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Electron microscopic observation of domain movement in reconstituted erythrocyte membranes

(concanavalin A receptor/colloidal gold/lipid monolayer/lateral flow)

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ABSTRACT **The movement of labeled concanavalin A (Con A) receptors in reconstituted human erythrocyte membranes was observed directly in an electron microscope, using an environmental stage that kept the sample fully hydrated at all experimental temperatures. Human erythrocyte membrane ghosts were spread on the air/water interface in a Langmuir trough. The surface monolayer film contained most native proteins and lipids of the erythrocyte membrane. The Con A receptors in the film were labeled with Con A-conjugated, 25-nm-diameter gold microspheres. Unsupported bilayer membranes were reconstituted by dipping a 1000-mesh grid through the labeled surface film. The reconstituted membrane samples were observed under low beam current and photographed by timed exposures with sensitive x-ray films. The total radiation per exposure was kept below the damage threshold of 5×10^{-4} coulomb/cm^2. The Con A–gold labels were observed to move in unison within local areas (domains) of the reconstituted membrane. The size of the domains and the velocity of the labels were measured as functions of temperature. The typical domain size was 10 μm^2 and the typical velocity of the labels was 7 nm/sec. The minimum domain size and velocity were found at 17°–28°C. Reduction of the amount of cholesterol in the precursor erythrocyte membrane caused the domain velocity at 7°C to decrease and the domain size to increase; the opposite effect was observed with cholesterol enrichment. The results indicate that the components of the erythrocyte membrane tended to form moving domains and that the motion was related to lipid phase separation in the bilayer.**

The lateral motion of various molecular components in biological membranes is a subject of wide biological and biophysical interest. Although the fact that molecules undergo lateral motion is well known, the motion of individual protein molecules has only recently been visualized by fluorescence microscopy (1). Relative displacement of domains, as well as changes of domain motion and domain structure with temperature, has not yet been visualized. The major difficulty in observing lateral movement of individual domains or molecules is the limitation of the spatial resolution of the light microscope. The dehydration requirement for specimens practically precludes the possibility of observing motion in an electron microscope. Use of an environmental stage (2) to keep the specimen fully hydrated during electron microscopic observation has overcome this obstacle to some extent. This technique, together with the method of forming molecular bilayers over the holes of fine-mesh grids (3), enabled the study of hydrated and unsupported lipid bilayers by electron diffraction (4–6) and the imaging of phase-separated domains by the diffraction contrast technique (7). A preliminary measurement of membrane motion has also been made (8). Such electron microscopic observations augment previous observations of phase-separated domains in model membranes by fluorescence microscopy (9), by freeze-fracture electron microscopic observations of lipid patterns (10, 11), and by the repartitioning of integral proteins resulting from membrane-lipid phase separation (5, 12–14).

In the work reported here, we adapted the method of Verger and Pattus (15) to spread biological membranes as monolayers and applied the dipping method (3) to reconstitute a planar membrane in order to study the domain structure and motion in a reformed human erythrocyte membrane. Concanavalin A-conjugated gold microspheres were used as electron-contrast markers to label Con A receptors. We report here our direct observation of the movements of labeled Con A-receptor molecules and domains in reformed erythrocyte membranes by electron microscopy. The results were quantitated as functions of temperature and of modified cholesterol levels of the reformed membrane.

METHODS

Preparation and Characterization of the Surface Films. Human erythrocyte ghosts were prepared according to the method of Dodge *et al.* (16). Ghost membranes were spread as monolayers at less than 10 dynes/cm surface pressure (1 dyne = 10 μN) on Hanks' balanced salt solution (pH 7.4) in a Langmuir trough at room temperature, by a method similar to that of Verger and Pattus (15). After spreading, the surface film was compressed to a surface pressure of 30 dynes/cm by using a mobile barrier. The surface tension was measured by a platinum plate connected to a Cahn electrobalance. The surface film was washed three times by moving it between two mobile barriers to adjacent washing troughs. A 2 × 3-mm piece of mica was dipped through the air-water surface, using a mechanical drive that moved at a rate of 1 cm/min. The film deposited on the mica by dipping was sandwiched against a copper plate and immediately frozen by being plunged into liquid propane. Freeze–fracture was carried out in a Polaron E7500 unit. Replicas were examined in a Siemens 101 electron microscope. For compositional analysis, the surface film was collected by aspiration to a glass collector. The surface film and the subphase buffer were analyzed by $NaDodSO_4$/PAGE for their protein content and by thin-layer chromatography for their lipid content.

