

# EFFECTS OF CHOLESTEROL ON LIPID ORGANIZATION IN HUMAN ERYTHROCYTE MEMBRANE

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## ABSTRACT

The molar ratio of cholesterol to phospholipid (C/P) in human erythrocyte membrane is modified by incubating the cells with liposomes of various C/P ratios. The observed increase in cell surface area may be accounted for by the addition of cholesterol molecules. Fusion between liposomes and cells or attachment of liposomes to cells is not a significant factor in the alteration of C/P ratio. Onset temperatures for lipid phase separation in modified membranes are measured by electron diffraction. The onset temperature increases with decreasing C/P ratio from 2°C at C/P = 0.95 to 20°C at C/P = 0.5. Redistribution of intramembrane particles is observed in membranes freeze-quenched from temperatures below the onset temperature. The heterogeneous distribution of intramembrane particles below the onset temperature suggests phase separation of lipid, with concomitant segregation of intramembrane protein into domains, even in the presence of an intact spectrin network.

The free cholesterol to phospholipids (C/P) ratio in the plasma membrane of eukaryotes is usually regulated to maintain proper membrane fluidity for the normal functioning of the cell. The C/P ratios are altered in some cells in pathological states. For instance, the membranes of leukemic cells have a lower C/P ratio than those of normal lymphocytes (19). The C/P ratio in the erythrocyte membranes of hypocholesteremic cells and of spur cells may differ from each other by an order of magnitude (3). Alteration of the cholesterol level in the erythrocyte membrane leads to changes in ionic permeability (3, 38), glycerol permeability (2, 14), fragility (2, 3), microviscosity (1, 4), lateral diffusion (34), and protein-lipid interaction (1). In spite of these findings, corresponding changes in membrane ultrastructure and in molecular organization have so far not been reported.

Free cholesterol is exchangeable *in vivo* between the erythrocyte membrane and serum lipoprotein. The C/P ratio of the erythrocyte membrane may also be controlled *in vitro* by incubating the cells with certain sera (12, 22), or with liposomes of a given C/P ratio (1-4). The exchange mechanisms are not completely known. Nevertheless, by applying the liposome method, the C/P ratio in the erythrocyte membrane has been increased up to three times its natural value (4).

We have investigated the ultrastructure and lipid phase changes in human erythrocyte membranes with modified C/P ratios ranging from 0.4 to 2.5 by *in vitro* liposome incubations. We first determined whether the C/P ratio alteration was a result of membrane-liposome fusion, liposome attachment to the membrane surface, or a net exchange of cholesterol between the membrane

and liposomes. The size and surface morphology of the modified cells were monitored by dark-field light microscopy and scanning and freeze-fracture electron microscopy. The ghost membranes of these cells were then studied by negative staining, freeze-fracture, and electron diffraction to follow the changes in ultrastructure and molecular organization as a function of temperature. Finally, the lipid was extracted from the membranes for C/P ratio determination and for further electron diffraction studies.

## MATERIALS AND METHODS

### *Modification of C/P Ratio in Erythrocyte Membrane*

Sonicated vesicles or liposomes were prepared according to Cooper et al. (3). D-1-phosphatidylcholine, dipalmitoyl (Sigma Chemical Co., St. Louis, Mo.), and cholesterol (Sigma Chemical Co.) at C/P molar ratios of 0.3, 1.0, and 2.0 were dissolved in chloroform, and the solvent was evaporated in vacuum. The lipids were suspended in 0.155 M NaCl and sonicated. After sonication, human serum albumin (mg/mg of lipid) was added, and the albumin-liposome mixture was centrifuged at 21,800 g for 30 min to sediment undispersed lipid. The liposome suspensions were used within 12 h, although they were stable for a few days at 4°C. Within this period, no cholesterol pattern was seen by x-ray diffraction.

