Biochimica et Biophysica Acta, 323 (1973) 43–54

BBA-76440

A FREEZE-ETCH STUDY OF THE EFFECTS OF FILIPIN ON LIPOSOMES AND HUMAN ERYTHROCYTE MEMBRANES

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(Received April 4th, 1973)

SUMMARY

Previous investigations have demonstrated the presence of circular negatively stained lesions in phospholipid–cholesterol liposomes and in the cell membranes of erythrocytes that have been treated with the polyene antibiotic, filipin; these lesions are morphologically similar to those found in erythrocyte cell membranes after immune lysis. Filipin has been shown also to cause release of both glucose and macromolecular enzyme markers from liposomes that contain cholesterol. In the present study, freeze-etching was employed to determine if these lesions are holes through the membrane. Structural alterations (pits, doughnut-shaped craters and protrusions), whose formation was dependent on the presence of cholesterol, were demonstrable in liposomes after incubation with the antibiotic; analogous changes were also produced by filipin in erythrocyte membranes. However, examination of outer and inner surfaces, and outer and inner fracture faces, did not indicate that these structural alterations could be equated with transverse holes. It is, therefore, unlikely that the negatively stained lesions represent stable open channels through liposomal bilayers and erythrocyte membranes which are responsible for the escape of marker compounds and cytoplasmic constituents.

INTRODUCTION

Negative staining has been frequently employed in an attempt to determine whether a variety of lytic compounds and treatments produce discrete lesions in cell membranes that can be detected by electron microscopy. The agents that have been most extensively examined by this technique include saponin[1], filipin[2], and antibody–complement[3]. These studies have shown that saponin and the polyene antibiotic produce different lesions in rat erythrocyte membranes[4] or phospholipid–cholesterol liposomes[2], although it is generally accepted that both compounds exert their lytic effect by initial interaction with sterols presents in susceptible membranes (reviewed in ref. 5). In contrast, the lesions found in a variety of natural cell membranes after immune lysis by antibody–complement[6] were quite similar to those produced by filipin with respect to size, shape and distribution.

The fact that the lytic action of filipin can be mimicked with appropriate liposomes as indicated by the release of trapped glucose marker[7] led to the development of several liposomal systems responding in an analogous manner to antibody–

complement (reviewed in ref. 8). This experimental approach to the study of humoral immune lysis was based on the similarity of the filipin- and complement-induced lesions and the possibility, which was consistent with various lines of indirect evidence[5,6], that the latter might correspond to transverse pores (holes) throught the cell membrane. However, other laboratories have subsequently obtained evidence that did not support this hypothesis. Knudsen *et al.*[9] were unable to detect negatively stained lesions in liposomes prepared from sheep erythrocyte lipids that had been incubated with anti-Forssman antibodies and guinea pig serum as source of complement. Employing purified complement proteins, Polley *et al.*[10] found that the lesions were already present on sheep erythrocytes after the reaction involving C5 (the fifth component of the complement sequence), although cells in this intermediate state still manifested an intact permeability barrier.

Recently, we have been able to confirm the findings of Knudsen *et al.*[9] using liposomes of defined composition that had been sensitized with either globoside or lipopolysaccharide as antigen[11]. No consistent indication of the presence of lesions could be obtained for liposomes that had been incubated with the appropriate antibody and guinea pig serum, whereas lesions were invariably found in liposomes that had been treated with filipin. In these experiments, failure to detect the lesions by negative staining seemed particularly significant because immune damage to lecithin liposomes, as well as incubation with filipin, resulted not only in the release of a small solute (glucose) but also in the parallel loss of a much larger marker (β-galactosidase); the size of this enzyme closely approximates the dimensions of the lesions in erythrocyte membranes after complement-dependent hemolysis. Furthermore, Seeman and coworkers[12,13] have recently examined sheep erythrocyte membranes after immune lysis by freeze-etching and concluded that the lesions probably do not extend through the membrane as a hole. These observations prompted a similar study of the effects of filipin on liposomes and erythrocyte membranes as described in this paper; while this manuscript was in preparation, we were kindly informed by Professor L. L. M. van Deenen that an analogous freeze-etch investigation has also been performed in his laboratory[14].

MATERIALS AND METHODS

Lipids and liposome preparation

The following were obtained from commercial sources and ran as pure compounds upon thin-layer chromatography in several solvent systems: beef brain sphingomyelin and egg lecithin (Pierce Chemical Co., Rockford, Ill.); cholesterol (Sigma Chemical Co., St. Louis, Mo.); dicetyl phosphate (Fisher Scientific Co., St. Louis, Mo.); Liposomes were usually prepared from mixtures containing phospholipid (lecithin and/or sphingomyelin), cholesterol, and dicetyl phosphate in molar ratios of 2:1.5:0.2. The dried lipid films were dispersed by agitation with a Vortex mixer in a sufficient volume of distilled water (or, in some experiments, 20% glycerol) to yield a 10 mM phospholipid suspension.

Erythrocyte preparation

Erythrocytes from fresh human citrated blood were washed three times in phosphate-buffered saline (10 mM potassium phosphate–145 mM NaCl, pH 7.4)

by centrifugation at 2000×*g* for 5 min; white cells were removed by aspiration. Immediately before incubation with the antibiotic, the erythrocytes were washed two additional times with isotonic saline (150 mM NaCl) and left in a packed condition.

