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EFFECTS OF LIPID-PHASE SEPARATION ON THE FILIPIN ACTION ON MEMBRANES OF ERGOSTEROL-REPLACED *TETRAHYMENA* CELLS, AS STUDIED BY FREEZE-FRACTURE ELECTRON MICROSCOPY

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Summary

The effects of lipid-phase separation on the filipin action on pellicle membranes of ergosterol-replaced *Tetrahymena pyriformis* cells were studied by freeze-fracture electron microscopy. The pellicle membranes with phase separations induced by chilling from 34°C (growth temperature) to lower temperatures (30, 22 and 15°C) were treated with filipin. This produced filipin-induced lesions ("pits") only in the particulated (liquid) regions along the margin between solid and liquid domains, while they were produced in the particle-free (solid) areas when membranes were chilled to 15°C. The pellicle membranes with lesions induced by filipin at 34°C were chilled to 22°C. This chilling raised larger particle-free areas and more condensed particle-aggregations on the membranes than on the membranes without the filipin treatment. These results suggest that the membrane fluidity affects induction and development of the ergosterol-filipin complex in the membrane.

Introduction

Polyene antibiotics such as filipin, pimaricin, nystatin and amphotericin B are of biological interest because they selectively attack fungi and other organisms possessing sterols in their membranes [1,2], and because their target-sterol molecules play an important role in maintaining the proper membrane fluidity and are referred as to membrane stabilizers [3]. Therefore, many investigations of the interaction between polyene antibiotics and sterols (mainly cholesterol) [4—6] have been concerned with both the mechanism of polyene action and the function of sterol in the membrane. However, very little is

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known about the nature of the polyene interaction with membrane-ergosterol, which is characteristic of fungal plasma membranes.

We began by examining polyene actions on fungal membranes [7,8]. One of the major difficulties encountered in studying the ergosterol-polyene interaction has been to isolate ergosterol-containing membranes from the fungal cells with rigid cell walls. As a consequence, most studies so far have been based on whole cells [9—12], cholesterol-containing membranes [13,14] or artificial membranes [9,12,15,16].

Recently, an alternative system for obtaining ergosterol-containing membranes has been developed in our laboratory using *Tetrahymena* cells. The native sterol-like molecules of the pentacyclic triterpenoid tetrahymanol could be completely replaced by ergosterol [17]. Since the *Tetrahymena* membrane system has proved to be an excellent model for studying the dynamic changes in membrane composition, physical state, and functions [18], it seemed reasonable to apply this system for studies of relationship between polyene-ergosterol complex formation and membrane dynamics.

In the present study, we examined the effect of lipid-phase separation on susceptibility of ergosterol-containing *Tetrahymena* membranes to a polyene antibiotic, filipin. In addition, we performed freeze-fracture, ultrastructural analyses of the molecular lesion in the membrane induced by the filipin-ergosterol complex formation. Filipin was employed because it exerts the most distinctive alterations on membrane ultrastructure compared to all other polyenes [8].

It is suggested that the local environmental fluidity participates in initiation and development of the filipin-induced molecular lesion in the membrane.

Materials and Methods

Growth of T. pyriformis and ergosterol-replacement

T. pyriformis WH-14 was grown at 34°C in an enriched proteose-peptone medium as described previously [20].

For the ergosterol-replacement experiment, the cells were grown in a medium supplemented with exogenous ergosterol. Ergosterol (2 mg/200 ml culture) was added to the growth medium at 50°C as an ethanolic solution (0.4 ml) before inoculation, and was thus dispersed homogeneously in the medium. The culture medium was inoculated with a 4-day-old cell culture which had already been adapted to ergosterol supplementation. Cells were harvested in the mid-log phase of growth.

Isolation of surface membranes from T. pyriformis

The surface membranes, pellicles and cilia were prepared from the native and ergosterol-replaced cells, according to the method using a high phosphate buffer (0.2 M K_2HPO_4/0.2 M KH_2PO_4/3 mM EDTA/0.1 M NaCl, pH 7.2) as previously described [20].

Lipid extraction and analysis

Lipids were extracted from membrane fractions by the method of Bligh and Dyer [21] and the resultant lipid solution was stored in chloroform/methanol (6 : 1, v/v) at −20°C.

