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Fracture-label: Cytochemistry of freeze-fracture faces in the erythrocyte membrane

(membrane proteins/membrane structure/erythrocyte antigens/integral proteins)

PEDRO PINTO DA SILVA, CLIFF PARKISON, AND NANCY DWYER

Section of Membrane Biology, Laboratory of Pathophysiology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

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ABSTRACT **A method—"fracture label"—is described for the cytochemical labeling of the membrane faces produced by freeze-fracture. Human erythrocytes embedded in a crosslinked matrix are frozen, fractured in liquid nitrogen, thawed, labeled, and cut into thin sections. Electron microscope observation of the fracture faces shows preferential partition of concanavalin A binding sites with the inner half of the membrane. This signifies that, during freeze-fracture, binding sites are dragged from the outer surface across the outer ("exoplasmic") half of the membrane and retained on the protoplasmic fracture face (face P). The fracture process results in exposure of new anionic sites on face P. Fracture-label can be applied to the cytochemical characterization of the cellular components exposed by freeze-fracture of isolated cells and tissues.**

Freeze-fracture splits membranes along their hydrophobic interior, following the juncture of end groups provided by a membrane continuum with bilayer organization (1–4). Although fracture of the bilayer produces smooth faces, in all biological membranes these faces are interrupted by particulate components ("membrane intercalated particles" or "intramembranous particles") and, in most, by subtler rugosities ["subparticles" (5)]. Combined use of immunochemical and cytochemical techniques with freeze-etching methods, and freeze-fracture observation of reconstituted membrane preparations, demonstrate that, in the systems used, the particles represent the sites of integral transmembrane proteins (6–12). Because of the qualitative similarity of freeze-fracture images produced by all biological membranes, the particles are assumed to represent local structural asymmetries in the fracture process provoked by integral membrane proteins and, possibly, their associated lipids (3, 4). In the erythrocyte membrane the particles represent the site of the two main integral transmembrane proteins (glycophorin and band III component) which bear AB(H) antigens, influenza virus receptors, wheat germ agglutinin and concanavalin A (Con A) binding sites, as well as anionic sites (8–11, 13–15).

Although splitting of the bilayer continuum by freeze-fracture (and, in consequence, the partition of peripheral membrane proteins) is established, less is known about the fracture behavior of integral membrane proteins (3, 16)—in particular, their antigens and lectin binding sites at the surface (17). Most particles are generally associated with the inner membrane half (1, 2)—i.e., they are observed on the protoplasmic or P face (18). This may be the result of a stochastic process or reflect individual differences of fracture behavior of the components in each particle or both. On face P the particles represent, at least in part, portions of integral proteins located at the outer (exoplasmic, E) membrane half. It is unknown whether the particles contain hydrophilic surface groups of integral proteins dragged, during fracture, from the outer surface. We have labeled freeze-fractured erythrocytes and report the presence of Con A and anionic binding sites on their fracture faces.

MATERIALS AND METHODS

Preparation of Freeze-Fractured Gels. Human erythrocytes (O, Rh-positive) were fixed in 1% (vol/vol) glutaraldehyde for 30 min at 4°C, embedded in 15% or 30% bovine serum albumin (hereafter referred to as albumin) and gelled with 1% glutaraldehyde for 30 min at 25°C. Gels were sliced into 1 × 2 × 2 mm pieces, impregnated with 30% (vol/vol) glycerol, and frozen in Freon 22 cooled by liquid nitrogen. The frozen pieces, immersed in liquid nitrogen, were freeze-fractured by crushing with a glass pestle, thawed in 1.5% glutaraldehyde in 30% glycerol for 15 min at 4°C, and deglycerinated in 310 milliosmolar sodium phosphate buffer/1 mM glycylglycine, pH 7.5. The gel fragments were treated with label and processed for thin section.

