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DIFFERENTIAL EFFECTS OF TEMPERATURE ON THE NUCLEAR AND PLASMA MEMBRANES OF LYMPHOID CELLS

A STUDY BY FREEZE-ETCH ELECTRON MICROSCOPY

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SUMMARY

1. We have used freeze-fracture electron microscopy to examine the effects of cooling on the core ultrastructure of the plasma and nuclear membranes of normal thymocytes and lymph node cells, as well as concanavalin A-treated thymocytes and mouse lymphoma cells.

2. Chilling below 22 °C produces smooth areas, free of intramembranous particles on both faces of both inner and outer nuclear membranes. This effect is reversible and can be prevented by glutaraldehyde fixation.

3. Plasma membranes, in contrast to the nuclear membranes, exhibit no change in freeze-fracture morphology upon cooling.

4. We hypothesize that the changes observed in the nuclear membranes represent thermotropic lipid phase transitions and that such transitions either do not occur in plasma membranes or are there constrained to very small regions.

INTRODUCTION

Physical studies on artificial membranes, as well as on biomembranes, show that the lipids of these systems can undergo reversible fluid–solid (disorder–order) transitions when the temperature is dropped below a critical level [1–11]. Depending upon the nature of the fatty acids, the phosphatide headgroups, the proportion of cholesterol, as well as other variables, the transitions may occur over a very narrow temperature range, or they may be too diffuse to be defined [10, 12, 13].

In biomembranes, fluid–solid transitions have in some cases been detected by freeze-etching electron microscopy. This technique exposes the apolar cores of membranes [14, 15] which, except in myelin, are populated by intramembranous particles. These are at least in part protein [16, 17] and, in erythrocyte membranes, bear certain blood group substances [18]. In the plasma membranes of *Acholeplasma laidlawii* [19, 20] and *Escherichia coli* [19, 21, 22], as well as in nuclear and alveolar membranes of *Tetrahymena pyriformis* [23–26], chilling produces a separation of smooth, particle-poor areas from particle-enriched areas during chilling; reheating yields a normal

particle distribution. This effect of cooling can be reasonably attributed to disorder–order transitions of the membrane lipids.

The possibility that membrane lipids are fluid at physiological temperatures and that membrane proteins can move rather freely through a lipid phase is often invoked in explanation of surface transposition phenomena in lymphocytes, e.g. 'capping' [27–32]. However, recent rigorous studies [33, 34] indicate that these processes involve redistribution of lymphocyte receptors on the external membrane surface rather than their diffusion within the membrane.

We have, accordingly, examined normal thymocytes and lymph node cells, as well as concanavalin A-treated thymocytes and mouse lymphoma cells, for thermal effects on the distribution of intramembranous particles. We find that, just as in the case of *Tetrahymena* [26], the nuclear membranes of lymphocytes exhibit clear evidence of reversible thermotropic phase separations, while their plasma membranes show no micromorphologic manifestations of such processes.

MATERIAL AND METHODS

Cells

We harvested normal thymic lymphocytes from C57 BL/10 mice using the sterile techniques detailed in ref. 35. The cells were finally transferred into Eagles minimal essential medium, 10 % in fetal calf serum and maintained in 25 cm^2 Falcon petri dishes at 37 °C in 10 % CO_2, 90 % air until further processing. In some experiments, the cells were cultivated under these conditions for 48 h in the presence of 5 μg/ml concanavalin A (Miles-Yeda). The C57 BL/EL4 lymphomas were carried in C57 BL/10 mice and were isolated and handled as the thymic cells. Pig lymph node cells, harvested as in ref. 36, were then treated as the thymic cells.

Temperature-shift studies

To determine whether the distribution of intramembranous particles in nuclear and plasma membranes varies with temperature, we cooled the cells in 5 °C steps from 37 °C to 12 °C, equilibrating for 5 min at 32, 27, 22, 17 and 12 °C, before fixing at the equilibrating temperature. We fixed for 15 min at the equilibration temperature using 2 % glutaraldehyde in isotonic saline buffered at pH 7.2; the fixative had been previously brought to the equilibration temperature.

