

# Freeze-fracture Cytochemistry: Partition of Glycophorin in Freeze-fractured Human Erythrocyte Membranes

PEDRO PINTO DA SILVA and MARIA ROSARIA TORRISI

*Section of Membrane Biology, Laboratory of Pathophysiology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205. Dr. Torrissi's present address is the Institute of General Pathology, Rome, Italy.*

**ABSTRACT** Thin-section and critical-point-dried fracture-labeled preparations are used to determine the distribution and partition of glycophorin-associated wheat germ agglutinin (WGA) binding sites over protoplasmic and exoplasmic faces of freeze-fractured human erythrocyte membranes. Most wheat germ agglutinin binding sites are found over exoplasmic faces. Label is sparse over the protoplasmic faces. These results contrast with previous observations of the partition of band 3 component where biochemical analysis and fracture-label of concanavalin A (Con A) binding sites show preferential partition of this transmembrane protein with the protoplasmic face. Presence of characteristic proportions of WGA and Con A binding sites over each fracture face is interpreted to indicate the operation of a stochastic process during freeze-fracture. This process appears modulated by the relative expression of each transmembrane protein at either surface as well as by their association to components of the erythrocyte membrane skeleton.

In freeze-fracture, splitting of the bilayer membrane continuum with differential partition of integral membrane proteins can be considered a process of structural dissection. Recently, we developed "fracture-label" techniques for the direct cytochemical investigation of the distribution and partition of integral and peripheral membrane components as indicated by the presence of chemical groups and lectin binding sites on the faces of freeze-fractured plasma and intracellular membranes. The results can be observed both in thin sections of freeze-fractured material ("thin section fracture-label") (1, 2) and in platinum-carbon replicas of critical-point-dried specimens after freeze-fracture ("critical point drying fracture-label") (3).

Initial application of thin-section fracture-label to the cytochemical investigation of the fracture faces of human erythrocyte membranes showed that concanavalin A (Con A) binding sites are preferentially labeled on the protoplasmic faces (1). Labeling of the P face by Con A-ferritin conjugates implies that band 3 component (the Con A binding transmembrane protein; see references 4-8) generally partitions with the inner membrane half, a process that must involve dragging of its Con A binding heterosaccharides across the outer membrane half. These results are consistent with the biochemical analysis of half-membrane preparations (9) and, in addition, they indicate that Con A labeling of the E face, although minor, appears significant.

Much less is known about the partition of glycophorin.

Biochemical studies indicate the presence of glycophorin molecules and shorter sialoglycopeptides in preparations of exoplasmic membrane halves (9). These studies, however, were not designed to assess the degree of partition of glycophorin with the inner membrane half, nor could this partition be inferred from our initial fracture-label studies (1) showing the presence of cationized ferritin or colloidal iron on both P and E faces, as these markers cannot identify the molecular species labeled. We report here the use of thin-section and critical-point-drying fracture-label to determine the pattern of distribution and partition of glycophorin as determined by wheat germ agglutinin (WGA) labeling of protoplasmic and exoplasmic faces of freeze-fractured human erythrocytes. Our results show preferential label of the exoplasmic faces in contrast to preferential partition of band 3 with the protoplasmic faces. A description and morphological analysis of the protoplasmic and exoplasmic fracture faces of human erythrocytes that, after freeze-fracture, are critical point dried and platinum replicated is included.

## MATERIALS AND METHODS

### *Freeze-fracture*

Human erythrocytes (O positive) were fixed in 1% glutaraldehyde in 310 mOsm phosphate buffer, pH 7.4, for 1 h at 4°C, embedded in 30% bovine serum albumin (BSA), and gelled with glutaraldehyde (1%, 30 min, at room temperature). The gels were diced into small pieces (~1 × 2 × 2 mm), impregnated

gradually in 30% glycerol in phosphate buffer as described above, and frozen in partially solidified Freon 22 cooled by liquid nitrogen. Specimens to be critical point dried were freeze-fractured in a petri dish filled with liquid nitrogen, using a pre-cooled scalpel. Specimens destined to the observation of thin sections of fractured membranes were prepared by transfer of frozen gels into a liquid nitrogen-filled glass container immersed in a slush of liquid nitrogen and solid carbon dioxide and were freeze-fractured by repeated crushing with a glass pestle pre-cooled in liquid nitrogen. All samples were thawed in a solution of 30% glycerol and 1% glutaraldehyde in 310 mOsm phosphate buffer, pH 7.4, de-glycerinated in 1 mM glycyl-glycine in the same buffer, and washed twice before labeling or future processing.