Cholesterol level manipulation was carried out according to the method of Cooper et al. (3). Fresh erythrocytes were washed three times with Hanks' balanced salt solution (BSS) and resuspended in a hematocrit of 10% in BSS that contained penicillin (1,000 U/ml). Erythrocyte suspensions were combined with equal volumes of the albumin-liposome mixtures (6 mg of lipid per ml) and incubated in a shaking water bath at 37°C for up to 24 h. Liposome-free control samples consisted of equal volumes of erythrocyte suspensions and 0.155 M NaCl buffer with albumin. After incubation, all samples were centrifuged at 1,000 g for 20 min and washed three times. Erythrocyte ghosts were prepared according to Dodge et al. (5). Spectrin-poor ghosts were prepared according to Elgsaeter and Branton (6) by incubating the ghost in low-ionic-strength buffer for 24 h, as previously described (27).

For C/P ratio determination, samples of erythrocyte ghosts were washed two times in distilled water to eliminate water-soluble phosphate. The method of Rose and Oklander (28) was used for total lipid extraction. The cholesterol was determined according to Zlatkis et al. (41), and the phosphate according to Fiske and Subbarow (8).

### *Microscopy*

The first step in measuring cell size is dark-field light microscopy. The washed cells were observed in BSS suspension. Five photographs of typical views of each sample were taken at  $\times 450$ . The exact magnification was calibrated by the use of latex spheres of known size. All cells within the fields of view were measured, and statistics were recorded.

Cell shape, size, and possible liposome attachment to the surface were observed by scanning electron microscopy. The washed cells were adsorbed on microscope slides and then fixed for 1 h in 2% glutaraldehyde, postfixed for 1 h in 1% OsO<sub>4</sub>, and

dehydrated in a graded series of ethanol (25). The specimens on the slides were dried in a critical-point drying apparatus with CO<sub>2</sub> as the transition fluid, rotary coated with a layer of vacuum-evaporated platinum/carbon, and examined in an ETEC Autoscan scanning electron microscope. Five representative photographs of each sample were taken. All cells within the fields of view were statistically analyzed.

Negative-staining electron microscopy was used as an alternative method for studying the extent of liposome attachment to the membranes. The negative-staining procedure follows the recommended method of McMillan and Luftig (23) for erythrocyte ghost membranes. The samples were adsorbed onto a carbon-coated grid and were fixed with 2% OsO<sub>4</sub> for 10 min. The grid was then washed 10 times with distilled water, stained with 1% uranyl acetate, and observed in a Siemens Elmiskop 1A.

In freeze fracture experiments, samples of cells or ghosts were suspended in 30% glycerol, transferred to Balzers gold cups (Balzers Corp., Nashua N. H.), and rapidly quenched in liquid Freon 22 (Pennwalt Corp., Philadelphia, Pa.). In controlled-temperature experiments, samples were equilibrated at the set temperature for 15 min before freeze-quenching (32). Freeze-fracture and replication was performed in a Polaron E7500 Freeze Fracture Module (Polaron Instruments Inc., Line Lexington, Pa.) at a vacuum of  $5 \times 10^{-7}$  torr, using an Ultek TNB-X ion pump system (Perkin-Elmer Corp., Palo Alto, Calif.). The specimens were fractured at  $-115^\circ\text{C}$  and replicated with platinum/carbon. Replicas were cleaned with sodium hypochlorite ("Clorox") for 1 h and examined in a Siemens 101 electron microscope. Twenty photographs of representative views were taken of each replica.

### *Electron Diffraction*

Preparations of ghost membranes and unsupported bilayers of extracted membrane lipids for electron diffraction studies have been described previously (18). The entire procedure was performed under saturated water vapor in a nitrogen atmosphere, and the wet grid was transferred to the environmental stage (17) of the electron microscope via a transfer chamber. The specimen, separated from the microscope vacuum by two sets of apertures (100- $\mu\text{m}$  and 200- $\mu\text{m}$ , respectively), was kept fully hydrated at all controllable temperatures between  $-5^\circ$  and  $50^\circ\text{C}$  by differentially pumping. The formation of large ice crystals at low temperature was retarded by the presence of salt in the buffer and by the fact that the grid is covered by a thin (500 Å) layer of vitreous water only (16). The grid was always normal to the incident electron beam. Selective area electron diffraction was achieved by limiting the electron beam to about 2  $\mu\text{m}$  in diameter at the specimen level. Wet membrane ghosts were sampled one by one as described previously (18). Diffraction of unsupported bilayers was obtained from areas away from the grid bars. The specimen was exposed to no more than  $2 \times 10^{-6}$  coulombs/cm<sup>2</sup> of electron dose in the entire procedure. This dosage is below the threshold of tolerable damage (16). The patterns were recorded on Kodak No-screen x-ray film. The camera length was calibrated in each experiment with gold and aluminum microcrystal films.

