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FREEZE-FRACTURE ULTRASTRUCTURAL ALTERATIONS INDUCED BY FILIPIN, PIMARICIN, NYSTATIN AND AMPHOTERICIN B IN THE PLASMA MEMBRANES OF *EPIDERMOPHYTON*, *SACCHAROMYCES* AND RED BLOOD CELLS. A PROPOSAL OF MODELS FOR POLYENE·ERGOSTEROL COMPLEX-INDUCED MEMBRANE LESIONS

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SUMMARY

The effects of chemically different polyenes on fungal membranes (*Epidermophyton floccosum*, a human pathogenic fungus, and *Saccharomyces cerevisiae*) and human red blood cell membranes were studied by freeze-fracture electron microscopy in order to elucidate the interaction of these antibiotics with ergosterol. Each type of neutral, small amphoteric and large amphoteric polyenes produces a distinct morphological effect on the fungal membranes:

(1) Pit formation type. Filipin, a neutral polyene, produces 250–300 Å diameter "pits" or "invagination" both in ergosterol-containing fungal plasma membranes and cholesterol-containing red blood cell ghost membranes.

(2) Network particle aggregation type. The small amphoteric polyene, pimaricin, produces a network of membrane particle aggregation which encloses 1000 Å diameter particle-free areas in fungal membranes. These areas are slightly elevated toward the outside of the cell.

(3) Random particle aggregation type. The large amphoteric polyenes, amphotericin B and nystatin, cause a random segregation of the fungal plasma membrane and the red blood cell ghost membranes into particle-free and aggregated areas. It is concluded that these morphological differences are due to different mechanisms of polyene-sterol interactions in which the different size of the macrolide ring in the antibiotic structure may be involved. Since all of these antibiotics, except filipin, cause no alterations on whole red blood cells detectable by negative staining and freeze-fracture electron microscopy, it is possible that they have a higher affinity to ergosterol than cholesterol in membranes.

Abbreviations: EF face, fracture face of exoplasmic half; PF face, fracture face of protoplasmic half.

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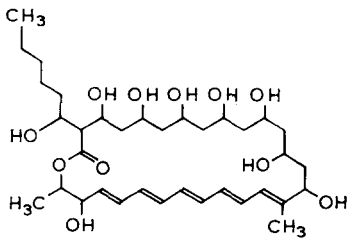
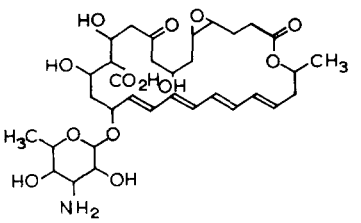
INTRODUCTION

The polyene antibiotics which are originally developed as antifungal agents have a high specific affinity to sterols in membranes [1–5] and exert an immense influence on the permeability and ultrastructure of artificial [6–11] and biological sterol-containing membranes [12–16]. Therefore, many studies have been made using these compounds in order to examine the roles that sterols play in the maintenance of membrane structure and function. These results are well reviewed by Kinsky [17] and others [18].

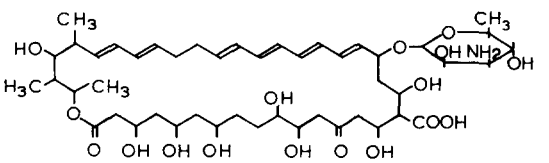
On the other hand, these polyene antibiotics have been widely used as a highly effective medicine for the treatment of systemic fungal diseases. However, they have severe side effects which may be due to their affinity to cholesterol in human cell



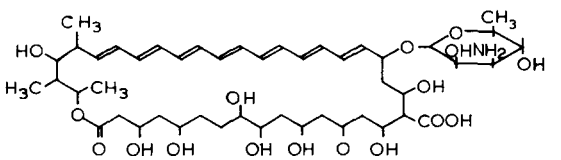
Filipin



Pimaricin



Nystatin



Amphotericin B

Fig. 1. Chemical structures of polyene compounds used in the present study.

membranes. Although the fungicidal activity of polyenes depends on the presence of sterols, little is known about their interaction with ergosterol in fungal plasma membranes. We have recently begun to examine the molecular mechanism of polyene · ergosterol complex formation in fungal plasma membranes. Since only amphotericin B has been known to have a stronger affinity to ergosterol than cholesterol [19], our previous work [20] was initiated by analysing the effects of this compound on the ultrastructure of the ergosterol-containing plasma membrane of a human pathogenic fungus, *Epidermophyton floccosum* by thin section and freeze-fracture electron microscopy. We have shown that amphotericin B induces a strong segregation of the membrane fracture plane into domains of intramembranous particle-free and densely particulated areas, which are followed by cell lysis, and confirmed that this polyene has a stronger affinity to ergosterol than cholesterol in membranes. We wish to extend this work by using a larger variety of polyenes with ergosterol-containing and cholesterol-containing membranes.