Incubation of liposomes and erythrocytes with filipin

Filipin complex was kindly provided by Dr George Whitfield, Upjohn Company Kalamazoo, Mich.; stock solutions (10 mg/ml of dimethylformamide) were prepared just prior to use. Liposomes were incubated 20 min at room temperature after the addition of 100 μl of filipin solution to 1000 μl of liposome preparation; this corresponds to a 0.15:1 molar ratio of filipin: liposomal phospholipid. These conditions were chosen on the basis of previous experiments[7] which have demonstrated that molar ratios in this range produced a rapid release of trapped glucose marker from liposomes prepared with cholesterol.

Erythrocytes were incubated 20 min at room temperature after thorough mixing of 9 vol. of filipin in isotonic saline (1000 μg antibiotic per ml) with 1 vol. of washed packed cells. Preliminary experiments indicated that this antibiotic: erythrocyte ratio was approximately 5 times the ratio required for 50% hemolysis under these conditions. The lysed membranes were washed by alternate centrifugation (25000×*g* for 20 min) and resuspension in cold distilled water until the supernatant solution was visibly free of hemoglobin, and finally dispersed in the minimum volume of water necessary to give a homogeneous suspension. Membranes from untreated erythrocytes were prepared in the same way from packed cells that had been initially lysed by the addition of 9 vol. of distilled water.

Negative staining procedure

Untreated (solvent control) or filipin-treated liposome preparations, and untreated (water-lysed) or filipin-treated human erythrocyte membrane suspensions, were diluted 5-fold with distilled water; an aliquot of the diluted samples was then mixed with an equal volume of 2% phosphotungstate (adjusted to pH 6.8 with KOH) containing 0.0025% bovine serum albumin. Formvar and carbon-coated grids were touched to droplets of this mixture and adhering material was drawn off with torn filter paper.

Freeze-etching procedure

Small drops of untreated or filipin-treated liposome preparations in either distilled water or 20% glycerol–phosphate-buffered saline mixture, and normal or filipin-treated membrane suspensions, were placed on gold alloy specimen carriers and frozen in liquid Freon 22 (E. I. DuPont de Nemours and Co., Wilmington, De.) cooled by liquid nitrogen. The specimens were freeze-cleaved at −100 °C and were deep-etched at −100 °C for times varying between 1 and 10 min. Shadowing with platinum–carbon was performed in a Balzers freeze-etching apparatus (Balzers High Vacuum Corp., Santa Ana, Calif.). The platinum–carbon replicas were floated off the specimens onto distilled water, cleaned with Clorox, and picked up on electron microscope grids.

Electron microscopy

All grids were examined in a Philips 300 electron microscope (Philips Electron

Instruments, Mount Vernon, N.Y.). Terms used to designate the various surfaces and cleavage (fracture) faces of freeze-cleaved and etched lipid bilayers and erythrocyte membranes are as follows: ES, the external surface of the bilayer or membrane revealed by etching of surrounding ice; IS, the internal surface of the bilayer or membrane, revealed by etching of ice contained within the structure; IFF, the inner fracture face, nearest to the internal surface of the bilayer or membrane, revealed by cleavage (fracture) of the frozen bilayer or membrane; OFF, the outer fracture face, nearest to the external surface of the bilayer or membrane, revealed by cleavage of the bilayer or membrane. All freeze-etch micrographs have been mounted with the direction of platinum shadowing from the bottom of the micrograph toward the top.

RESULTS

Negative staining of liposomes and erythrocyte membranes

Prior to examination of the freeze-etched specimens, control experiments were routinely carried out to confirm that filipin had produced the characteristic negatively stained lesions; their appearance in liposomes and human erythrocyte membranes is shown in Figs 1 and 2, respectively. The properties of these lesions have been described in detail elsewhere[2] and, accordingly, only a brief summary seems necessary as an introduction to the observations made on freeze-etched