Labeling of Glycoproteins in the Surface Film. Colloidal gold–Con A conjugates were used as markers to label Con A-receptor sites. The colloidal gold suspension was prepared by the method of Frens (17). The resultant gold particles were about 25 nm in diameter. The gold suspension was conjugated to Con A at pH 8.8 according to the method of Goodman *et al.* (18). After the removal of large aggregates and unconjugated Con A by differential centrifugation, the conjugated

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gold–Con A was resuspended in 50 mM Tris Cl, pH 7.0/150 mM NaCl/1 mM $MgCl_2$/1 mM $MnCl_2$/1 mM $CaCl_2$ to one-tenth of the original volume. The gold–Con A was introduced into the subphase of the surface film, which was maintained at a surface pressure of less than 10 dynes/cm for labeling. After 2 hr of labeling at 10°C, the surface film was washed twice and compressed to 30 dynes/cm. An unsupported bilayer of the compressed film was formed on a 1000-mesh electron microscope grid by the dipping method (3).

Modification of the Cholesterol/Phospholipid Ratios in Erythrocyte Membrane. The ratios of cholesterol to phospholipid in erythrocytes were modified by a method similar to that of Cooper *et al.* (19). Small unilammellar vesicles of dipalmitoylphosphatidylcholine (Avanti Polar Lipids) and cholesterol (Sigma) at cholesterol/phospholipid molar ratios of either 0 or 2.0 were made by sonication. After sonication, heat-inactivated human serum albumin (1 mg/mg of lipid) was added, and the albumin/liposome mixture was centrifuged at 20,000 × *g* for 30 min to sediment undispersed lipid. Washed fresh erythrocytes were resuspended to a hematocrit of 10% in Hanks' balanced salt solution containing penicillin (1000 units/ml) and then were combined with an equal volume of the albumin/liposome mixtures (6 mg of lipid/ml) and incubated in a shaking water bath at 37°C for up to 24 hr. After incubation, all samples (including liposome-free control samples) were centrifuged at 1000 × *g* for 20 min and then were washed three times. Erythrocyte ghosts were then prepared. For cholesterol/phospholipid ratio determination, samples of erythrocyte ghosts were washed three times in distilled water to eliminate water-soluble phosphates. The lipids were extracted with chloroform/methanol (2/1, vol/vol), and cholesterol and phosphate were determined according to Yeagle *et al.* (20).

Observation of Con A–Gold Label Movement. The unsupported, hydrated bilayer was observed in an environmental stage in a Siemens 1A microscope (2). All operations were performed under an atmosphere saturated with water vapor at all temperatures from −6°C to 35°C. Thus, the samples were kept fully hydrated at all times. The 80-kV electron beam current was kept below 30 $\mu A/cm^2$ at the specimen level by fully energizing the first condenser lens and using a 5-μm aperture for the second condenser lens. The images of the unsupported bilayer membranes were extremely dim at this low beam current. Focusing was made in an area adjacent to the area of interest, at a slightly higher beam current density. The electron micrographs were taken at a magnification of 6500, with exposure times from 5 to 30 sec and at different specimen temperatures from −6°C to 37°C, on Kodak No-Screen x-ray film. To distinguish the natural movement of Con A receptors from an artifact of the electron beam irradiation, the following experiments were conducted. One previously unexposed area of the specimen was exposed to the electron beam for 5 sec to record a micrograph. Then the beam was turned off for 20 sec. The same area was again exposed for 5 sec to record a doubly exposed image in order to measure the "innate" movement of gold–Con A microspheres during this time interval. The same area of the membrane was then continuously irradiated by the electron beam for two additional 30-sec intervals to record a second and a third image. The displacement of the labels measured from the three exposures was used to determine the irradiation effect. The electron radiation dose was measured by a Keithley electrometer connected to an insolated and calibrated screen (21). This allowed the determination of the threshold electron-beam dose for influencing the innate movement of the labels.

