lamellar to micellar phase transition[2,11,12]. The existence of micelles is suggested by the finding that at high resolution, the light rings surrounding each pit appear to contain discrete substructures (Fig. 8). It is perhaps worth noting that the pit shown in Fig. 8 is bounded by two light rings.

Chemical composition of the light rings

All of the experiments described above were carried out by Procedure A. This method, however, has disadvantages when used to determine whether filipin has a visible effect on lipids, such as cholesterol, which are extremely difficult to disperse in an aqueous environment. Procedure B was employed for this purpose. The rationale behind this method is that, as the organic solvents evaporate first, a transient monolayer of lipid forms on the grid surface with which the antibiotic could possibly interact. Using the latter method, we were not able to demonstrate any effect of filipin when lecithin alone was present, thus confirming the observations made with Procedure A.

The antibiotic did, however, have a visible effect on cholesterol in the absence of lecithin as demonstrated by the following observations. Fig. 9 is a picture of

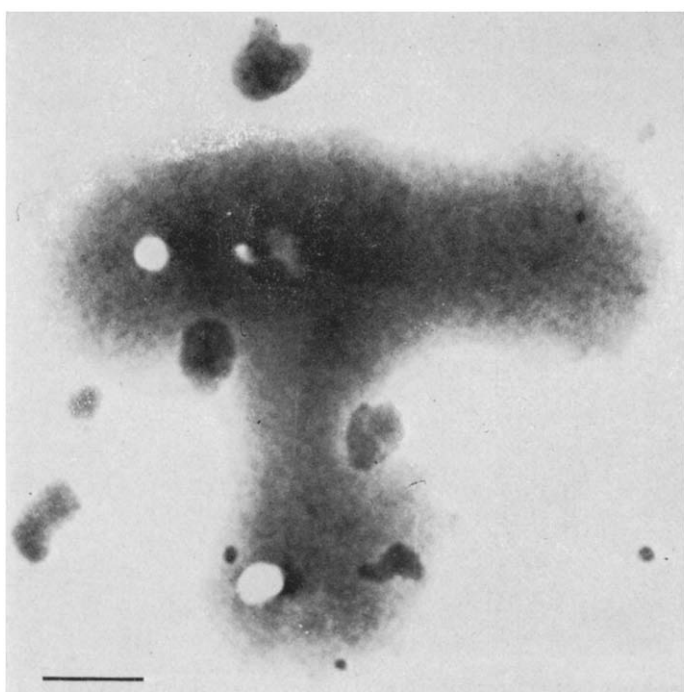

Fig. 9. Filipin (Procedure B). See text for additional details. Marker indicates 1000 Å.

filipin (no cholesterol); no pronounced structure is apparent. At the present time we are reluctant to interpret this picture as indicating the existence of filipin in micellar aggregates although this possibility has been suggested indirectly by other experiments[5]. Fig. 10 is a picture of cholesterol (no filipin); this micrograph is similar to the one of a cholesterol 'microcrystal' published previously by LUCY AND GLAUERT[13]. Figs. 11 and 12 are both pictures of filipin *plus* cholesterol. In Fig. 11 there may have