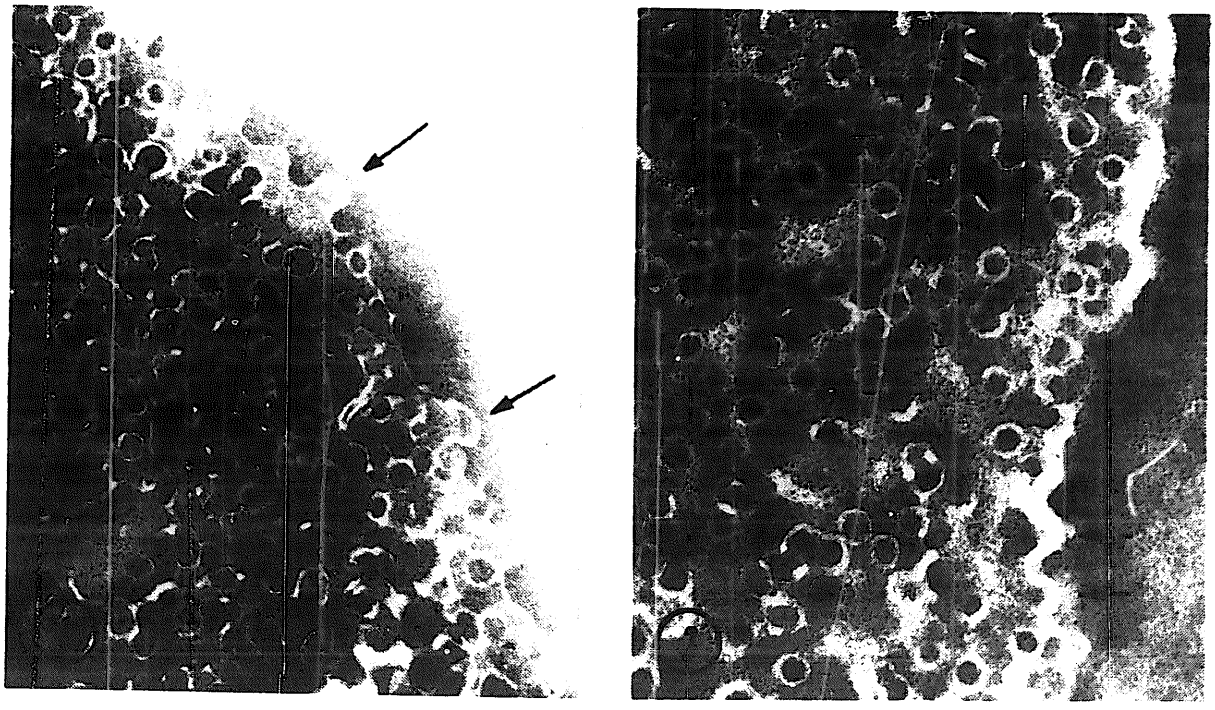


Fig. 1. Liposomes (containing lecithin, sphingomyelin, cholesterol and dicetyl phosphate in molar ratios of 1.5:0.5:1.5:0.2) treated with filipin and negatively stained. Circular lesions are scattered over the liposome and protrusions (arrows) are apparent at the edges of the liposome. Magnification × 130000.

Fig. 2. Human red blood cell membrane treated with filipin and negatively stained. Circular lesions are present on the membrane and protrusions (arrows) are seen at the edge of the ghost. Magnification × 130000.

preparations. In liposomes, the dark centers of the lesions average 170 Å in diameter and the total diameter of the dark center *plus* surrounding white rim measures approximately 250 Å; in the membranes, the corresponding dimensions are 150 Å for the dark center and 280 Å for the total diameter of the lesion. A significant aspect of filipin treatment, which has not been emphasized previously, is the presence at the edges of negatively stained liposomes of protrusions measuring approximately 300 Å in diameter (Fig. 1). Protrusions were also apparent on the edge of the erythrocyte membrane as it turned in on itself (Fig. 2), although they were more difficult to define than on liposomes.

Freeze-etching of untreated and filipin-treated liposomes

Untreated liposomes showed a multilaminated "onion skin" appearance when examined by freeze-etching (Fig. 3). Deep etching allowed the external and internal surfaces of the liposomes to be visualized in addition to the fractured bilayer faces. The external and internal surfaces, and fracture faces, of liposomes that had not been incubated with filipin were smooth.