## Cytochemical Labeling

For fracture-label with WGA, gel fragments were incubated in a solution of 0.25 mg/ml WGA in 0.1 M Sorensen's phosphate buffer, 4% polyvinylpyrrolidone pH 7.4 for 30 min at 37°C. Controls were preincubated in 0.4 M *N*-acetyl-D-glucosamine for 15 min at 37°C followed by incubation in a WGA solution in the presence of 0.4 M *N*-acetyl-D-glucosamine. The lectin-treated fragments were washed twice in Sorensen's phosphate buffer with 4% polyvinylpyrrolidone and incubated in ovomucoid-coated colloidal gold (10, 11) for 60 min at room temperature. For labeling of freeze-fractured gels with Con A, gel fragments were incubated with the lectin (1 mg/ml in Sorensen's phosphate buffer) for 30 min at 37°C. Controls were preincubated in 0.4 M methyl- $\alpha$ -D-mannopyranoside for 15 min at 37°C followed by incubation in a solution of the lectin in the presence of sugar as described above. After washing, the lectin-treated fragments were incubated in a suspension of horseradish peroxidase-coated colloidal gold (10, 11) for 60 min at room temperature. Gel fragments, not treated with either lectin, were also incubated in suspensions of either ovomucoid-coated colloidal gold or peroxidase-coated colloidal gold.

## Processing for Electron Microscopy

Fracture-labeled specimens were fixed in 1% osmium tetroxide (in veronal acetate buffer, pH 7.4 for 30 min at room temperature), dehydrated in ethanol, and critical point dried in ethanol/carbon dioxide. After critical point drying, the fractured specimens were replicated by deposition of platinum (2 nm) evaporated from a platinum-carbon electron gun and reinforced with carbon. Some gels were also dehydrated in acetone or in amyl acetate and critical point dried in carbon dioxide; other fractured gels were freeze-substituted in a solution of 3% osmium tetroxide in acetone (overnight in liquid nitrogen, 90 min at -40°C, 90 min at -10°C, and 1 h in ice), washed in 100% acetone at room temperature, and critical point dried in acetone/carbon dioxide. The replicas were cleaned in sodium hypochlorite, washed in distilled water, and mounted on Formvar-coated grids.

For thin sections, labeled fragments were fixed in 1% osmium tetroxide in veronal acetate buffer pH 7.4 for 2 h at 4°C, stained en bloc with uranyl acetate (5 mg/ml), dehydrated in acetone, and embedded in Epon 812. Thin sections were examined unstained or poststained with uranyl acetate and lead citrate.

## RESULTS

Cytochemical labeling of erythrocyte fracture faces requires thawing of gel fragments followed by additional glutaraldehyde fixation. These steps appear to induce changes in the organization of membrane components partitioned with each fracture face. In thin sections where, after labeling, the fractured membranes are exposed to osmium tetroxide fixation, acetone dehydration, and resin impregnation, the fracture faces are seen in favorable sections as interrupted trilaminar (i.e., unit membrane) profiles (see references 1 and 2 for ultrastructural detail). In platinum replicas of gel fragments that, after thawing, glutaraldehyde fixation (labeling, if any) and dehydration, are critical point dried and replicated, the appearance of protoplasmic and exoplasmic fracture faces is also different from that seen in conventional freeze-fracture replicas. Membrane particles are absent from both faces. The protoplasmic faces (face P, Figs. 1 and 2) appear as a mosaic in which a relatively smooth, particle-free, surface is uniformly interrupted by numerous small depressions apparently erratic in shape and pattern of distribution (Fig. 2). The depressions appear shallow (probably <10 nm) (Fig. 2) and their exact outline is difficult

to follow, especially that of the edge proximal to the source of shadow. While some depressions are continuous with each other, the raised smooth face appears to form a continuum locally interrupted by the erratic depressions (Fig. 2). In some cases, a narrow band with smooth texture is observed at the periphery of the protoplasmic faces contiguous with and frequently covered by extensions of the embedding medium (Fig. 1, arrowheads). The exoplasmic faces (face E) are easily distinguished from P faces as they display a rough, yet uniform texture not very different from that of the cross-fractured BSA (Fig. 1, right). In these thawed specimens, the aspect of the fracture faces is independent of the solvent used for dehydration and critical point drying (ethanol, acetone, amyl acetate). However, if the specimens are not thawed but, instead, are freeze-substituted in acetone/osmium tetroxide solutions, the P faces appear densely granular (Fig. 3) with a texture more akin to that observed in conventionally freeze-fractured erythrocytes.