RESULTS AND INTERPRETATIONS

The collected surface films retained most proteins of the original ghost membranes, as judged by $NaDodSO_4$/PAGE (Fig. 1*a*). The glycoprotein glycophorin, which is likely to be the major Con A receptor, comigrated with band 3 proteins

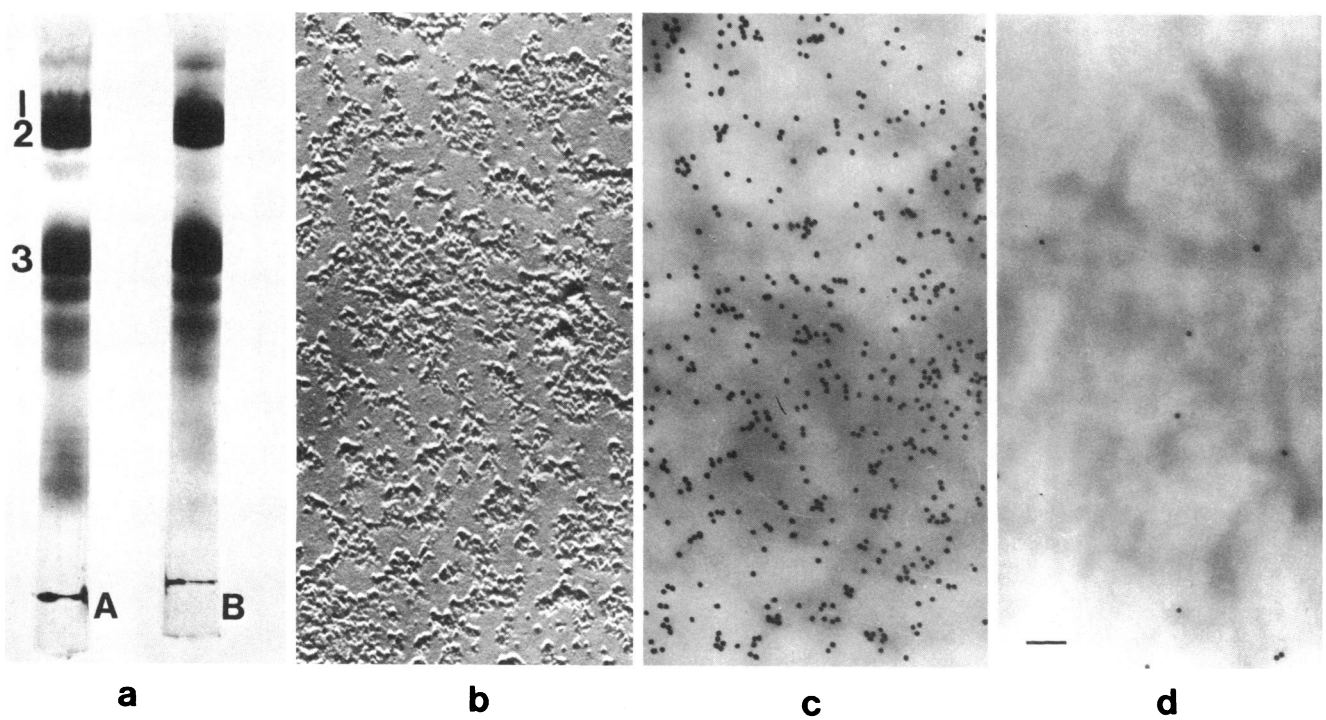

FIG. 1. (*a*) $NaDodSO_4$/PAGE analysis showing protein compositions of erythrocyte membrane ghosts (gel A) and of a surface-film spread from the ghost membranes (gel B). The protein bands 1–3 are labeled according to Fairbanks *et al.* (22). The proteins were visualized by staining with Coomassie blue. (*b*) Freeze–fracture electron micrograph of reformed membrane from the surface film. (*c*) Surface film labeled for 2 hr with 25-nm Con A–gold microspheres and deposited on a Formvar substrate. (*d*) Surface film labeled with unconjugated gold microspheres by procedures otherwise identical to those used for the film shown in *c*. (Bar = 0.1 μm.)

(22). There are negligible amounts of protein in the subphase (data not shown). The appearance of intramembranous particles in the membrane reconstituted from the surface film (Fig. 1*b*) was similar to that in ghost membranes (5), indicating that the surface film contained similar visible integral proteins in the ghost membrane. After labeling with 25-nm Con A–gold particles, the surface film deposited on a Formvar substrate showed gold microspheres "randomly" distributed over most of the sample area (Fig. 1*c*). Control experiments employing unconjugated gold microspheres with otherwise identical procedures showed negligible label (Fig. 1*d*). The final subphase supporting the film from which the samples were made was also found to contain no label. Evidently the attachment of gold microspheres to the film was Con A-mediated.