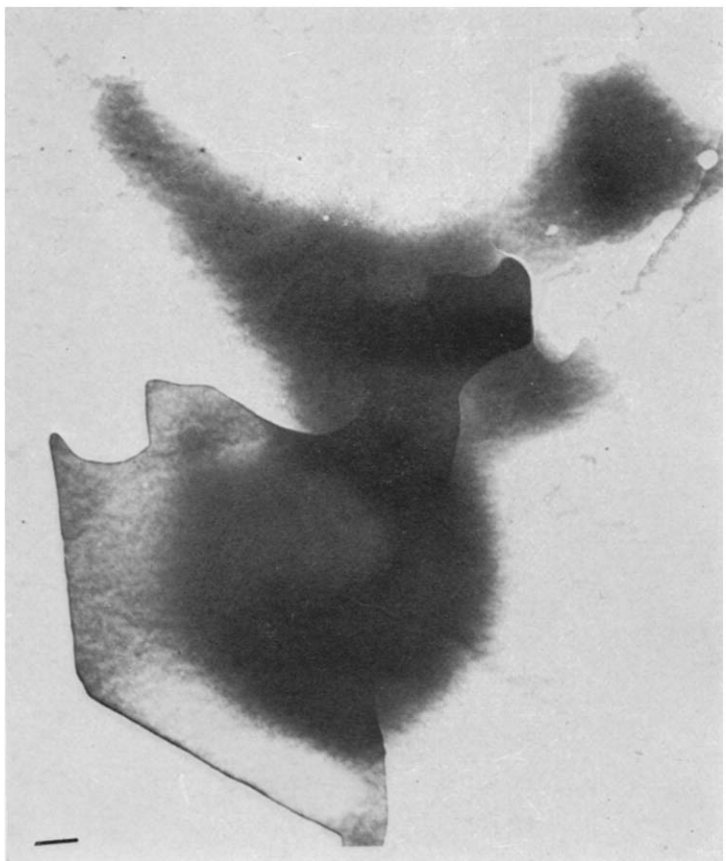

Fig. 10. Cholesterol (Procedure B). See text for additional details. Marker indicates 1000 Å.

been a localized excess of filipin since forms corresponding to the antibiotic appear in the background (*cf.* Fig. 9). It should be noted that pits are present and, particularly, numerous structures which could be interpreted as side views of pits. Conversely, in Fig. 12, there may have been a localized excess of cholesterol since forms corresponding to the sterol are apparent (*cf.* Fig. 10). In this case, pits are also visible especially along the edges and on the surface of the sterol microcrystal. These observations thus suggest that the light rings surrounding each pit in the filipin-treated erythrocyte membrane may consist predominately, if not exclusively, of cholesterol in combination with filipin.