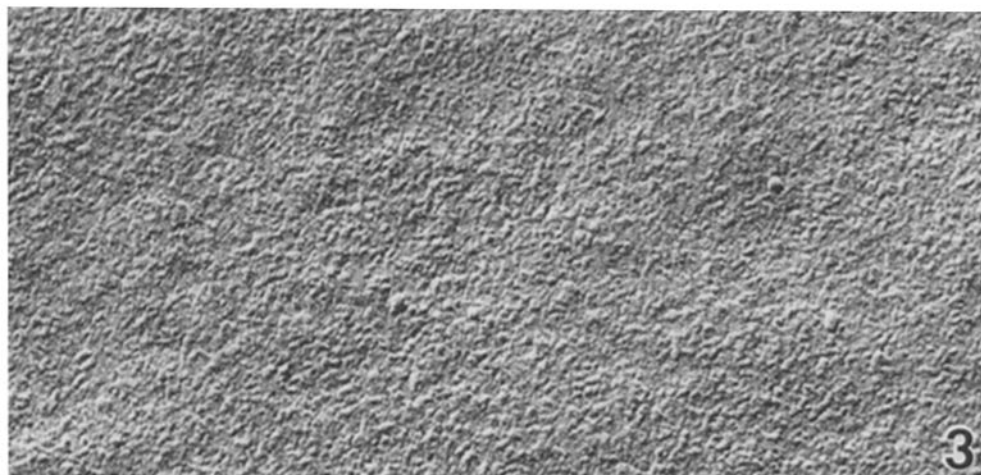
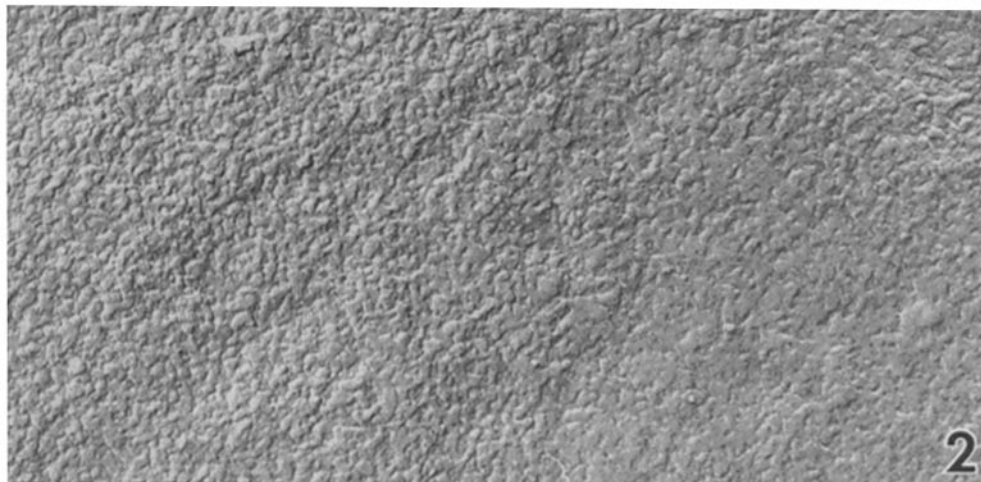
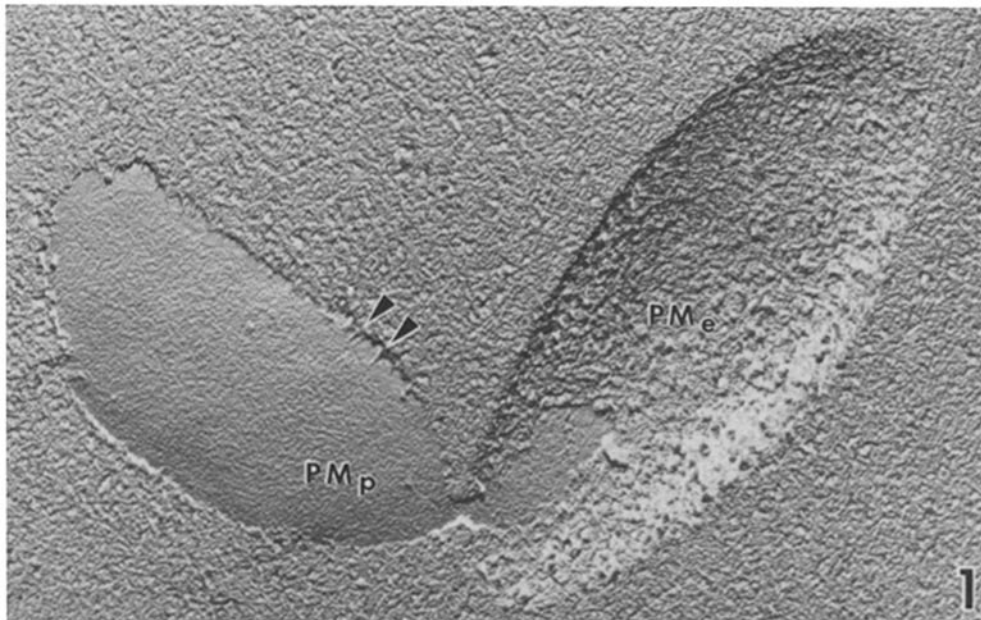
## Cytochemical Labeling

In replicas of critical-point-dried specimens, colloidal gold particles are easily identified because they are not altered by hypochlorite cleaning of the replicas and do not detach from their platinum-carbon casts. They are observed as black (electron-opaque) circles (diameter, 13–16 nm) with which a white cone of shadow is generally associated. Preliminary experiments showed that neither uncoated colloidal gold nor ovomucoid-coated colloidal gold attaches to the membrane faces of freeze-fractured erythrocytes.

