TRANSLATIONAL MOBILITY OF THE MEMBRANE INTERCALATED PARTICLES OF HUMAN ERYTHROCYTE GHOSTS

pH-Dependent, Reversible Aggregation

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ABSTRACT

This paper demonstrates the translational movement along the plane of the human erythrocyte ghost of the membrane particles exposed by freeze-fracture. The membrane particles can be aggregated by incubation of the ghosts in media with a pH in the vicinity of 5.5 or 3.5. The particles are disaggregated in neutral and alkaline media (pH 9.5) and also at pH 4.5. Aggregation of the particles at pH 5.5 is reversible, prevented by prefixation in glutaraldehyde and by media of high ionic strength. Particle aggregation occurs within 2–4 min. These results are consistent with the concept that the erythrocyte ghost membrane is a planar fluid domain formed by a bilayer membrane continuum which is interrupted by localized, yet mobile, proteic intercalations.

INTRODUCTION

Freeze-fracture provides extensive face views of a cleavage plane probably formed by the juncture of the acyl chains in lipid bilayers of biological membranes and lamellar phases of lipids (5, 11, 29). In most membranes the planar continuity of the fracture plane is interrupted by numerous particles whose size, number, and array show considerable variation according to the membrane type (6). The chemical nature of the particles of most plasma membranes is not known. However, recent work suggests that the particles of erythrocyte ghosts and sarcoplasmic reticulum membranes may represent protein-containing sites. Reconstitution experiments have shown that particles are absent on the fracture faces of lamellar phases of lipids (12) or pure lipid extracts from erythrocyte ghost membranes but present on the fracture faces of lipid vesicles or lamellar lipid phases containing erythrocyte ghost or sarcoplasmic reticulum membrane proteins (23, 30). Moreover, etching experiments have shown that the particles contain A or B antigens (28, 31, 32), influenza virus, and wheat germ agglutinin receptors (37). Consequently, the membrane particles must include the glycoprotein and/or glycolipid responsible for these surface characteristics. At present, however, it is uncertain whether or not the particles of a given membrane type are chemically uniform and whether each particle consists of a single macromolecule or corresponds to a supramolecular entity.

Because the fracture face represents a plane through the hydrophobic interior of the membrane, it has been concluded that the particles represent localized interruptions of the bilayer

membrane continuum (29). The membrane particles could thus be considered as a morphological representation of protein-containing sites which are intercalated, but not necessarily sequestered, in the hydrophobic matrix of the bilayer membrane continuum Because surface properties are associated with the particles (A or B antigens, influenza virus, and wheat germ agglutinin receptors), they probably contain regions which protrude to the outer membrane surface.

When erythrocyte ghost membranes are fractured, most of the particles remain attached to the convex fracture face (the inside half of the bilayer membrane) (14) This attachment probably reflects deep penetration of this membrane half by the membrane particle and suggests that regions of the particle may also be in contact with the hydrophilic components at the inner surface of the erythrocyte membrane. If so, the particles are not only intercalated into the apolar matrix of the membrane but represent a structural unit which extends to the membrane surface and traverses the hydrophobic spaces of the bilayer membrane continuum (28).

It was reasoned that if the particles represent localized proteic intercalations in a bilayer membrane and are thus in polar continuity with membrane surface components, their distribution in the membrane might be affected by ionic changes in the composition of the medium. My experiments show that the membrane intercalated particles of human erythrocyte ghosts can indeed be aggregated through exposure to media of suitable pH and ionic strength and that this aggregation is reversible. I propose that aggregation and redissociation of the particles occurs through translational movement within the plane of the membrane.

MATERIALS AND METHODS

Erythrocyte ghosts were prepared from fresh human blood (O, Rh positive) drawn in Alsever's solution. The ghosts, produced by hemolysis in 20 mosmols of potassium phosphate buffer of pH 7.5, were hemoglobin-free and gray-white in color (13). The ghost suspensions were stored in ice not longer than 3 days.

For each experiment 50 (or 120) μl of packed ghosts were added to 8 (or 25) ml of the desired buffer solution and incubated. The composition of the incubation media as well as periods and temperatures of incubation are detailed in Table I. The pH of the buffers was measured on a Beckman Zeromatic pH meter using reference and glass electrodes. Variation of the pH during the experiment was minimal (less than 0.1 unit).

In experiments designed to test the effect of very low incubation temperature, 100 μl of a suspension of ghosts in 50% glycerol were rapidly mixed into buffer precooled to -16°C. The ghost suspensions were incubated at -16°C for 30 min and made to 1% glutaraldehyde Suspensions were fixed for 32 hr at -16°C, transferred to ice, incubated for 20 hr, diluted to 25% glycerol, pelleted, and frozen

In experiments designed to determine the effect of a short incubation time, a ghost suspension (obtained by centrifugation of the ghosts at 20,000 *g*) in 8 mM potassium phosphate buffer at pH 7.5 was rapidly titrated under vigorous stirring with 8 mM phosphoric acid containing 2.5 mM $CaCl_2$. After stabilization of the pH at 5.5, portions were rapidly frozen at intervals as indicated in Table I.