Quantitative analysis of tetrahymanol and ergosterol was performed following the method described previously [22], using cholesterol as an internal standard. Care was taken not to expose ergosterol-containing samples too long to light and air.

Filipin treatment of isolated surface membranes

Membrane suspensions in the phosphate buffer were rewarmed to 34°C (growth temperature). Part of the membrane sample was incubated with filipin (Upjohn, Kalamazoo, Mich., U.S.A.) at a concentration of 50 μg/ml at 34°C for 30 min and then fixed at 34°C with 1% glutaraldehyde solution buffered with sodium phosphate buffer (0.1 M, pH 7.2) for 15 min in preparation for freeze-fracturing. Other aliquots of the membrane suspension were chilled to 30, 22, and 15°C over 4 min and incubated with filipin at a concentration of 50 μg/ml at 30, 22, and 15°C for 30 min, respectively. The membrane suspensions were then fixed at these temperatures by quickly adding an equal volume of 2% glutaraldehyde, pre-cooled to the required temperature.

The remaining aliquots of the membrane suspensions were first incubated with filipin at the same concentration, then chilled to 30, 22, and 15°C over 4 min, and kept for 30 min. These suspensions were subsequently fixed as above.

Freeze-fracture electron microscopy

The fixed samples were transferred to increasing concentrations of glycerol, terminating with a 24-h incubation with 30% glycerol in 0.95% NaCl solution. The cells were pelleted in a clinical centrifuge, frozen in liquid Freon-12 and kept in liquid nitrogen until ready for fracturing in a Hitachi HFZ-1 freeze-etching device. Replicas, prepared by platinum-carbon followed by carbon shadowing, were floated onto distilled water, and cleaned by adding several drops of hypochlorite, followed by soaking in 75% H_2SO_4 for 24 h. The replicas were then given three rinses in water prior to collection on 400-mesh grids. Electron-microscopic observations were performed using a JEM 100-U electron microscope.

Results

Phase separation in untreated pellicle and cilia membranes of ergosterol-replaced Tetrahymena cells

The *Tetrahymena* pellicle consists of the plasma membrane, and the outer and inner alveolar membranes [19] while cilia are surrounded by the plasma membrane alone. Freeze-fracture replicas reveal two faces of the cytoplasmic leaflet and the exoplasmic leaflet of individual membranes due to separation of the apposed hydrophobic ends of the phospholipid bilayers. The former face is henceforth referred to as the PF face and the latter as the EF face [23]. Identification of membranes and their fracture faces in the *Tetrahymena* pellicle system was described previously [19,24,25].

Completion of ergosterol-replacement was confirmed by gas-liquid chromatography. The saponified lipid fraction extracted from *Tetrahymena* membranes showed a single peak of ergosterol instead of tetrahymanol after a 24-h

incubation in the ergosterol-supplemented medium.

Replicas of ergosterol-replaced *Tetrahymena* pellicle membranes showed no significant difference from those of native cells containing tetrahymanol [24, 25]. The PF face of the outer alveolar membranes was randomly associated with a larger number of particles (≈110 Å in diameter) than the EF face of the membranes [19].

Phase separation was induced by rapid chilling from the growth temperature (34°C) to 30, 22 and 15°C. Whereas chilling to 30°C produced a mild phase separation, the membranes cooled to 15°C revealed much larger phase separations. Thus, the phase separation of membrane lipids is visualized in freeze-fracture replicas as a segregation of the membrane into smooth regions devoid of particles and particle-aggregated areas (Fig. 1). Fig. 1 showed the phase separations observed on the PF face of an outer alveolar membrane, which were induced by chilling cells to 22°C. These observations have been discussed in more detail in other papers [18,24—26].

