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MEMBRANES OF *TETRAHYMENA*

II. DIRECT VISUALIZATION OF REVERSIBLE TRANSITIONS IN BIOMEMBRANE STRUCTURE INDUCED BY TEMPERATURE

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SUMMARY

Rapid temperature changes cause reversible structural transitions in the alveolar membranes of the poikilothermic eukaryote *Tetrahymena* as revealed by freeze–etch electron microscopy. At an optimal growth temperature of 28 °C, 115-Å particles are randomly distributed on the outer faces of the fractured alveolar membranes and apparently corresponding holes are seen on the inner faces. After chilling the cells to 5 °C, these particles and holes are largely aggregated. Reheating the cells to 28 °C causes a random redistribution of particles and holes. This temperature-induced phenomenon of reversible particle aggregation is discussed with respect to movement of membrane components. We conclude that membrane components move translationally and/or normally to the membrane plane which may be important for transport processes within and across biomembranes.

INTRODUCTION

Though biomembranes play an important role in nearly all fundamental biological processes, their molecular organization is not clear. However, current hypotheses of membrane structure envisage these entities to comprise mobile arrays of proteins associated with characteristic lipids, held together by noncovalent hydrophobic bonds. The apolar domains of membrane lipids and proteins presumably form the core, while their polar domains relate to the two surfaces and to possible aqueous transmembrane channels[1–3].

Biomembranes respond to variations in environment; for instance, temperature modifies essential membrane characteristics such as permeability and excitability[4–7]. Such functional effects imply changes of molecular membrane organization and are reflected by the fact that the differential thermal calorimetry signals from Mycoplasma membranes are reversibly altered by temperature[8] (*cf.* also refs 9, 10). The changes observed are ascribed to reversible liquid crystalline⇌crystalline transitions of phospholipid bilayer arrays within the membrane of this organism.

In this context one is eager to learn whether the reversible membrane transitions detected by indirect, averaging methods such as calorimetry, can also be revealed

directly. Freeze–etch electron microscopy is particularly appropriate for this purpose since it reveals large areas of the hydrophobic membrane matrix; at cleavage, the membrane is split and two hydrophobic faces of the membrane core are exposed[11–12]. These fractured faces are usually covered with randomly distributed particles. Though the real nature of these particles is uncertain, there is indirect evidence that they represent proteins and/or glycoproteins complexed with lipids[13–15] and that some are actually 'channel proteins'[16].

We have accordingly utilized freeze-etch electron microscopy to identify temperature-sensitive components and domains in membranes and herein demonstrate a temperature-induced redistribution of membrane-intercalated particles in cytomembranes of the poikilothermic eukaryote *Tetrahymena pyriformis* GL.

MATERIALS AND METHODS

The ciliate protozoan *Tetrahymena pyriformis* amicronucleate strain GL, was propagated axenically in the "steady-state" logarithmic growth phase at 28 °C in culture flasks containing 400 ml of 2% proteose peptone – 0.4% liver extract medium. To chill the cells, the flasks were cooled directly to 5 °C within 4 min,