For freeze-fracture, samples of pelleted ghosts were placed on 3 mm cardboard disks and quenched in the liquid phase of partially solidified chlorodifluoromethane (Freon 22) cooled in liquid nitrogen. Freeze-fracture was carried out in a Balzers device with a stage temperature of -115°C (no etching) (5, 26). The micrographs are mounted with the shadow from the bottom to the top and, with few exceptions, show the particle-rich face (convex fracture face, see Ref. 29 for interpretation) Shadows are white.

RESULTS

The results of each experiment are presented in Table I. The following scale was used to evaluate the degree of aggregation of the membrane particles: D, "dissociated"—defined as the particle distribution on the convex fracture face of ghost exposed to 8 mM buffer at pH 7.5; A, definite aggregation; AA, intense aggregation. To a limited extent, the aggregation of the particles was variable, occasionally even within a single ghost. The results presented in Table I and the micrographs are representative. The degree of aggregation of the membrane particles does not completely describe the alterations in the freeze-fracture morphology of the ghosts. Description of the results and analysis of the micrographs (Figs. 1–14) is necessary to evaluate morphological modifications which cannot easily be tabulated.

pH

In ghosts incubated at pH 7.5 and 9.5 the membrane particles are dissociated. Dispersed particles and small groups of particles coexist; there are no organized patterns (Fig. 1 a). Be-

TABLE I

Outline of Experiments

Type of Test	First incubation				Second incubation				Degree of particle aggregation‡	Fig no
	Composition*	pH	Temperature	Period	Composition	pH	Temperature	Time period		
			°C	min			°C			
pH	T	9 5	35	30					D	—
	T	7 5	35	30					D	1 a, 1 b
	P	7.5	35	30					D	—
	P	6 2	35	30					A	2 a, 2 b
	P	5 5	35	30					AA	3 a, 3 b
	Ac	5.5	35	30					AA	4
	P	5 0	35	30					AA	5
	P	4.8	35	30					A	—
	P	4.5	35	30					D	6
	P	4.0	35	30					A	7
	P	3.5	35	30					AA§	8
	P	3 0	35	30					AA§‖	9
Reversibility	P	5 5	35	30	T	7.5	35	30 min	D	10 b
	P	5.5	35	30	P	7 5	35	"	D	—
	P	4.8	35	30	P	7.5	35	"	D	10 a
	P	3.5	35	30	P	7.5	35	"	D§¶	—
Glutaraldehyde	P, Glu	7.5	25	30	P	5.5	35	30 min	D	11 a
		5.5	35	30	P, Glu	5.5	25	"	AA	11 b
Time	P	7.5	25	10					D	—
	P**	5.5	25	2–4					AA‡‡	12 a
	"	"	25	5–7					AA	12 b
	"	"	25	30–35					AA	12 c
Temperature	P, Gly§§	7 5	−16	30	P, Gly, Glu	7 5	−16 4	32 hr 20 "	D	—
	P, Gly	5.5	−16	30	P, Gly; Glu	5 5	−16 4	32 " 20 "	A	13 b
	P; Gly	5.5	35	30	P, Gly, Glu	5.5	−16 4	32 " 20 "	A	13 c
	P	5.5	4	30/60/120‖‖					AA	13 a
Ionic strength	P; .14 M NaCl	7.5	35	30					D	14 b
	P, .14 M NaCl	5.5	35	30					D	14 a
	P; 1 M NaCl	7.5	35	30					D/A	—
	P, 1 M NaCl	5.5	35	30					D	14 c

* All buffers 8 mM. Abbreviations: P, potassium phosphate, T, tris/HCl; Ac, sodium acetate; Glu, 1% glutaraldehyde; Gly, 50% glycerol. All solutions containing 1 mM $CaCl_2$ except phosphate buffer at pH 7 5.

‡ D, dissociated, A, definite aggregation; AA, intense aggregation.

§ Apparent structural damage.

‖ Particles coalescent in a portion of the aggregates (Fig. 9).