This paper presents a freeze-fracture electron microscopical analysis on the apolar membrane core alterations of ergosterol-containing membranes of *E. floccosum* and *Saccharomyces cerevisiae*, and cholesterol-containing human red blood cell ghost membranes, which are induced by three chemically different groups of polyenes: (1) neutral (filipin), (2) small amphoteric (pimaricin) and (3) large amphoteric polyenes (amphotericin B and nystatin) (Fig. 1). We proposed on the basis of these ultrastructural observations the tentative models of ergosterol (or cholesterol)· polyene complex-induced alterations in the fungal plasma membranes.

MATERIALS AND METHODS

Growth of organism

Saccharomyces cerevisiae were grown for 1 day and *Epidermophyton floccosum* TEF-30 for 5 days at 28 °C in shake flasks containing Sabouraud's medium (4 % glucose, 1 % polypeptone, and 0.5 % yeast extract). The cells were harvested when they reached the mid-logarithmic phase.

Preparation of erythrocyte membrane

Isolation of erythrocyte membranes was carried out essentially according to the method of Dodge et al. [21]. Washed erythrocyte suspension was hemolysed in 20 volumes of 20 mosM veronal-HCl buffer (pH 7.4) and ghosts were sedimented by centrifugation at 20 000 × *g* for 20 min. Then the ghosts were washed with the same buffer 3 times subsequent to hemolysis.

Incubation of fungal cells and red blood ghost membranes with polyenes

The harvested cells were incubated for 10 min–2 h at 28 °C with Filipin (Upjohn), Amphotericin B (Squibb), Nystatin (Squibb) and Pimaricin (Torii) at final concentration of 50 μg/ml. In some cases 12.5 μg/ml concentration was also used. The antibiotics were dissolved in dimethylsulphoxide at a concentration of 20 mg/ml. Whole red cells and the ghost membranes obtained from 10 ml of fresh human blood were incubated with polyenes for 30 min at 37 °C. As control cells, the specimens were incubated with dimethylsulphoxide at the same concentration as the experimental use.

Freeze-fracture electron microscopy

Samples were prefixed for 5 h at 28 °C for fungi and 37 °C for red blood cell ghost membranes and red blood cells with 2 % glutaraldehyde buffered with 0.1 M cacodylate, pH 7.4, and transferred to 40 % glycerol in 0.85 % NaCl. The specimens, on brass holder, were rapidly frozen in Freon 12 at liquid N_2 temperature. Freeze-fracturing and etching were carried out using a Hitachi HFZ-1 freeze-etching device. Replication was performed with platinum-carbon and the replicas were coated with carbon. After floating on distilled water the replicas were cleaned in a hypochlorite solution, followed by soaking in 75 % H_2SO_4 for 24 h. After this cleaning procedure, the replicas were given three rinses in water prior to collection on 400-mesh grids. The electron microscopic observation was performed with a Hitachi HS-8 electron microscope.

Negative staining electron microscopy

Control or polyene-treated red blood cell ghost membranes were diluted 5-fold with distilled water, one drop of which was placed on a formvar and carbon-coated grid. After the drop of suspension was drawn off with torn filter paper, 1 % phosphotungstate (adjusted to pH 6.8 with KOH) was dropped on the grid, followed by drawing filter paper. The specimen grids dried in air were examined with a Hitachi HS-8 electron microscope.

RESULTS

Control membranes

The inner half of the plasma membrane of *E. floccosum* and red blood cell ghost membrane viewed from the outside of the cell (PF face, fracture face of protoplasmic half [22]) contains large numbers of randomly distributed intramembranous particles measuring 85–105 Å in diameter, while the outer half of the membranes viewed from the inside (EF face, fracture face of exoplasmic half [22]) contain only a small number of particles (Fig. 2a–d). These observations are consistent with those reported elsewhere [19, 23].

In contrast, the plasma membrane of *S. cerevisiae* exhibits a specific hexagonal arrangement of particles [24] surrounded by other (Fig. 2e arrow,) randomly distributed particles and long, shallow invaginations on the PF face (Fig. 2e). The EF also shows small particle aggregations, with fewer randomly distributed particles (Fig. 2f, arrows). This freeze-fracture appearance is also identical to that previously reported [24].

Effect of filipin

Filipin produces pronounced alterations of the membrane structure in *E. floccosum*, *S. cerevisiae* and red blood cell ghost as revealed by freeze-fracture electron microscopy. Fig. 3 shows a freeze-fractured red blood cell ghost membrane treated with filipin. On the EF face, small round protrusions of 250–300 Å in diameter are arranged in rows. These rows contain three to ten protrusions which maintain a constant distance between each other (Fig. 3, arrows). Some protrusions have a central depression of about 70 Å in diameter (Fig. 3, thick arrows) and this was first described as "doughnut shaped craters" by Tillack and Kinsky [13]. On the PF