Cytochemical Labeling: (*i*) *Cationized ferritin.* Gel fragments were incubated with cationized ferritin (9, 19) (Miles; 1 mg/ml) at pH 7.5 and pH 4.0 in 310 milliosmolar phosphate buffer for 15 min at 4°C. (*ii*) *Colloidal iron.* Gels were incubated 15–30 min at 25°C with colloidal iron (20). (*iii*) *Con A.* Gels were incubated with Con A-ferritin (250 μg/ml) for 30 min at 25°C; controls were preincubated in 0.1 M methyl α-D-glucopyranoside. In some experiments erythrocytes were pretreated with neuraminidase (*Clostridium perfringens*, type VIII, Sigma), at 1 unit/ml in 310 milliosmolar phosphate buffer, pH 7.5, for 5 min at 4°C and 30 min at 37°C. Gel fragments were washed three times before dehydration.

RESULTS

Ultrastructure

Thin sections of freeze-fractured gels show numerous erythrocytes embedded in the crosslinked albumin. The margin of the gel fragments shows that fracture followed the erythrocyte membrane or caused crossfracture of the erythrocyte (Fig. 1*a*). In order to distinguish freeze-fractured erythrocytes from those exposed by gel cutting, we observed the surfaces of 15% albumin gels cut or snapped at room temperature. Here, the erythrocytes were not crossfractured and cleavage followed the albumin gel or the interface between gel and erythrocyte surface, with short incursions into adjoining albumin (Fig. 1*g*). Thus, crossfractured erythrocytes along a gel fragment margin indicated that the cells, on that gel face, were freeze-fractured.

Thin section of the fracture faces of erythrocyte membranes revealed P faces as a series of short segments with unit mem-

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Abbreviations: Con A, concanavalin A; P face, protoplasmic membrane face; E face, exoplasmic face.