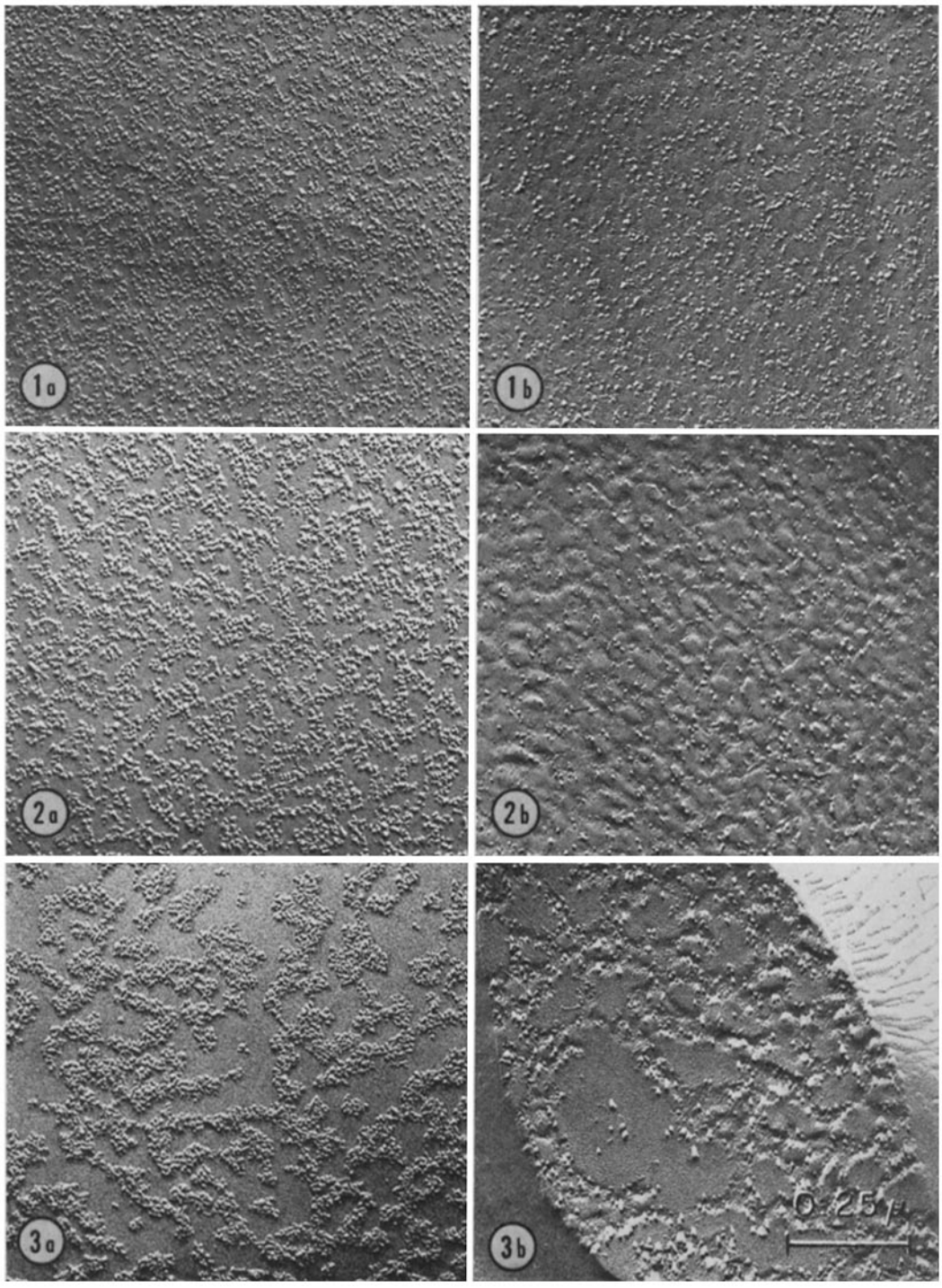
¶ Difficult to evaluate due to structural damage.