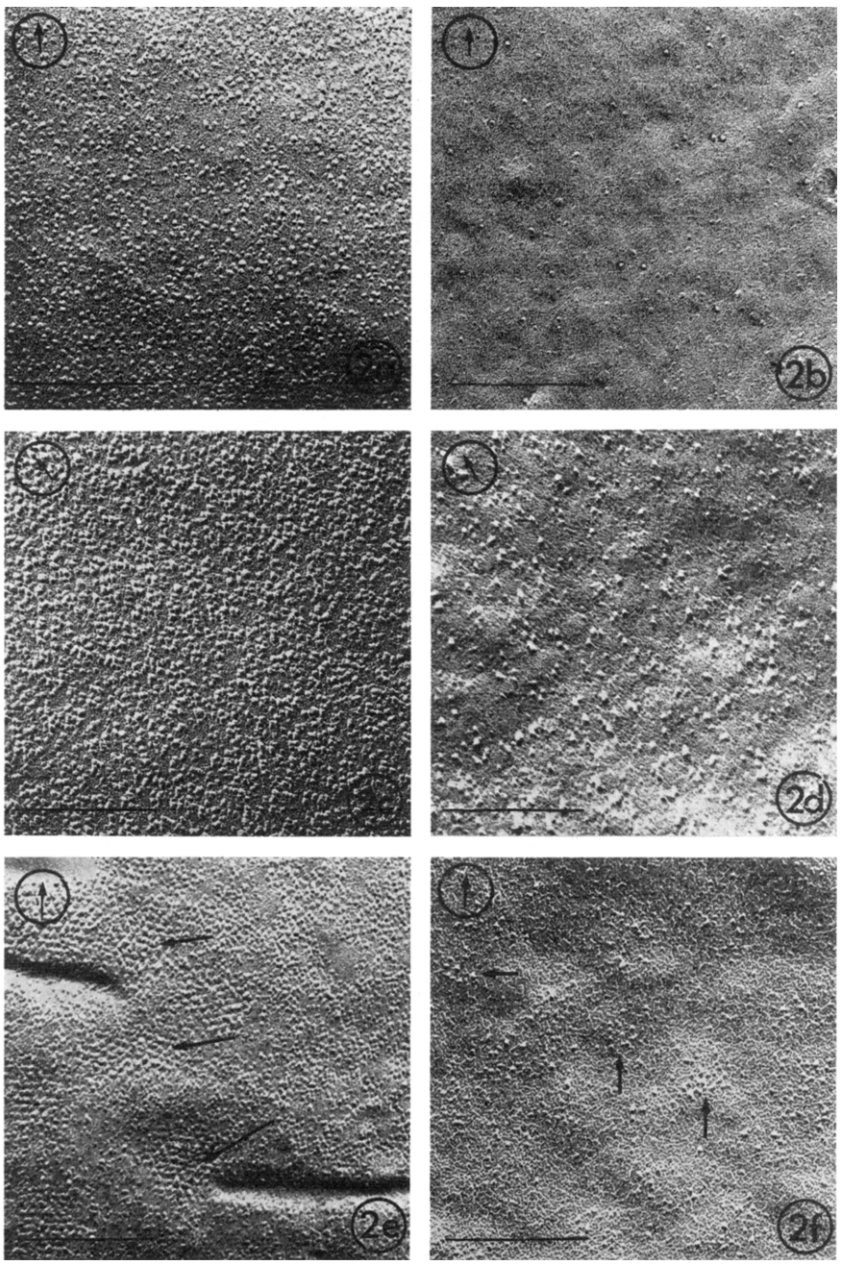


Fig. 2. Freeze-fracture electron micrographs of control membranes. The outer half of the fractured plasma membrane viewed from the outside of the cells (PF face) demonstrates a random distribution of many intramembranous particles in *E. floccosum* (a) and red blood cell ghost (c). In contrast, the inner half of the plasma membrane viewed from the inside of the cells (EF face) shows fewer random particles (b, d). In *S. cerevisiae*, the PF face of the plasma membrane (e) shows characteristic, long shallow invaginations and a hexagonal particle (arrows) aggregated areas with many random particles, while the EF face shows small particle aggregation (arrows) with fewer random particles (f). Shadowing directions are from bottom to the top in all figures shown in this article. Magnification: ×72 000.