## RESULTS

### *C/P Ratio*

Liposomes of C/P ratios of 0.3, 1.0, and 2.0 were used to modify the C/P ratio of human

erythrocyte membrane from an initial value of 0.95 (30) to final values approaching those of the liposomes (3). Cell samples were taken after 2, 6, and 24 h of incubation in five repeated experiments. The C/P ratios of the total lipids extracted from the membrane of these cell samples are presented in Fig. 1. Error bars represent variations between experiments plus individual experimental error. After 10 h of incubation, the C/P ratio of the cell membrane was about half way between the original value of 0.95 and that of the liposomes used. The rates of change approximately agree with those from experiments in which tetrahydrofuran (31) and serum lipoprotein (22) were used. Depletion of cholesterol from the membrane also led to increased fragility (3) and resulted in some apparent hemolysis. In one enrichment experiment, human serum albumin was not added and the rate of cholesterol enrichment was significantly reduced (Fig. 1). This suggested that serum albumin facilitated, but was not totally responsible for, the cholesterol transfer between liposomes and erythrocyte membranes.

#### Morphology and Surface Area Measurement

The apparent diameters of cells and ghosts in aqueous suspension measured by dark-field light microscopy agree with those measured by scanning electron microscopy after critical-point drying. At low C/P ratios, some of the cells become spherocytes, whereas, at high C/P ratios, most of the cells have a flattened, "pancake" shape (Fig.

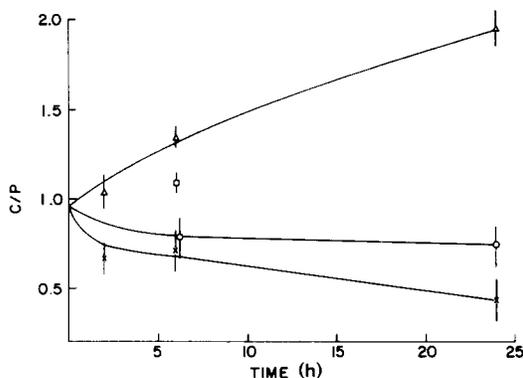


FIGURE 1 Alteration of C/P ratio in erythrocyte membrane at various incubation times with liposomes composed of cholesterol and dipalmitoylphosphatidylcholine at C/P ratios of 0.3 (x), 1.0 (o), and 2.0 ( $\Delta$ ). ( $\square$ ) Sample incubated with liposomes of C/P = 2.0 but in the absence of serum albumin. Bars represent experimental error plus variations between repeated experiments.

2). The latter form has been seen by Cooper et al. (3). Because the cells are irregular in shape, precise measurement of cell surface area is not possible. We approximated the surface areas of these cells by using spherical, biconcave, and disk models (36). The surface area of cells is found to increase with increasing C/P, which is in agreement with the fragility measurement (3). At C/P ratios of 0.4 and 1.95, the mean surface areas per cell were estimated to be  $126 \pm 30$  and  $195 \pm 40 \mu\text{m}^2$ , respectively, as compared with a controlled value of  $140 \pm 25 \mu\text{m}^2$  at C/P = 0.95. The net increase in area after cholesterol enrichment approximately equals the surface area of the additional cholesterol molecules ( $39 \text{ \AA}^2/\text{molecule}$ ), assuming a constant surface area for the existing lipid and protein molecules (3, 4). At low C/P ratios (<0.5), this simple relation between the surface area and cholesterol molecules does not hold, possibly because of the condensation of unsaturated phospholipids by cholesterol molecules (11).

High magnification scanning electron micrographs show that most cells have a smooth surface. About 10% of the cholesterol-poor cells have one to two small ( $500 \text{ \AA}$ ) vesicles attached to the surface. The cells incubated with liposomes having C/P = 1.0 are mostly biconcave, about 3% of the cells having vesicles attached. Cells incubated in liposome-free media have no attached vesicles. In samples of cholesterol-rich cells, 2% of the cells are associated with one or two elongated "vesicles" or rods about  $0.1 \mu\text{m}$  long. These elongated objects could be a form of aggregation of cholesterol-rich lipid mixtures.