Effects of filipin on the ergosterol-replaced membranes

Numerous lesions induced by filipin were identified as "pits", or small invaginations of membranes viewed from the exoplasmic to cytoplasmic side. The pits gave the appearance of small, round protrusions measuring 250—300 Å in diameter on the EF faces of the pellicle membranes (Fig. 2) and depressions of 170 Å diameter on the PF faces [8]. They usually appear in clusters while individual pits maintain a constant distance (approx. 300 Å) from each other forming equilateral triangles, as indicated by solid lines in Fig. 2. The larger

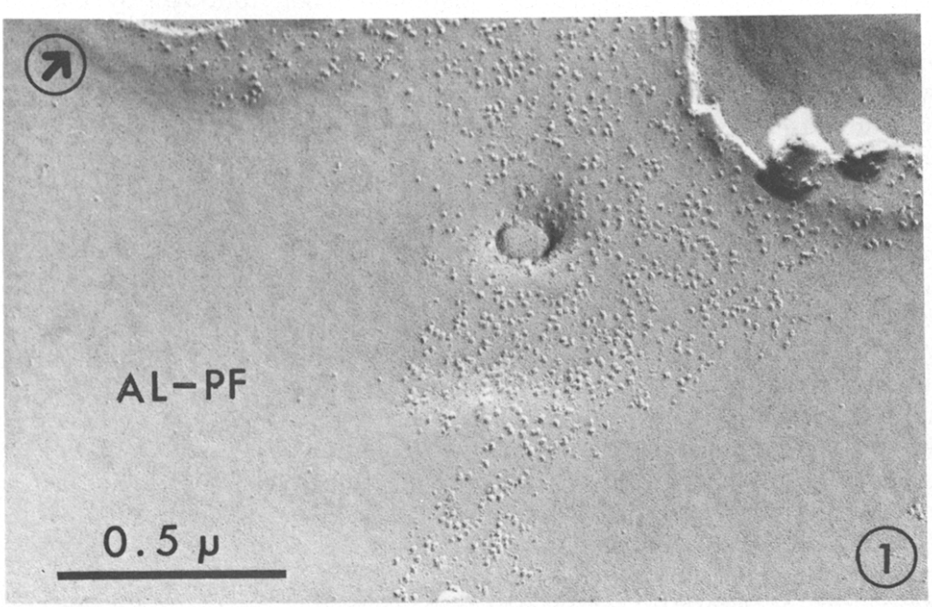

Fig. 1. Phase separation exerted in the outer alveolar membrane by chilling cells from 34 to 22°C. Membrane particles were excluded to produce the particle-free (gel-phase) and particle-dense (liquid-phase) areas. AL-PF, PF face of the outer alveolar membrane.

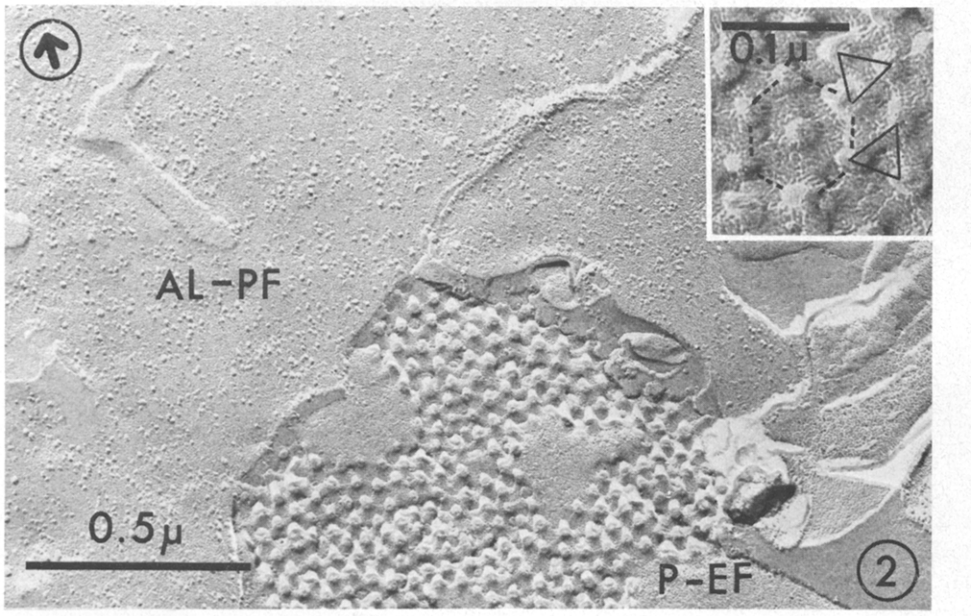

Fig. 2. Effects of filipin on the plasma membrane. Small protrusions were produced in clusters on the EF face of the plasma membrane (P-EF). Alveolar membranes are not yet affected. AL-PF, PF face of the outer alveolar membrane. Insert: individual protrusions are approx. 250 Å in diameter and apparently maintain a contant distance of approx. 300 Å from each other so that equilateral triangles (solid lines) and a hexagonal pattern (broken lines) can be traced.