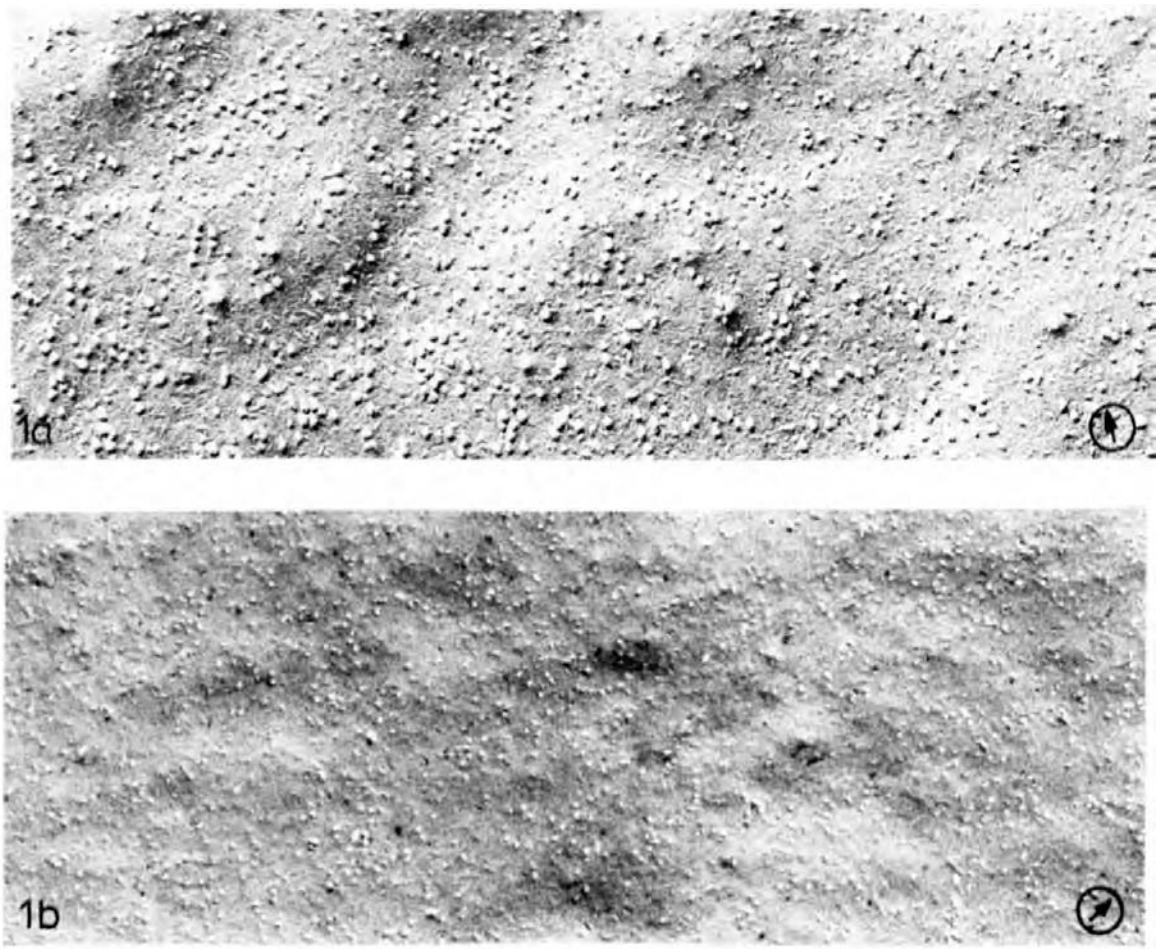

Fig. 1. *Tetrahymena* cells grown at 28 °C. Randomly distributed 115-Å particles on the outer (a) and obviously corresponding holes on the inner face (b) of the fractured alveolar membranes. Pre-fixed with glutaraldehyde and glycerinated. 80000×.

by use of an ice–salt mixture. Samples for freeze-etching, were taken at 28 °C, 22 °C, 18 °C, 15 °C, 12 °C, 10 °C and 5 °C. Alternatively, the cells were pelleted and, after 10 min at 28 °C transferred into precooled 5 mM Tris–HCl 10 mM magnesium acetate (pH 6.8) (Tris–Mg^{2+} medium). The latter procedure effects the temperature change within a few seconds. The cells were then kept at 5 °C for 10 min and subsequently warmed within 25 s to the optimal growth temperature of 28 °C. *Tetrahymena* cells survive these manipulations without severe damage. Samples for freeze-etching were taken from the cultures just prior and immediately after the temperature shift to 5 °C, as well as at the beginning and end of the exposure to 5 °C.