In additional experiments, cells stepped down to 12 °C and equilibrated for 5 min at this temperature, were reheated to 37 °C over a 3 min period, equilibrated for 5 min at this temperature and then fixed at 37 °C.

To determine whether chemical fixation would stabilize membranes against temperature-induced changes, we equilibrated cells at 37 °C, fixed with glutaraldehye for 5 min at 37 °C, equilibrated at 4 °C for 15 min and then proceeded with glycerolization and freeze-etching. The control cells for this experiment were fixed at 4 °C after prior equilibration at this temperature.

After fixation, we washed the cells twice in buffered saline and transferred in stepwise fashion to 25 % glycerol over a period of 2 h at room temperature. We then pelleted the cells at 3000 $g \cdot$ min and froze them on cardboard discs in Freon 22 cooled by liquid N_2. We carried out fracturing, etching (1 min at −100 °C) and replication with a Balzers freeze-etch apparatus (Model BA 360 M; ref. 37). The replicas were examined in a Siemens Elmiskop (Model Ia), calibrated with a standard

grating replica. We evaluated nuclear membrane areas, particle numbers and pore numbers on cut-outs of calibrated positives. In evaluating particle numbers, we counted only particles with well-defined shadows and diameters exceeding 50 Å (measured perpendicular to the direction of the replicating beam). Shadowing direction on the micrographs is indicated by the arrows in the right hand corner.

Except in the concanavalin A studies, the temperature shift experiments were conducted within 1 h after cell isolation.

RESULTS

Plasma membranes

Freeze-fracturing cleaves plasma membranes internally, parallel to the membrane surface. This exposes an inner fracture face oriented toward the cytoplasm and an outer fracture face oriented toward the extracellular space. (Figs 1 and 2). The inner fracture face reveals numerous, uniformly distributed intramembranous particles, ranging between 30 Å and 140 Å in diameter (mean $\approx$ 85 Å). Not infrequently, one observes two particles in close proximity (Fig. 2).

The outer fracture face shows fewer particles (Fig. 1). All of the lymphoid cells exhibit the same freeze-etch appearance, and this appearance does not change significantly with temperature (Figs 2 and 3). No particle aggregation is observed at 37, 32, 27 or 22 °C and only in very rare instances can one observe aggregates or linear arrays of 3–6 particles at 17, 12 and 4 °C. No particle aggregation could be detected at any temperature in thymocytes treated with concanavalin A.

The fracture face topology of cells equilibrated and fixed at 37 °C, and then cooled at 4 °C is indistinguishable from those of cells fixed at 37, 32, 27 and 22 °C.

Nuclear membranes

The nuclear envelope of the lymphoid cells consists of an outer membrane, bordering on the cytoplasm, and an inner membrane adjacent to the nucleoplasm. Upon fracturing, both membranes cleave internally, parallel to the membrane surface, each revealing an outer fracture face and an inner fracture face. The inner fracture faces of the outer and inner nuclear membranes orient towards the cytoplasmic and nuclear spaces, respectively, whereas the outer fracture faces orient toward the cisternal space between the two membranes of the nuclear envelope. The inner fracture faces of the two nuclear membranes appear similar in all the lymphoid cells examined. They bear 1000–1900 uniformly distributed, intramembranous particles per μm^2. These range in size between 30 Å and 120 Å (mean $\approx$ 75 Å). The outer fracture faces show fewer particles but numerous, uniformly distributed depressions.