At 7°C, the mean velocities of labeled Con A receptors as a function of beam exposure showed that the mean velocity remained 6 nm/sec as long as the cumulative dose was less than 10^{-3} coulomb/cm^2. The velocity changed abruptly when the electron beam exceeded this cumulative dose threshold (results not shown). Nearly identical results were obtained from three experiments. The cumulative dose of 5×10^{-4} coulomb/cm^2 (31 electrons/nm^2) was considered to be the high dose limit for our experiment. The motion of labels was apparently not influenced by the electron beam for cumulative doses less than this limit, barring an undetectable change of velocity during the recording of the first micrograph.

The loci of movement of labeled glycoproteins were photographed at various time intervals (Fig. 2 *a* and *b*). Within each sector of the observed field, the labels moved more or less in unison. Independent movement of individual labels (diffusive motion) within a domain was uncommon. The collective movement of the labels ranged from unidirectional to vortex-like. The velocities of labels on different sectors of the observed field were very different, suggesting that each observed field should be divided into several local domains according to their common particle velocity. The boundaries between domains were discernible in most micrographs. The variation of domain areas with temperature was measured from 54 micrographs; a histogram is presented in Fig. 3 *Left*. The mean size and the dispersion of sizes of the domain tended to decrease with increasing temperatures up to 17°C. At 37°C, larger areas of higher velocities were formed. The trend indicates a general breaking-up and reformation of the larger domains as temperature rises, with the minimal domain size at the temperature range of 17°–30°C. At any given instant, there is no apparent difference in the spatial distribution of labels from domain to domain; therefore, the domains are not recognizable in static micrographs.

Because the velocities of individual labels were similar within each domain, the mean drift velocity of labels in each domain should represent the drift velocity of the entire domain. The domain velocities were measured for a total of 306 domains at various temperatures. The mean velocity was about 7 nm/sec at all temperatures tested except for −6°C, at