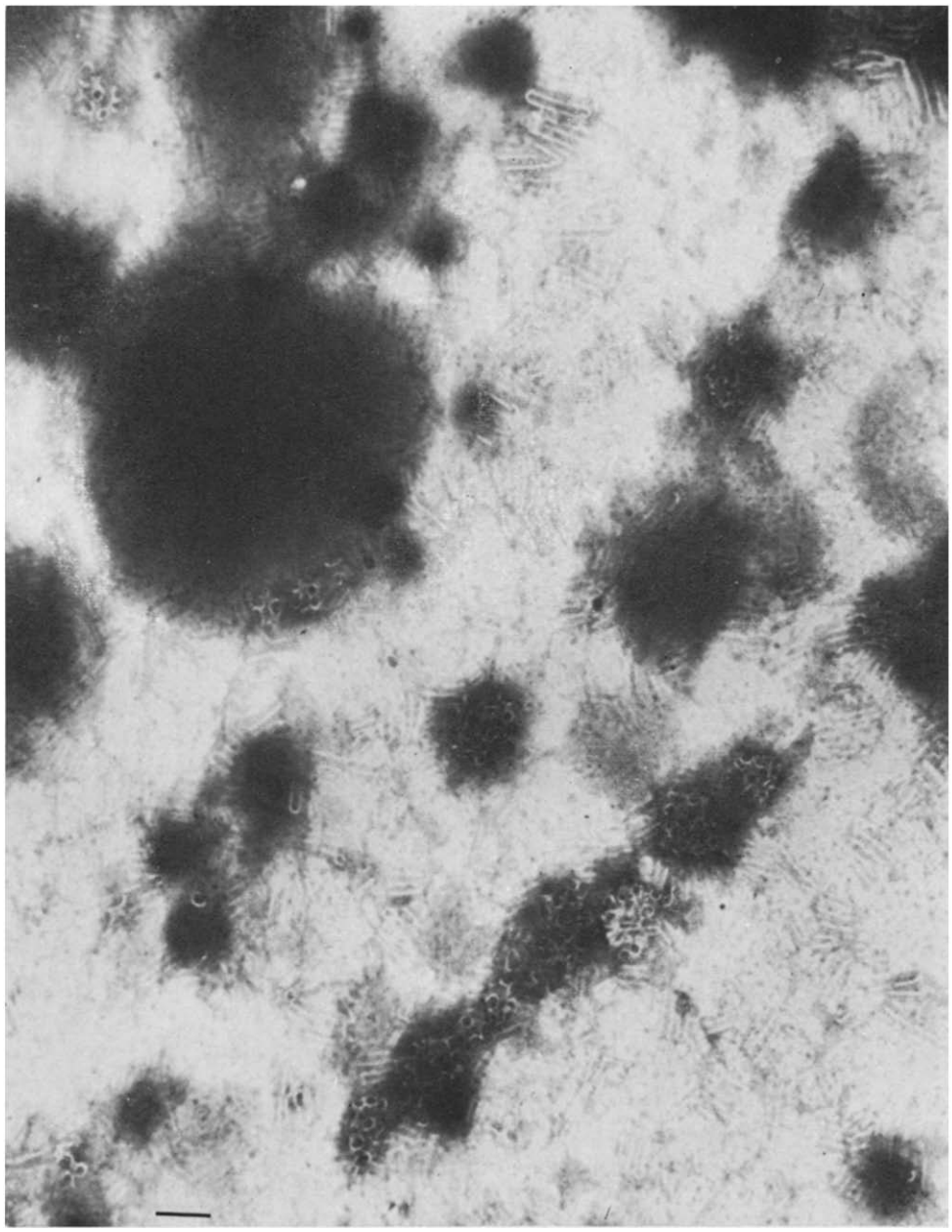

Fig. 11. Filipin and cholesterol (Procedure B). See text for additional details. Marker indicates 1000 Å.

Stabilization of lipid bilayers by cholesterol

The preceding experiments further suggest that filipin interacts with regions of natural and artificial membranes which contain a relatively high proportion of