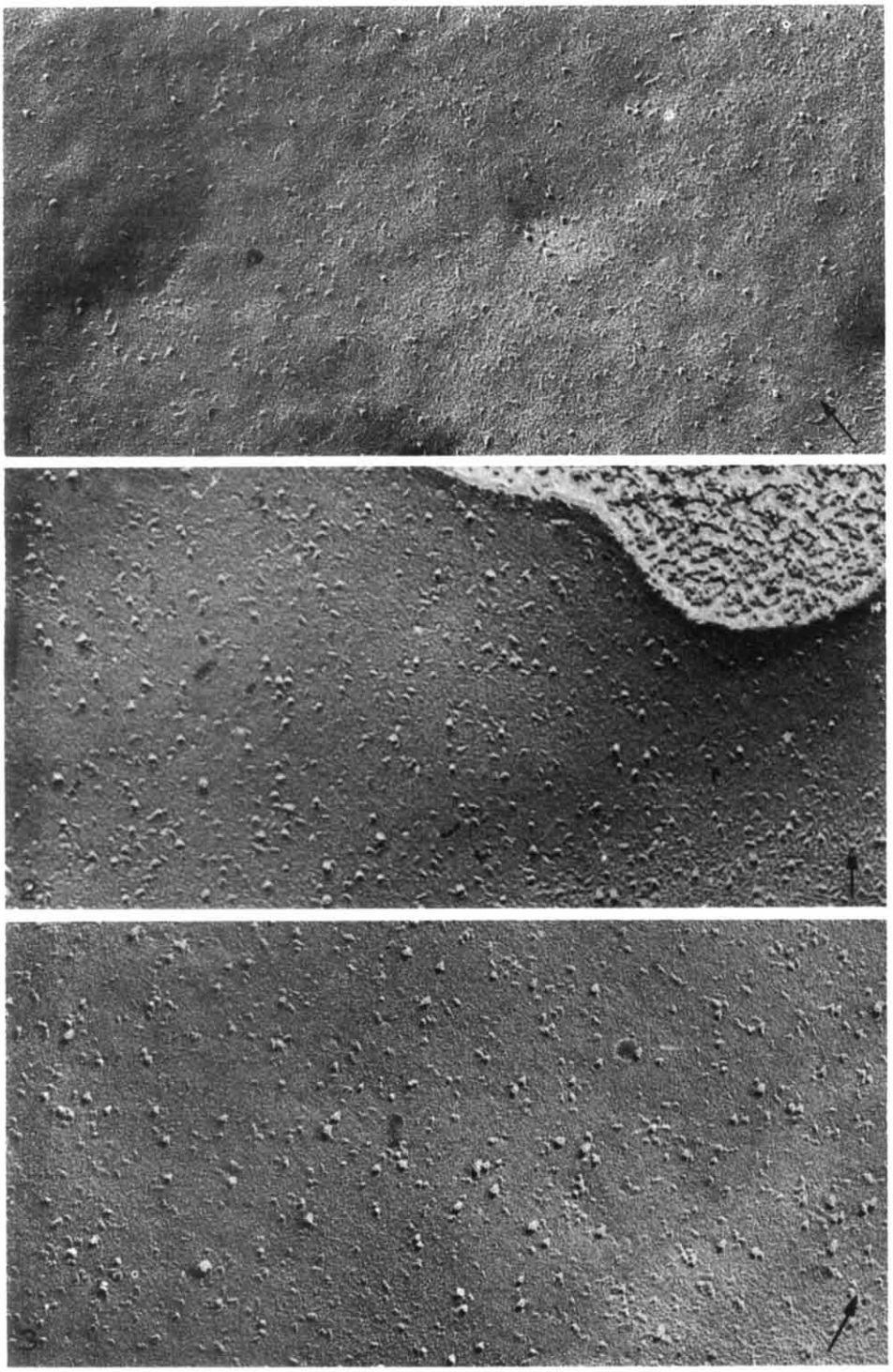
When fixed at 37, 27 and 22 °C, all lymphoid cells examined show a uniform distribution of particles or depressions on the inner and outer fracture faces, respectively, of the two nuclear membranes (Figs 4 and 5).

In contrast, the freeze-fracture characteristics of the nuclear membranes

Fig. 1. Pig lymphocyte plasma membrane at 37 °C. Outer fracture face, i.e. oriented to the cell exterior. Uniform distribution of intramembranous particles. × 100 000.

Fig. 2. Pig lymphocyte plasma membrane at 37 °C; inner fracture face bearing more particles than outer fracture face, but also in uniform distribution. × 100 000.

Fig. 3. Inner fracture face of a pig lymphocyte plasma membrane at 12 °C. There is no change in particle distribution. × 100 000.