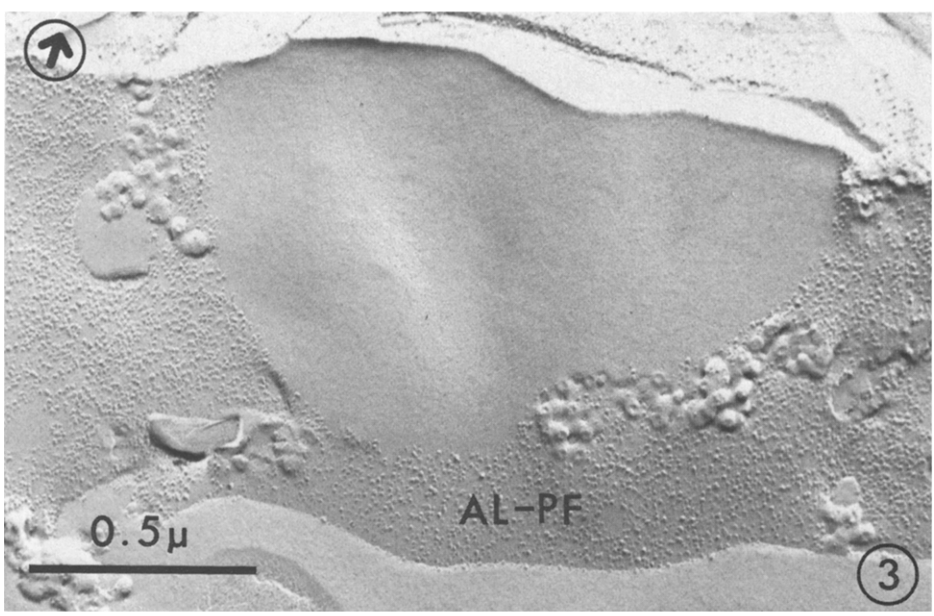

Fig. 3. Effects of filipin on the moderately phase-separated alveolar membrane. Pellicles were chilled from 34 to 30°C over 4 min, then treated with filipin. Pits were formed along the margin between the particle-rich and the particle-free regions.

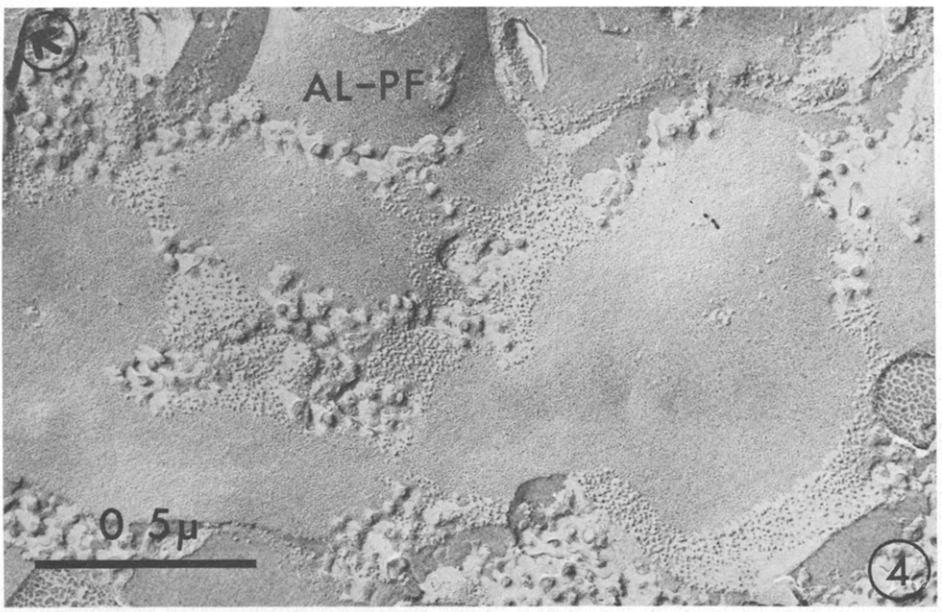

Fig. 4. Effects of filipin on the phase-separated alveolar membrane. Pellicles were chilled from 34 to 22 C, then treated with filipin. Pits were produced much more than the case shown in Fig. 3, and seen only in the particulated area (liquid-phase area).