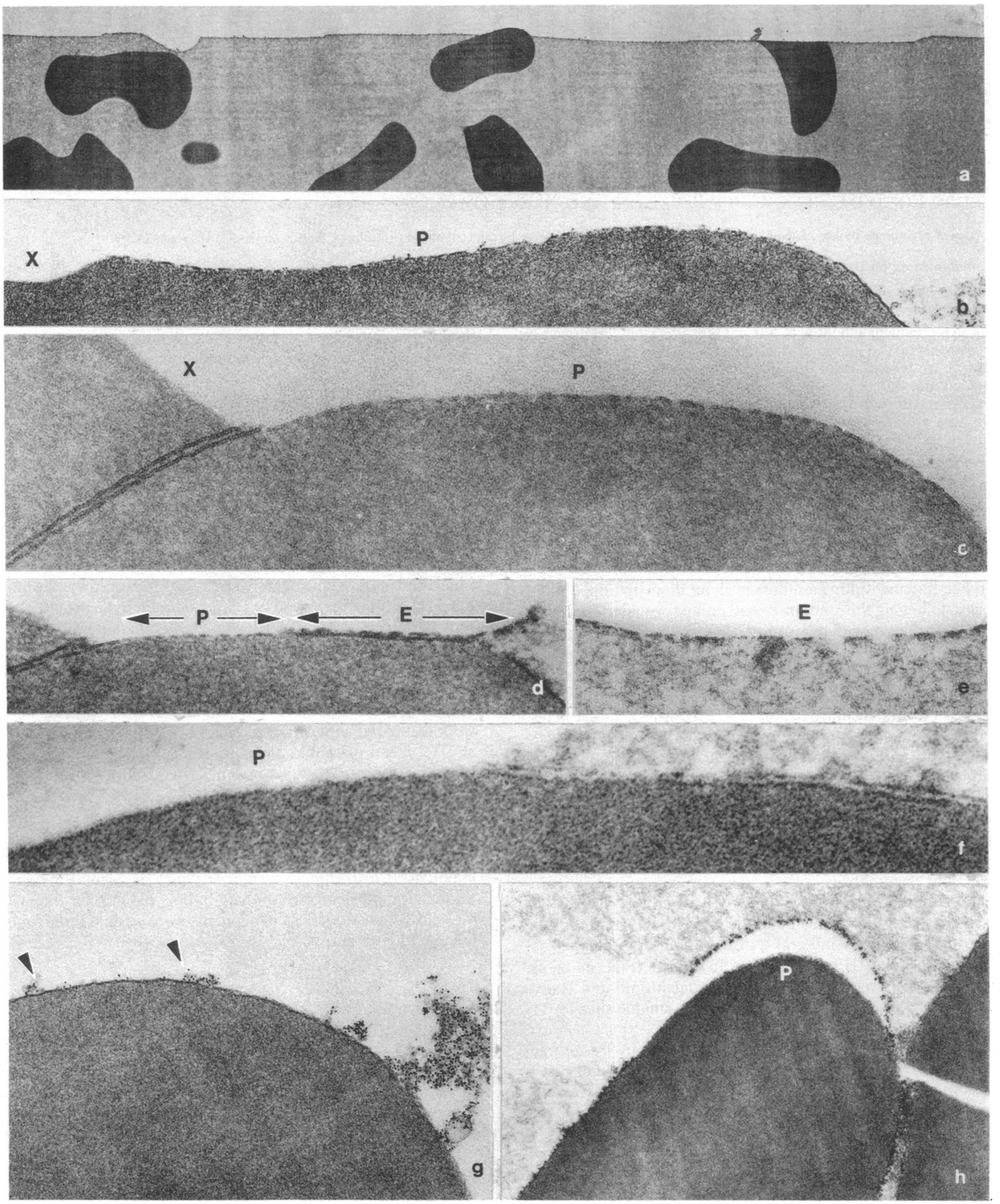

FIG. 1. (*a–e*) Fractured erythrocytes thawed in glycerol. In *a* are shown, on the fractured edge (left to right) the site of an E face, a P face, and a crossfracture (labeled X in *b* and *c*). Fracture faces P and E appear as interrupted unit membrane profiles (*b–e*). (*b*) Erythrocyte treated with Con A-ferritin in the presence of α-methyl glucoside (compare with Fig. 2 *f* and *g*). (*c–e*) Erythrocytes treated with polylysine. (*a*, ×3000; *b*, ×50,000; *c*, ×100,000; *d* and *e*, ×75,000.) (*f*) Freeze-fractured, freeze-substituted, face P appears as a single leaflet. (×245,000.) (*g*) Face of cut gel labeled with cationized ferritin at pH 7.5. Ferritin is clustered and associated with remnants of the gel on the membrane-surface (arrowheads). (×50,000.) (*h*) Fracture ("crack") of erythrocytes prelabeled with cationized ferritin. Label remains on the surface of the membrane (exoplasmic half) and is absent from face P. (×40,000.)