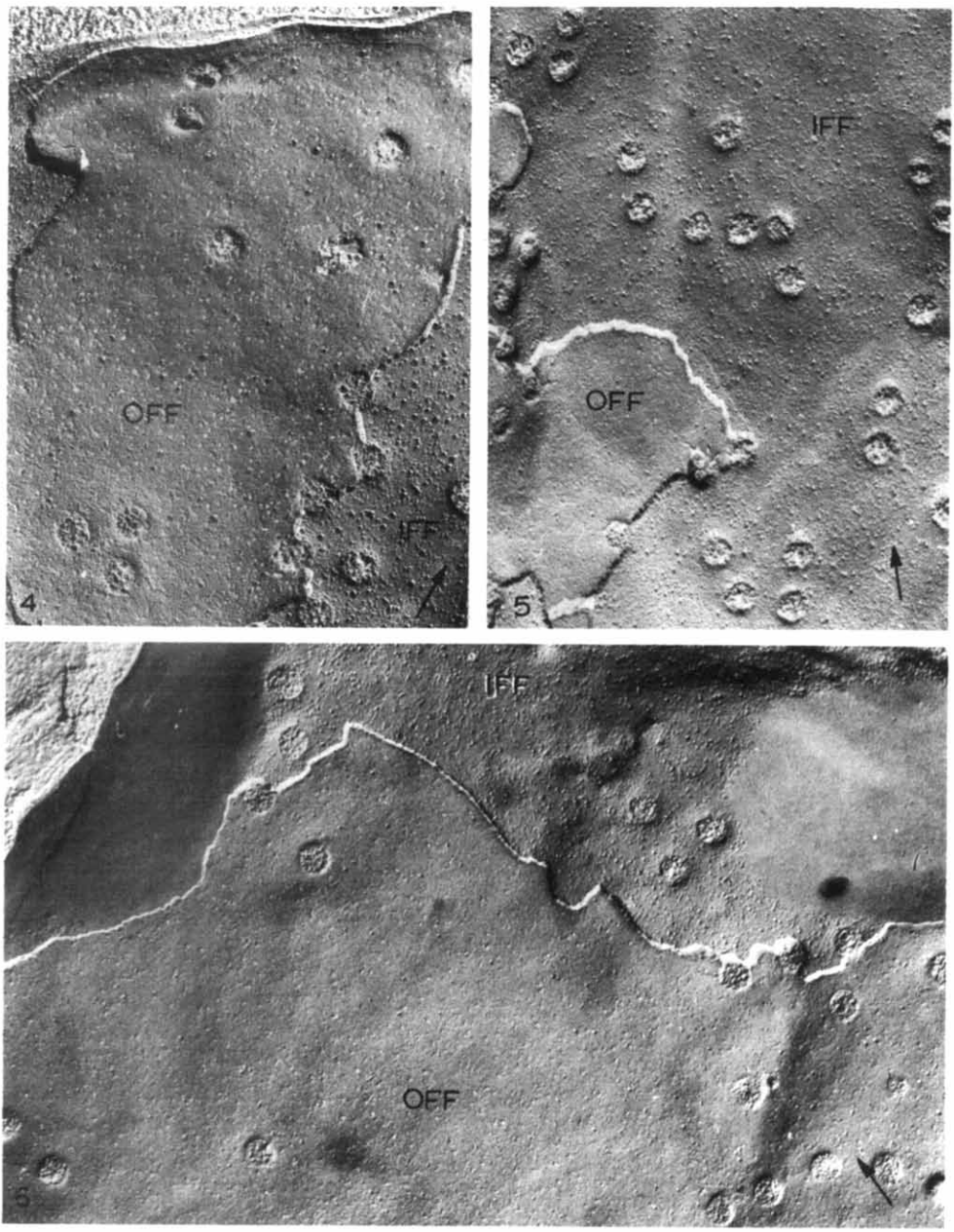


Fig. 4. Concanavalin A-treated C-57 lymphocyte at 37 °C. IFF, inner fracture face of the outer nuclear membrane oriented toward cytoplasm. OFF, outer fracture face of the inner nuclear membrane oriented toward cytoplasm. Note uniform particle distribution. × 50 000.