In other experiments, *Tetrahymena* cells grown in proteose peptone–liver extract medium were washed with distilled water 3 times at 28 °C for about 40 min, and incubated with distilled water at 5 °C. In general, the samples were fixed for 10–30 min at isolation temperature using 2.5% glutaraldehyde dissolved in Tris–Mg^{2+} medium. The following controls were performed to exclude fixation artifacts: (a) samples were fixed at 28 °C with 2.5% glutaraldehyde, immediately cooled to 5 °C and all further manipulations carried out at this temperature. In addition, some

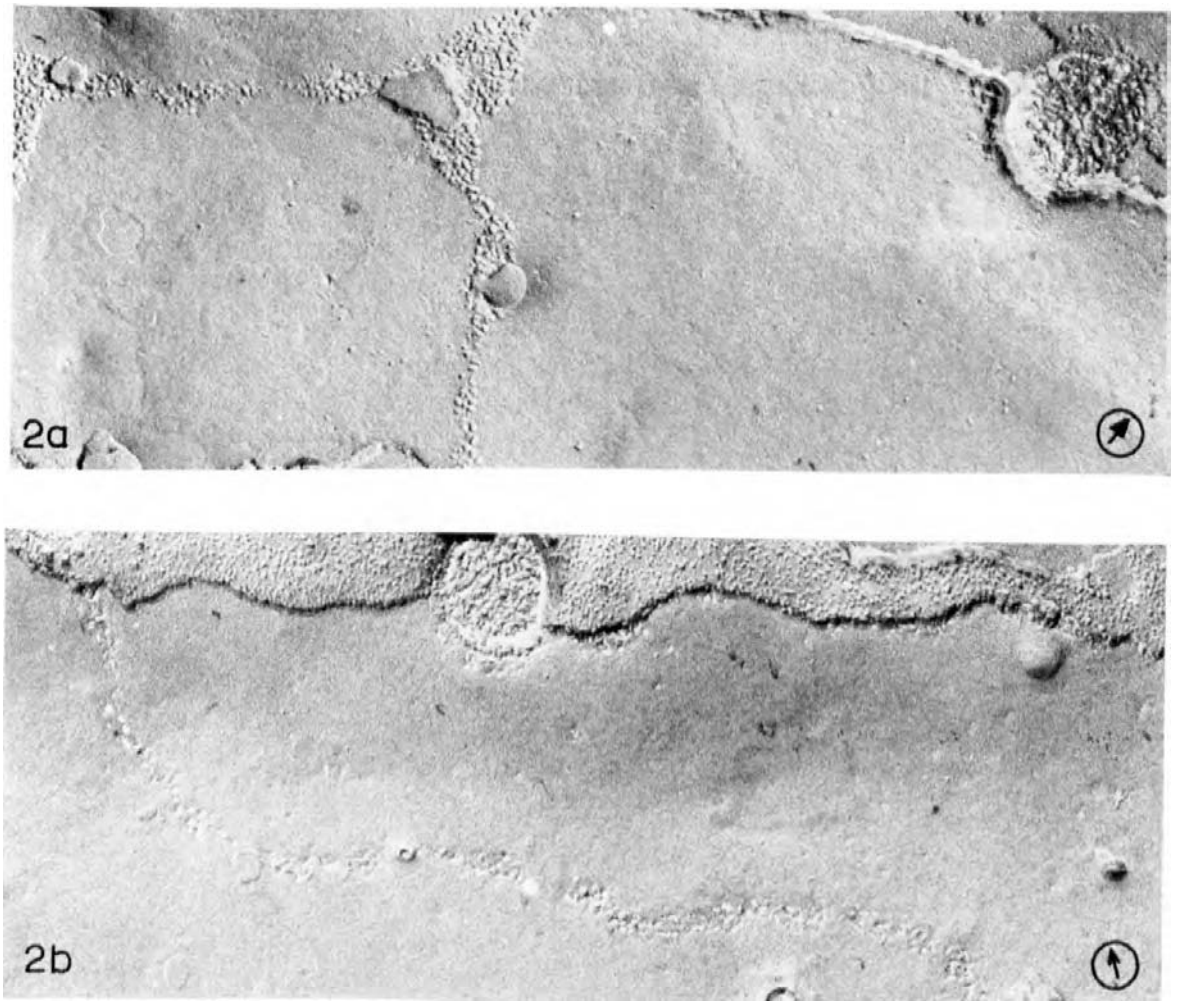

Fig. 2. *Tetrahymena* cells slowly chilled to 5 °C. Particles on the outer (a) and holes on the inner face (b) of the fractured alveolar membrane are strongly aggregated. A significant decrease in particle number is observed. In the upper part of b, the inner face of the fractured plasma membrane is revealed. Pre-fixed with glutaraldehyde and glycerinated. 80000 ×.

cells grown at 5 °C or 28 °C were incubated in glycerol solutions of the same temperatures without any fixation.

After washing out the glutaraldehyde, the cells were incubated in graded solutions of glycerol at concentrations up to 25% in Tris–Mg^{2+} medium for 2–3 h. The glycerinated cells were then rapidly frozen in liquid Freon 22. Fracturing, etching and replicating was in a Balzers Model BA 360 M machine at –100 °C; etching was for 1 min. The replicas were examined in a Siemens Elmiskop IA. Encircled arrows indicate shadowing direction on the micrographs.