** Ghost suspension titrated with 8 mM phosphoric acid, 2.5 mM $CaCl_2$

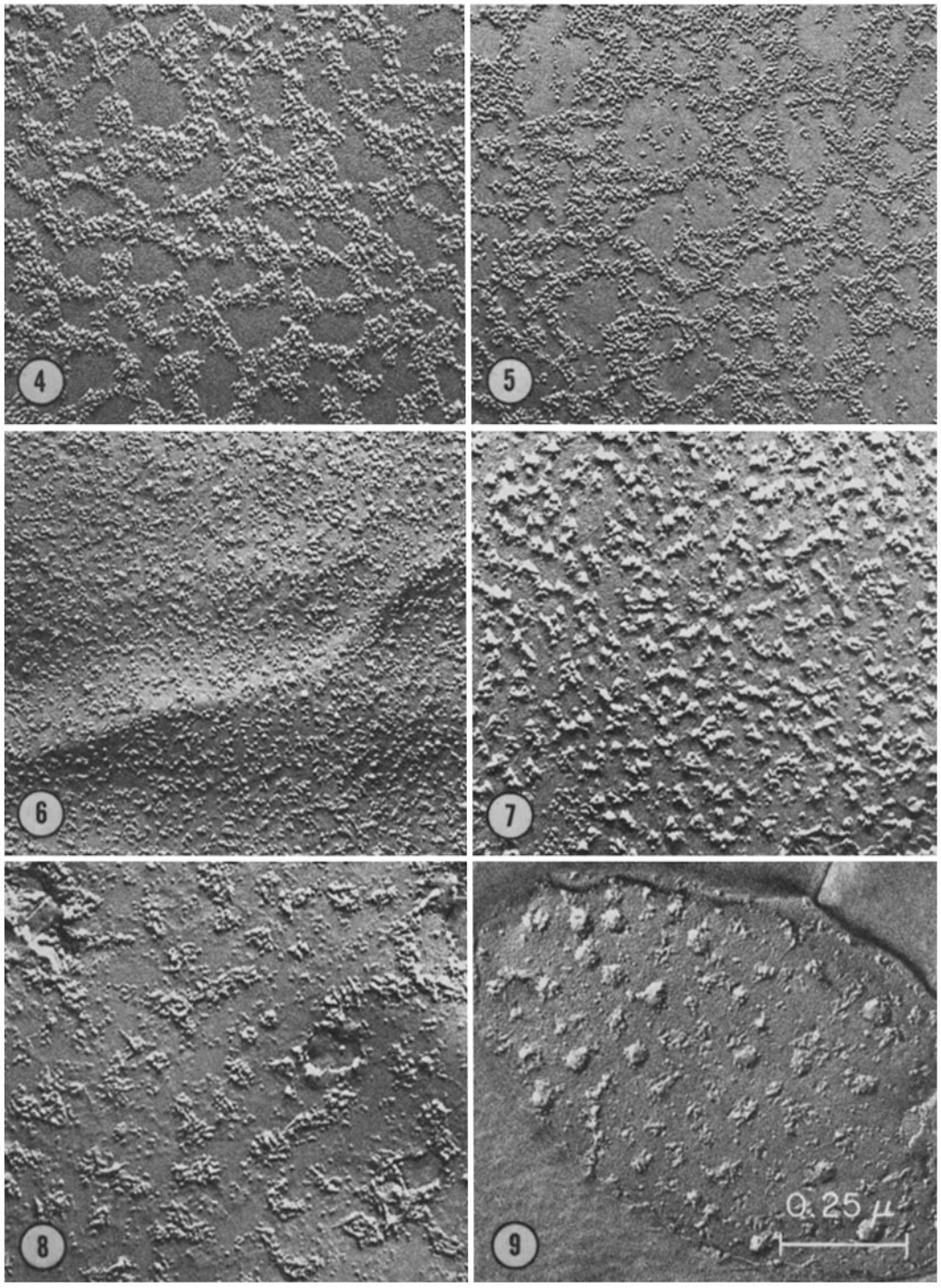
‡‡ One of the replicas shows a portion of the fracture faces with less particle aggregation and, eventually, dissociated particles.

§§ Please see Materials and Methods for experimental details.

‖‖ Total time elapsed from starting of incubation period to freezing of the samples.



FIGURES 1-9 Fracture faces of erythrocyte ghost membranes exposed to media with pH ranging from 7.5 to 3.0. The particles are dissociated at pH 7.5 (Fig. 1 a, convex fracture face; Fig. 1 b, concave fracture face). At pH 6.2 the particles are slightly aggregated (Fig. 2). Aggregation is intense at pH 5.5 (Fig. 3, in phosphate buffer; Fig. 4, in acetate buffer) and pH 5.0 (Fig. 5). The particles are dispersed at pH 4.5 (Fig. 6); clumped in small aggregates at pH 4.0 (Fig. 7); intensely aggregated at pH 3.5 (Fig. 8); and aggregated and coalescent at pH 3.0 (Fig. 9). Gross complementarity of convex and concave fracture faces is difficult to establish if the particles are dissociated (Figs. 1 a and 1 b) but is clear if the particles are aggregated (Figs. 2 a and 2 b; 3 a and 3 b). All figures, × 70,000.



FIGURES 4–9 See legend under Figs. 1 a–3 b.

cause of this randomness it is very difficult to recognize the depressions left on the concave fracture face (Fig. 1 b) by the particles which have been fractured away with the other membrane half (convex fracture face, Fig. 1 a). At pH 6.2 the particles are clearly, although not intensely, aggregated (Fig 2 a); most particles on the convex fracture face are organized into a discontinuous network of aggregates. The patterns of aggregation are also, and often more clearly, recognizable by the array of depressions left on the concave fracture face (Fig. 2 b). At pH 5.5 and pH 5 0 the aggregation of the particles is intense (Figs. 3 a, 4, and 5) The depressions left on the concave fracture face are clear and demonstrate the gross complementarity of convex and concave fracture faces produced by a single plane of fracture (14) (Fig. 3 b). The fewer particles present on the concave fracture face are almost exclusively found in depressed areas, showing that the particles are aggregated into a single pattern and not into two unrelated patterns. At pH 4.8 the particles are still clearly aggregated but at pH 4.5 the particles are again dissociated (Fig. 6). At pH 4.0 the particles are organized in small tight aggregates (Fig. 7). At pH 3.5 aggregation of the particles is very intense (Fig. 8), occasionally resulting in the largest aggregates observed. At pH 3.0, strong aggregation of the particles is observed but the particles begin to coalesce (Fig. 9),