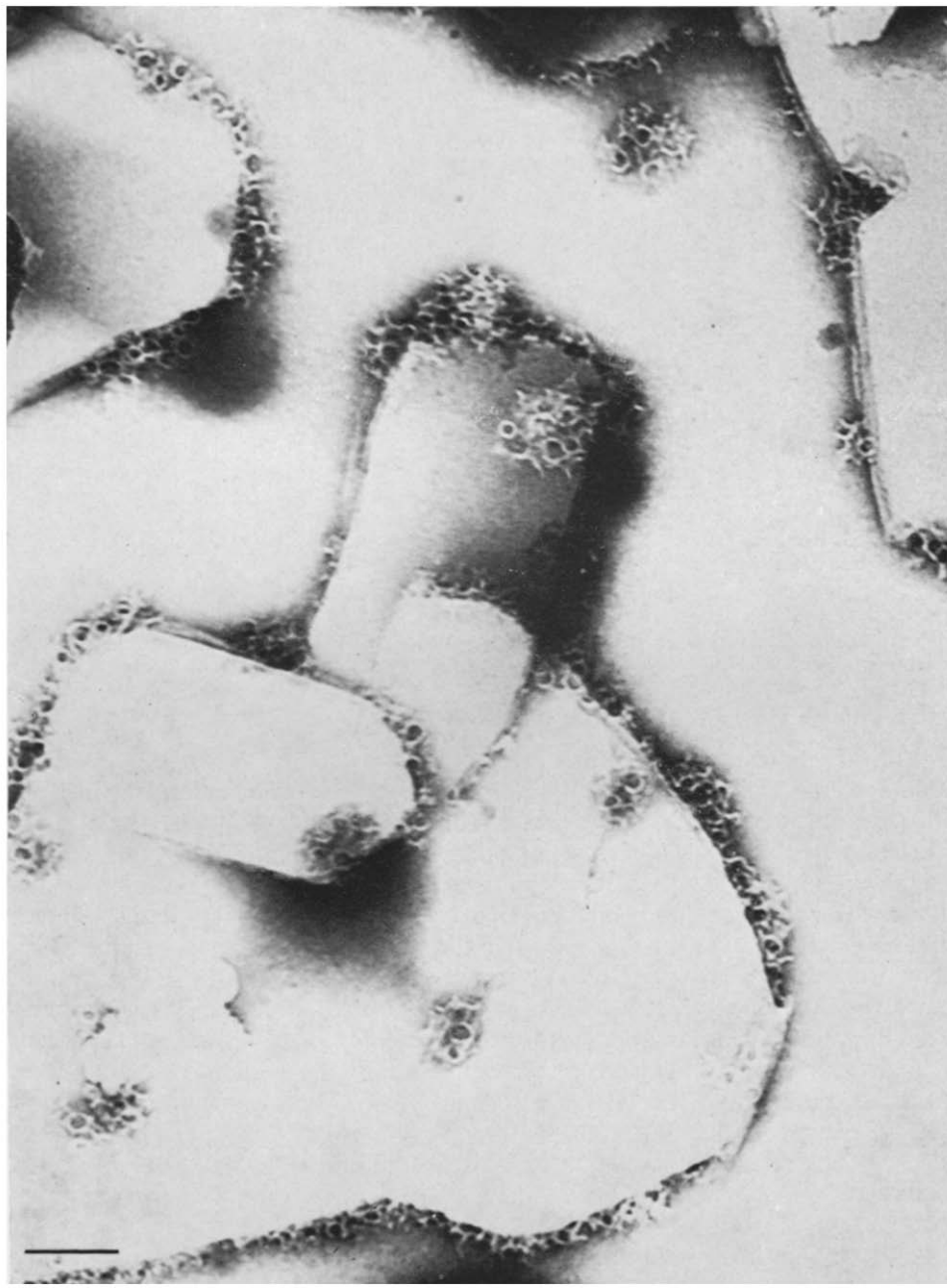

Fig. 12. Filipin and cholesterol (Procedure B). See text for addition details. Marker indicates 1000 Å.

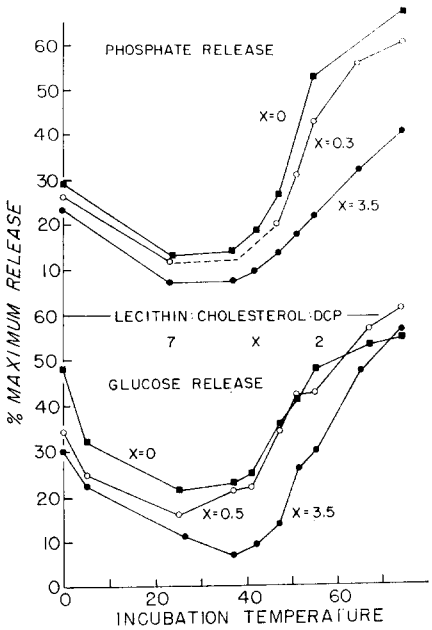

Fig. 13. Effect of incubation temperature on release of inorganic phosphate or glucose from liposomes. Liposomes were prepared from lipid mixtures containing lecithin and dicetylphosphate (DCP) in a molar ratio of 7:2. Varying quantities of cholesterol (X) were added as indicated in the figure. See text for experimental details.

sterol. It seems logical to assume that, as a consequence of combination with the antibiotic, the sterol can no longer perform its function as a membrane constituent. A possible clue to the role of sterols in membrane systems is provided by the experiment described in Fig. 13. In this experiment, the effect of temperature on the leakage of trapped compounds (either glucose or inorganic phosphate) from liposomes, containing various quantities of cholesterol, was determined by the method described previously. It is apparent that less marker is released at a given temperature from liposomes which contain a high molar ratio of cholesterol. This experiment therefore provides additional support for the contention that membrane sterols may function to stabilize the bilayer configuration of lipids. It should also be noted that, under the conditions of the above experiment, the liposomes show a maximum stability near 37°.