In order to check the possibility that we might have overlooked the internalized liposomes and the liposomes detached from the membrane during the critical-point drying process for SEM, we used freeze-fracture and negative-staining electron microscopy in comparative morphological studies. Random sampling of freeze-fractured cross sections of cells showed that about 5% of low cholesterol cells had one to two liposomes appearing within the cell cross section. Rod-shaped inclusions were found in cross sections of about 2% of the high cholesterol cells. Negatively stained membrane ghosts have a predominantly smooth periphery with occasional, small vesicular features in the center. It cannot be determined whether these vesicular features are internal or external to the ghost because stain readily penetrates the membrane ghost. The infrequent appearance of attached and internalized liposomes in cells indicates

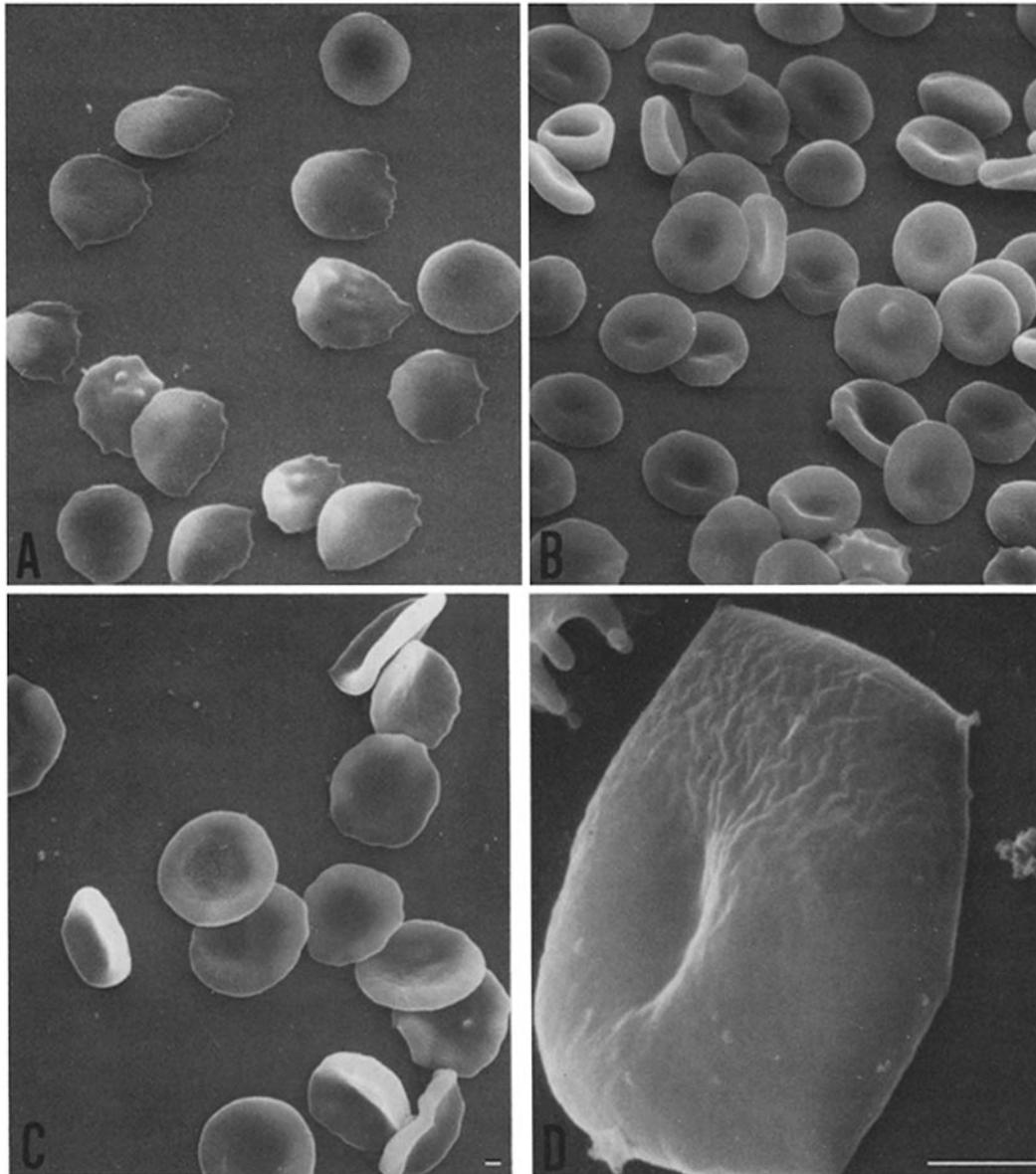


FIGURE 2 Scanning electron micrographs of erythrocytes modified by cholesterol exchange. The resultant C/P ratios of the membrane lipids are (A) 0.47, (B) 0.80, and (C) 1.95. Cells with attached liposomes are rare. An example is shown in D at C/P = 0.4. Bars, 1.0  $\mu\text{m}$ . (A–C)  $\times 2,000$ . (D)  $\times 15,000$ .

that the errors caused by liposomes in the determination of the C/P values of the membrane are insignificant. To account for the C/P ratio alteration after 24 h of incubation solely by liposome attachment and inclusion, using the 300 Å liposomes as described, the total surface area of the attached and internalized liposomes would have

to be at least five times the initial surface area of the cell. This would require  $2.3 \times 10^5$  liposomes, which, if attached to the cell surface, would completely cover the surface. If included in the cell interior, these liposomes would occupy about 10% of the volume of the cell, or 20% of any randomly fractured cross section of the cell. Our electron

