

Effect of Membrane Splitting on Transmembrane Polypeptides

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Abstract. We investigated the effect of membrane splitting on the primary structure of human erythrocyte membrane polypeptides. Monolayers of intact, chemically unmodified cells were freeze-fractured and examined by one-dimensional SDS PAGE. Silver-stained gels revealed all major polypeptides that stain with Coomassie Blue as well as all bands that stain with periodic acid Schiff's reagent. Both nonglycosylated and glycosylated membrane polypeptides could be detected at concentrations of only a few nanograms per band. Membrane splitting had no effect on the position or number of bands. Monolayers of intact erythrocytes that had been enzymatically radioiodinated with lactoperoxidase were examined by electrophoresis, fluorography, and liquid scintillation counting. Radioactivity was quantified before and after monolayer formation and splitting, and at sev-

eral stages of gel staining, drying, and fluorography. Although overexposed fluorographs revealed several minor radioiodinated bands in addition to band 3 and the glycoporphins, no new bands were detected in split membrane samples derived from intact cells. These observations support the conclusion that neither the band 3 anion channel nor the glycoporphin sialoglycoproteins are fragmented during freeze-fracturing. Although both band 3 and glycoporphin partition to the cytoplasmic side of the membrane, preliminary quantitative observations suggest an enrichment of glycoporphin in the split extracellular "half" membrane. We conclude that the process of membrane splitting by planar monolayer freeze-fracture does not cleave the covalent polypeptide backbone of any erythrocyte membrane protein, peripheral or integral.

MANY permeability and signalling functions appear to be associated with membrane-spanning polypeptides. In the human red blood cell (RBC),¹ for example, ion and glucose transport are mediated by integral transmembrane polypeptides (22, 39, 41). For other membranes, signalling functions have been explained in terms of structural models that require the interaction of two or more diffusible elements inserted into one or the other "half" of the lipid bilayer (6). Membrane-splitting by planar freeze-fracture could be used as a physical fractionation technique to establish the validity of such models, if the effects of splitting on membrane polypeptides were known.

Current biochemical and cytochemical information about the effect of freeze-fracturing on membrane polypeptides is limited and somewhat contradictory. Some investigators who have studied freeze-fractured, radioisotopically labeled RBC membranes (9, 10, 28) have concluded that membrane spanning glycoproteins fragment during splitting. Others have used cytochemical methods to examine reactive sites on fracture faces (33, 34) and concluded that reactive groups could be pulled through the membrane while also acknowledging that

the observations "could also be accounted for by the breakage of the amino acid chain" (34). However, both the biochemical and cytochemical approaches required enzymatic or chemical modification of the membrane and specific reactive sites. Thus several questions remain. What is the effect of splitting on integral membrane polypeptides in membranes that have not been chemically labeled or fixed? Do heavily glycosylated proteins, such as red cell glycoporphin A, which contains a single membrane-spanning polypeptide chain (20, 25), behave differently from minimally glycosylated ones, such as the anion channel band 3, which spans the bilayer several times (36)? How can one explain the additional bands that have been seen in split radioiodinated membrane preparations?

To answer questions about the effect of splitting on membrane proteins, we used planar freeze-fracture (13) of both unlabeled and lactoperoxidase radioiodinated (9) RBCs to produce split membrane polypeptides for analysis by one-dimensional SDS gel electrophoresis, silver-staining (27, 35), fluorography, and liquid scintillation counting. In contrast to previous studies (9, 28, 30), our approach was to quantify membrane splitting (19) by examining all polypeptides from both "halves" of the split membrane preparation and measure their recovery and distribution. We compared unlabeled proteins separated on gels and stained with silver to proteins

¹ HVG, heavy-glass fraction; PAS, periodic acid Schiff's reagent; PLG, PL-glass fraction; PL-glass, polylysine-treated coverglasses; RBC, red blood cell; RBCG, RBC white ghosts; TCA, trichloroacetic acid; WCM, whole cell monolayers.

labeled by lactoperoxidase radioiodination and analyzed by fluorography and liquid scintillation counting. Silver staining has the sensitivity to detect the nanogram levels of polypeptides produced by planar freeze-fracture (17, 27, 35). Fluorography and scintillation counting are not only sensitive but also allow reproducible quantitation. For chemically modified membranes, we used lactoperoxidase-catalyzed radioiodination, since it is known to label the major transmembrane proteins of the red cell, notably band 3 and glycophorin A (9, 29).