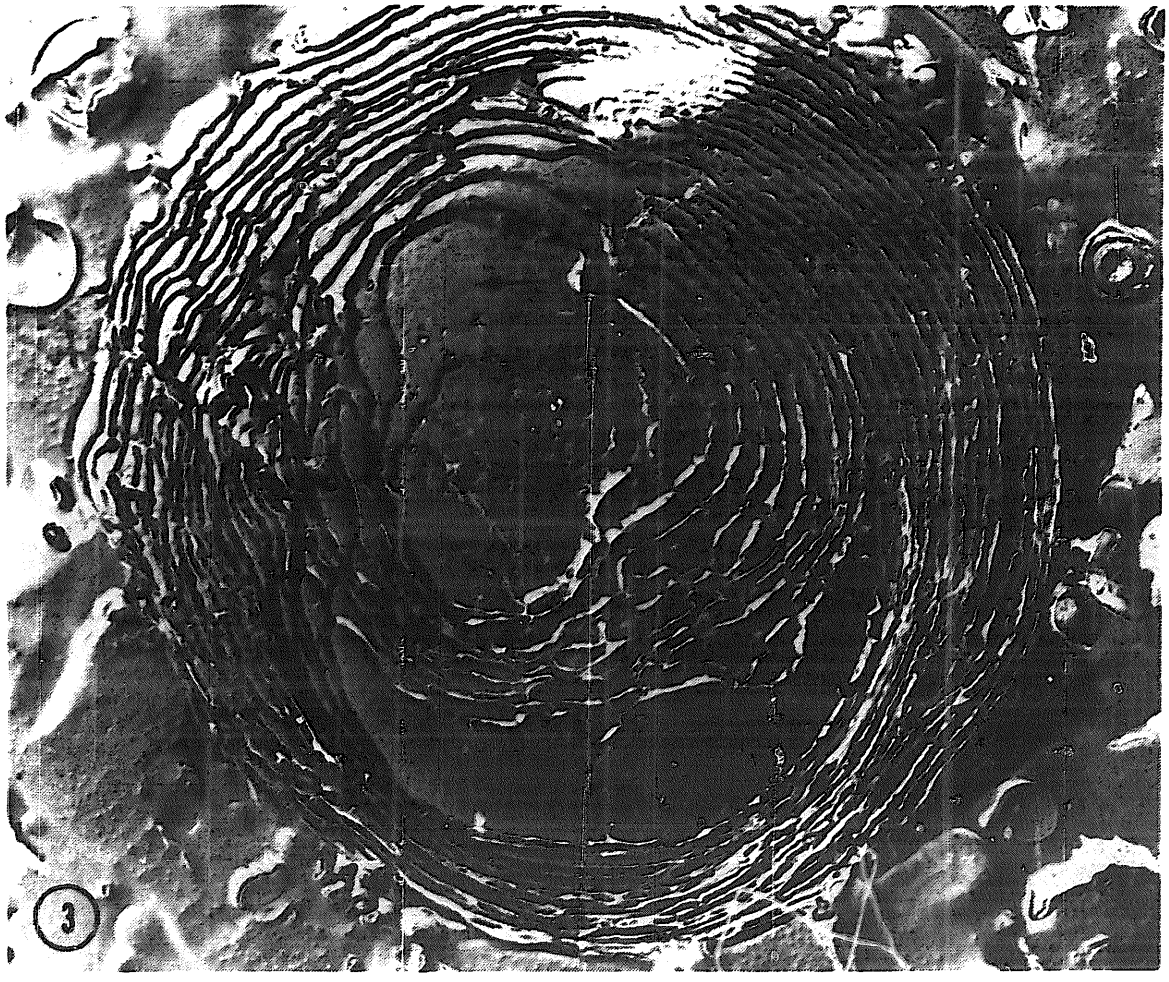
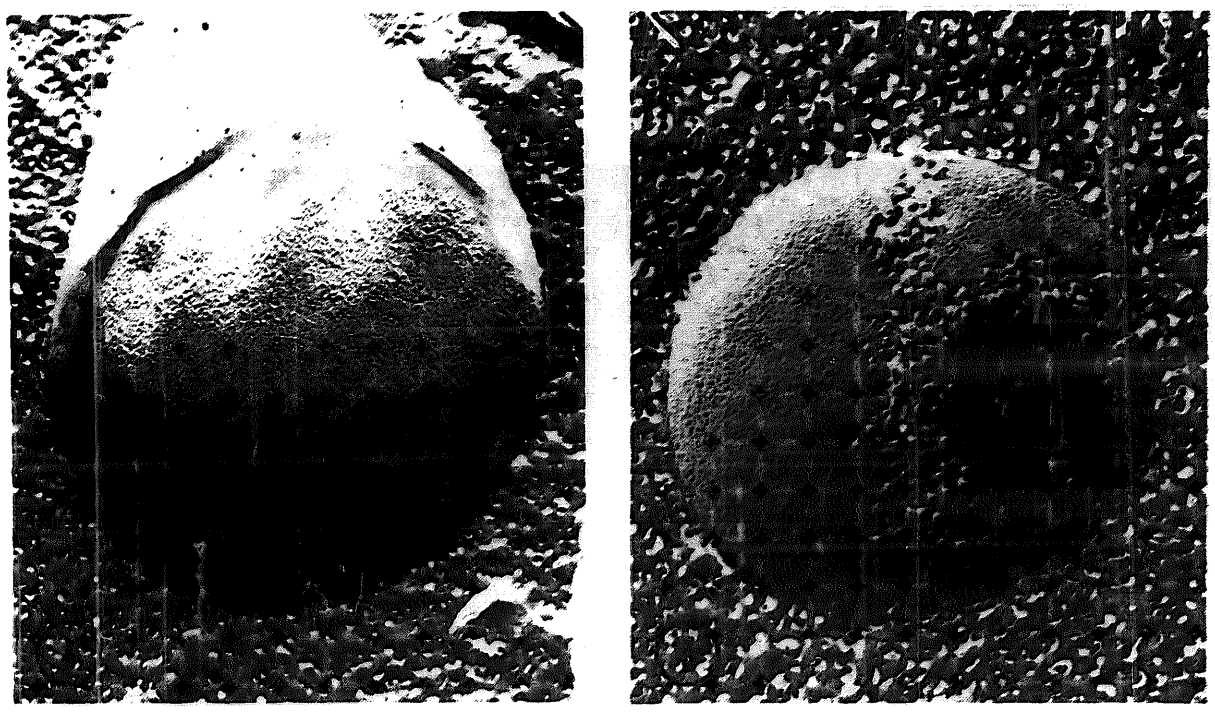


Fig. 3. Platinum–carbon replica of an untreated liposome containing sphingomyelin, cholesterol, and dicetyl phosphate in molar ratios of 2:1.5:0.2). The liposome consists of multiple laminated compartments. Freeze-cleaving and etching exposes inner surfaces of the lipid bilayers (arrows). Magnification × 9000.



Figs 4 and 5. Replicas of liposomes prepared without cholesterol (containing lecithin and dicetyl phosphate in molar ratio of 2:0.2; Fig. 4) or with cholesterol (containing lecithin, cholesterol, and dicetyl phosphate in molar ratios of 2:1.5:0.2; Fig. 5). Lesions are present only in liposomes containing cholesterol. These liposomes are suspended in 20% glycerol magnification in phosphate-buffered saline; therefore, only cleaved faces of the lipid bilayer are seen. Fig. 4, magnification 42000×; Fig. 5, magnification ×35000.

Liposomes in which cholesterol was not incorporated showed no ultrastructural alterations when examined by freeze-etching after treatment with filipin (Fig. 4). However, ultrastructural changes were produced upon incubation with the antibiotic of either lecithin or sphingomyelin liposomes that contained varying amounts of the sterol (10–50 mole percent chlolesterol) (Figs 5–8). Three morphologically different alterations were seen on both external surfaces and fracture faces of the filipin treated liposomes. These lesions were not uniformly distributed over the liposomal layers but were present in patches; innermost layers usually had fewer lesions than outer layers.