Observation of platinum replicas of critical-point-dried freeze-fractured erythrocytes treated with WGA and labeled with ovomucoid-coated colloidal gold (Figs. 4 and 5) shows that 70–90% of the label is spread over the exoplasmic face and 10–30% is associated with the protoplasmic face. Over both faces the distribution of the label appears uniform, with some clusters of particles that may represent aggregated gold micelles not completely removed during washing and concentration of the colloidal gold suspensions.

Partition of WGA binding sites is also well illustrated in thin-sectioned preparations (Figs. 6–8). Colloidal gold particles labeled intensely the exoplasmic faces (Figs. 6 and 8), whereas they were sparse over the protoplasmic faces (Fig. 7). Asymmetry in the partition of WGA binding sites was clearly illustrated in areas where "cracks" in the gel provided complementary views of the labeling relative to a single segment of fractured membrane (Fig. 8). Controls showed that labeling of fractured gels preincubated with *N*-acetyl-D-glucosamine and treated with WGA in presence of the same sugar resulted in virtual absence of colloidal gold particles over both protoplasmic and exoplasmic faces. In all experiments, colloidal gold label was rare over either the fractured BSA gel or the cross-fractured erythrocyte.

To compare, in the same set of experiments, the partition of glycophorin-associated WGA binding sites with that of band 3 component as expressed by labeling of Con A binding sites, we treated fractured gels with this lectin followed by incubation in peroxidase-coated colloidal gold. Here, as expected, partition of the label was reversed, with most colloidal gold particles present over the protoplasmic faces (Fig. 9; see reference 1 for thin-section observations). Specificity of the label was demonstrated by the lack of nonspecific adhesion of peroxidase-coated colloidal gold and by drastic reduction of the label on both P and E faces in gels pretreated with methyl- $\alpha$ -D-man-



FIGURES 1-3 Ultrastructure of fracture faces of human erythrocyte membranes thawed (Figs. 1 and 2) or freeze-substituted (Fig. 3) before critical point drying and platinum-carbon replication. In thawed specimens, P faces appear relatively smooth and are interrupted by numerous erratic depressions (see Fig. 2 for higher magnification). E faces (Fig. 1, right) are densely granular. In Fig. 1, the edge of face P is smoother, covered by occasional projections (arrowheads), and may represent a portion of the actual membrane surface exposed during limited retraction of the gel. In freeze-substituted erythrocytes (Fig. 3), face P has a particulate texture. Fig. 1,  $\times 27,000$ ; Figs. 2 and 3,  $\times 77,000$ .