The combination of techniques described above enabled us to investigate nonglycosylated polypeptides and sialoglycopeptides derived both from chemically unmodified and enzymatically modified membranes and "half" membranes. The data show that the covalent backbones of all native or labeled membrane polypeptides, whether peripheral, integral, transmembrane, glycosylated, or unglycosylated, are not detectably modified by the freeze-fracture process. Importantly, these data show for the first time that membrane splitting can be used for analysis of functionally important transbilayer proteins without the analytical complications of covalent-bond cleavage.

Materials and Methods

Cell and Monolayer Preparation

Human erythrocytes (A*) were washed (15), and white ghosts (RBCGs) were prepared by hypotonic lysis (7). Polylysine-treated, 11 × 22-mm coverglasses (PL-glasses) were prepared as previously described (16), except here they were initially cleaned by immersion in hot (100°C) Isoclean detergent (Isolab Inc., Akron, OH), 2% (vol/vol). Pelleted cells or ghosts were diluted with 1/2 volume buffer, 50 µl were applied to each PL-glass, and unattached cells were removed by washing (15).

Planar Monolayer Freeze-fracture

Cell monolayers on PL-glass were sandwiched against a piece of heavy glass (12 × 24 × 0.6 mm), frozen in freezing liquid Freon-22 and fractured as described previously (15) to produce two fractions: the heavy-glass fraction (HVG) enriched in cytoplasmic membrane "halves," and the PL-glass fraction (PLG) enriched in extracellular membrane "halves."

Neuraminidase Treatment

Before monolayer formation, a portion of the washed RBCs (10% hematocrit) were treated at 37°C for 60 min with *Clostridium perfringens* neuraminidase (Sigma Chemical Co., St. Louis, MO), 4 µg/ml, in 130 mM NaCl buffered with 20 mM acetate to pH 5.5.

Radioiodination

Intact washed RBCs were radioiodinated with lactoperoxidase, according to the procedure of Mueller and Morrison (29) as cited by Edwards et al. (9). In a typical experiment, 2.0 ml of 310 mM phosphate-buffered saline, pH 7.4 (PBS), contained 400 µl washed pelleted RBCs (20% hematocrit), 500 µCi ¹²⁵I (New England Nuclear, Boston, MA), and 1.95 × 10⁻⁷ M lactoperoxidase (Sigma Chemical Co.). Freshly prepared H₂O₂ (5 mM, 20 µl) was added once each minute for 10 min. Cells were washed 3× with PBS, then 5× with Hepes-buffered saline, HBS (10 mM Hepes, 142 mM NaCl, 5 mM Mg²⁺, pH 7.4). Cells were routinely labeled with FITC-concanavalin A (15, 18), but processing for gel electrophoresis precluded the use of the double-labeled membrane splitting technique (15) to quantify the degree of membrane splitting.

Lipid Analysis

To evaluate the distribution of radioactivity, labeled cells were solubilized in SDS and analyzed using the Bligh and Dyer lipid extraction procedure (3). Aqueous and chloroform phases and the interface between the two phases were transferred to vials and dried under N₂ at 50°C. The residue was solubilized in 500 µl SDS borate with 15-s sonication before adding 10 ml Aquasol II (New

England Nuclear). Extraction tubes were washed with an additional 500 µl SDS borate, and the washes were transferred to vials. Silanized conical glass centrifuge tubes and untreated glass scintillation vials were used throughout. Radioactivity was measured using a Beckman LS7000 Liquid Scintillation Counter (Beckman Instruments Inc., Fullerton, CA).