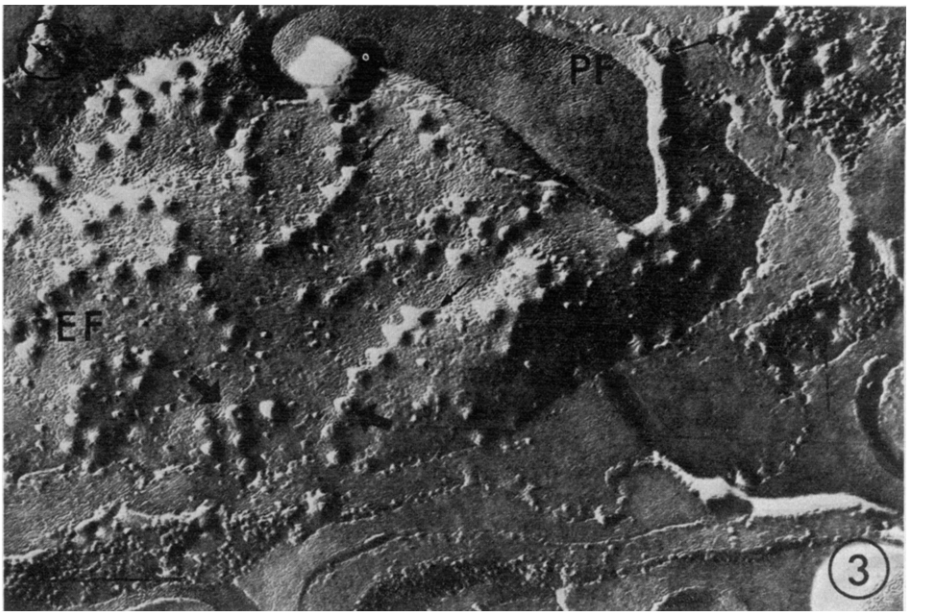


Fig. 3. Effects of filipin on red blood cell ghost membranes revealed by freeze-fracture electron microscopy. The EF face (EF) shows small round protrusions measuring 250–300 Å in diameter, arranged in rows (arrows). Some protrusions have a central depression of about 70 Å in diameter (thick arrows). The PF face (PF), in contrast, displays small excavations (200–250 Å in diameter, dotted arrows) and a segregation of the membrane plane into particle-free areas and aggregated areas. Magnification: ×80 000.

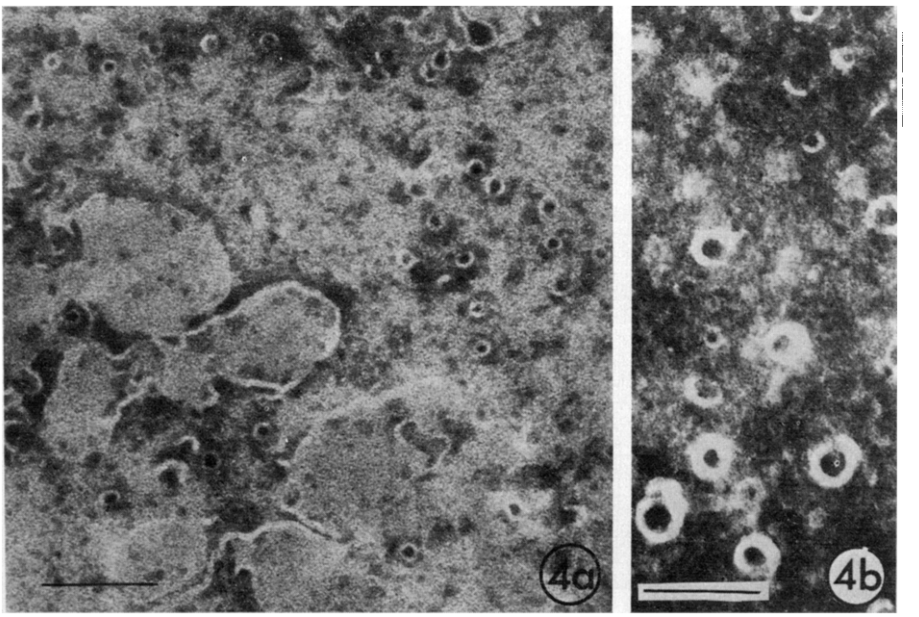


Fig. 4. Negatively stained alteration of red blood cell ghost membranes caused by filipin treatment. "Rings", ranging from 250 to 300 Å, with the dark center measuring about 170 Å in diameter are seen. Magnification (a) ×80 000; (b) 160 000.

face there are many excavations which may correspond to the protrusions on the EF face (dotted arrows). A segregation of particle-free zones and particle-aggregated areas are also produced on the PF face. Fig. 4a and Fig. 4b show the characteristic negatively stained lesions consisting of dark centers, averaging 170 Å in diameter, and surrounding white rims, which form the 250–300 Å rings. These observations on the ghost membranes correspond to those of liposomes and red blood cell membranes which were described by Tillack and Kinsky [13], Kinsky et al. [12] and of *Acholeplasma* by Verkleij et al. [14].

Ergosterol-containing plasma membranes of *E. floccosum* [20] were also strongly affected with filipin. The PF face displays many excavations (Fig. 5a, arrows), while no aggregation is apparent (Fig. 5a). Fig. 5b shows many protrusions on the EF face. They appear to be almost in the same size and shape as those seen on the EF face of red blood cell ghost membrane. These protrusions and excavations seem to fit each other to form many small "pits" or "invaginations" of the plasma membrane. Alterations of the plasma membrane of *S. cerevisiae* are similar to those seen on that of *E. floccosum* (not shown).

In general, filipin produces small "pits" or "invaginations" which are always protruded toward the cytoplasmic side, but not holes through the membrane. These characteristic lesions are almost identical between three natural membranes containing even different sterols.