One type of lesion was a pit measuring approximately 150 Å in diameter (Fig. 6). In regions containing pits, they frequently occurred in evenly spaced arrays and were usually seen on the outermost layer of the liposomes. Linear depressions, which seemed to connect the pits, were often present. Freeze-cleaved liposomal membranes showing the outer fracture face (OFF) indicated that these pits were not holes through the membrane, but rounded depressions with bottoms (Fig. 7).

A second type of lesion, closely related to the pits, were doughnut-shaped structures with central depressions and elevated rounded edges (Fig. 6). These craters were frequently seen on the inner fracture faces (IFF) and on inner layers of the liposomes, although some were also present on the external surface of the outermost liposomal layer. The depression at the center of the doughnut-shaped

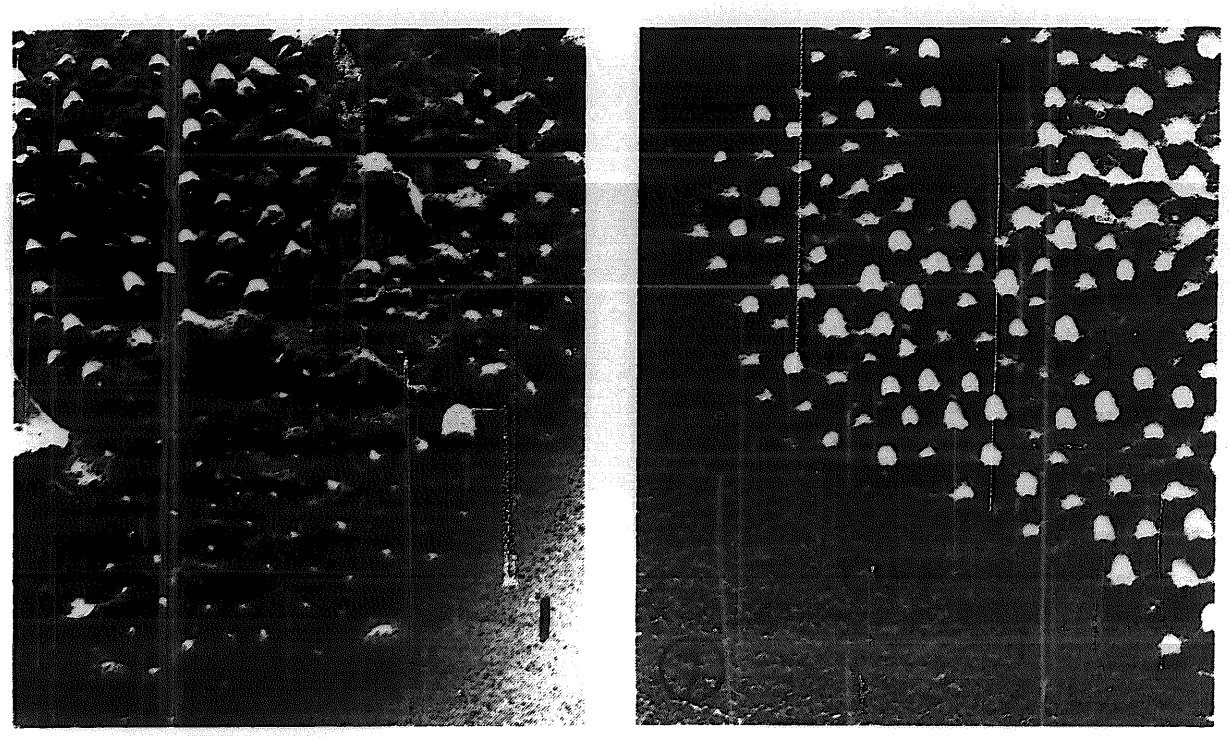


Fig. 6. Replica of a filipin-treated liposome (containing lecithin, cholesterol, and dicetyl phosphate in molar ratios of 2:1.5:0.2). Deep-etching of the ice (I) exposes the external surface (ES) of the outermost layer of the liposome and this surface is covered by pits. Fine linear depressions connect some of the pits. Cleavage of the lipid bilayer (arrow indicates cleavage line) exposes inner fracture faces (IFF) that contain doughnut-shaped lesions. Magnification × 85000.

Fig. 7. Replica of a freeze-cleaved filipin-treated liposome (containing lecithin, cholesterol, and dicetyl phosphate in molar ratios of 1:1:0.2) showing the outer fracture face (OFF). The OFF is the cleaved bilayer face nearest to the external surface of the liposome and shows rounded protrusions connected by elevated ridges. Magnification × 90000.

lesions was also approximately 150 Å in diameter and the total diameter of the depression *plus* rim averaged 400 Å.

The third type of lesion seen in filipin-treated liposomes was a rounded protrusion that measured approximately 350 Å in diameter in liposomes that contained primarily lecithin as phospholipid (Fig. 8). Even larger protrusions, some measuring as much as 1500 Å in diameter, were seen in liposomes that contained primarily sphingomyelin (not shown).