DISCUSSION

The present investigation has demonstrated that filipin causes pit formation in erythrocyte membranes and lipid dispersions which contain cholesterol. Since pits were not obtained with derivatives of filipin, which had little or no hemolytic activity, these observations suggest that pit formation may represent the terminal stage in the mechanism by which the antibiotic affects cell membrane structure.

The use of negative staining to recognize alternative configurations of lipids has been discussed by BANGHAM AND HORNE[14] who emphasize that this method

probably preserves the lipids as though they were in the presence of water. Our electron micrographs are at least consistent with the hypothesis that filipin acts by inducing a lamellar to micellar phase transition. Limitations of this method, however, do not yet warrant the conclusion that the pits are actually pores in the membrane, surrounded by lipid micelles, through which cytoplasmic material can leak out of the cell; nevertheless, this possibility must be considered seriously.

Regardless of the interpretation, this investigation has produced visible evidence that filipin can interact with cholesterol. These results are in accord with previous studies showing that the presence of sterol in the cell membrane is apparently a necessary, although not a sufficient condition, for filipin sensitivity[3,4]. They are also in agreement with studies showing that the polyene antibiotics preferentially penetrate lipid monolayers containing sterol[11] and disrupt lipid bilayer films prepared in the presence of cholesterol[12]. However, WEISSMANN AND SESSA[15], studying the effect of filipin on the release of various markers (phosphate, chromate, and glucose) from liposomes prepared with and without cholesterol, reported that sterol was not required and that pre-incorporation of cholesterol into the liposomes did not promote more extensive release by the antibiotic. Thus, leakage can occur under experimental conditions which do not favor pit formation*. Their results also raise the possibility that current concepts regarding the mode of action, and basis for the selective toxicity, of filipin and other polyene antibiotics may require modification. The alternative, however, that liposomes are less appropriate models for studying the mode of polyene antibiotic action than either lipid monolayers or bilayer films must also be taken into consideration. This alternative explanation is examined in detail in the following papers[16,17].

It is of some interest to compare the present electron micrographs with those obtained using other lytic agents. BANGHAM AND HORNE have published a picture of a lecithin–cholesterol dispersion which had been treated with lysolecithin (see Plate IX of ref. 14). The resulting pattern is analogous to that produced by perhydrofilipin (Fig. 6). These observations suggest that reduction of the conjugated double bond sequence may have converted filipin into a derivative which produces lysis by an entirely different mechanism, perhaps related to the mechanism by which lysolecithin acts. ROSSE, DOURMASHKIN AND HUMPHREY[18] have recently shown that pits are present in human erythrocyte membranes prepared by immune lysis in the presence of complement. These pits resemble those produced in human erythrocytes after lysis with filipin (Fig. 3). In pointing out this similarity, we are not implying that the antibody-complement system has the same receptor(s) in the membrane as do the polyenes. Indeed, BLADEN, EVANS AND MERGENHAGEN[19] have demonstrated that *Eschericia coli* protoplast membranes treated with appropriate antisera in the presence of complement also contain pits and it is, of course, well documented that sterol is absent in these organisms. It is, however, possible that immune and polyene induced lysis produce the same end result, *i.e.*, an alteration of membrane lipid structure.

* The lipid dispersions used in the present investigation do not contain dicetylphosphate whereas this compound is a constituent of the liposomes employed by WEISSMANN AND SESSA[15]. We have repeated the electron microscopic experiments with dicetylphosphate added to the lipid mixtures used in preparation of the dispersions, and obtained results identical to those described in the text, *i.e.*, the presence of dicetylphosphate did not alter the morphologic effects induced by filipin.