Reversibility

The fracture faces of ghosts incubated at pH 7.5 after a preincubation at pH 5 5 or 4 8 showed that the membrane particles were redispersed (Figs. 10 a, 10 b). Redispersion was difficult to establish in ghosts preincubated at pH 3 5 due to the structural damage caused by exposure to this low pH.

Glutaraldehyde

Prefixation with glutaraldehyde at pH 7.5 prevented particle aggregation (Fig 11 a) Intense particle aggregation was, however, observed on the fracture faces of ghosts incubated at pH 5.5 before fixation with glutaraldehyde (Fig 11 b).

Time

Most fracture faces of the ghosts exposed for no longer than 2–4 min to a medium of pH 5 5 at 25°C showed intense particle aggregation (Fig. 12 a) similar to that observed in ghosts exposed for longer periods (Figs 12 b, 12 c)

Temperature

Particle aggregation at pH 5.5 was not prevented in ghosts incubated at 4°C (Fig 13 a) or even at −16°C (Fig. 13 b).

Ionic Strength

The fracture faces of ghosts incubated in phosphate buffer at pH 5 5 and 7 5 containing 0.15 or 1.0 M NaCl showed the particles mostly dissociated (Figs 14 a, 14 b, 14 c).

DISCUSSION

The present results show that the membrane particles of human erythrocyte ghosts can be aggregated and that this aggregation can be reversed. Aggregation and redispersion must occur through translational movement of the membrane particles along the plane of the membrane. Since erythrocyte ghosts are simple membrane systems without cytoplasm, hypothetical alternatives (15) which invoke particle synthesis and/or metabolic turnover or the integration of previously synthesized particles into the membrane can be ruled out Still another alternative (the removal of particles from their original positions and subsequent reinsertion at a different location in the membrane [15]) is unlikely because it would probably result in substantial loss of particles, which was not observed. Furthermore, particle removal would involve the rupture of extensive hydrophobic associations between the particle and the bilayer membrane.

It is clear that glutaraldehyde fixation stabilizes the particle distribution since ghosts incubated and fixed at pH 7.5 no longer exhibit particle aggregation upon incubation at pH 5.5. Intense particle aggregation is observed in membranes which, after incubation at pH 5.5, were stabilized with glutaraldehyde It can therefore be concluded that aggregation is not a freezing artifact (eutectic formation).

Particle aggregation at pH 5.5 occurred within 2–4 min, including the titration of medium and membranes which took approximately 1 min and the establishment of new ionic gradients at the membrane surfaces. In fact, it is likely that aggregation of the particles occurs within a period