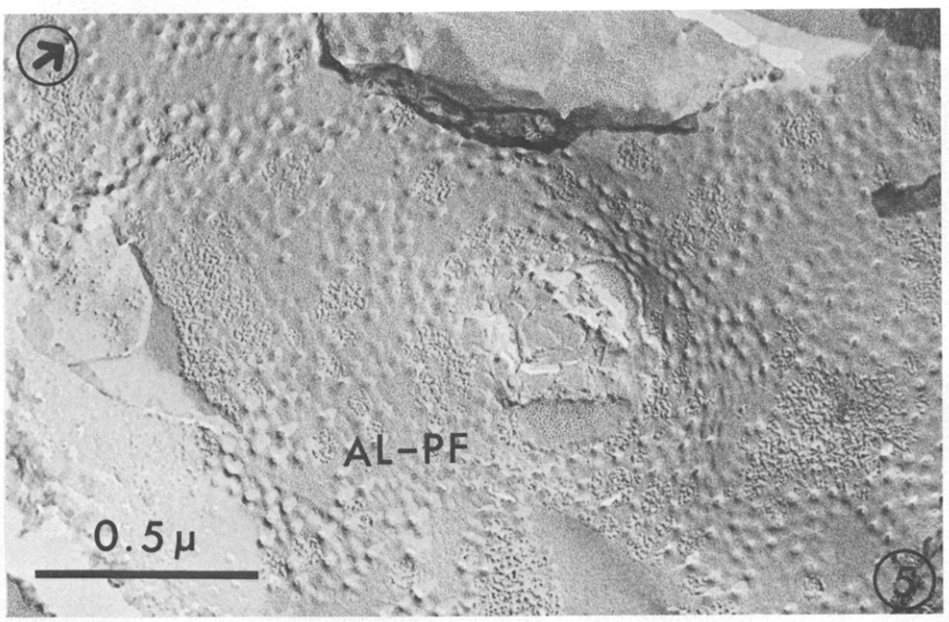

Fig. 5. Effects of filipin on the prominently phase-separated alveolar membrane. Pellicles were chilled from 34°C to 15°C over a 4-min period, then treated with filipin. Pits were produced in the particle-free area (gel-phase area).

clusters reveal that the pits were arranged systematically so that a hexagonal pattern was recognizable, as traced with broken lines on Fig. 2.