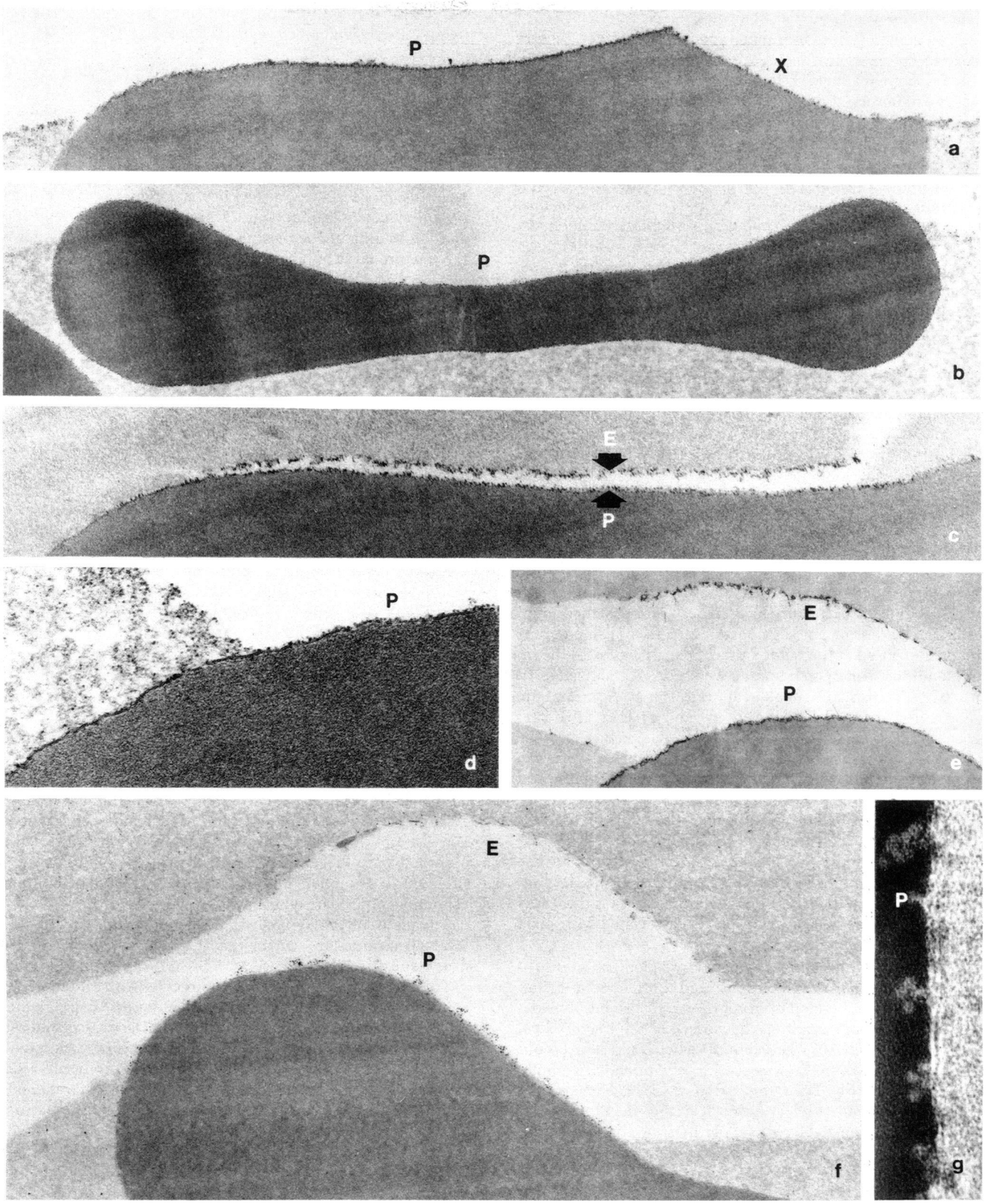

FIG. 2. (*a*) P face labeled by cationized ferritin at pH 7.5 (sparse label on the crossfractured hemoglobin, X). (×30,000.) (*b* and *c*) Fractured erythrocytes show intense label by cationized ferritin at pH 4.0 on both P and E faces (see crack in *c* and, in 15% albumin gels, also on the embedded erythrocyte surface (*b*). (*a*, ×20,000; *b*, ×35,000.) (*d*) Erythrocyte pretreated with neuraminidase, fracture-labeled with cationized ferritin at pH 4.0. Label is intense on face P but weak on the embedded surface. (×55,000.) (*e*) Colloidal iron label on P and E faces. (×20,000.) (*f* and *g*) Complementary aspects of Con A-ferritin label in a gel crack. *g* is printed as a negative for better contrast. See Fig. 1*b* for methyl glucoside control. (*f*, ×35,000; *g*, ×285,000.)

brane profile overlying the hemoglobin-rich interior (Fig. 1 *b–d*). In general, these unit membrane segments were lighter than intact membranes, and clearest "on end," upon orientation with a goniometer stage. E faces, similar but generally less distinct, were observed in 30% gels overlying the crosslinked albumin or in 15% gels of polylysine-coated cells (Fig. 1 *d* and *e*), the latter providing adjoining aspects of P and E faces (Fig. 1*d*). The expected thin section appearance of a "split," freeze-fractured membrane, namely elimination of one of the dark leaflets of the unit membrane, was never observed, even when the fractured gels were thawed in osmium or osmium/glutaraldehyde. When the fractured gel fragments were freeze-substituted in acetone/osmium the unit membrane segments were not visible and only the inner dense line was suggested (Fig. 1*f*).

Thin sections of gels with erythrocytes prelabeled (i.e., before inclusion in albumin) with cationized ferritin at pH 7.5 and fixed with glutaraldehyde showed dense label of the concave areas correspondent to the exoplasmic membrane half, and absence of label on face P, as observed in conventionally fractured preparations (2, 21). These results were clearest in cracks (i.e., incomplete fractures) of the gel as quasi-complementary aspects (Fig. 1*h*). Conventional freeze-fracture (−110°C, Balzers 301) of erythrocyte–albumin gels produced fracture faces (not illustrated) similar to those of erythrocytes in aqueous suspension.