Fig. 5 Concanavalin A-treated C-57 lymphocyte cooled to 12 °C and reheated to 37 °C. No smooth areas are visible. IFF and OFF as in Fig. 4. × 50 000.

Fig. 6. Thymic lymphocyte chilled to 4 °C. Large smooth areas are seen on the inner fracture face (IFF) of the outer nuclear membrane, i.e. adjacent to cytoplasm. Note that the particles in the particle bearing areas are not aggregated. OFF, outer fracture face of the inner nuclear membrane. × 50 000.

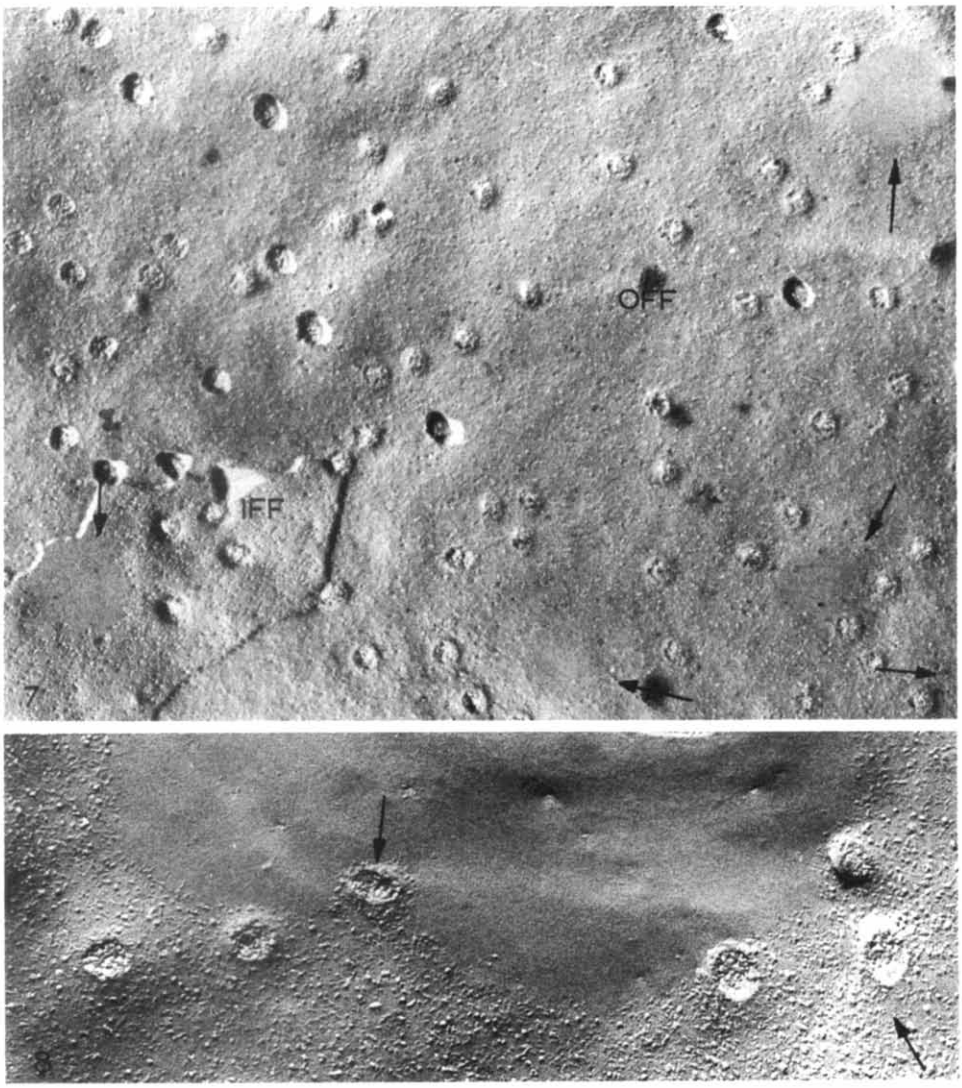


Fig. 7. Outer fracture face of inner nuclear membrane of C-57 lymphocyte at 4 °C, revealing large smooth area. × 75 000.