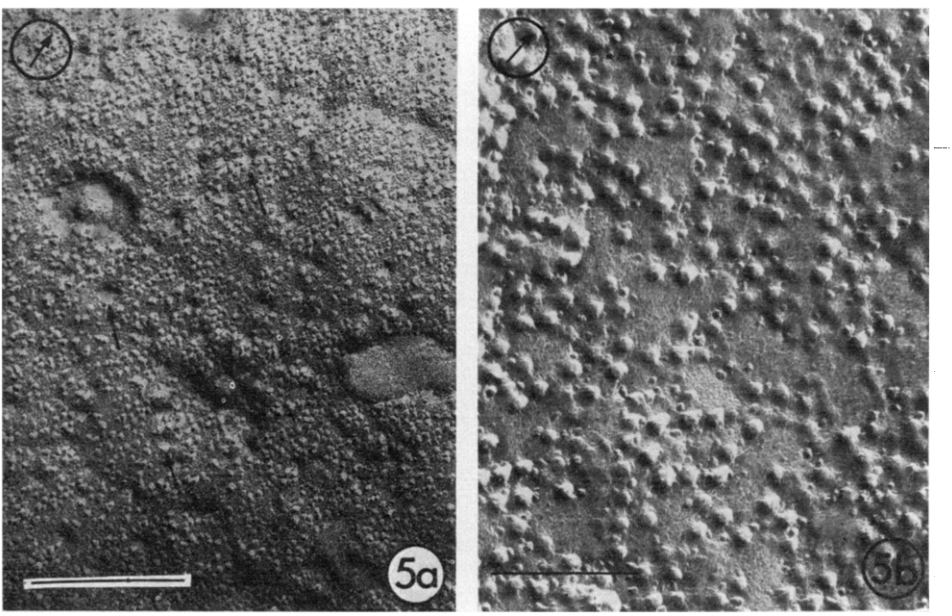


Fig. 5. Effects of filipin on *E. floccosum* as revealed by freeze-fracture electron microscopy. (a) The PF face of the plasma membrane shows small excavations ranging 200 to 250 Å with randomly distributed particles. (b) The EF face reveals many small protrusions (250–300 Å) among fewer random particles. Magnifications: 80 000.

Effect of pimaricin

A pronounced effect of pimaricin was exerted on the plasma membranes of *E. floccosum* as shown in Fig. 6a-b. Replicas of the PF face reveal that the plasma membrane is segmented into uniform, circular particle free-areas measuring approximately 1000 Å in diameter, which are defined by lines of particles and thus produce a network appearance (Fig. 6a). These round particle-excluded areas appear to form slight elevations. As a consequence, the lines of particles are situated in the furrows between those low "elevations". Particle-free areas are also created on the EF face, but, in contrast, they give a slightly depressed appearance (Fig. 6b). The particle lines here seem to be situated on the elevated ridges of these shallow depressions and appear to be composed of fewer particles than those found on the PF face. Similar findings are found with *S. cerevisiae*. However, the original hexagonal patterns are intact (Fig. 7, thick arrow). In some cells, the membrane was almost transformed into small vesicles ranging from 1000 to 1500 Å, thus cell lysis seems to start.

Red blood cell ghost membranes treated with pimaricin display a strong particle aggregation on both fracture faces (Fig. 8), but no network appearance as seen on fungal membrane fracture faces. Negative staining electron microscopy revealed no detectable alterations. It is of some interest that whole red blood cells are not affected morphologically by pimaricin treatment while ghost membranes are altered as described above (not shown).

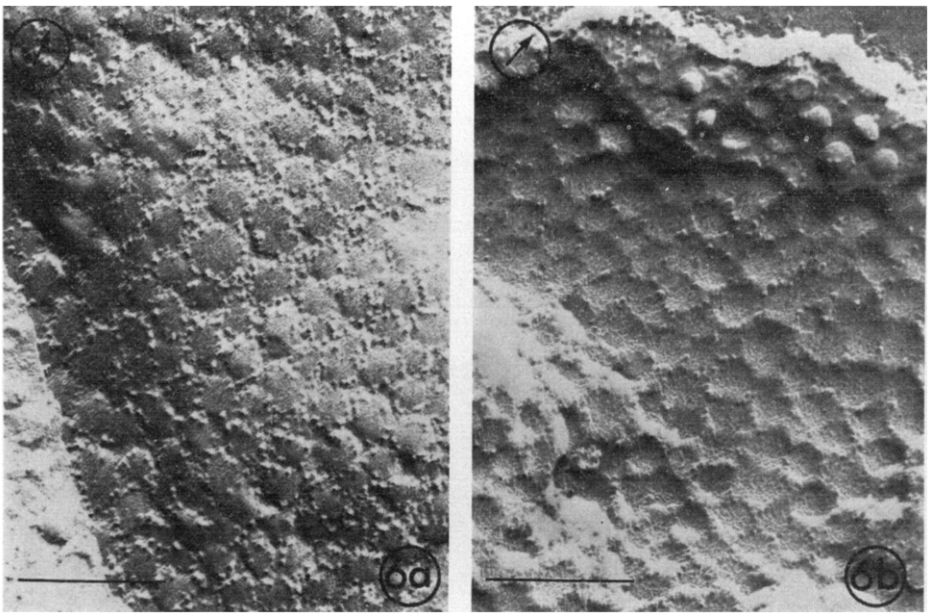


Fig. 6. Effects of pimaricin on the *E. floccosum* plasma membranes as visualized by freeze-fracture. (a) The PF face is segmented into smooth particle-free circular areas, ranging 800–1000 Å in diameter, which are defined by the particle lines. These particles appear to be excluded from the smooth areas into the borders between these smooth areas, thus forming a network appearance. These smooth circular areas are slightly elevated. (b) The EF face shows also smooth circular areas, but they are here depressed. The particles are also transmoved to the elevated borders, which show a network appearance. Magnification: ×80 000.