Cytochemical labeling of the fracture faces

These experiments aimed at an initial characterization of the surface chemistry of fracture faces. They were designed to investigate whether or not segments of integral transmembrane proteins, exposed at the outer surface, partitioned with the inner membrane half upon fracture. We used: (*i*) cationized ferritin at pH 7.5 for anionic sites and at pH 4.0 for acidic groups; (*ii*) colloidal iron for strongly acidic groups; and (*iii*) Con A to detect surface sites associated with band III component (10, 22).

Cationized Ferritin. Labeling of fractured erythrocyte–albumin gels with cationized ferritin at pH 7.5 showed that face P was intensely and continuously labeled (Fig. 2*a*). This contrasted with the appearance of the embedded erythrocyte surfaces, which remained unlabeled, although in 15% gels, and to a limited extent in 30% gels, ferritin penetrated the albumin. Label on cross-fractured erythrocytes was less intense (Fig. 2*a*). On the faces of cut gels only occasional clusters of ferritin were present on erythrocytes, associated with small fragments of albumin (Fig. 1*g*).

In erythrocyte–albumin gels labeled by cationized ferritin at pH 4.0, the label on face P was dense and continuous (Fig. 2*b*). The surfaces of embedded erythrocytes were also intensely labeled even at 10–20 μm from the surface of the fractured gel (not illustrated). In 30% gels intense label could also be observed on the sites of E faces. Gel cracks provided complementary aspects (Fig. 2*c*). Observation of 15% gels made with neuraminidase-pretreated erythrocytes labeled, after freeze-fracture, with cationized ferritin at pH 4.0 showed intense label on the P face but sparse label on embedded membrane surfaces (Fig. 2*d*).

Colloidal Iron. Labeling with colloidal iron revealed the presence of strongly acidic groups on both fracture faces and, also, on the surface. Cracks in the gel provided complementary views (Fig. 2*e*).

Con A. Labeling of fractured erythrocyte–albumin gels with Con A-ferritin conjugates showed strong label on face P (Fig. 2 *f* and *g*) and approximately 25% of total label on face E. This was clear in cracks of the gel (Fig. 2*f*). Surface labeling of erythrocytes embedded in 15% albumin was minimal (Fig. 2*f*). Fractured gels labeled in the presence of 0.1 M methyl α-D-glucopyranoside showed much reduced label (Fig. 1*b*).

DISCUSSION

Our results demonstrate that Con A binding sugar residues associated with an integral erythrocyte membrane protein at the outer surface can be identified on the protoplasmic face (face P) of freeze-fractured membranes. The process of fracture appears also to cause exposure of new anionic sites. The application of cytochemical labeling techniques to the identification and localization of chemical groups and other binding sites on the fracture faces of biological membranes is, therefore, possible.

The process of fracture that occurs upon crushing in liquid nitrogen appears qualitatively similar to that of conventional freeze-fracture. Between −100°C and −269°C the appearance of fractured membranes is unchanged (23). Fracture of *prelabeled* erythrocytes shows that, as in conventional freeze-etch studies (2, 8–11, 21), macromolecular labels at the outer surface are absent from face P. In addition, conventional freeze-fracture shows that embedding of erythrocytes in albumin does not alter their fracture appearance. Our experiments do not prove membrane splitting; splitting of the membrane bilayer regions is *assumed* because of previous investigations (1–3) and induced from the similarity between crushing in liquid nitrogen and conventional freeze-fracture methods.

Ultrastructural observation of thin-sectioned fracture faces showed an interrupted, subtle, yet distinct unit membrane profile (Fig. 1 *b–e*). This unexpected result, which was obtained even in fractured erythrocyte gels thawed in osmium or glutaraldehyde/osmium solutions, suggests limited reorganization of membrane components during thawing, before glutaraldehyde or glutaraldehyde/osmium fixation. It is likely that reorganization mainly involves membrane lipids and occurs during an attempted and natural process of reconstruction of the bilayer after freeze-fracture. Redistribution of integral membrane proteins is unlikely because glutaraldehyde fixation, before and after fracture, results in cross-linking of integral proteins, peripheral proteins at the inner surface, and hemoglobin. The unilamellar profile expected from a split bilayer membrane was observed only in gels freeze-substituted in osmium/acetone (Fig. 1*f*)—i.e., under conditions of maximal lipid stabilization: osmium fixation starts at a much lower temperature; acetone is an adequate solvent for apolar lipid chains exposed in a split bilayer. Unfortunately, cytochemical labels cannot be used in freeze-substituted preparations.