microscopic results show that the probability of liposome inclusion or attachment is five orders of magnitude below this estimate.

### Temperature-dependent Properties

The onset temperatures of lipid phase separation in ghost membranes, as indicated by the highest (onset) temperature at which wide-angle diffraction rings corresponding to a gel phase of acyl chain packing are observable (18), were measured by electron diffraction. Fig. 3 (left) shows a trace of this ring at a spacing of 4.2 Å that disappeared as the temperature was raised above the onset temperature. Fully hydrated membrane ghosts derived from both high and low cholesterol cells were studied. The onset temperatures are plotted as functions of their respective C/P ratio in Fig. 4. The scattering of points is in part the result of cell-to-cell variations and in part the result of the difficulty in pinpointing the onset of the appearance of a faint diffraction ring against the more intense, diffuse diffraction band corresponding to the bulk of the lipid in the liquid crystalline state (Fig. 3). The statistical best-fit curve in Fig. 4 indicates that low cholesterol membranes tend to have a higher onset temperature, a finding suggested by calorimetric measurement of extracted lipids (20, 35). Our electron diffraction results from

bilayers of extracted lipids from selected samples of ghost membranes agree with those from the original membranes.

Freeze-fractured ghost membranes quenched from temperatures much above the onset temperature (Fig. 5A and B) appear to have a "random" distribution of intramembrane particles (IMP) on both the P and E faces, whereas those quenched from temperatures much below the onset temperature (Fig. 5C) appear to have aggregated parti-

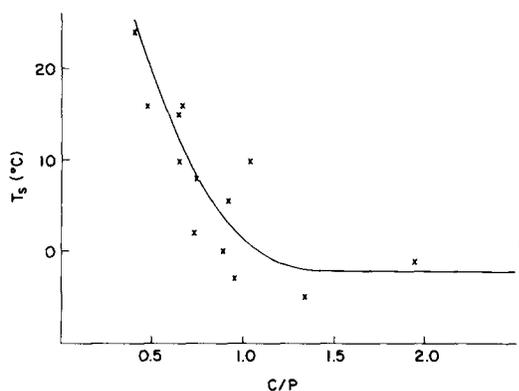


FIGURE 4 Onset temperatures of lipid phase separations in ghost membranes at various C/P ratios. The onset temperatures are determined by electron diffraction of hydrated ghosts.