Protein Analysis

To evaluate radioactivity bound to protein, RBCs (100 µl) freshly labeled by lactoperoxidase iodination and column purified (15) were added to HBS (300 µl) in a microfuge tube. Trichloroacetic acid (TCA) (50%, 100 µl) was added with vortexing. Samples were incubated in an ice bath for 10 min and centrifuged in a Beckman Air-fuge at 16,000 g for 1 min. Supernatant (500 µl) was decanted into tubes, and pellets resuspended in SDS sample buffer (500 µl) with sonication. Tubes were washed with SDS, radioactivity measured by scintillation counting as described above, and total protein by the method of Lowry et al. (24).

SDS PAGE

Frozen cell monolayers, both unsplit and split, were either directly transferred from liquid nitrogen to Laemmli sample buffer (23) in teflon-capped glass scintillation vials, or first lyophilized and then transferred. Lyophilization (warming glasses from -196°C to 25°C *in vacuo*) facilitated quantitative recovery; dry samples neither diluted the sample buffer nor cooled it to temperatures favoring proteolysis. However, lyophilization often produced aggregates that failed to enter the separation gel. In a typical experiment, 500 µl Laemmli sample buffer was preheated in a glass scintillation vial for 3 min to 75°C or 95°C. One to six monolayers were dropped one at a time into the hot buffer and swirled, and the vials were tightly capped. After heating for 5 min, vials were cooled to 20°C, sonicated for 5 s (15), and centrifuged to remove liquid from between glasses for 3 min at 3,000 rpm (Sorvall SS-34 rotor [Beckman Instruments, Inc.] with #4 corks as cushions). Samples were transferred to conical tubes and each volume measured and adjusted to 600 µl. Aliquots of 50 or 80 µl were transferred to 4 × 8 × 0.8-mm wells in a stacking gel (4.5% acrylamide, pH 6.8) overlying an 11.5% (or as indicated), pH 8.8, separation gel. After electrophoresis, gels were fixed and stained with Coomassie Blue (12), periodic acid Schiff's (PAS) reagent (12), or silver (27, 35), and photographed on a light box with Polaroid Type 55 film. Negatives or gels were scanned with a Mark III CS double-beam recording microdensitometer (Joyce-Loebl, Gateshead, England), and optical density peaks were integrated as previously described (13).

Fluorography

Gels were rinsed in distilled water, placed under water on top of a sheet of cellophane, 22 × 35 mm (Bio-Rad cat. no. 1650963 [Bio-Rad Laboratories, Palo Alto, CA]), supported by plate glass, 25 × 25 cm. A second sheet of cellophane was placed on top of the gel and overlaid with a frame of 2.5-cm wide strips of 6.4-mm thick glass. We clamped the entire sandwich under water, carefully avoiding air bubbles and stretching the cellophane tight. The clamped apparatus was removed from the water and dried horizontally for 2 to 3 h in a laminar air flow chamber, or overnight in a hood. Dry gels were exposed to Kodak X-Omat AR-5 x-ray film between Cronex Lightning Plus intensifying screens (Dupont Nemours and Co., Wilmington, DE) for 2 to 7 d at -100°C, warmed to 20°C, developed in Kodak GBX developer for 5 min, stopped in 2% acetic acid for 30 s, fixed in Kodak GBX fixer for 5 min, and rinsed in distilled water for 10 min, all at 20°C.

Quantification of Radioactivity in Gels

To evaluate loss of radioactivity during gel processing after electrophoresis, labeled cells were electrophoresed in 0.8-mm thick slab gels as described above, and radioactivity was monitored during processing as described below.

Untreated Gel Immediately after Electrophoresis (Fig. 3A). Entire gel lanes, including the stacker, were cut into 5-mm wide strips and transferred to 600 µl H₂O₂ (30%) in vials. The vials were tightly capped (teflon liners), heated at 75°C overnight, and cooled to 20°C. Aquasol II (10 ml) was added, and samples were counted in a Beckman LS7000 Liquid Scintillation Counter. Counts were corrected for background and quench.

After Acetic Acid/Methanol Fix and Wash (Fig. 3B). Gels were transferred to 10% acetic acid in 50% methanol and stored for 12 to 15 h at 4°C. Solution volumes for this and subsequent treatments were 400 ml, temperature 20°C. Samples were cut and radioactivity was counted as in the section on Fig. 3A above.