Labeling of freeze-fractured erythrocytes with cationized ferritin at pH 7.5 revealed numerous anionic sites. On face P their origin can be twofold: (*i*) sites at the outer surface associated with integral proteins that, during freeze-fracture, are pulled through the outer membrane half and remain associated with the inner half; (*ii*) sites sequestered within the membrane, necessarily associated with integral membrane proteins, and exposed by freeze fracture.

The presence of intense and continuous label on face P of fractured erythrocytes labeled by cationized ferritin at pH 4.0 demonstrated the presence of numerous acidic groups. These anionic sites appear to be revealed, at least in part, by the fracture process. This was shown by the observation of fractured erythrocytes that were exposed to neuraminidase prior to gelling. In these preparations, labeling of face P remained intense although it was much decreased on the surface (compare Fig. 2 *b* and *d*). These results made it impossible to determine whether anionic surface sites associated with integral membrane components (i.e., sialic acid in glycophorin and possibly

also in band III) are partitioned with the inner membrane half during fracture. Labeling with colloidal iron at pH 1.8 illustrated the presence of strongly acidic groups on face P but could not establish whether they represented sialic acid groups dragged, during fracture, from the outer surface.

The appearance of new anionic groups caused by fracture is compatible with membrane splitting. The process of fracture either exposes new domains within the membrane interior or causes distortion of protein chains and concomitant exposure of additional anionic groups. Acidic groups may exist in the membrane interior (13, 15) either free (e.g., lining a putative hydrophilic channel) or titrated by other groups.

Labeling of the P face with Con A showed that, during freeze-fracture, oligosacharides originally exposed at the outer surface of the erythrocyte and associated with a major transmembrane protein [band III, the anion transport protein (13, 15, 22)] can be dragged across the outer half of the membrane and partition with the inner half, where they retain their binding specificity. This conclusion assumes the absence of other Con A binding sites—i.e., sites not originally exposed at the outer surface but made available by the process of fracture or thawing; investigation of the chemistry and conformation of erythrocyte membrane proteins indicates that the sugar residues associated with the two main integral proteins are exposed at the outer surface (13–15). The proportions of Con A-ferritin molecules and of membrane-intercalated particles that partition with the P and E faces are similar. Interpretation of our results does not require, nor does it conflict with, the possibility of partial breakage of covalent bonds within integral membrane proteins during freeze-fracture and is compatible with biochemical evidence for partition of band III during fracture (16).

The label on the exoplasmic half of the erythrocyte membrane is difficult to interpret. In 15% gels this face is not stabilized, and 30% gels do not provide as firm a base as that found at the inner erythrocyte surface against crosslinked hemoglobin. It is possible that an unknown proportion of the label was attached, through defects on the fracture face, to components at the outer surface of the membrane.

Current views of the structure and dynamics of biological membranes, including the acceptance of membrane splitting, rest, in no small measure, on the experimental analysis of freeze-fractured and etched membranes. Exceptions to consensus (24) result from misunderstanding the concept of membrane splitting to imply that all of the membrane splits. Our experiments demonstrate that this is not so: membrane splitting must be confined, as originally interpreted (1, 2), to the bilayer continuum of biological membranes. The fracture plane is deflected at the site of each particle and it is this deflection that morphologically determines the particle itself: i.e., the membrane particles are defined relative to, and in contradistinction to, the bilayer continuum of biomembranes. Although in every successfully investigated case integral membrane proteins (and, in consequence, their tightly associated lipids) were shown to be components of the membrane particles, the presence of integral proteins is not, in principle, a necessary condition; micellar subphases sequestered within the polar matrix of the bilayer can also appear, in freeze-fractured preparations, as "particles"—i.e., as local deviations of the fracture plane, albeit with a characteristic morphology (25, 26). In many membranes, however, and particularly in specialized membranes (e.g., chloroplast grana) or membrane regions (e.g., gap junctions) interruptions of the bilayer continuum may account for most of the membrane area. Thus, *sensu stricto*, the proportion of membrane area in which bilayer splitting occurs can be smaller, even much smaller, than that occupied by interrupting integral proteins and their associated lipids. The underlying assumption is that, even in these cases, the bilayer continuum still exists and guides the process of fracture.