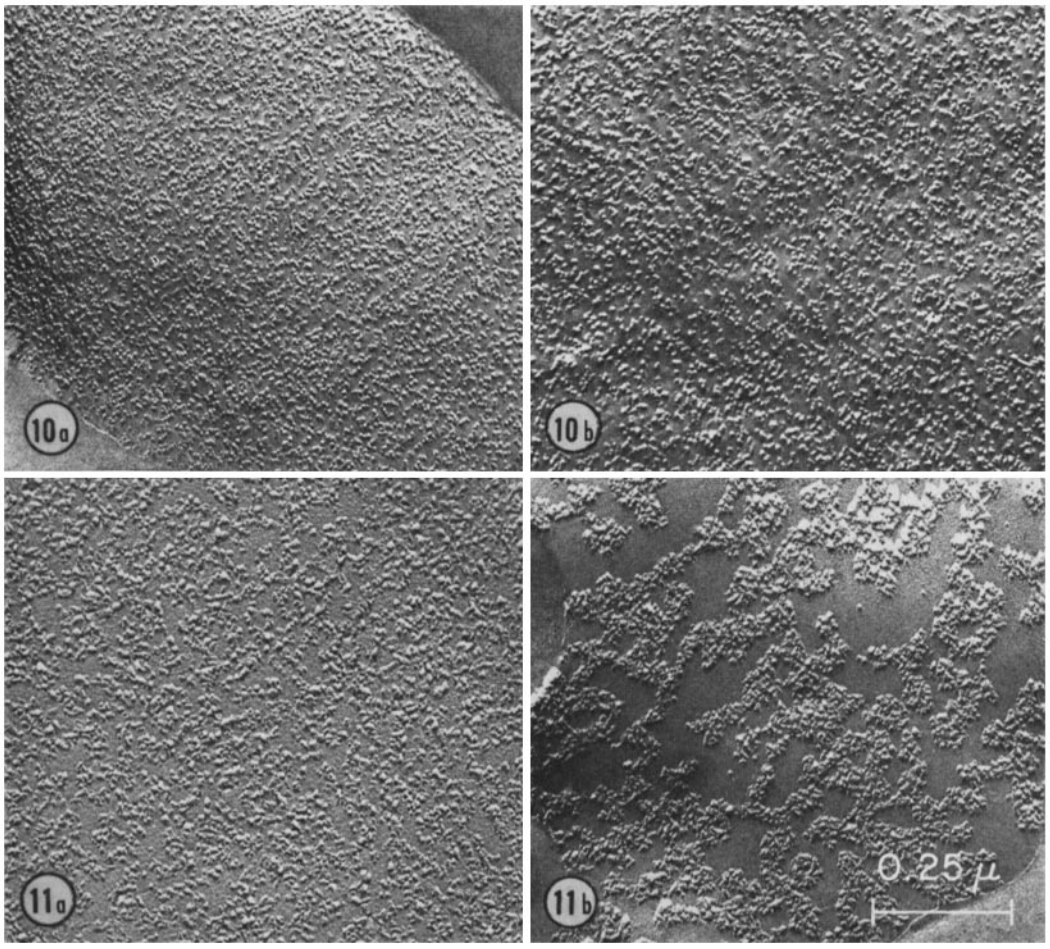


FIGURE 10. Reversibility of particle aggregation. After aggregation of the particles at pH 4.8 and 5.5, dissociation can be obtained by incubation in buffer at pH 7.5 (Fig. 10 a, 4.8–7.5, Fig. 10 b, 5.5–7.5) × 70,000.

FIGURE 11. Effect of glutaraldehyde fixation on particle aggregation at pH 5.5. If the membranes are prefixed in glutaraldehyde, exposure to pH 5.5 does not result in aggregation of the particles (Fig. 11 a). If the membranes are postfixed in glutaraldehyde after exposure at pH 5.5, intense aggregation results indicating that the particles are aggregated during the incubation and not during rapid freezing of the sample (Fig. 11 b). × 70,000

much shorter than 2–4 min because (*a*) translational movement of the particles involves two-dimensional diffusion, a process much faster than diffusion in three dimensions (1); and (*b*) aggregation of the particles can be achieved through very short translations (less than 1000 A). In this respect, the short period of rotation obtained for a rhodopsin molecule within the plane of a retinal rod disc membrane, about 10^{-5} sec (10), clearly suggests the possibility of very rapid translations in the absence of stringent steric restrictions ("the total energy of a system is divided equally among the different degrees of freedom," principle of the equipartition of energy [16]).

Aggregation of the membrane particles occurred from −16° to 35°C. This is not surprising because (*a*) variations in absolute temperature to which the rate of diffusion is linearly related (2) were relatively small (less than 20%), and (*b*) for red cell membranes no sharp transition temperature below which the mobility of the lipid components would be much reduced has yet been