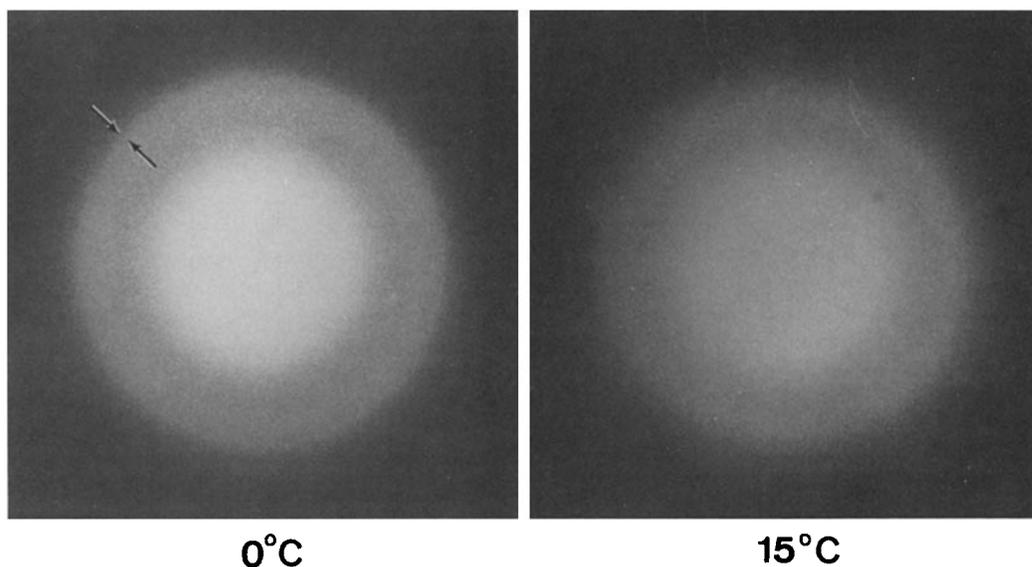


FIGURE 3 Electron diffraction patterns of hydrated ghost membranes at C/P = 0.65. The faint but sharp edge (arrows) seen at 0°C is an indication of the existence of lipid gel phases, with a diffraction spacing at 4.2 Å. The edge disappears at temperatures above the onset temperature. The diffuse ring has a spacing of 4.6 Å.