In summary, our results show that, during freeze-fracture, Con A binding sites—i.e., surface groups associated with band III component, an erythrocyte transmembrane protein—are preferentially partitioned with the inner, protoplasmic, half of the membrane and that new anionic sites are revealed by the process of fracture. Our results illustrate also the feasibility of the combined application of cytochemical and freeze-fracture techniques to study the topochemistry of plasma membranes, intracellular membranes, and the cytoplasm.

1. Branton, D. (1966) *Proc. Natl. Acad. Sci. USA* **55**, 1048–1055.
2. Pinto da Silva, P. & Branton, D. (1970) *J. Cell Biol.* **45**, 598–605.
3. Fisher, K. A. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 173–177.
4. Pinto da Silva, P. (1979) in *Freeze-Fracture: Methods, Artifacts and Interpretation*, eds. Rash, J. & Hudson, S. (Raven, New York), pp. 646–672.
5. Pinto da Silva, P., Martinez-Palomo, A. & Gonzalez-Robles, A. (1975) *J. Cell Biol.* **54**, 538–550.
6. Grant, C. W. M. & McConnell, H. M. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 4653–4657.
7. Hong, K. & Hubbell, W. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 2617–2621.
8. Pinto da Silva, P., Douglas, S. D. & Branton, D. (1971) *Nature (London)* **232**, 194–196.
9. Pinto da Silva, P., Moss, P. & Fudenberg, H. H. (1973) *Exp. Cell Res.* **81**, 127–138.
10. Pinto da Silva, P. & Nicolson, G. L. (1974) *Biochim. Biophys. Acta* **363**, 311–319.
11. Tillack, T. W., Scott, R. E. & Marchesi, V. T. (1972) *J. Exp. Med.* **135**, 1209–1227.
12. Yu, J. & Branton, D. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 3891–3895.
13. Marchesi, V. T. (1979) *Semin. Hematol.* **16**, 3–20.
14. Steck, T. L. (1974) *J. Cell Biol.* **62**, 1–19.
15. Steck, T. L. (1978) *J. Supramol. Struct.* **8**, 311–324.
16. Edwards, H. H., Mueller, T. J. & Morrison, M. (1979) *Science* **203**, 1343–1345.
17. Rash, J. E., Hudson, C. S., Ellisman, M. H. (1978) in *Cell Membrane Receptors for Drugs and Hormones*, eds. Straub, R. W. & Bolis, L. (Raven, New York), pp. 47–68.
18. Branton, D., Bullivant, S., Gilula, N. B., Karnovsky, M. J., Moor, H., Mühlethaler, K., Northcote, D. H., Packer, L., Satir, B., Satir, P., Speth, V., Staehelin, L. A., Steere, R. L. & Weinstein, R. S. (1975) *Science* **190**, 54–56.
19. Danon, D., Goldstein, L., Marikovsky, Y. & Skutelsky, E. (1972) *J. Ultrastruct. Res.* **38**, 500–510.
20. Hyat, M. A. (1970) *Principles and Techniques of Electron Microscopy: Biological Applications* (Van Nostrand & Reinhold, New York), Vol. 1, pp. 298–299.
21. Tillack, T. W. & Marchesi, V. T. (1970) *J. Cell Biol.* **45**, 649–653.
22. Findlay, J. B. C. (1974) *J. Biol. Chem.* **249**, 4398–4403.
23. Kirchanski, S., Elgsaeter, A. & Branton, D. (1979) in *Freeze-Fracture: Methods, Artifacts and Interpretation*, eds. Rash, J. & Hudson, S. (Raven, New York), pp. 149–152.
24. Sjöstrand, F. S. (1979) *J. Ultrastruct. Res.* **69**, 378–420.
25. De Kruijff, B., Verkleij, A. J., Van Echteld, C. J., Gerritsen, W. J., Mombers, C., Noordam, P. C. & de Gier, J. (1979) *Biochim. Biophys. Acta* **555**, 200–209.
26. Pinto da Silva, P. & Nogueira, M. L. (1977) *J. Cell Biol.* **73**, 161–181.