Freeze-etching of filipin-treated human erythrocyte membranes

Ultrastructural alterations, induced by filipin, were detected on both the external and cytoplasmic surfaces, and on both fracture faces, of the erythrocyte membrane. The external surface as revealed by deep-etching showed craters as well as protrusions (Fig. 9). The internal diameter of the craters was approximately 150 Å in diameter and the rounded protrusions averaged 300 Å in diameter. The inner fracture face of the freeze-cleaved membrane also showed craters and protrusions (Figs 9 and 10); the craters were even better defined than on the external surface of the membrane and appeared to have an intact bottom. The characteristic 70-Å intramembranous particles on the inner fracture face were no longer randomly distributed but were in a reticulated arrangement between the filipin-induced lesions. The outer

Fig. 8. Replica of a filipin-treated liposome (containing lecithin, sphingomyelin, cholesterol and dicetyl phosphate in molar ratios of 1.5:0.5:1.5:0.2). External surfaces (ES) of the liposome exposed by deep-etching of the ice (I) show rounded protrusions, sometimes with slight dimpling. Inner layers of this liposome are free of lesions (arrow). Magnification × 67000.

fracture face and the inner surface of the filipin-treated erythrocyte membrane revealed numerous protrusions and some craters (Fig. 11) which were similar to those seen on the external surface.

DISCUSSION

The present experiments indicate that filipin treatment induces at least two distinguishable types of ultrastructural lesions (pits and protrusions) in liposomes and erythrocyte membranes that are detectable by both negative staining and freeze-etching. It seems particularly significant that the antibiotic induced pits in freeze-etched human erythrocyte membranes are morphologically similar to the pits or surface rings initially described by Seeman[12] in freeze-etched sheep erythrocyte membranes after immune lysis and which have been equated by him with the negatively stained lesions. A subsequent detailed study by these investigators[13] have led them to conclude that these pits were not holes because the center of the rings appeared to be on the same level as the plane of the membrane.

This conclusion is also supported by their earlier observation[12] that the pits were mainly present on the exterior etch face and rarely on the interior etch face of

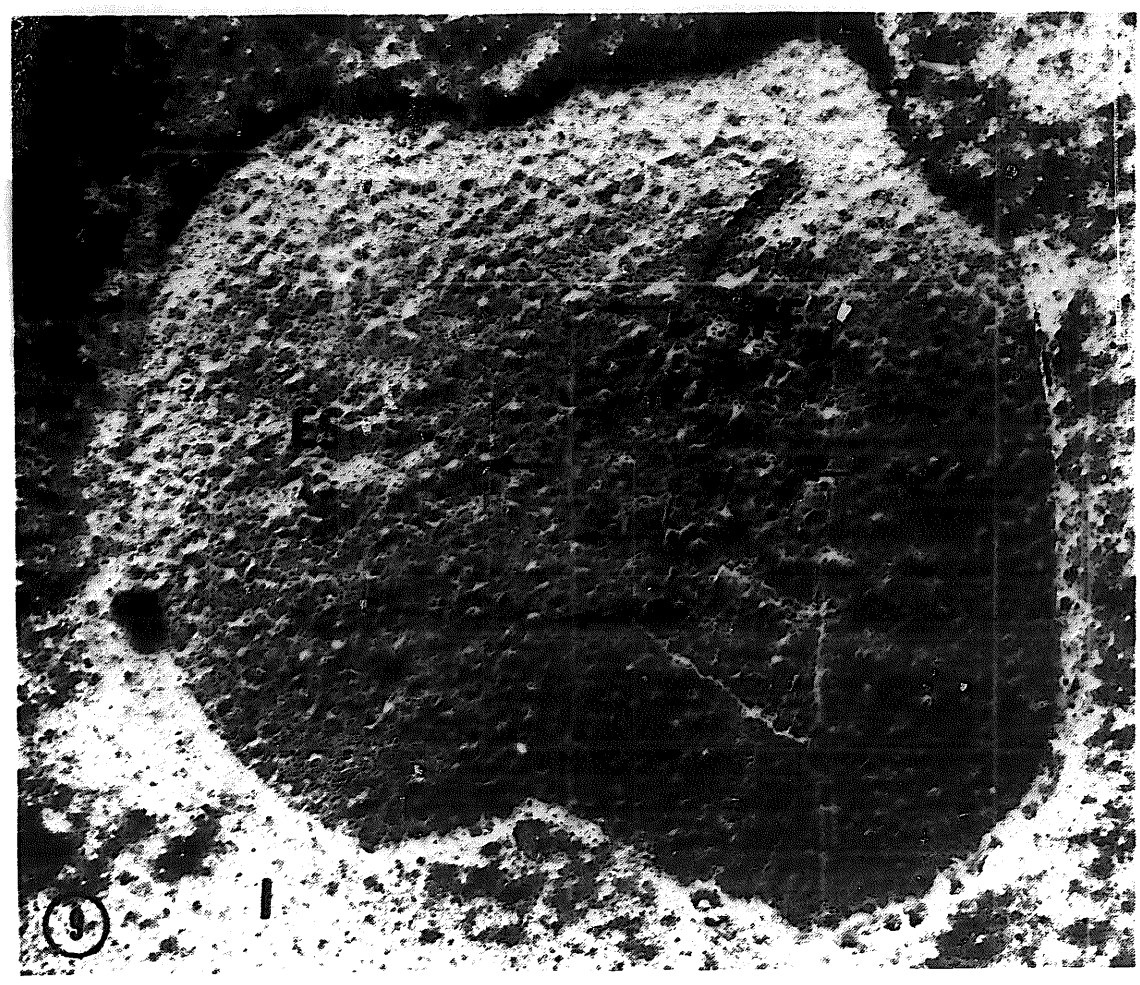


Fig. 9. Replica of a membrane from an erythrocyte that was lysed with filipin. The external surface (ES) of the erythrocyte membrane, revealed by etching of surrounding ice (I), is covered with craters (small arrows) and protusions (large arrows). The inner fracture face (IFF) of the membrane also showed craters and protrusions. Magnification × 45000.