The particle numbers on the fractured faces were evaluated on arbitrary 0.2 μm^2 areas of at least 10 different cells using calibrated positives.

RESULTS

At 28 °C, all freeze-fractured membranes of *Tetrahymena* normally show a random distribution of membrane-associated particles. At 5 °C, however, the particles aggregate to a variable degree. Because this is most dramatic in the membranes of the alveolar sacs lying just below the plasma membrane, we have concentrated on these. This membrane type contains about 3 times as much protein as phospholipid[17].

At 28 °C, the outer fractured alveolar membranes reveal about 1100 ± 200 randomly distributed 115-Å particles per μm^2 on the membrane face oriented toward the plasma membrane (+face; *cf.* Fig. 1a) and about 1100 ± 200 smaller holes on the face oriented toward the interior of the alveolar sacs (−face; Fig. 1b; *cf.* also ref. 18). Obviously, the deposition of the shadowing material tends to increase the apparent size of particles and decrease the apparent size of holes. Moreover, a random distribution of particles and holes is revealed in cells at 22 °C and 18 °C. At 15 °C, however, particles and holes begin to aggregate (Fig. 3). The membranes of organisms slowly cooled to 5 °C reveal a large degree of aggregation of the particles and holes into mostly linear arrays and some small plaques at 10 °C and 5 °C (Figs 2a and 2b); at the same time, the number of particles decreases significantly to 10–600 per μm^2 at 5 °C.

Particle aggregation is blocked by glutaraldehyde and is not seen in cells which were fixed with glutaraldehyde at 28 °C before cooling (Fig. 7). The membranes of