After Glutaraldehyde Fix and Methanol Wash (Fig. 3C). Gels were fixed

with 10% glutaraldehyde for 30 min, then washed five times with 15% methanol, 20 min per wash. Samples were cut and radioactivity counted as in the section on Fig. 3A above.

After Silver Stain (Fig. 3D). Gels were stained with silver according to Poehling and Neuhoff (35). Briefly, gels were washed with glass-distilled water, 10 min; stained with 0.047 M silver nitrate, 7 min; washed in glass-distilled water, 6 min; developed in citric acid-formaldehyde, 4 min; stopped in 1% acetic acid, 15 min; and washed and stored in glass-distilled water, 12–15 h. Samples were cut and radioactivity was counted as in the section on Fig. 3A above.

After Drying (Fig. 3E). Silver-stained gels were placed between two pieces of cellophane and dried on glass overnight as described above. Dried gels were cut and radioactivity was counted as in the section on Fig. 3A above.

To quantify the radioactivity in the experiments reported in this paper, fluorographs were used as templates to mark radioactive regions on the dry gels for cutting. Cut dry strips were placed in 200 μ l H₂O at 75°C, and after 5 min, 600 μ l H₂O₂ was added and samples were processed as in the section on Fig. 3A above.

Preliminary accounts of selected aspects of this study were presented at the American Society for Cell Biology Meetings in San Antonio, TX, November, 1982 (17), and Kansas City, MO, November, 1984 (19).

Results

Gel Electrophoresis/Silver-Staining

To determine if the silver-stain techniques of Merrill et al. (27) and Poehling and Neuhoff (35) would be suitable for analysis of nanogram quantities of RBC membrane polypeptides, silver-stained gels of RBCG concentration series were evaluated (Fig. 1). Even at total membrane protein loads as low as 0.12 μ g, all major bands typical of the Coomassie Blue-stained gels of human RBCG membranes could be seen with both methods. For bands 5, 6, and 7, which account for 4.5%, 5.5%, and 3.4% of the total protein respectively (41), this represented a sensitivity of 3 to 5 ng, and for glycophorin, PAS 1 and 2 (PAS 1 monomer), which make up 6.7% of the total membrane protein, a sensitivity of \sim 7 ng. At loads in excess of 1 μ g, numerous other bands could be seen. Although the Merrill et al. (27) technique was used in initial studies, the Poehling and Neuhoff (35) method produced a clearer background with less longitudinal streaking. In addition, the sialoglycoprotein bands appear gray and nonglycosylated bands appear yellow-brown.

Some bands between bands 4.2 and 5 (Fig. 1, asterisks) were present at the lower protein loads and even in the absence of protein (sample buffer only, not shown) or in other protein mixtures, such as molecular weight standards. Systematic deletion of sample buffer components revealed that these bands appeared when the buffer contained 2-mercaptoethanol. Substitution of dithiothreitol for 2-mercaptoethanol showed similar artifactual bands. The origin of these bands is controversial (32, 37). Although RBCG membrane solubilized in sample buffer lacking mercaptoethanol lacked the spurious bands, the reducing agent prevented high molecular weight aggregates, sharpened most bands, and thus was routinely included at a concentration of 2% in all sample buffers.

Fig. 2 compares positions and relative intensities of silver-stained bands (Fig. 2, E and F) to those stained by Coomassie Blue (Fig. 2, A, B, G, and H). All bands stained by Coomassie Blue were also stained by the Merrill et al. (27) or Poehling and Neuhoff (35) techniques. The relative intensities of all bands were similar but not identical; for example, compare intensities of silver-stained bands 1, 2, and 3 to the other bands and note the higher relative intensities of the Coomassie Blue-stained bands 1–3. Moreover, in the silver gel several