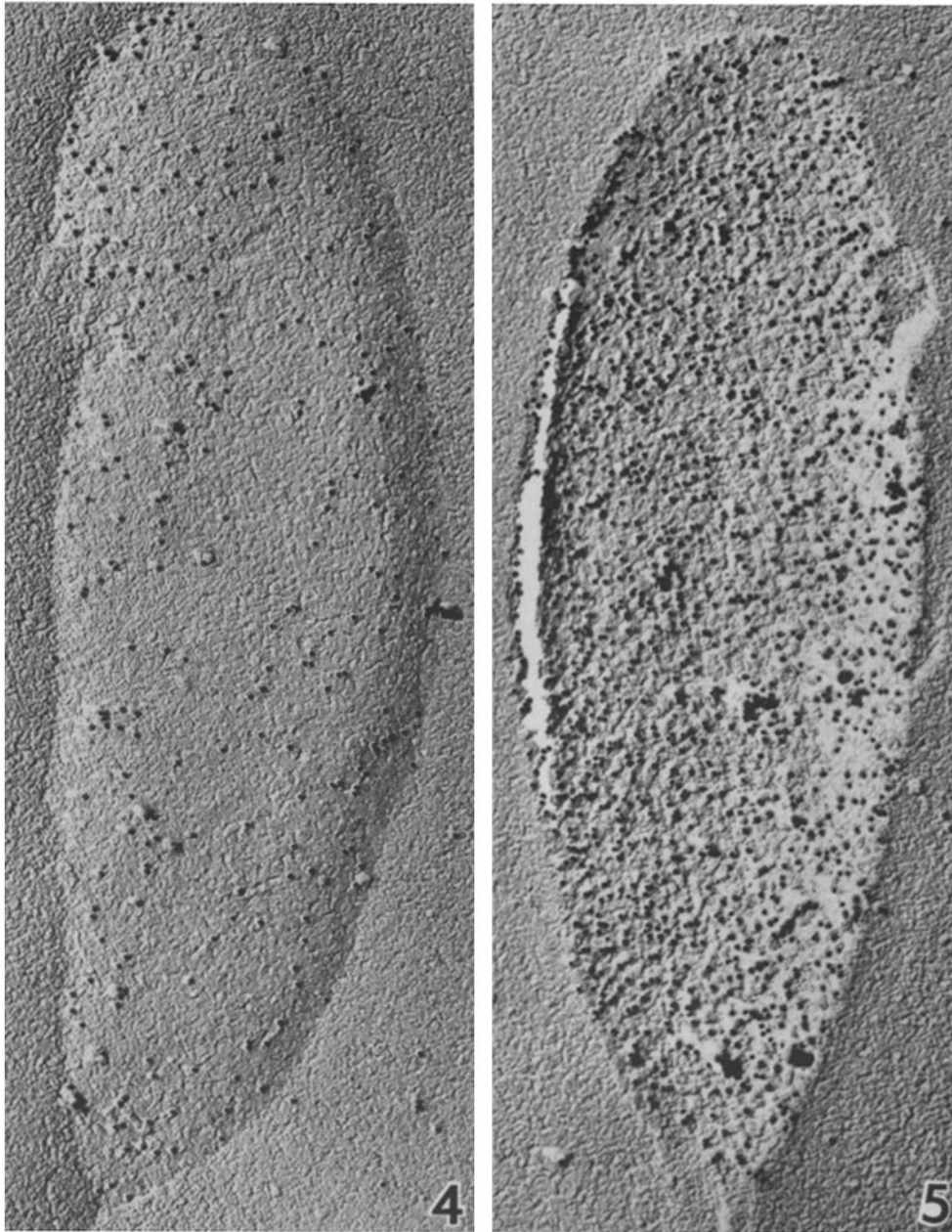
nopyranoside and treated with Con A in presence of the same sugar.

#### DISCUSSION

Description and analysis of the morphology of fracture faces after critical point drying and platinum replication is required

to understand the molecular reorganizations that appear to occur upon thawing of freeze-fractured membranes and may affect the interpretation of cytochemical labeling results.

Upon critical point drying and Pt/C replication, the protoplasmic faces comprise smooth regions interrupted by multiple erratic fissures and small depressed platforms, whereas exoplasmic faces display a uniformly coarse texture. We believe