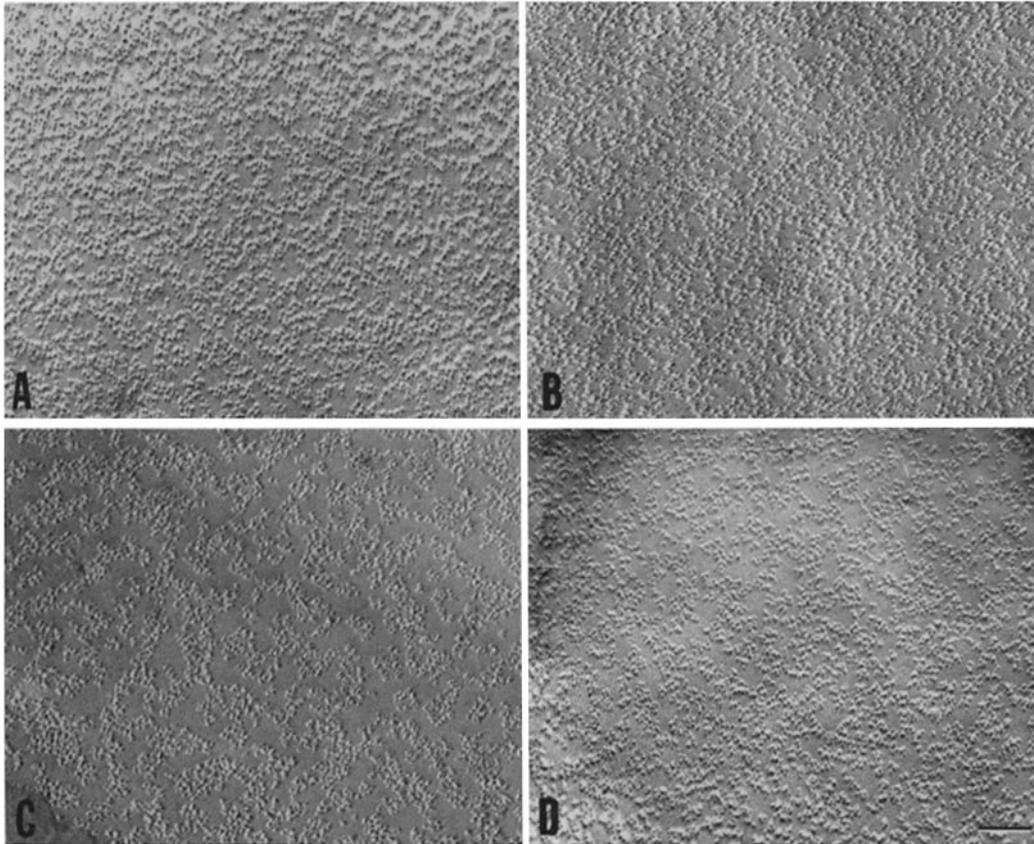


FIGURE 5 Freeze-fracture electron micrographs of erythrocyte ghost membranes at various C/P ratios and quenching temperatures (A) C/P = 0.75, 21°C; (B) C/P = 0.90, 21°C; (C) C/P = 0.75, 4°C; and (D) C/P = 0.90, 4°C. Bar, 100 nm.  $\times 68,000$ .

cles. Those membranes quenched from temperatures near the onset temperature consist of mixed populations of ghost membranes at different stages of particle aggregation and ghosts showing slightly aggregated IMP (Fig. 5 D). The temperature-dependent effects are reversible. The distribution of IMP in these samples has been quantitated mathematically (27). The fact that a mixed population of cells shows different degrees of particle aggregation supports the interpretation that a heterogeneous population of cells is partially responsible for the scattering of points in Fig. 4.

To examine the influence on IMP distribution of the cytoskeletal control from the spectrin network, we studied by freeze-fracture intact erythrocytes, fresh erythrocyte ghosts, and ghosts partially depleted of spectrin (6, 7). In spectrin-poor ghosts, 25% of spectrin was removed as indicated by SDS gel electrophoresis, whereas all spectrin

was retained in control samples after 24-h incubation. Similar quenching-temperature-dependencies of particle distribution were observed in all these samples. However, if low spectrin ghosts were resuspended at pH 5, gross particle aggregations occurred, as reported previously (7, 27). Rapid quenching without glycerol gives similar results. Therefore, it is unlikely that the thermal effects are artifacts caused by the cryoprotectant. From previous experience with freeze-fracture and calorimetric experiments (32), we know that the effect of 30% glycerol on the lipid phase transition is limited to a shift of  $<2^{\circ}\text{C}$ .

#### DISCUSSION

When liposomes are used to alter the C/P ratio of erythrocyte membranes, the question remains as to whether the liposomes fuse with the membrane as has been suggested (26, 29, 36) or whether the

liposomes merely attach to the cell (25), leading to a false C/P ratio determination of the membrane. If the changes in C/P ratio are mainly due to the fusion of liposomes to the membrane, both cholesterol and phospholipid molecules of the fused liposomes would have to be added to the membrane. To achieve a given final C/P ratio using liposomes of C/P = 0.3 and 2.0, as we did in our experiment, would mean a many-fold expansion of the surface area of the cell. It is obvious that fusion alone cannot account for the observed changes in the C/P ratio. Liposome inclusion-attachment was also found to be insignificant in our experiments, which verifies a report that uncharged liposomes do not attach to erythrocytes (24). (Positively charged liposomes [24] and serum lipoprotein LP-X [36] do attach to the erythrocyte surface through electrostatic forces, a process that leads to a rapid incorporation of both cholesterol and phosphatidylcholine at the same rate [36].) Our results can only be explained by assuming a much faster exchange rate for cholesterol than for phosphatidylcholine (3, 22). Serum albumin seems to facilitate this exchange.