the membrane. The latter finding is in contrast to the situation obtained with filipin where ultrastructural lesions were detected on both the outer and inner surface of the freeze-etched membranes. However, on the basis of the following considerations, we do not believe that this situation is inconsistent with the morphological evidence that the filipin-induced lesions do not represent holes through the membrane. The initial receptor for the antibiotic is cholesterol and it is probable that this abundant membrane component (which accounts for nearly 50% of the lipid in mammalian erythrocytes) is present on both sides of the cell membrane. Accordingly, we cannot exclude the possibility that many (if not all) of the lesions on the inner surface may have been formed after cellular lysis had rendered this surface accessible to the antibiotic. On the other hand, in the case of immune lysis of sheep erythrocytes, complement activation occurs after initial binding of antibody to Forssman antigen. Forssman antigen, a ceramide glycolipid, is a minor component of sheep erythrocyte membranes (0.1% or less of the total lipids) and may resemble other antigens in being exclusively located on the outer surface.

Finally, it should be emphasized that in many respects this investigation has

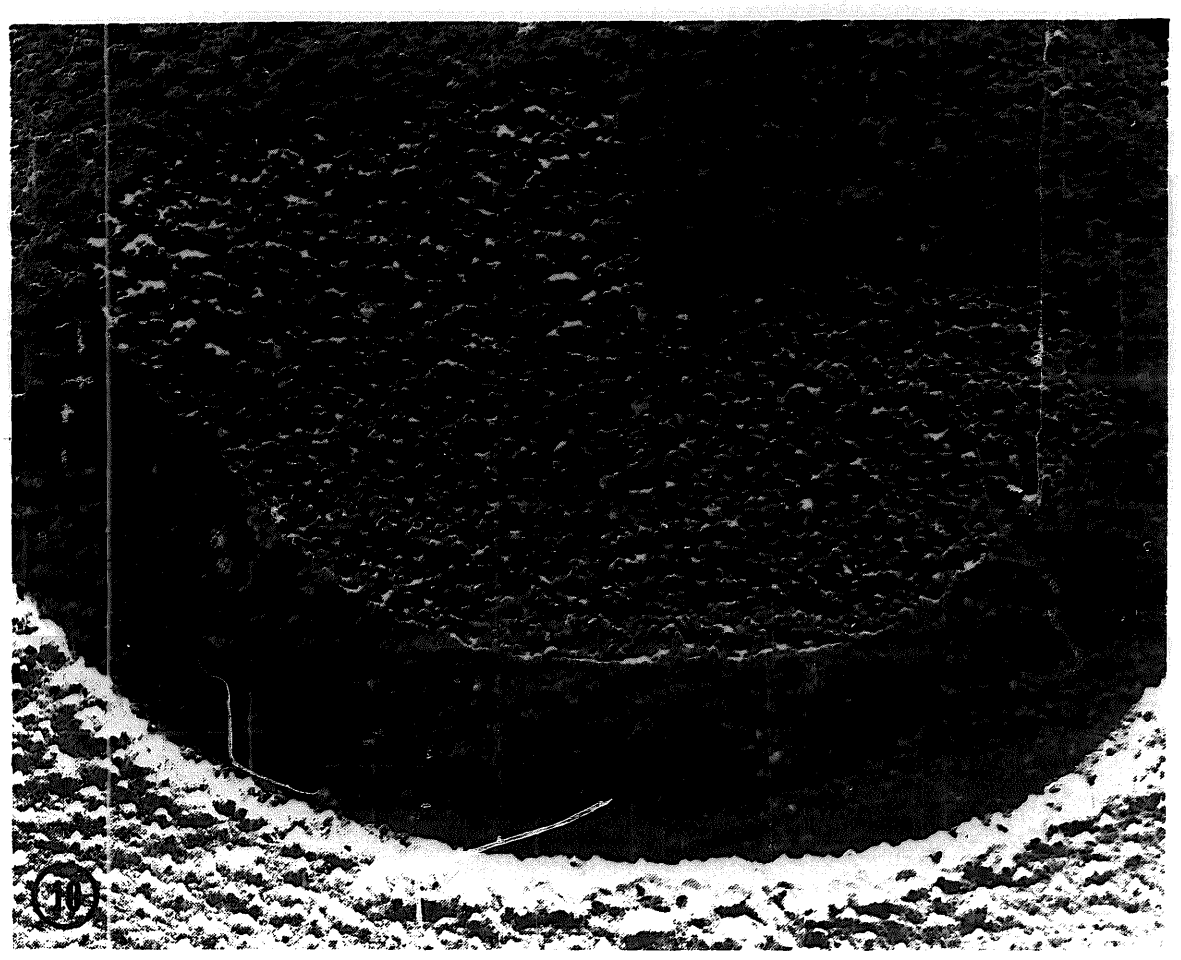


Fig. 10. Replica of a filipin-lysed erythrocyte membrane. The inner fracture face (IFF) contains numerous craters (arrows). The 70-Å intramembranous particles are in a reticulated arrangement between the craters. Craters and protrusions are less conspicuous on the external surfaces (ES) of this heavily shadowed membrane than on the lightly shadowed membrane in Fig. 9. Magnification × 70000.

raised more questions than it has answered. For example, it is not known whether the three types of ultrastructural alterations observed in freeze-etched membranes arise independently or if they are causally related (as in the possible sequence: protrusions→doughnut structures→pits). An even more important question concerns the relation of these ultrastructural lesions to the ultimate mechanism by which filipin destroys the ability of natural and model membranes to act as permeability barriers. In this regard, it is significant that the production of the various lesions depends on the presence of sterol as does the sensitivity of different membranes to damage by the antibiotic. Although these lesions may not correspond to true holes, the possibility remains that they may represent intermediate stages leading to a change in membrane structure that has so far not been (or cannot be) detected by either negative staining or freeze-etching. Alternatively, it is possible that these lesions may represent a final stage indicative of resealed membrane regions that, after antibiotic complexation with sterol, possess a molecular organization different from untreated membranes.