Effects of filipin on the pellicular membrane with phase separation

The pellicles were first chilled from 34°C to lower temperatures, and kept

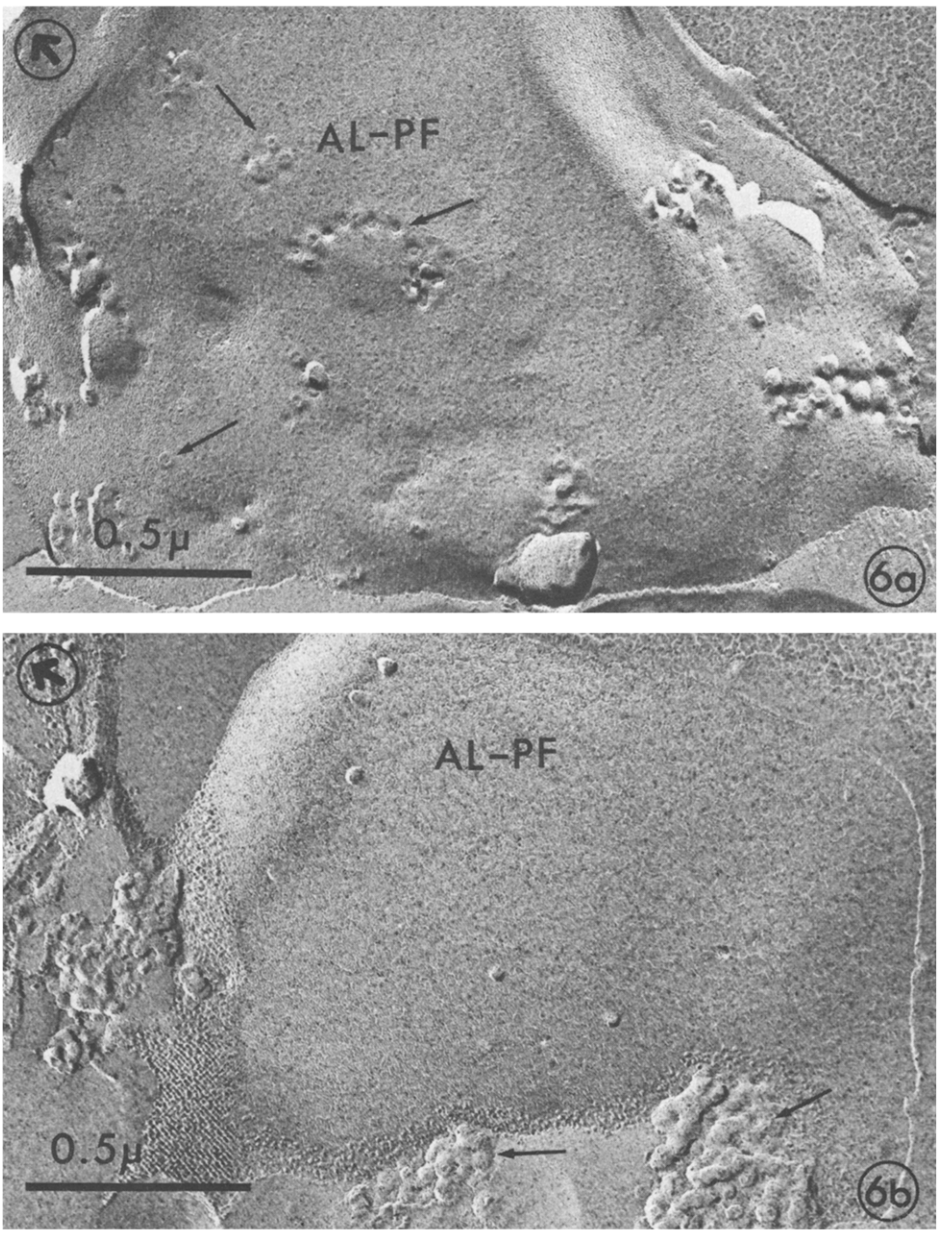

Fig. 6. Effects of chilling on the filipin-induced lesions in alveolar membranes. Pellicles were treated first with filipin, then chilled from 34 to 22°C over a 4-min period. (a) Pits were decreased in their depth, suggesting to be fading out (arrows). (b) Particles were densely aggregated. Aggregation of pits is seen on the particulated area (arrows), and extremely wide particle-free areas were produced.

for 4 min, filipin was then added as described earlier. The clusters of pits produced by filipin treatment were observed along the margin between the particle-free areas and the particle-studded regions of membranes in 30°C (Fig. 3) and 22°C chilled (Fig. 4). These "pits" were observed to spread only into the particulated area (Fig. 3 and 4). By contrast, no "pit" lesions were detected in the particle-free regions. The density of particles observed between "pit" lesions in particle-rich regions is higher for 22°C-treated than for 30°C-treated membranes. When the membranes were chilled to 15°C the particles are much more condensed and the pits were absent from the particle-packed areas and were, instead, observed in the particle-free regions of the membrane (Fig. 5). Individual pits were less prominent, having a rather smooth appearance, in contrast to the pits produced in particulated areas at higher temperatures (22, 30°C; Fig. 3 and 4).

Effects of rapid chilling on the membrane-lesions caused by filipin

Membranes with lesions induced by incubation with filipin for 30 min at 34°C were exposed to rapid chilling at 22°C, thus forming a distinct phase separation. The filipin-treated membranes demonstrated further morphological alteration due to this phase separation (Fig. 6). The particle-free areas expanded and particles aggregated more densely than in filipin-free specimens fixed at the same chilling temperature (22°C). In the particle-free regions, the pits and protrutions became less prominent and shallow "ring-like" structures were occasionally observed (Fig. 6a, arrows). On the other hand, the lesions in the particulated areas seemed to increase drastically in their depth, height and size of the clusters (Fig. 6b, arrows).