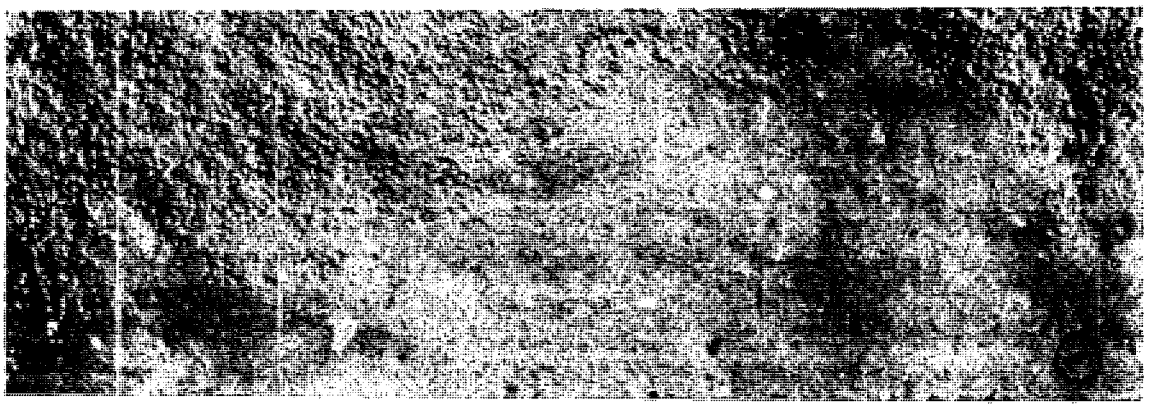

Fig. 3. *Tetrahymena* cell at 15 °C. Particles on the outer face of the alveolar membrane begin to aggregate. Pre-fixed with glutaraldehyde, glycerinated. 50000×.

cells cooled to 5 °C within 5 s also show particle (hole) aggregation, but into a net-like pattern without immediate reduction in particle number (Figs 4a and 4b). However, after 10 min at 5 °C the particle number does diminish to about 500–1000 per μm^2. Within 30 s after reheating to 28 °C the alveolar membranes of many *Tetrahymena* cells again exhibit the normal random distribution and number of particles (Fig. 5). However, other cells still show a little particle aggregation. This aggregation is similar to that of cells grown in distilled water at 5 °C. In this case, the particles are not so largely aggregated on cooling as in cells grown in proteose peptone–liver extract medium at 5 °C (Fig. 6).

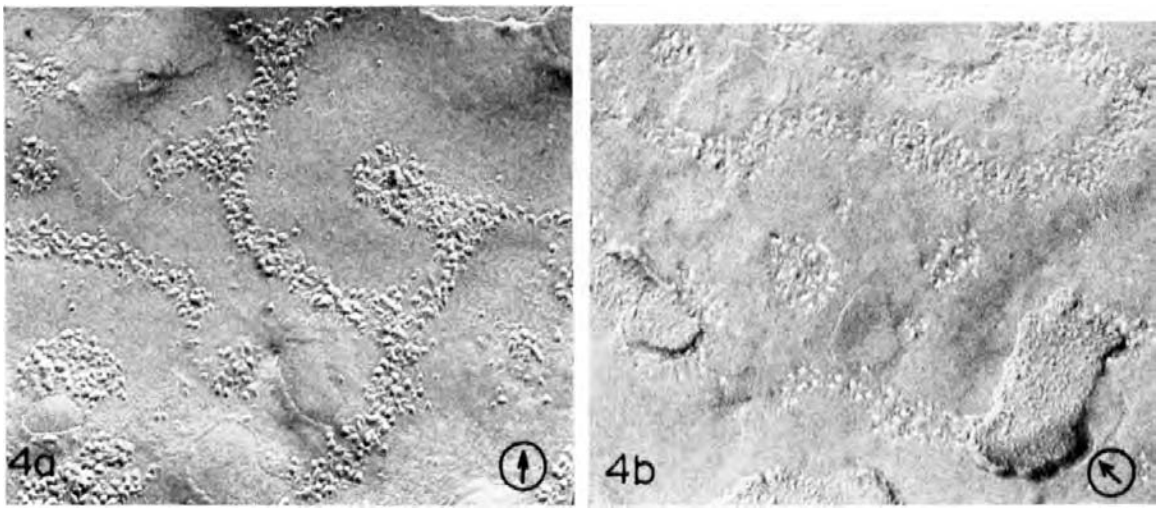

Fig. 4. *Tetrahymena* cells quickly chilled to 5 °C. Particles on the outer (a) and holes on the inner face (b) of the fractured alveolar membranes are aggregated. No significant decrease in particle number is observed. Pre-fixed with glutaraldehyde, glycerinated. 80000 ×.