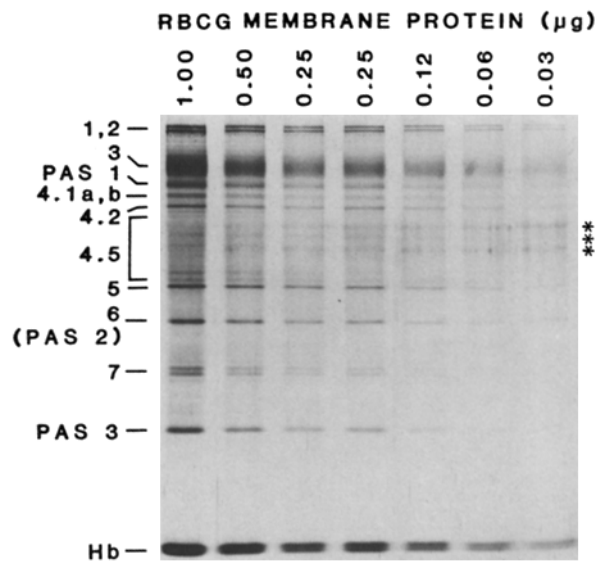


Figure 1. Silver-stained gel of RBCG membrane dilution series. Total protein loads in micrograms indicated at the top of the gel. Major RBC polypeptide bands are designated at the left. Asterisks, region of spurious bands; *Hb*, hemoglobin. Laemmli slab gel, 0.8 mm thick; 4.5% acrylamide stacking gel, pH 6.8; 10.5% acrylamide separation gel, pH 8.8; 30 μ l sample applied. Stacker electrophoresis 8 mA for 1 h; separation gel, 16 mA for 2 h. Merrill et al. (27) silver-stain.

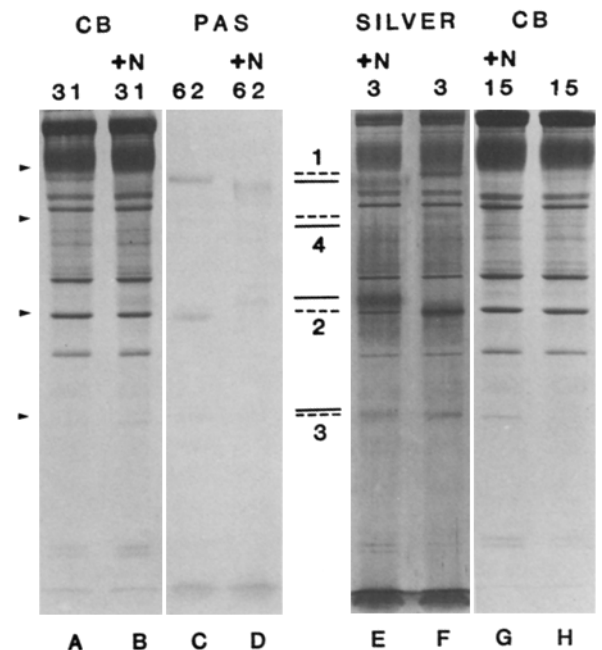


Figure 2. Gels of control and neuraminidase-treated RBC membranes. Portions of two slab gels are shown and each was divided after electrophoresis for staining with either Coomassie Blue (lanes A and B) and PAS (lanes C and D) or silver (lanes E and F) and Coomassie (lanes G and H). Total membrane protein applied indicated in micrograms at the top of each lane. The positions of glycosylated polypeptides before (broken lines) and after (solid lines) neuraminidase treatment (N) are indicated. Note that all four PAS-positive bands and their silver-stained counterparts lose intensity and shift in position after neuraminidase treatment. Slab gel parameters as in Fig. 1, except separation gels 12.5% acrylamide; left gel (lanes A–D) 1.5 mm thick; right gel (lanes E–H) 0.8 mm thick. Merrill et al. (27) silver-stain. On 12.5% gels, PAS 2 co-migrates with band 6 before neuraminidase treatment.