Perhaps, even more significant than the similarities noted above is the lack of similarity apparent when filipin is compared with saponin. It has long been known that saponins are potent hemolytic agents and that their lytic activity can be antagonized by free cholesterol. In 1937, SCHULMAN AND RIDEAL demonstrated that saponins preferentially penetrate sterol monolayers[20,21]. The fact that the polyene antibiotics behave very much like the saponins is a perplexing problem since there is little structural resemblance between the two classes of compounds. The present investigation provides some basis for the view that the detailed mode of action of the polyenes and saponins may not be identical although both can interact with membrane sterol. Fig. 14 is a picture of an equimolar lecithin–cholesterol dispersion

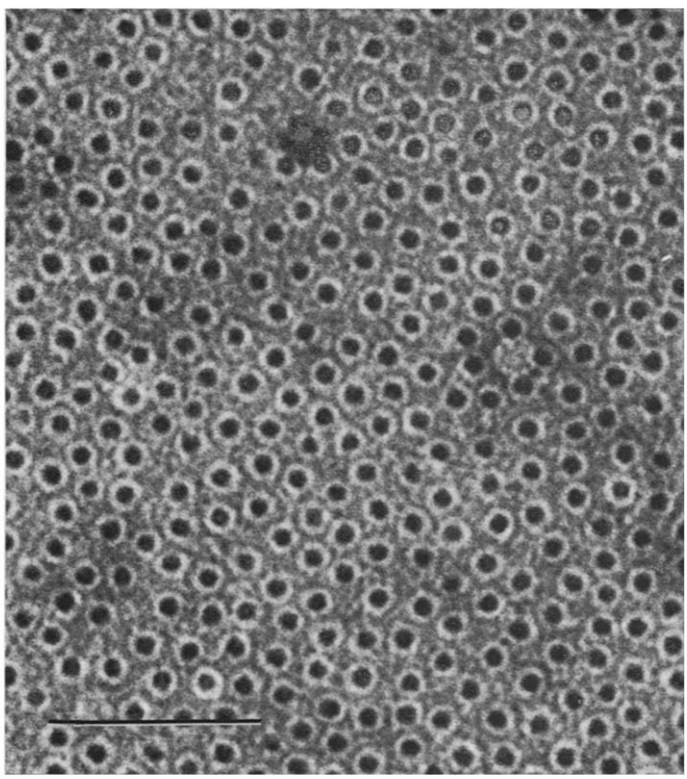

Fig. 14. Lecithin–cholesterol dispersion (1:1 molar ratio) treated with 200 μg of saponin instead of filipin (Procedure A). Marker indicates 1000 Å.

treated with saponin; pictures like this were originally described and interpreted by LUCY AND GLAUERT[13] and BANGHAM AND HORNE[14]. Unlike those produced by filipin (*cf.* Fig. 4), the pits produced by saponin are more hexagonal in shape. Furthermore, the pits produced by saponin are not isolated, *i.e.*, they appear in regular array, whereas the pits caused by filipin are distributed more or less randomly. This difference was also observed in mammalian erythrocyte membranes which had been prepared by lysis with saponin or filipin[2]. The basis for this difference is not yet known

but may be due to a difference in the type of complex formed between the lytic agent and the membrane lipids. LUCY AND GLAUERT have suggested that the light rings surrounding the pits produced by saponin may consist of two types of micelles containing cholesterol–saponin and lecithin–cholesterol[13]. The results of the current investigation suggest that the light rings surrounding the pits produced by filipin may consist solely of sterol which had complexed with the antibiotic. The above observations do indicate, in any case, that there is no *a priori* basis for the assumption that all lytic agents which can interact with sterol need produce the same morphological effect on cell membranes and lipid dispersions.

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