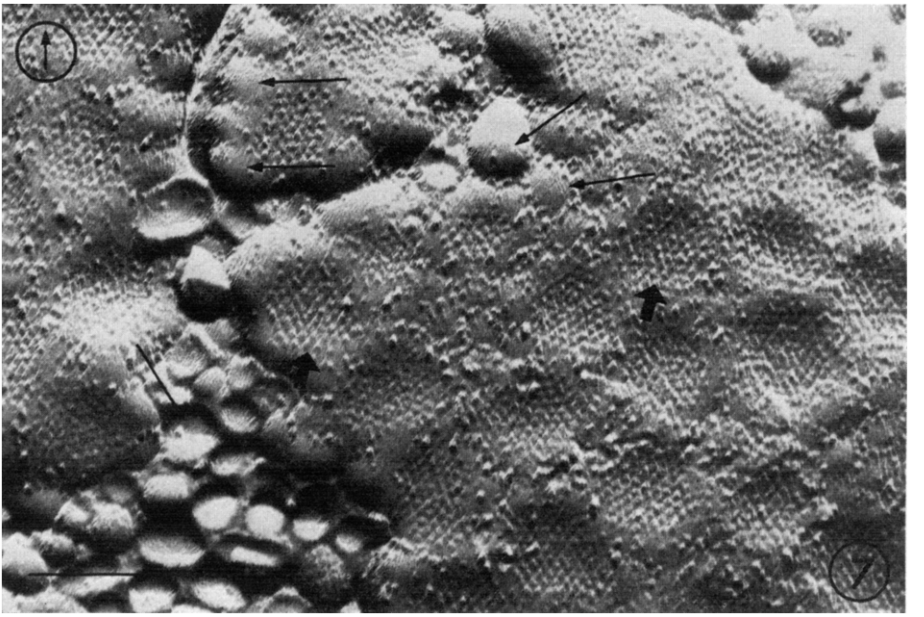


Fig. 7. The PF face of the freeze-fractured *S. cerevisiae* plasma membrane treated with pimaricin. Smooth circular areas measuring 800–1000 Å (arrows) are seen surrounded with membrane particles. 1000–1500 Å vesicles are produced in some cells. Intact hexagonal patterns of particle arrangement were seen (thick arrows). Magnification: ×80 000.

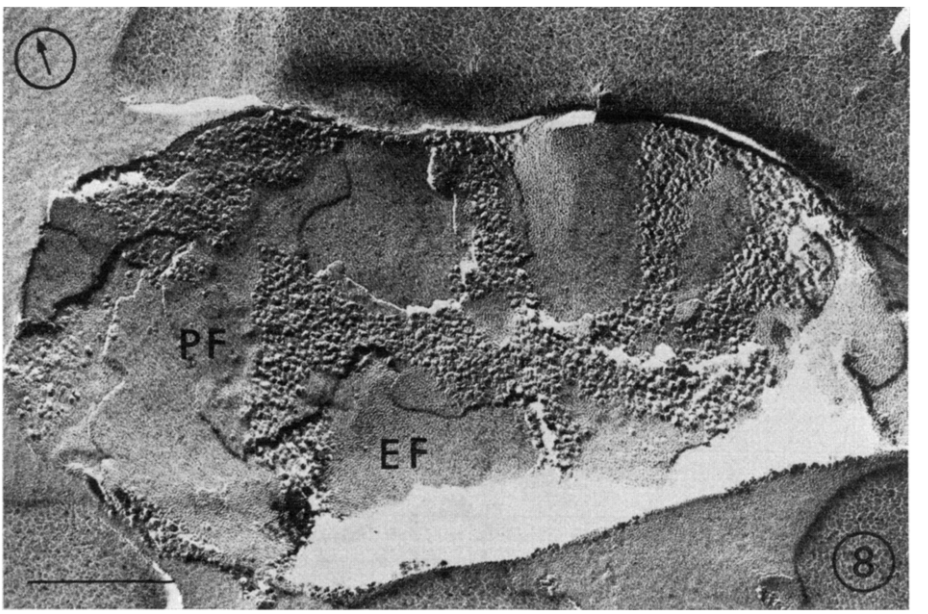


Fig. 8. Effects of pimaricin on the red blood cell ghost membranes as revealed by freeze-fracture. The membrane particles are strongly aggregated and smooth particle-free areas are formed on PF face (PF). Particle aggregations are also seen on the EF face (EF). Magnification: ×80 000.