Discussion

Using ergosterol-supplemented *Tetrahymena* cell, we were able to study the effects of filipin on the ergosterol-containing membranes isolated from this free-living organism. The ultrastructural alterations (formation of pits and protrutions) in the ergosterol-containing membranes induced by filipin were apparently identical to those observed in the cholesterol-containing liposomes and red blood cell membranes reported by Tillack and Kinsky [15].

The uniformly spaced arrangement of the "pits" producing hexagonal patterns suggests a systemic molecular interaction between polyene and ergosterol, as proposed earlier by de Kruijff and Demel [12]. Our observation that lesions were not produced solitarily but always in clusters led us to suggest that the first pit creates a microenvironmental membrane alteration around itself which leads to formation of adjacent pits.

Of significance were the effects of membrane-fluidity changes on the filipin actions in the temperature-sensitive alveolar membrane of *Tetrahymena*. The alveolar membrane is located on the inner side of the plasma membrane and has been shown to be so temperature-responsive that the change in a few degrees in environmental temperature induces lipid-phase separation of the membrane [24]. The particle-free areas revealed in freeze-fracture replicas would correspond to the crystallized lipid (gel-phase) areas induced by chilling below the lipid-phase transition temperature [24,28,29]. The membrane particles are

excluded from the solid-crystalline areas and move into the fluid areas, where the particles aggregate. Also, such emergence of particle-free areas might partially result from perpendicular movement of membrane particles, as described in endoplasmic reticulum of *Tetrahymena* [26]. Therefore, the particle-free areas are interpreted to represent the gel-phase regions of the membrane while the particulated areas represent the liquid-phase domains in freeze-fracture replicas [24,27,29].

Lesions along the margin between particle-free and particle-rich areas were produced when membranes were treated with filipin after chilling from 34 to 30°C. This is particurally interesting because it is plausible that the lateral compression in the gel-phase regions opens a loose space between the two physically different phases. In this regard, it is relevant to note Shimshick and McConnell's concept [30] that the coexistence of two lipid phases facilitates membrane transport through the extended liquid-phase regions of membranes, which are induced by compression of the crystalline domains. This idea may also explain the reason why the lesions developed in particulated (liquid-phase) areas, but not in particle-free (gel-phase) regions (Fig. 3 and 4). The liquid-phase domains are extended enought to let filipin molecules insert into lipid bilayers easier than the solid compressed area. However, the possibility that ergosterol-filipin complexes might be formed which were not visualized as pits in the freeze-fracture picture [11] cannot be completely ruled out.

When the membrane was phase-separated by drastic chilling to 15°C, the liquid-phase area was conversely more compressed than the gel-phase area because the protein particles aggregated tightly at the liquid-phase region. Thus, filipin-lesions occurred in the solid (gel-phase) domains (Fig. 5). The formation of the lesions on the gel-phase area may indicate that the sterol molecules were not squeezed out from the gel-phase area, rather remained, while the protein particles were excluded.

As for the effects of physical state of membrane phospholipids, Blau and Bittman [31] reported that no discontinuity in Arrhenius activation energy for filipin-cholesterol complexation in liposomes was found at the lipid-phase transition temperature. Although no change in affinity of filipin for ergosterol was detected in the present study, it should be pointed out that the filipin-induced lesions occurred only in liquid phase domains, but not in the gel-phase area of the phase-separated membrane (Fig. 3 and 4). This suggests that the lipid physical state probably affects the mechanism of filipin-ergosterol interaction in membranes.

In *Acholeplasma laidlawii*, polyene antibiotics including filipin shift back the discontinuity in the Arrhenius plot of the ATPase activity toward the temperature observed in absence of cholesterol [11]. This suggests that dissociation of cholesterol molecules from membrane phospholipid molecules changes the membrane fluidity. The results of our experiments showed that filipin treatment of the alveolar membrane altered its fluidity. Much wider particle-free areas were produced than in membranes without filipin treatment at the same chilling temperature (22°C), as shown in Fig. 6. However, we could not quantify the intensity of these changes of phase separation with and without filipin treatment. This suggests that the filipin treatment made the membrane more sensitive to temperature-induced phase separation, which

probably means an alteration of the membrane fluidity.

The disappearance of the lesions (pits or protrusions) could be due to vertical movement of the filipin-sterol complex or filipin molecules alone rather than their lateral displacement into liquid domains, since a random distribution of the incomplete pits and protrusions were observed in particle-free, solid areas (Fig. 6a).

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