Table I. Distribution of Radioactivity after Lipid Extraction

Sample	Expt. 1 (n = 3)		Expt. 2 (n = 4)		Expt. 3 (n = 3)		Overall %
	Radioactivity	% Total	Radioactivity	% Total	Radioactivity	% Total	Distribution
	<i>cpm*</i>		<i>cpm*</i>		<i>cpm*</i>		%
Water/MetOH	23,567 ± 3,644	30	16,409 ± 1,138	31	20,801 ± 2,900	42	34 ± 13
Interface	2,859 ± 538	4	1,521 ± 538	3	1,865 ± 2,358	4	4 ± 1
Chloroform	5,476 ± 380	7	2,079 ± 198	4	2,959 ± 806	6	6 ± 3
Tube surface	45,548 ± 4,418	59	32,871 ± 2,772	62	23,332 ± 3,564	48	56 ± 15
Total	77,450		52,880		48,957		

* Mean ± 2 SD.

distinct additional bands are present (Fig. 2F, broken lines). In 12.5% acrylamide gels, one migrates just below band 3, one below band 4.2, one in the region of band 6, and one between band 7 and hemoglobin. The positions of these bands are similar to those of the major sialoglycoproteins of the RBC membrane: PAS 1, 4, 2, and 3 (from top to bottom) (26).

Sialoglycoprotein Visualization

To determine if these additional bands contained carbohydrate, gels were stained with PAS (Fig. 2, C and D). The positions of the four PAS-positive bands corresponded to the positions of four of the additional bands revealed by Merrill et al. (27) silver-staining (Fig. 2, E and F). In Coomassie Blue-stained gels, these bands were faintly visible only in overloaded gels (Fig. 2A, arrows). Parallel controls cut from this same gel and stained with Schiff's reagent without periodic acid pretreatment showed no bands.

To determine if the PAS- and silver-stained bands contained sialoglycopeptides, we used neuraminidase to cleave *N*-acetyl neuraminic acid (sialic acid) residues from accessible oligosaccharides of intact RBCs before electrophoresis (11). Representative examples of the polypeptide and carbohydrate patterns of neuraminidase-treated membranes are shown in Fig. 2, B, D, E, and G. Coomassie Blue staining (Fig. 2, A, B, G, and H) showed slight but detectable differences between neuraminidase-treated and untreated samples. Neuraminidase treatment removed a faint band just below band 3 and enhanced the staining of a band migrating near *M_r* 26,000 (Fig. 2, B and G). Otherwise, the gels appeared identical. The positions of bands on 1.5-mm thick gels (Fig. 2, A and D) with 31- or 62- μ g total protein loads were very similar to those on 0.8-mm gels (Fig. 2, E and H) with 3- or 15- μ g loads enabling a comparison of the heavily loaded, PAS-stained, thick gels (Fig. 2, C and D) with minimally loaded, silver-stained, thin gels (Fig. 2, E and F). Neuraminidase treatment altered the positions and the relative intensities of all four PAS bands (Fig. 2D). Although the PAS-positive bands were barely discernable after neuraminidase treatment (Fig. 2, solid lines), they could easily be seen after silver staining at only 5% of the protein load required for PAS staining: 3 μ g (Fig. 2E) vs. 62 μ g (Fig. 2D). Although the PAS 2 (predominantly PAS 1 monomers) bands of non-neuraminidase-treated cells co-migrated with band 6 in these 12.5% gels, they migrated more slowly than band 6 in 7.5% to 11.5% gels and could thus be independently resolved (Figs. 1 and 3).

Table II. Distribution of Radioactivity after TCA-Precipitation

Sample	Radioactivity	Distribution
	<i>cpm*</i>	% total
Supernatant	7,064 ± 477	9
Pellet	66,923 ± 6,961	89
Tube	1,352 ± 860	2
Total	75,339	

* Mean ± 2 SD; n = 4.

Distribution of Radioactivity

The distribution of radioactivity in lactoperoxidase-radioiodinated cells was analysed by lipid extraction and protein precipitation. The results of lipid extraction are shown in Table I. Approximately 6% of the radioactivity partitions with the chloroform phase. A significant fraction of denatured protein (56%) also adsorbed to the walls of the glass tube. In experiment 1, the sum of the counts of individual fractions was compared to untreated controls: total recovery of activity was 97.5%.

Table II shows that after TCA precipitation, 9% of the total radioactivity remained in the aqueous phase. This percentage was much higher if cells were not passed through an agarose column, suggesting the presence of free or noncovalently bound iodine immediately after labeling. The data in Tables I and II are consistent, given the following interpretation. In Table II, 91% of the radioactivity is TCA-precipitable and adsorbed. In Table I, adsorbed and interfacial activity account for 60% with the remaining 30% derived from the water/methanol fraction (presumably from the sialoglycoproteins).