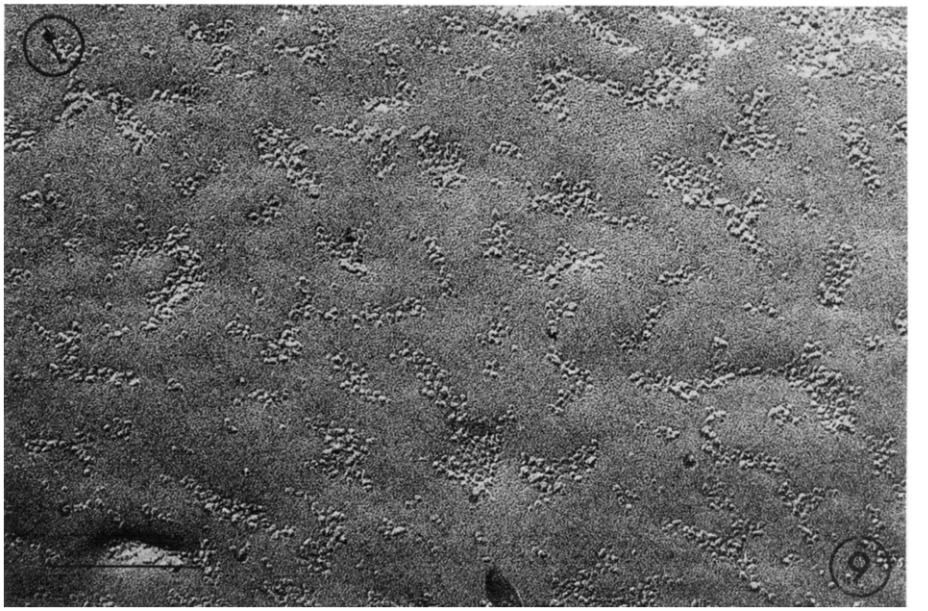


Fig. 9. Effects of amphotericin B on the freeze-fractured *E. floccosum* plasma membrane. Membrane particles aggregate into small particle islands surrounded with particle-free areas. Magnification: ×80 000.

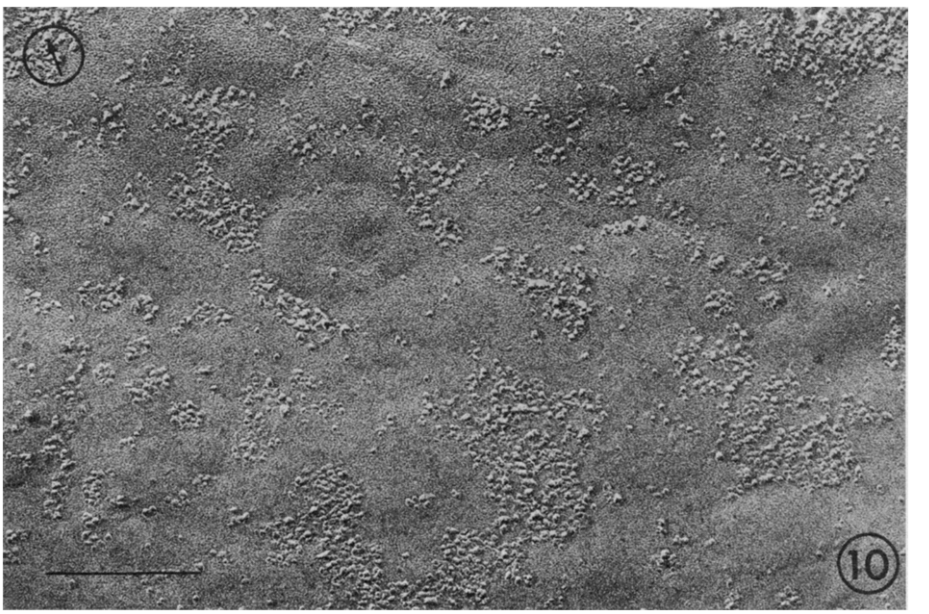


Fig. 10. Effects of nystatin on the freeze-fractured *E. froccosum* plasma membranes. Pronounced particle aggregation and wide particle-free areas are seen on the PF face. Magnification: ×80 000.

Fig. 11. Freeze-fracture micrograph of *S. cerevisiae* treated with nystatin showing the PF face of the plasma membrane. The particles aggregate around the original hexagonal patterns (arrows), which become unclear in some patterns. Magnification: × 80 000.

Effect of amphotericin B and nystatin

Both amphotericin B and nystatin clearly segregate the PF face of the plasma membrane of *E. floccosum* into particle-aggregated areas and free areas (Fig. 9 and Fig. 10), although they do not induce any significant alterations of the EF face. Since both of these polyenes have similar structures (Fig. 1), it is reasonable to expect that they produce similar effects, which, in turn, differ from those produced by filipin and pimaricin. Both polyenes also produce the particle aggregation on the PF face of *S. cerevisiae* plasma membrane. It is interesting to note that the particles aggregate around the original hexagonal patterns of particles, thus the patterns become unclear in some cells (Fig. 11, arrow).

The effects of these polyenes on red blood cells and the ghost membranes were also examined by negative staining and freeze-fracture electron microscopy. Similar particle aggregations to those seen on fungal membranes are revealed on the ghost membranes by freeze-fracture (not shown), while no alteration is detected by negative staining. It should be pointed out that whole red blood cells are hardly affected on the ultrastructure of the membrane with these polyene treatments, although their ghost membranes are easily influenced.