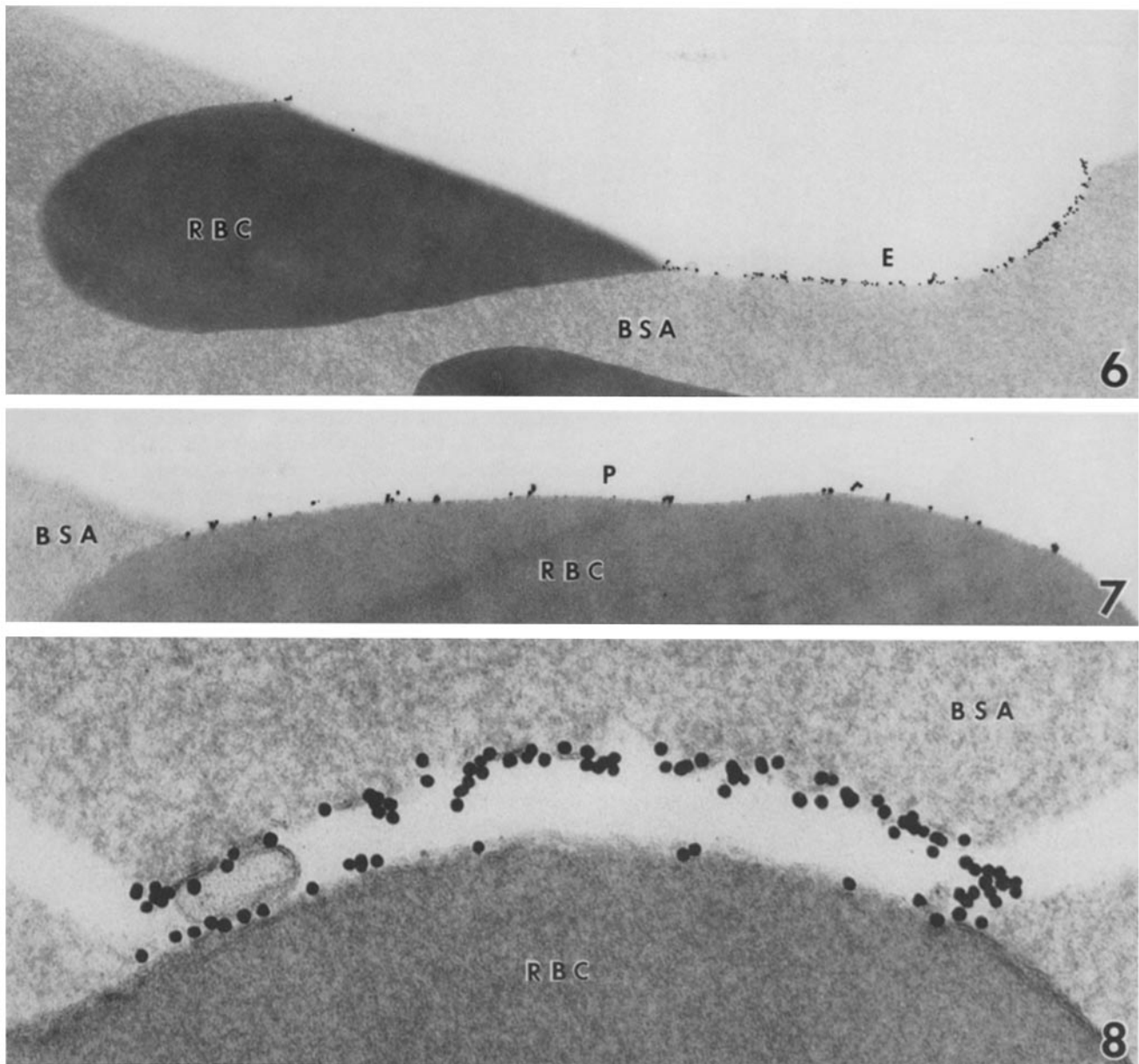


FIGURES 4 and 5 Partition of WGA binding sites between P and E faces of freeze-fractured erythrocytes treated with WGA and labeled with ovomucoid-coated colloidal gold, critical point dried, and platinum/carbon replicated. Label is sparse on face P (Fig. 4) and heavy on face E (Fig. 5). Figs. 4 and 5,  $\times 47,000$ .

that these deviations from the appearance of conventional freeze-fracture preparations result from reorganization post-fracture of membrane components, principally membrane lipids. In freeze-substituted preparations where osmium fixation and acetone dehydration occur at low temperature, these processes of reorganization appear to be minimized as replicas of freeze-substituted preparations are quite similar to those of conventionally freeze-fractured erythrocytes. These results parallel previous thin-section observations of protoplasmic faces as these appear as interrupted trilaminar profiles, except if they are freeze-substituted in osmium tetroxide/acetone where they are seen as the continuous single lamellae expected from split membrane bilayers (1, 2). It is possible that the continuum of relatively smooth areas observed in replicas of critical-point-dried P faces represents a view of the reorganized bilayered regions, and the erratic fissures stand for interruptions of the bilayer, as observed in thin sections. In exoplasmic faces, reorganization appears less well controlled and structural damage more pronounced, particularly that which may be associ-

ated with critical point drying (P and E faces look similar in thin section but are different in replicas after critical point drying). Exoplasmic faces appear more vulnerable, as they lack the firm support and opportunities for cross-linking provided to components of the protoplasmic face by the hemoglobin and the membrane skeleton (12, 13). In addition, the heterosaccharides at the outer surface offer less opportunities for successful cross-linking with the embedding BSA.

Our results show that, upon freeze-fracture, most WGA binding sites are found over exoplasmic faces, whereas a significant minority is also observed over the protoplasmic faces. Specificity of the label over both P and E faces is demonstrated by drastic reduction of label in preparations incubated in the presence of a competing sugar and by lack of nonspecific adsorption of colloidal gold or of ovomucoid-coated colloidal gold to either face. This partition contrasts with that of Con A binding sites. These are preferentially labeled over protoplasmic faces as seen here in replicas of critical-point-dried fracture-labeled preparations and previously observed in thin



FIGURES 6-8 Partition of WGA binding sites between P and E faces, as observed by thin-section electron microscopy. Label is strong over face E (Fig. 6), weak over face P (Fig. 7). Crack in Fig. 8 shows partition of label between complementary regions in the same membrane. Fig. 6,  $\times 25,000$ ; Fig. 7,  $\times 39,000$ ; Fig. 8,  $\times 137,000$ . RBC, erythrocyte; BSA, gel.

sections of fractured erythrocytes (1). The experiments show also the technical advantages of using Pt/C replicas of critical-point-dried fracture-labeled preparations in studies of the distribution and partition of surface binding sites. They allow the observation of numerous fractured membranes and permit a better and easier assessment of the pattern of distribution over entire fracture faces. Thin-section fracture-label is, however, of particular value when it is necessary to identify the type of cell or cellular organelle being labeled (14).