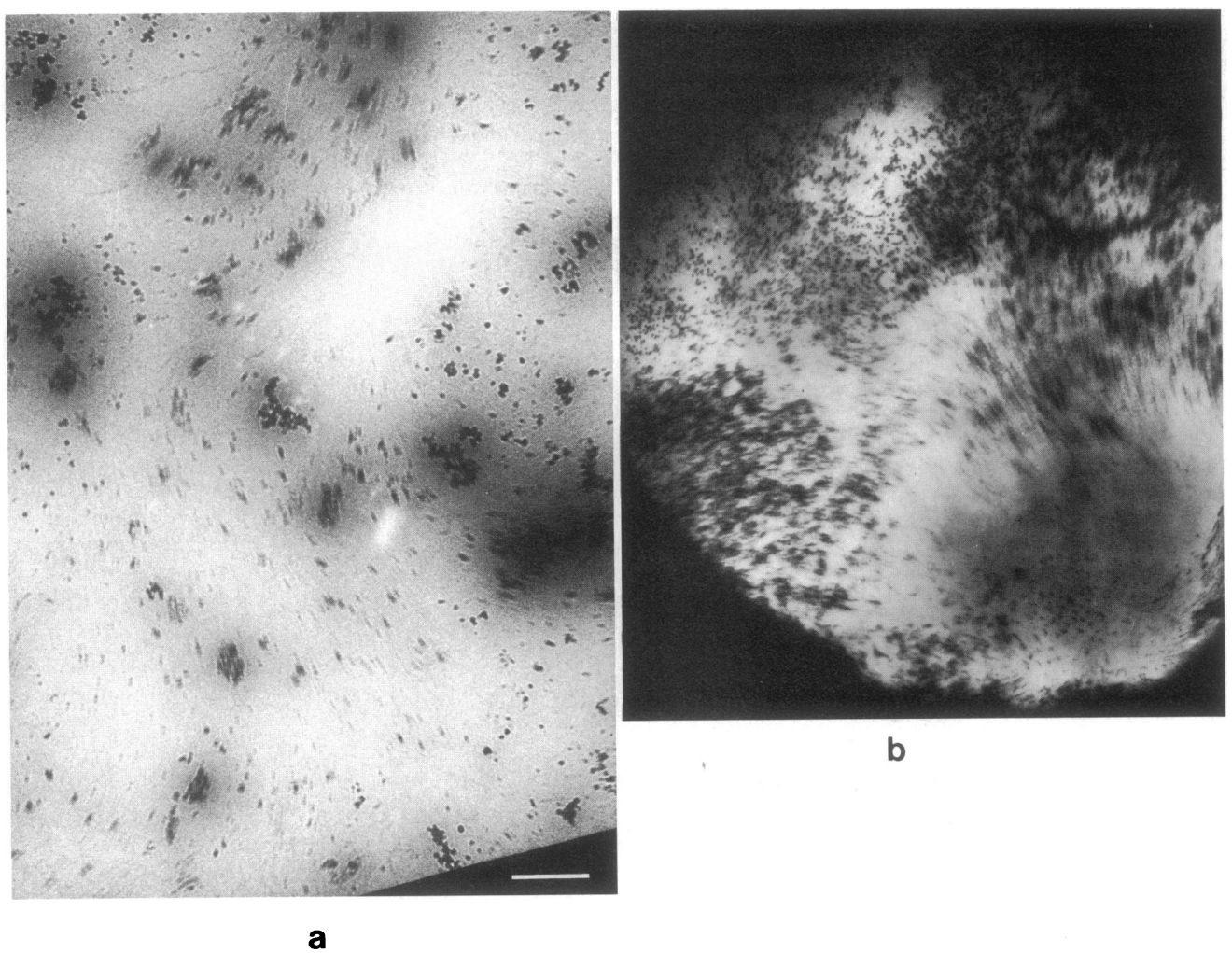

FIG. 2. Electron micrographs of the movement loci of Con A–gold labels in unsupported, reformed membranes at 7°C (*a*) and 12.5°C (*b*) in an atmosphere saturated with water vapor. Exposure times were 15 sec and 8 sec, respectively. (Bar = 1 μm.)