Fig. 8. Outer fracture face of inner nuclear membrane of C-57 lymphocyte chilled and fixed at 4 °C, revealing large smooth area. Arrow indicates a nuclear pore complex still surrounded by intramembranous particles. × 75 000.

change drastically when the cells are equilibrated at temperatures below 22 °C (Figs 6, 7 and 8). Thus, both nuclear membranes of all the lymphoid cells equilibrated at 17 °C reveal smooth planar areas (3000 Å or greater in diameter) on 40–55 % of their inner and outer fracture faces (Fig. 7; Table I). The proportion of fracture faces is greater in cells fixed at 12 °C (Table I) and, in the case of cells fixed at 4 °C, the smooth areas become enormous (Figs 6 and 8) and occur on at least 90 % of the fracture faces.

TABLE I

PERCENTAGE OF NUCLEAR MEMBRANE FRACTURE FACES WITH SMOOTH AREAS AT DIFFERENT FIXATION TEMPERATURES

'Smooth areas' are regions essentially free of intercalar particles with a diameter of 300 nm and over. At least 20 fracture faces in at least three replicas were evaluated for each temperature point.

Fixation temperatures (° C)	C-57 BL/10 lymphocytes (%)	C-57 BL/10 lymphocytes (concanavalin A-stimulated) (%)	Pig lympho-cytes (%)	C-57 BL/EL4 lymphoma (%)
37 (control)	0	0	0	0
32	0	0	0	0
27	0	0	0	0
22	0	0	0	0
17	45	40	40	55
12	55	50	50	67
37 (reheated from 12 °C)	0	0	0	0

The smooth areas on the inner fracture faces occasionally exhibit intramembranous particles. Significantly, the number of particles in the particle-bearing areas in the nuclear membranes of cells cooled to 4 °C prior to fixation, (e.g. where we find large smooth areas) does not exceed that found in cells fixed at 37 °C; we find 800–1600 particles/μm^2 in the particle-bearing domains. This means that we do not observe particle aggregation. On the outer fracture faces, which reveal few particles, but numerous depressions at 37 °C, the smooth areas show occasional particles and/or depressions.

The distribution of nuclear pore complexes does not change, and when the smooth areas are large, one finds pores in regions completely free of particles. Not infrequently, nuclear pore complexes lying in the particle-rich area are partly surrounded by a narrow band of particles, which extends into the smooth areas (Fig. 8).

The formation of the smooth areas is reversible. Thus, when cells first equilibrated at 12 °C are reequilibrated at 37 °C prior to fixation, no smooth regions can be observed on the nuclear membrane fracture faces (Fig. 5; Table I).

TABLE II

FREQUENCY OF NUCLEAR PORE COMPLEXES IN DIFFERENT LYMPHOCYTES

Lymphocytes	Number of nuclear pore complexes/μm^2 ($\pm$ S.D.)	Area of nuclear envelope evaluated (μm^2)
C-57 BL/10 lymphocytes	3.30 $\pm$ 0.21	232.5
C-57 BL/10 lymphocytes; Concanavalin A-stimulated	5.27 $\pm$ 0.22	326.1
Pig lymphocytes	3.82 $\pm$ 0.24	344.9
C-57 BL/EL lymphoma	9.28 $\pm$ 0.29	1075.0

Cells fixed at 37 °C prior to cooling to 4 °C do not reveal smooth areas on their nuclear membrane fracture faces. We could detect no difference in temperature response between the cell types tested.

The nuclear membranes of the four lymphocyte types reveal some differences in pore frequency (cf. Table II). Thus, the pore frequency in the concanavalin A-treated thymocytes is significantly greater than that of control cells (cf. also ref. 38), and the malignant lymphocytes exhibit more than twice as many pores per uni tarea of nuclear surface than normal cells. However, in none of the cells does pore frequency or pore distribution vary with temperature.