As WGA binds mainly to the sialic acid residues (15) in glyophorin, and Con A to mannosyl residues in band 3 component (7, 8), fracture-label can distinguish the partition of heterosaccharides from the main erythrocyte integral transmembrane proteins. Biochemical analysis of membrane preparations obtained by freeze-fracture of monolayers of human erythrocytes attached by polylysine to glass cover slips indicates the presence of glyophorin in fractions enriched in exoplasmic

membrane "halves" (9). However, in these qualitative biochemical studies the presence and amount of sialoglycoprotein partitioned with the inner, protoplasmic face was not established. Upon fracture of erythrocyte membranes, the cells are separated into two fractions: one is enriched in exoplasmic membrane halves adherent to the glass cover slips; the other comprises the remaining part of the erythrocytes, namely the protoplasmic half of fractured membrane regions, the unfractured membranes, and the contents of the cell lumen. Thus, in the absence of a quantitative interpretation (see reference 16 for outline of method) of the analysis of those membrane fractions, it was impossible to establish the mode of partition of glyophorin, upon fracture. Clearly, analysis of our results is subject to the limitations normally encountered in attempts aiming at the quantitative interpretation of cytochemical data. Yet, our findings demonstrate, in their essence, the contrastive mode of partition of the main erythrocyte transmembrane





FIGURE 9 Freeze-fractured erythrocyte treated with Con A labeled with horseradish peroxidase-coated colloidal gold. Most of the label is associated with face P.  $\times 50,000$ .

proteins: they establish that whereas the majority of WGA and Con A binding sites remain associated, upon fracture, with E and P faces, respectively, significant minorities are labeled over the complementary fracture faces. This contrastive partition accords with the relative distribution of these proteins across the bilayer. While glycoprotein has over half of its amino-acid chain exposed at the outer surface and only 30% (or 40 amino acids) at the cytoplasmic surface (reviewed in reference 17), band 3 component is relatively less exposed at the outer surface and has a large segment ( $\sim 40,000$  daltons) at the inner surface (5, 6). Preferential partition of Con A binding sites with the protoplasmic face may also reflect the association of band 3 component to spectrin via an ankyrin (band 2.1) bridge (reviewed in references 12 and 13). In consequence, it appears that preferential partition of surface binding sites with the inner (protoplasmic) half of the membrane is favored in those transmembrane proteins that are preferentially expressed and/or anchored at the cytoplasmic surface. As WGA and Con A binding sites can partition with either fracture face, it follows that partition of each molecule is a stochastic event in which the probability of partition with either face reflects general rules governing the transmembrane attitude of the protein but also local conditions prevailing in the molecular environment of each integral protein at the moment of fracture. Opposite partition of WGA and Con A binding sites occurs despite the possible association of glycoprotein and band 3 component into oligomeric units (8, 18). While partition of Con A binding sites with the P face implies dragging of the entire chain up to the terminal saccharide across the exoplasmic half of the membrane, partition of WGA binding sites with the exoplasmic face does not necessarily imply dragging of its cytoplasmic segment across the protoplasmic membrane half, as it could

also be accounted for by the breakage of the amino acid chain (9).

The presence on the exoplasmic face of lectin binding sites originally exposed at the outer surface is difficult to interpret. As discussed, exoplasmic faces are less well stabilized after fracture and, although their thin sections indicate partial reconstruction of a bilayer, the process appears obscure, especially regarding the possible involvement of membrane proteins. Presence of the label can be accounted for by reorganization of membrane components postfracture and/or orientation reversal of glycoprotein heterosaccharides dragged during fracture by band 3 component molecules with which they appear to be associated. We must note, however, that lectin labeling experiments cannot be used to decide whether the molecules labeled remained structurally intact or whether covalent bonds in polypeptide chains were ruptured during fracture as suggested by previous biochemical analysis of the exoplasmic half of erythrocyte membranes (9). Yet, while labeling of outer surface sites on exoplasmic faces appears intriguing and obscures the molecular rearrangements that make it possible, it corresponds to a reliable experimental fact observed in a variety of plasma and intracellular membranes (2, 3, 14) and advantageous in the study of partition of membrane components upon fracture.

In conclusion, fracture-label permits structural and cytochemical dissection of membrane surface sites, in particular the identification of those associated with transmembrane protein or oligomeric units that are preferentially associated with the inner membrane half. Our results confirm that band 3 component molecules are the principal protein of the membrane-intercalated particles as observed on freeze-fractured erythrocyte membranes (19, 20). On the exoplasmic faces, the particles may reflect the minority of band 3 component molecules and/or glycoprotein that appear to partition with the outer membrane half.

We thank Drs. H. H. Edwards, K. A. Fisher, P. M. Gullino, and M. Morrison for discussions, and Messrs. Charles Brown and Clifford Parkison for excellent technical assistance.