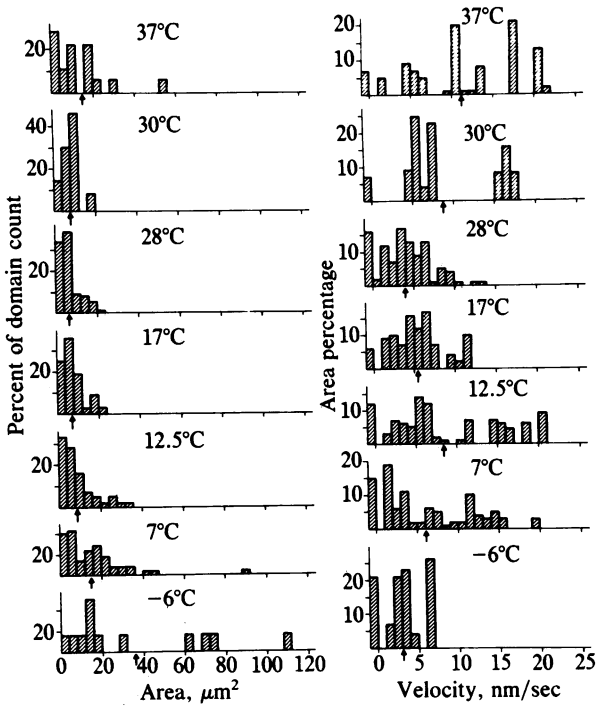

FIG. 3. (*Left*) Histogram of domain counts in reformed membrane at various temperatures, grouped according to the area per individual domain. Arrows indicate mean domain areas. (*Right*) Similar histogram of area percentage grouped according to domain velocity. Arrows indicate mean velocities.

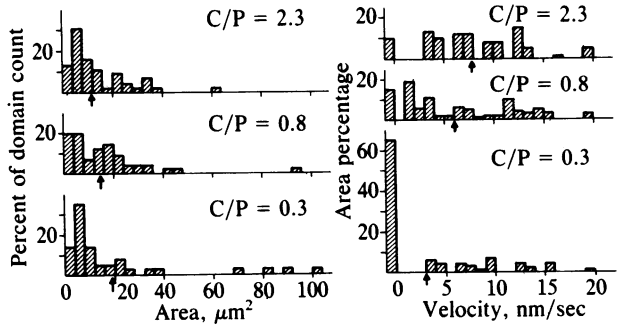

FIG. 4. (*Left*) Histogram of domain count versus area per domain in reformed membranes with modified cholesterol/phospholipid molar ratios (C/P) at 7°C. Arrows indicate mean domain areas. (*Right*) Similar histogram of area percentage grouped according to velocity. Arrows indicate mean velocities.

which a mean velocity of 3 nm/sec was measured (Fig. 3 *Right*). Generally speaking, domains gained higher velocity as temperature increased, except in the interval 17°–28°C, which is also the temperature range with the minimum domain size.

Correlation plots of domain size and velocity at each temperature showed that there were only weak positive correlations with linear regression, with correlation coefficients for samples at −6°, 7°, 12.5°, 17°, 28°, 30°, and 37°C being 0.33, 0.09, 0.59, 0.02, 0.03, 0.14, and 0.42, respectively.

Changes in domain area and velocity in membranes with modified cholesterol/phospholipid ratios at 7°C are presented in Fig. 4. At a reduced cholesterol/phospholipid ratio of 0.3, approximately 65% of the domain area had zero velocity. Many of these immobile domains were >70 nm² in area. The area percentage of immobile domains was reduced to 15% and 10% as the cholesterol/phospholipid ratio increased to 0.8 and 2.3, respectively. As the ratio increased, the mean domain size decreased while the mean velocity increased. There is no significant correlation between velocity and domain size at each cholesterol/phospholipid ratio.

DISCUSSION

There have been many measurements of movements of protein and lipid molecules in cell membranes (35). Whether the molecules move in unison or as individual entities could only be determined by inference. In the work described here, we directly observed the movement of single molecules or groups of molecules by electron microscopy. Our method permits direct measurements of molecular motion in membranes, reduces the spatial averaging processes inherent in many other methods, and extends the resolution limit of observation to the nanometer scale. However, the specimens we used are components of a biological membrane rendered in the form of "black membrane." Even though they contain almost all the original molecular components, their organization could have been altered by the spreading process. We recognize such limitations and aim to study the properties of these membranes as reconstituted membranes rather than as intact cell membranes.