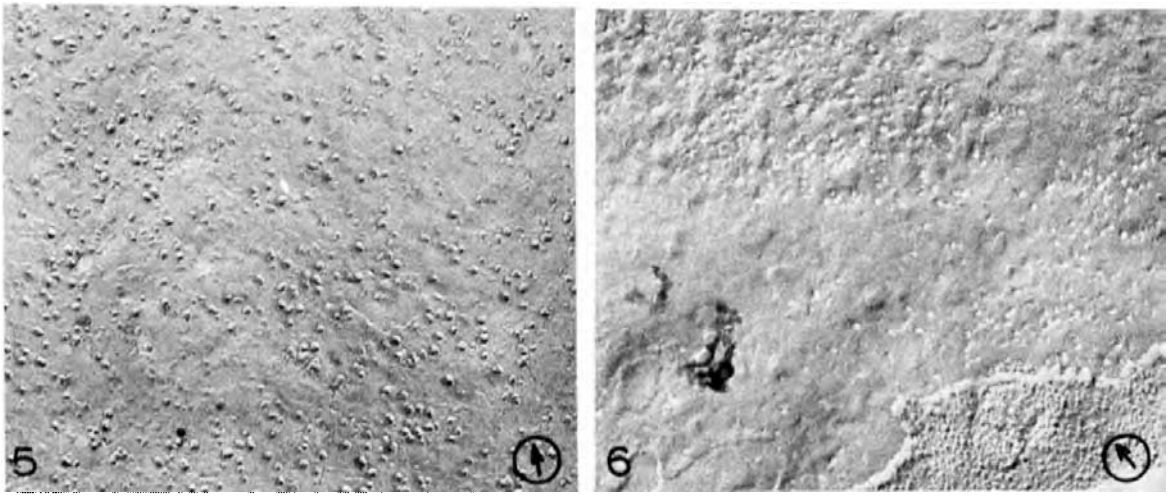

Fig. 5. *Tetrahymena* cell quickly chilled to 5 °C, left at 5 °C for 10 min, and then reheated to 28 °C. The original membrane state with randomly distributed particles is restored. Pre-fixed with glutaraldehyde, glycerinated. 80000 ×.

Fig. 6. *Tetrahymena* cell grown in distilled water and quickly chilled to 5 °C. Holes on the inner face of the fractured alveolar membrane are not strongly aggregated. Pre-fixed with glutaraldehyde, glycerinated. 80000 ×.

Unfixed cells, processed at 5 °C or 28 °C until freezing, show the same structural features on the membranes as comparable glutaraldehyde-fixed cells, excluding severe glutaraldehyde effect on the freeze–etch appearence of these membrane types.

Fig. 7. *Tetrahymena* cell grown at 28 °C, fixed with glutaraldehyde and immediately chilled to 5 °C. The particles on the outer face of the fractured membranes of two adjacent alveolar sacs are not aggregated. Glycerinated. 80000×.

DISCUSSION

We have shown by freeze–etch electron microscopy that temperature changes can quickly and reversibly alter biomembrane topology in *Tetrahymena*. This phenomenon elucidates some problems concerning the disposition and behaviour of membrane-associated particles in freeze–etched membranes though the real nature of these particles still remains uncertain.

Normally the membrane-associated particles on the +face and the corresponding holes on the −face are randomly distributed. However, at low temperatures both the particles on the +face and the holes on the −face of the fractured alveolar membranes lose their random distribution and aggregate. These clumps revert to the normal, irregular distribution upon return to optimal growth temperature. Moreover, the particle number and the hole number per μm^2 of fractured alveolar membrane return to the same range. This suggests that the particles and holes correspond, *i.e.* that the particles associated only with the +face protrude significantly into the opposite −face. Thus, we assume that the observed particles penetrate the entire membrane core of the alveolar membranes.

The phenomenon of particle aggregation in freeze-etched membranes has been previously observed in freeze-etched erythrocyte ghosts after phospholipase C treatment[19] and after massive trypsination[13]. Very long exposure to trypsin causes a significant loss of erythrocyte membrane-associated particles (ref. 20; Speth, V., unpublished). These findings have been interpreted as evidence that membrane-associated particles are at least partly proteins. This partly proteinaceous nature of particles is also indicated by our finding that glutaraldehyde, which reacts essentially with the protein moieties of membranes, hinders the aggregation of membrane-intercalated particles. Conceivably, cross-linking of a connecting network of various membrane proteins would hinder particle aggregation.