DISCUSSION

These freeze-fracture studies presented in this communication indicate that neutral, small amphoteric and large amphoteric polyenes produce different characteristic alterations on the plasma membranes of *E. floccosum*, *S. cerevisiae* and red

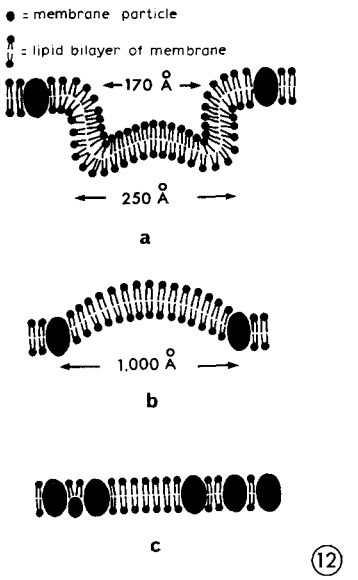


Fig. 12. Hypothetical scheme of the membrane alterations induced by three chemically different types of polyenes: (a) Pit-formation type ; Filipin, a neutral polyene, produces this type of alteration on fungal membranes and red blood cell membranes. (b) Network particle-aggregation type; Pimaricin, a small amphoteric polyene, produces this type of alteration on fungal plasma membranes. (c) Random particle-aggregation type; Amphotericin B and Nystatin, the large amphoteric polyenes, cause this type of alteration on fungal plasma membranes.

blood cell ghosts. Hypothetical models of three different lesions are proposed in Fig. 12 and designated as (1) pit-formation type, (2) network particle-aggregation type, and (3) random particle-aggregation type, so that these differences could be accounted for more clearly. Fig. 12a shows a model of filipin-induced alterations (pit-formation type). The dark center of the "ring" revealed by negative staining may correspond to the inside of the small pit or "invagination" protruding to the cytoplasmic side and the white "rim" of the "ring" to the membrane portion of the side "wall" of the "invagination". Intramembranous particles are excluded from these lesions. This model differs from that presented by De Kruijff [25], in that they do not show any "pit" in their model of cholesterol-filipin complex-induced membrane alterations. We feel that it is more likely that a "pit" occurs in the lesion, because the PF face of red blood cell and fungal membranes always show 150–170 Å excavations in freeze-fracture specimens. Our present findings also confirmed an observation of Tillack and Kinsky [13, 25] that these "pits" could not be equated with transverse holes. In contrast, pimaricin produces mild extrusions or elevations toward the outside of the cells (Fig. 12b), excluding intramembranous particles into the borders between these extrusions and forming a network structure seen on the fracture membrane planes (network particle-aggregation type). Amphotericin B and nystatin, on the other hand, appear to cause a translateral movement of the particles along the membrane plane in the broad areas (Fig. 12c), bringing about a random distribution of particulated areas and particle-free areas (random particle-aggregation type).

Since it is in the size of the macrolide ring that these compounds differ from

each other, these differences in membrane alterations could be caused by the difference in the size of this hydrophobic portion of the polyene structure.

It is also important to mention that these polyenes except filipin caused no detectable membrane particle rearrangement in whole red blood cells at the concentrations which are enough to produce the profound alterations of fungal cells, indicating that they have a higher affinity to ergosterol than cholesterol in membranes. Similar conclusions were derived by Archer and Gale [19] and Archer [26] from the investigations of K^+ release from *Candida* and *Mycoplasma* cells. They indicated that amphotericin methyl ester has a higher affinity for ergosterol than for cholesterol while filipin has reverse affinities. It seems likely that the existence of amino sugar base in the molecules is strongly related to their affinity to ergosterol, because filipin with less affinity to ergosterol has no sugar base. It seems, therefore, reasonable to use these polyenes in the clinical treatment against systemic fungal diseases. These polyenes, including even filipin, show a tendency to create more pronounced alterations in fungal membranes than in red blood cell membranes as long as they are treated in whole cells. This suggests that the sterol distribution in phospholipid bilayer membrane matrix is largely different between fungal plasma membranes and red blood cell membranes, so that ergosterol in fungal membranes could be more susceptible to polyenes than cholesterol in red blood cell membranes. Alternatively it could be suggested that the higher susceptibility to ghost membrane than whole red blood cells is caused by the membrane sidedness which has been recently accepted [27].

It is plausible that all these alterations are caused by "inactivation" of sterol function as a membrane fluidity stabilizer, due to the formation of sterol · polyene complexes in membranes, because the sterol-polyene interaction is considered to be stronger than the sterol-phospholipid interaction [14], and it does not change the amount of sterols in membranes [19]. Thus, the alteration of fluidity e.g. liquid crystalline-gel phase, could be induced and it may be the main mechanism of these polyene-induced lesions. It should be pointed out that filipin-induced lesions are morphologically similar to "rings" produced by treatment with O-haemolysin of *Clostridium perfringes* [28], streptolysin O [29], and θ-toxin (Nozawa et al., in preparation) which form specific complexes with cholesterol in membranes. Furthermore, similar alterations have been described by Seemen [30] in freeze-etch and negative stained erythrocyte membrane after immune lysis.

Since the proposed models in the present study could not show localization of the polyene · sterol complexes within the membrane, to make this point clear further detailed studies by physico-chemical technique should be necessary, therefore being now in progress.

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