A surprising finding is that we did not observe general diffusive motion of the labels. Most of the motion we observed was of a convective nature. This is different from a previous observation of latex microsphere movements on lecithin bilayers (8). The difference may be that the reformed membranes we used have more components, including cytoskeletal proteins (bands 1 and 2, Fig. 1*a*), and that the labels we used are linked to the membrane molecules by specific binding. There may be more than one Con A molecule attached to each gold microsphere, so that crosslinking by the labels may occur. This would impede the diffusive motion of individual molecules as well as that of extremely small domains. The crosslinking of receptors and the consequent linkage to the cytoskeleton may also impede the innate two-dimensional Brownian motion of individual receptors. The mass of the gold microsphere is also a heavy burden to the movement of individual molecules.

The most striking finding of our experiments is the existence of clearly defined motional domains in these reformed membranes. The typical size of a domain is about 10 μm². The typical velocity of a domain is 7 nm/sec, and the maximal velocity we observed was 22 nm/sec, which is just the upper limit set by fluorescence measurement of diffusion velocity for glycoproteins in erythrocyte membranes (23). We realize that the unsupported, reformed membranes we studied are physically different from the erythrocyte membranes in a fluid environment and that the two velocities are not analogous. Neither can we claim from our results that motional domains exist in intact erythrocyte membrane. Nonetheless, our observation of domains with velocities similar to that of erythrocyte membranes shows that there is a tendency of the membrane components to form separate domains that move at comparable velocities.

The variations of domain velocity and domain area with temperature show that at any experimental temperature, there is always a mixture of mobile and immobile portions of the reformed membrane, even at temperatures as low as −6°C. Assuming that the immobile domains represent domains containing gel-phase lipids, and the mobile ones fluid-phase lipids, then lipid phase separation occurs throughout our experimental temperature range. At −6°C some immobile or slow-moving (<5 nm/sec) domains become very large (>35 μm²). Such large gel-phase domains could be responsible for the observation of gel-phase lipid diffraction at low temperatures (4, 5). Our observation agrees with electron diffraction (4) and NMR (24) results that at temperatures greater than −5°C, large solid domains in erythrocyte membrane are not detected. The domains become smaller as

temperature increases. A possible explanation for the trend reversal at 17°–28°C is that the broken-up and increasingly mobile domains observed at 7°C and 12.5°C are in fact fragments of larger gel-phase domains. Their motion is similar to that of icebergs floating in a viscous medium. As these "icebergs" break into smaller entities, boundary areas increase and the motion of the labels is no longer in unison, resulting in an apparent "traffic jam" and a reduction of the mean domain velocity. At higher temperatures (>30°C), fluid domains become larger and eventually predominate. Labels again flow uniformly within the fluid domains, leading to a higher average velocity. The temperature range 17°–28°C thus represents the midpoint of a broad phase-transition, where the domain size is at a minimum and the boundary area is at a maximum (25, 26). Interestingly, the midpoint of such transition coincides with many functional discontinuities observed at about 20°C, as reported by some authors (27–31).

We have observed a general reduction of mean drift velocities along with many large immobile domains at 7°C in cholesterol-depleted (cholesterol/phospholipid ratio = 0.3) erythrocyte membranes. This result agrees with the report (32) that the diffusion of the lipid probe was 50% slower in cholesterol-depleted erythrocyte membrane than in non-depleted membranes at low temperature. The removal of a portion of the cholesterol from the erythrocyte membrane is known to sharpen the phase transition and thus render the membrane more solid at temperatures just below the phase-transition temperature (5). Our findings are analogous to those observed in oriented dimyristoyl phosphatidylcholine/cholesterol multilayers, where increasing cholesterol content led to increasing lateral diffusion rate of a lipid probe (33, 34) below the phase-transition temperature. Therefore, we believe that the variations we observe in domain size and velocity with temperature and cholesterol content are determined mainly by the physicochemical properties of membrane lipids.

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