The net increase or decrease of cholesterol in the membrane would be expected to cause considerable changes in the organization of lipid molecules in the membrane (1, 13, 21, 29, 34, 36). At temperatures just below 0°C, a phase transition in erythrocyte membrane has been observed by Raman spectroscopy (37) and by electron diffraction (18). The onset of a phase transition becomes detectable at higher temperatures if the C/P ratio is reduced. Upon removal of cholesterol from the lipid extracts of erythrocyte membranes, a broad calorimetric transition extending from -20° to 27°C has been observed (35). Gel-state lipid diffraction was observable up to 20°C if the cholesterol in the lipid extract was reduced to 3% (13). Studies of ghost membranes by fluorescent depolarization (4) and photobleaching techniques (34) have shown that the fluidity and the lateral mobility of lipids decrease more rapidly with temperature in cholesterol-depleted ghosts. It may be reasoned that the erythrocyte phospholipids (36) have an intrinsic broad-phase transition. The addition of cholesterol reduces the degree of cooperativeness among phospholipid molecules, thereby reducing the likelihood of gel-state domain formation at higher temperatures. Further reduction of the weak, gel-state diffraction signal at the high temperature margin of the broad transition causes an apparent shift to a lower temper-

ature of the detectable onset of the transition. Our results and those from x-ray diffraction of extracted lipids (13) support this hypothesis. The observable onset temperature of phase separation is indeed a function of C/P ratio, starting from 25°C at C/P = 0.4 and decreasing to 2°C at C/P = 1.0 (Fig. 4). Increase in the onset temperature resulting from the presence of free fatty acid and lysophospholipids, as has been pointed out (18), is unlikely in this case inasmuch as these products do not vary significantly with C/P ratio, and serum albumin is present in all incubations. The similar transition onset temperatures in ghost membranes and in lipid extracts of similar C/P ratio indicate that protein molecules in the erythrocyte membrane have little effect on the phase property of its bulk lipids (18). This analysis may not apply to those temperature-dependent functions thought to be related to the phase states of "boundary" lipids (37, 40).

The heterogeneous distribution of IMP at temperatures below the onset temperatures is likely a consequence of lipid-phase separation inasmuch as this effect is sensitive both to temperature and to C/P ratios. A recent study of the temperature-dependent IMP aggregation in erythrocyte membrane (10) also attributes the cause of particle aggregation to lipid phase separation, although a role for spectrin aggregation has been suggested (7). Although our study does not settle the question as to spectrin effects on IMP aggregation, we have shown that IMP distribution is at least partly controlled by the state of lipid organization. Possibly, the formation of phase-separated lipid microdomains leads to a preferential partitioning of intramembrane proteins (21) that have some freedom of lateral motion, even in the presence of a spectrin network. The onset temperatures of lipid domain formation are moderated by the cholesterol level of the membrane. At a given temperature, the lower the C/P ratio, the more likely is phase separation leading to nonrandom IMP distribution. The thermal effect on the distribution of IMP in the erythrocyte membrane is similar to, though not as pronounced as, that observed in model membranes (21) and in low cholesterol membranes such as nuclear membranes (39) and mitochondrial membranes (15). To draw a conclusion from model bilayer studies (21), the open areas in freeze-fracture micrographs of erythrocytes below the transition onset temperature represent small lipid bilayer domains in the gel state. These domains are stable features and not tran-

sient "scars" left from cell-liposome collision (36) inasmuch as any transient scars 500 Å in diameter would have been completely recovered in 0.6 s, assuming a lateral diffusion coefficient of protein to be  $10^{-11}$  cm<sup>2</sup>/s (9). These gel-state domains, like islands in a sea of lipids, rigidify the bilayer, thereby reducing the overall lateral mobility of lipids (33, 34). The heterogeneous lipid states could also lead to increased osmotic fragility (3) and could be responsible for the nonuniform distribution of fluorescent dyes at low temperatures (34). This heterogeneity is removed by adding more cholesterol or by raising the temperature.

Cholesterol is believed to be a moderating component in biomembranes. Its role is predicted by numerous studies on model bilayers. Our experiments demonstrate its effects on the plasma membrane of eukaryotes. At a low C/P ratio, the bilayer shows some degree of phase separation. Isolated domains exist at temperatures below the onset temperature. Above the onset temperature, the membrane is homogeneous. With the addition of cholesterol, this distinction is gradually diminished. Thus, among other factors, lipid-phase separation and cholesterol level definitely influence the molecular arrangement of the plasma membrane.

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