DISCUSSION

Our experiments demonstrate that chilling induces the reversible emergence of smooth, particle-free areas on the fracture faces of lymphocyte nuclear membranes. However, plasma membranes of these cells do not exhibit this phenomenon. By analogy to other systems [19–26], we reason that the appearance of the smooth areas, induced in the fracture faces of nuclear membranes, reflects a disorder–order transition of the membrane lipids. However, unlike the situation observed with *Acholeplasma* membranes and the alveolar membranes of *Tetrahymena*, the emergence of smooth areas on the fracture faces of the lymphocyte nuclear membranes does not cause an increase in the particle number or an aggregation of particles in the particle-containing areas. These observations cannot be explained by a lateral displacement of intramembranous particles into fluid areas by an expanding solid–lipid phase. Therefore, lipid-ordering obviously squeezes intramembranous particles asymmetrically out of the fracture plane; indeed, this has also been observed in the alveolar membranes of *Tetrahymena* ([23] as well as in the plasma membranes of *Echerichia coli* [19, 22]). Of course, we cannot tell whether the particles move first parallel to the membrane plane and then perpendicular to it. Also, we cannot exclude the possibility that one membrane type exhibits exclusively lateral particle movement and another only normal particle displacement.

Significantly, the temperature-induced nuclear membrane changes can be fully prevented by glutaraldehyde fixation. This reagent crosslinks proteins over a distance of not more than 6 Å. This distance is much smaller than the average minimum distance between the membrane particles. Since the membrane particles are not aggregated by glutaraldehyde, the stabilization induced by glutaraldehyde cannot be attributed to crosslinking between particles. A more reasonable hypothesis is that glutaraldehyde links the particles to a protein framework, which cannot be seen by our electron-microscopic methods [23].

Our data fit those of Jost et al. [39] whose electron paramagnetic resonance studies show that 96–97 % of spin labels covalently bound to the membrane proteins in nerve bundles of the walking legs of *Homarus americanus* is immobilized by glutaraldehyde, while the membrane lipids are not immobilized. We therefore suspect that, when lymphoid cells are glutaraldehyde fixed at 37 °C and cooled at 4 °C, lipid ordering still occurs but cannot produce particle displacement.

At present, freeze-fracture electron microscopy can reveal disorder–order transitions of membrane lipids only when these cause particle displacement. The detection sensitivity of particle displacement (formation of smooth areas) depends

on the minimum distance between particles. The smaller the mean interparticle distance, the smaller the smooth area which can be detected. Taking a diameter of 80 nm for a smooth area and assuming that it represents only ordered lipid, we can detect disorder–order transitions in clusters of $2 \cdot 10^4$ phosphatide molecules (taking a surface area projection of 50 $Å^2$ per packed phospholipid).

Unlike the membrane particles in nuclear membranes, the nuclear pore complexes do not become dislocated concomitantly with the formation of smooth areas. This suggests that the location and distribution of the pores is stabilized by a non-thermotropic mechanism within or adjacent to the nuclear membranes. This could involve the type of protein network extending throughout some nuclei, e.g. isolated rat liver nuclei [40, 41].

The dramatic, temperature-induced reorganization observed in lymphocyte nuclear membranes cannot be observed in lymphocyte plasma membranes. These reveal an invariant freeze-fracture pattern at all temperature steps between 37 °C and 4 °C. The lack of apparent thermotropic response in these membranes can be explained in at least two ways: first, the plasma membrane lipids may not undergo a defined phase transition upon chilling. This is true also for erythrocyte lipids and may relate totally or in part to the high cholesterol : phospholipid ratio ($\approx$ 1.0 in lymphocyte plasma membranes [36] and $\approx$ 0.25 in nuclear membranes [42]); (high cholesterol : phospholipid ratios tend to "smear out" order–disorder transitions in lipids [12, 13]). A second possibility is that phase transitions do occur but that dislocation of the particles is prevented by association with an unvisualized framework; this would be analogous to the case of the glutaraldehyde-fixed nuclear membranes. An obvious third possibility is that the membrane particles cannot move rapidly and that the lipids do not undergo defined phase transitions.

Our data suggest that the intramembranous particles in the plasma membranes of lymphocytes are relatively immobile even at physiological temperatures. The data are consistent with the suggestions in refs. 33 and 34 that surface transpositions in lymphocytes, such as 'capping', do not necessarily represent events occurring within the 'inner' membrane and do not necessarily involve redistribution of membrane core components represented by or related to intramembranous particles.

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