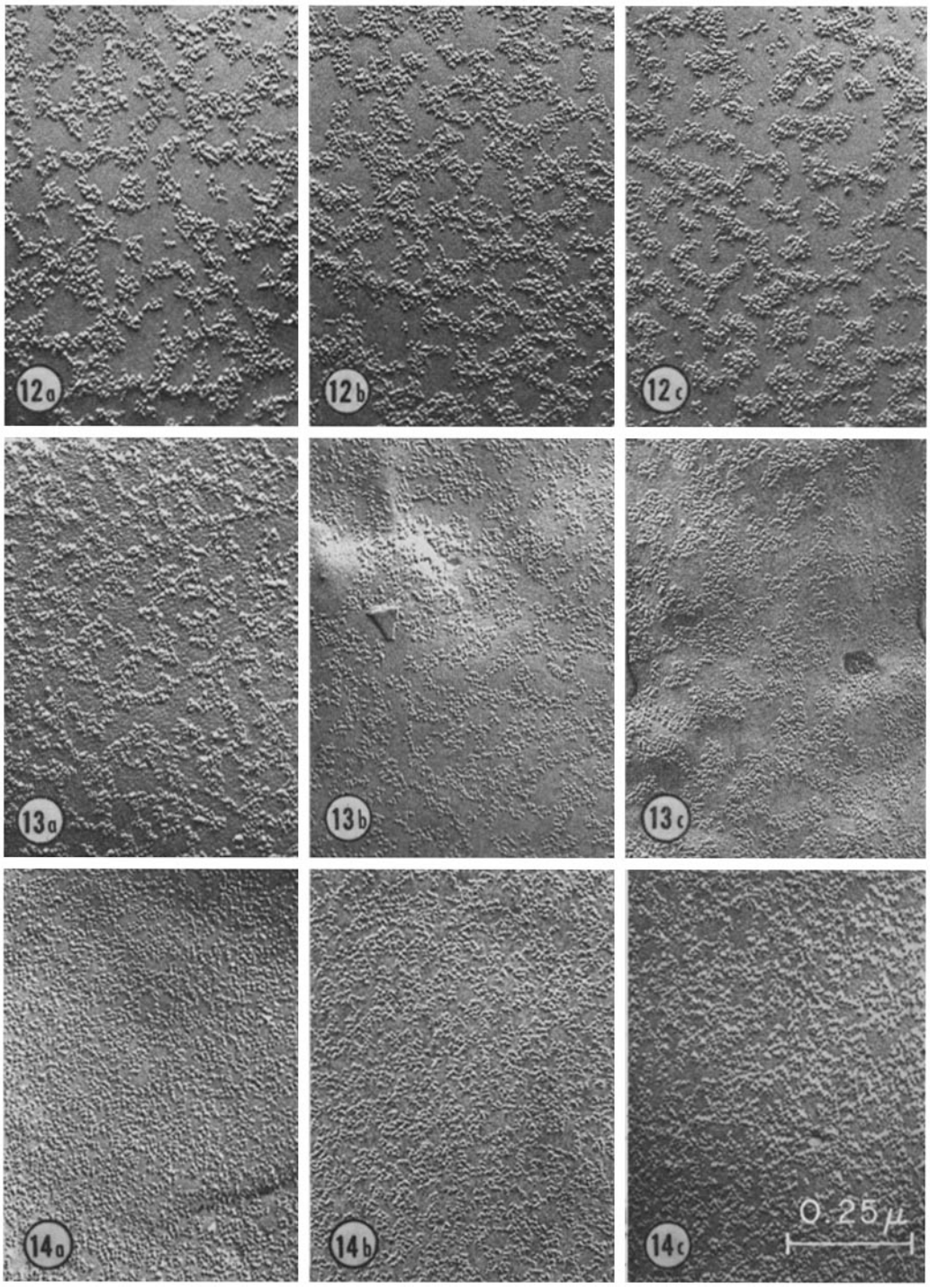


FIGURE 12 Effect of time on particle aggregation at pH 5.5. Aggregation occurs within 2–4 min from initiation of titration (Fig. 12 a) and is similar to that observed after 5–7 min (Fig. 12 b) or 30–35 min (Fig. 12 c). × 70,000.

FIGURE 13 Effect of low temperature on particle aggregation at pH 5.5. Aggregation is clear in membranes incubated at 4°C (Fig. 13 a). In 50% glycerol incubation at −16°C does not prevent aggregation (Fig. 13 b) which is similar to that observed at 35°C (Fig. 13 c). Nonparticulate areas in Fig. 13 a show cobblestone appearance, a result of contamination produced by gradually increasing the voltage for shadowing, instead of firing electrodes at a preset voltage. × 70,000.

FIGURE 14 Effect of ionic strength on particle aggregation at pH 5.5. Aggregation at pH 5.5 is prevented in media containing 0.14 M NaCl (Fig. 14 a; Fig. 14 b, control at pH 7.5) or 1 M NaCl (Fig. 14 c). × 70,000.

demonstrated. Although temperature dependence has been observed in the light microscopy immunofluorescence study of the intermixing of surface antigens of virally fused cells (15), the low resolution of immunofluorescence techniques makes it impossible to determine whether the apparent mixing of fluorescence results from translational movement of antigen molecules *per se* or whether it is caused by intermixing of larger, antigen-containing membrane domains. In the present experiments, however, the higher resolution of the freeze-etch technique (about 20 A) and the close packing of the aggregated particles demonstrate that particle movement occurred relative to neighboring membrane components. These results are consistent with a concept which envisages the membrane of the erythrocyte ghost as a planar fluid domain (4, 21) formed by a bilayer membrane continuum which is interrupted by localized, yet mobile, probably proteic intercalations.[1]