To determine loss of radioactivity during gel processing, 0.8-mm thick slab gels were cut into strips and solubilized, and the radioactivity was measured by liquid scintillation counting. Fig. 3 summarizes the results; 56% of the total activity is removed during gel processing and staining. In a separate set of experiments, the total activity loaded per lane was compared to the summed activities of sliced lanes immediately after electrophoresis. Recovery was 86 ± 7% (mean ± 2 SD, n = 3).

Because of the significant loss of radioactivity during processing, several untreated gels were dried immediately after electrophoresis and fluorographed. Comparison of fluorographs of untreated control gels to those of silver-stained gels showed that controls possessed the same major bands as stained gels. Although there was a general loss of radioactivity

in stained gels, there was no selective loss during processing. Second, controls showed the same location of radioactivity as stained gels as well as additional minor bands. These minor bands were also detected in over-exposed stained gels and

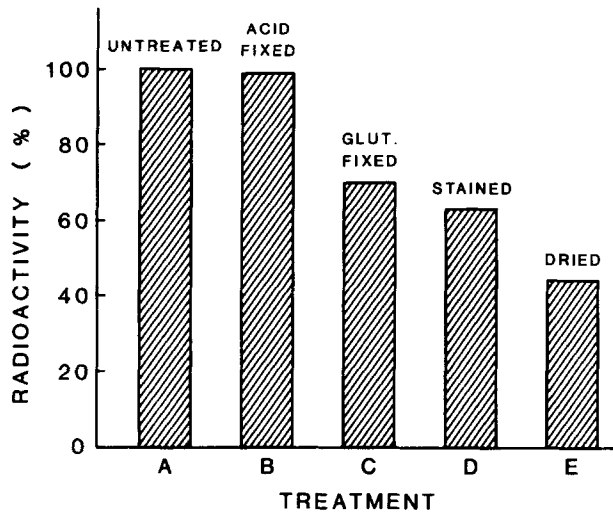


Figure 3. Loss of radioactivity during gel processing. (A) Untreated gel immediately after electrophoresis. (B) After acetic acid/methanol fix and wash. (C) After glutaraldehyde fix and methanol wash. (D) After Poehling and Neuhoff (35) silver stain. (E) After drying.

were present in both split and unsplit samples. Third, controls showed the same relative intensity among labeled bands as stained gels (also verified by scintillation counting); i.e., loss of radioactivity was proportionally the same for all bands. Given these findings, gels were silver-stained, dried, and processed for fluorography, and the bands were cut out for scintillation counting.

Split, Unlabeled Cell Monolayers: Silver-Staining

The effects of freeze-fracture on unlabeled cell monolayers were identical to those on lactoperoxidase-radioiodinated RBC monolayers (Fig. 4). Triplicate samples of unsplit whole cell monolayers (WCM) and paired complementary PLG fractions enriched in extracellular fracture faces, E-faces (4), and HVG fractions enriched in cytoplasmic fracture faces, P-faces, are shown flanked by molecular weight standards. Numerous polypeptide bands in addition to membrane bands can be seen. Hemoglobin migrates near the front of the gel and serves as a marker of the percentage of splitting. Note the reduced intensity of all bands on the PLG side relative to the HVG side; i.e., most of the cell and membrane protein remains with the cytoplasmic side of the split. The presence of hemoglobin on the PLG side of the split represents intact, unsplit cells. Although there were minor differences in the intensities of specific bands relative to each other, all major bands were present in both extracellular and cytoplasmic