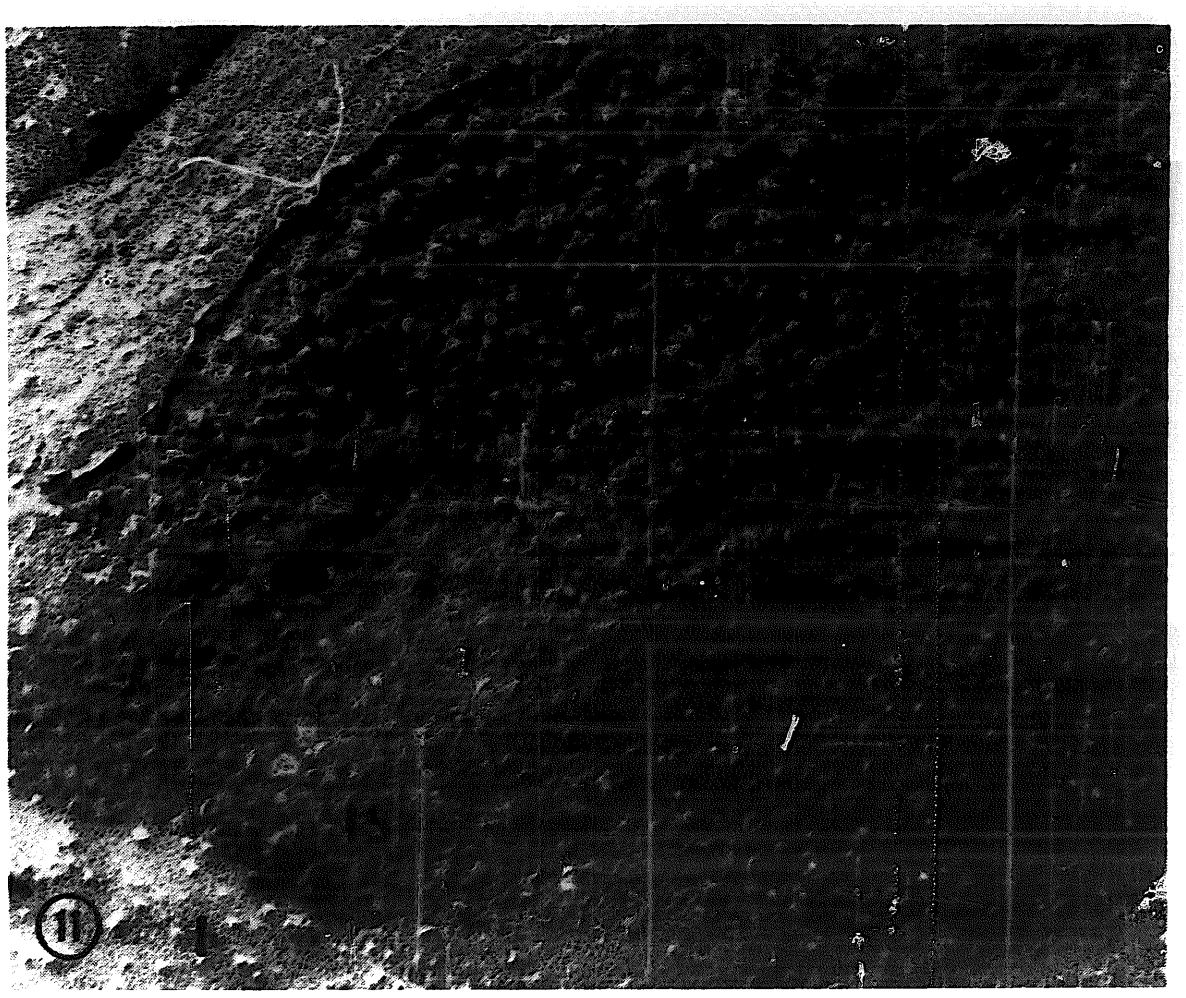


Fig. 11. Replica of a filipin-lysed erythrocyte membrane showing the inner surface (IS) of the membrane exposed by etching of ice (I) and the outer fracture face (OFF). Numerous rounded protrusions (large arrows) and some craters (small arrows) are present on both the IS and the OFF. Magnification × 60000.

ACKNOWLEDGEMENTS

Research supported by: Brown and Williamson Tobacco Corporation; Larus and Brother Company, Inc.; Liggett and Myers Incorporated; Lorillard, a Division of Loews Theatres Inc.; Philip Morris Incorporated; R. J. Reynolds Tobacco Company; United States Tobacco Company; and Tobacco Associates, Inc. to Thomas W. Tillack and grant No. AI-09319 to Stephen C. Kinsky.

We wish to acknowledge the skillful technical assistance of Shirley Carroll.

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