What may be the factors which are responsible for the stabilization of the particle within the plane of the membrane and yet allow for its translational freedom? Particle aggregation was dependent on the pH and ionic strength and could be prevented by glutaraldehyde (a fixative acting on the polar regions of the membrane). This dependence would be difficult to accommodate were the particles entirely sequestered within the hydrophobic milieu. If the particles are the morphological representation of protein-containing sites which are intercalated, but not sequestered, in the hydrophobic matrix of the bilayer membrane, they must be exposed to a heterogeneous environment which is, successively, hydrophilic/hydrophobic/hydrophilic. Because this exposure would be topologically distinct from that provided by a homogeneous milieu (either polar or apolar), it demands in principle a particular topological organization of the hydrophilic and hydrophobic regions of such sites. A general topological solution would not sequester the hydrophobic regions within the core of a membrane particle but concentrate them equatorially (38, 39) in a torus in register and interacting with the hydrophobic matrix of the bilayer membrane (Fig. 15a, b).[2] Such a topological solution would thus account for the observed translational motion of a particle since translational (and also rotational) motion of a particle would merely involve the exchange of equivalent, noncovalent bonds. However, remarkable vertical stability of the intercalated particles (i.e. motion perpendicular to the plane of the membrane) is expected as vertical deviations would probably result in the

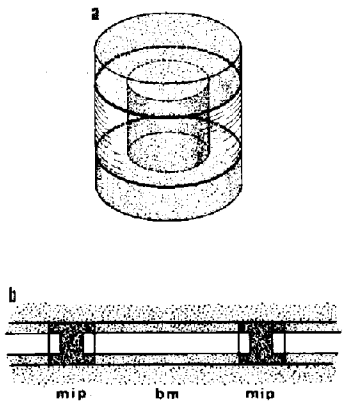


FIGURE 15 Fig. 15 a, Hypothetical topological relationships proposed for the hydrophilic and hydrophobic spaces of a membrane intercalated particle. The hydrophobic residues (not shaded) are equatorially distributed in a torus in register with the hydrophobic matrix of the bilayer membrane (see Fig. 15 b). Axial organization of residues (shaded) could provide hydrophilic continuity across the membrane. Fig. 15 b, Schematic interpretation of the hydrophilic and hydrophobic spaces in a bilayer membrane (*bm*) with membrane intercalated particles (*mip*). Translational movement of the particles along the plane of the bilayer membrane involves only the exchange of equivalent, noncovalent bonds. These diagrams are intended only to facilitate the interpretation of possible topological relationships. They should not be interpreted as actual structural models of any membrane.

[1] The importance of hydrophobic lipid-protein interactions as determinants of membrane structure has been repeatedly acknowledged (17, 19, 20, 22, 39). Recently, it has been proposed that in erythrocyte ghosts some of the proteins span the entire membrane (3, 8, 35). At present, however, no more than circumstantial evidence can associate such proteins with the membrane particles.

[2] It is clear that this topological organization can include structural and functional asymmetries (7). In an 85 A particle it could also allow (although not necessarily) for the axial and terminal distribution of its hydrophilic regions which might provide a hydrophilic continuum across the membrane (Figs. 15 a, 15 b). An extreme instance in which hydrophilic continuity may be provided by membrane particles is the gap junction (25, 27, 33).

reduction of polar and apolar interactions and in an entropy reduction as apolar residues are exposed to a hydrophilic environment

My results with red blood cell ghosts should not be indiscriminately extrapolated to other membrane systems where the translational movement of the intercalated particles may be limited by other membrane surface components or even by cytoplasmic effectors of intramembrane mobility (36, 40). These experiments are insufficient to explain the physical-chemical events which determine particle aggregation or particle dissociation. Because aggregation of the particles is reversible, it cannot be attributed to the selective removal of membrane components during incubation [3] pH and ionic strength dependence and reversibility suggest that this mechanism is electrostatic. It is tempting to speculate that particle aggregation represents an intramembrane precipitation analogous to that normally associated with the isoelectric point of most proteins.[4] However, it is also possible that aggregation is due to the contraction or aggregation of other membrane components to which the membrane particles might be intimately associated. Clearly, definition of the molecular mechanisms responsible for particle aggregation must await the results of future experiments, including selective removal or modification of membrane components

CONCLUSIONS

I The membrane particles of human erythrocyte ghosts can be aggregated. This aggregation is pH- and ionic strength–dependent, occurs within less than 2–4 min, is reversible, and can be prevented by glutaraldehyde fixation

II. Translational movement of the membrane particles must mediate their aggregation and redispersion.

III. The erythrocyte ghost membrane can be envisaged as a planar fluid domain formed by a bilayer membrane continuum which is interrupted by localized yet mobile, probably proteic, intercalations

[3] Removal of membrane components does occur during the preparation and storage of the ghosts and the experimental manipulations (18).

[4] Aggregation at two different pH ranges does not necessarily contradict the concept of isoelectric precipitation (24) because it may reflect spatial heterogeneity in the distribution of chemical groups (e g. at the outer and inner surfaces). In any case, irreversible structural changes caused by exposure to high hydrogen ion concentrations and concomitant loss of components, including bound cations (9, 34), make it difficult to relate particle aggregation at both pH ranges to the same system.

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