M. R. Torrisi was supported by a fellowship from the Istituto Pasteur-Fondazione Cenci Bolognetti (Italy).

Received for publication 15 July 1981, and in revised form 28 December 1981.

## REFERENCES

- Pinto da Silva, P., C. Parkison, and N. Dwyer. 1981. Fracture-label: cytochemistry of freeze-fracture faces in the erythrocyte membrane. *Proc. Natl. Acad. Sci. U. S. A.* 78:343-347.
- Pinto da Silva, P., C. Parkison, and N. Dwyer. 1981. Freeze-fracture cytochemistry: thin sections of cells and tissues after labeling of fracture faces. *J. Histochem. Cytochem.* 29:917-928.
- Pinto da Silva, P., B. Kachar, M. R. Torrisi, C. Brown, and C. Parkison. 1981. Freeze-fracture cytochemistry: replicas of critical point dried cells and tissues after fracture-label. *Science (Wash. D. C.)* 213:230-233.
- Steck, T. L., B. Ramos, and E. Strapazon. 1976. Proteolytic dissection of band 3, the predominant transmembrane polypeptide of the human erythrocyte membrane. *Biochemistry*. 15:1154-1161.
- Fukuda, M., Y. Eshdat, G. Tarone, and V. T. Marchesi. 1978. Isolation and characterization of peptides derived from the cytoplasmic segment of band 3, the predominant intrinsic membrane protein of the human erythrocyte. *J. Biol. Chem.* 253:2419-2428.
- Steck, T. L. 1978. The band 3 protein of the human red cell membrane: a review. *J. Supramol. Struct.* 8:311-324.
- Findlay, J. B. C. 1974. The receptor proteins for concanavalin A and lens culinaris phytohemagglutinin in the membrane of the human erythrocyte. *J. Biol. Chem.* 249:4398-4403.
- Pinto da Silva, P., and G. Nicolson. 1974. Freeze-etch localization of concanavalin A receptors to the membrane intercalated particles of human erythrocyte membranes. *Biochim. Biophys. Acta.* 363:311-319.
- Edwards, H. H., T. J. Mueller, and M. Morrison. 1979. Distribution of transmembrane peptides in freeze-fracture. *Science (Wash. D. C.)* 203:1343-1345.
- Geoghegan, W. D., and G. A. Ackerman. 1977. Adsorption of horseradish peroxidase, ovomucoid and anti-immunoglobulin to colloidal gold for the indirect detection of

- concanavalin A, wheat germ agglutinin and goat anti-human immunoglobulin G on cell surfaces at the electron microscope level: a new method, theory and application. *J. Histochem. Cytochem.* 25:1187-1200.
11. Horisberger, M., and J. Rosset. 1977. Colloidal gold, a useful marker for transmission and scanning electron microscopy. *J. Histochem. Cytochem.* 25:295-305.
  12. Lux, S. E. 1979. Spectrin-actin membrane skeleton of normal and abnormal red blood cells. *Semin. Hematol.* 16:21-51.
  13. Branton, D., C. M. Cohen, and J. Tyler. 1981. Interaction of cytoskeletal proteins on the erythrocyte membrane. *Cell.* 24:24-32.
  14. Pinto da Silva, P., M. R. Torrissi, and B. Kachar. 1981. Freeze-fracture cytochemistry: localization of wheat-germ agglutinin and concanavalin A binding sites on freeze-fractured pancreatic cells. *J. Cell Biol.* 91:361-372.
  15. Bhavanandan, V. P., and A. W. Katlic. 1979. The interaction of wheat germ agglutinin with sialoglycoproteins. The role of sialic acid. *J. Biol. Chem.* 254:4000-4008.
  16. Fisher, K. 1976. Analysis of membrane halves: cholesterol. *Proc. Natl. Acad. Sci. U. S. A.* 73:173-177.
  17. Marchesi, V. T. 1979. Functional proteins of the human red cell membrane. *Semin. Hematol.* 16:3-20.
  18. Nigg, E. A., C. Bron, M. Girardet, and R. J. Cherry. 1980. Band 3-glycophorin A association in erythrocyte membranes demonstrated by combining protein diffusion measurements with antibody-induced crosslinking. *Biochemistry.* 19:1887-1893.
  19. Bächli, T. H., K. Whiting, M. J. A. Tanner, M. N. Metaxas, and D. J. Anstee. 1977. Freeze-fracture electron microscopy of human erythrocytes lacking the major membrane sialoglycoprotein. *Biochim. Biophys. Acta.* 464:635-639.
  20. Gahmberg, C. G., G. Tauren, I. Virtanen, and J. Wartiovaara. 1978. Distribution of glycophorin on the surface of human erythrocyte membranes and its association with intramembrane particles: an immunochemical and freeze-fracture study of normal and En(a) erythrocytes. *J. Supramol. Struct.* 8:337-347.