We observe that particle aggregation is far less in alveolar membranes of cells grown in distilled water at 5 °C instead of in the proteose peptone–liver extract medium. We tend to correlate the severe intracellular ion-concentration deficit of cells grown in distilled water (Wunderlich, F. and Speth, V., unpublished) with the impaired aggregation process at low salt levels. However, ions, being very polar, are always concentrated into aqueous domains, although electrostatic attractions and repulsions between ions are greater in apolar, non-aqueous media, than in media of the ionic composition we usually employ. Our experiments therefore do not allow us to pinpoint the loci of the salt effects.

The reversible particle aggregation we see relates to Pinto da Silva's[16] observation that membrane-intercalated particles of freeze-fractured human erythrocyte ghosts can aggregate reversibly within 2–4 min as a function of pH. However, he did not find a diminution of particle number and suggests that membrane-intercalated particles move tangentially within the membrane plane. Indirect evidence for such movement has also been reported by Frye and Edidin[21] who find that, shortly after formation of human–mouse heterokaryons, the species-specific membrane antigens, revealed by the indirect fluorescent antibody method, remain in the different halves of the fused cells' membranes. However, after about 40 min at 37 °C, these antigens appear randomly redistributed over the surface of the heterokaryon. Such tangential translation may also apply to our finding that particles aggregate within 5 s after cooling without essential decrease in the particle numbers observed. Moreover, our finding, that after 10 min at low temperature the particles are still aggregated, is also consistent with the finding of Frye and Edidin[21], since in their fused human–mouse heterokaryons the re-intermixing of the cell membrane components is not influenced by protein synthesis inhibitors or ATP formation, but sharply decreases below 15 °C.

How is it that after 10 min at low temperature, or upon slow cooling, the number of particles per unit area in *Tetrahymena* membranes diminishes? Possible removal and subsequent reinsertion of membrane particles seems unlikely since the original membrane state can be restored within at least 30 s. We appear to be dealing with a reversible reorganization of the membranes without quantitative chemical alteration of membrane components. Therefore we suggest that the temperature-induced decreases in particle number arises from their movement normal to the membrane plane out of the − face into the + face.

To allow movement of membrane-intercalated particles, the observed mobility parallel or normal to the membrane plane demands that the membrane core is rather fluid. In fact, there is accumulating evidence that the lipids of natural membranes contain some fluid domains (e.g. refs 8–10, and 22–26). Particularly noteworthy is the electron spin resonance work of Träuble[27], who has found a strong correlation between the mobility of hydrocarbon chains and the onset of lateral diffusion of membrane components in artificial dipalmitoyllecithin monolayer vesicles. At low temperatures, labeled steroids are aggregated in clusters in these artificial monolayers. When the temperature is raised above the lipid phase transition point, however, the hydrocarbons chains become more mobile, allowing the steroids to diffuse randomly in the lateral membrane plane. This would appear to provide a good analogy to our findings that particle aggregation begins at about 15 °C and is essentially completed at 10 °C; this indicates that the alveolar membrane cores of *Tetrahymena* cells exhibit a temperature-sensitive transition point at about 15 °C. This membrane

transition point might be mainly ascribed to the lipid moiety since the lipids make up the essential part of membrane cores.

However, the most intriguing question does arise with respect to the "mechanism" by which temperature induces particle aggregation and reaggregation in *Tetrahymena* membranes. Though speculative, we suggest the following straightforward explanation: First, most membrane lipids are normally in the liquid crystalline state. Upon cooling, they crystallize, proceeding from an initiation point with crystallization centers extending two-dimensionally. At the expanding phase boundary the particles are moved into more liquid regions, until these are occupied and fully packed. At elevated temperatures, when the lipids again become fluid, aggregated particles can redistribute normally.

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