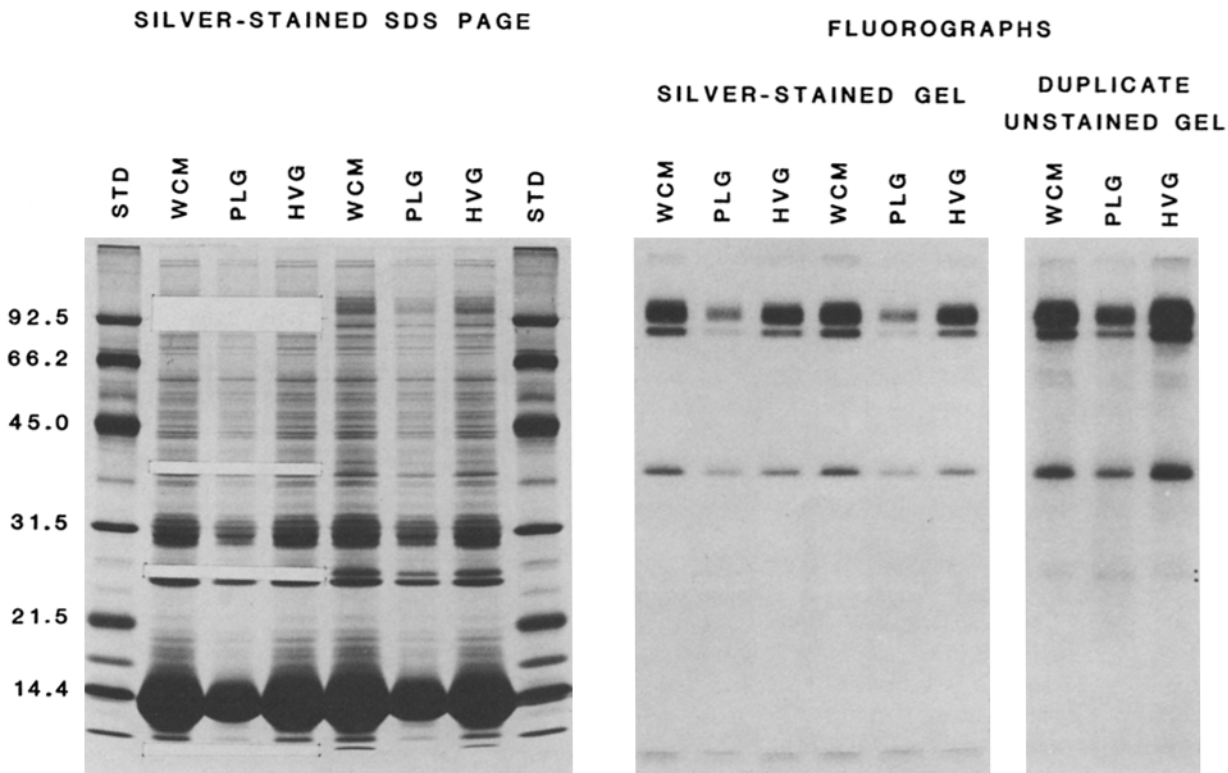


Figure 4. Gel and autoradiographs of lactoperoxidase-radioiodinated intact and freeze-fractured human red cells. Silver stained gel, left, its matching fluorograph, center, and duplicate unstained-gel fluorograph, right. Gel lanes show duplicate sets of unsplit whole cell monolayers (WCM) and split complementary polylysine-glass (PLG) fractions, and heavy-glass (HVG) fractions, flanked by molecular weight standards (STD). Radioactive regions of the left gel set were cut for scintillation counting using the center fluorograph as a template. Note that the anion channel, band 3, and glycophorin bands, PAS 1 and 2, are heavily labeled; and all bands in the split fractions have matching counterparts in the unsplit control, whether silver-stained or radiolabeled. The right fluorograph is of a gel co-electrophoresed with the left silver-stained gel, but dried immediately after electrophoresis and before staining. For identical exposure, band intensities are higher and minor bands visible, but no new bands can be detected in the split samples relative to the unsplit WCM.

fractions, and no new bands could be detected. Both PAS 1 and PAS 2 regions are more discernible in this gel than in this black and white print, since the sialoglycoprotein bands stain gray, whereas most of the bands stain yellow-brown in the Poehling and Neuhoff (35) procedure. We have also examined single membrane monolayers produced from cell or ghost monolayers that were lysed, hydraulically sheared, and freeze-fractured (Fisher and Yanagimoto, manuscript submitted for publication). In those studies as well, gels clearly show the presence of all original membrane bands and absence of any new bands. Inclusion of protease inhibitors such as phenylmethylsulfonyl fluoride in all solutions, including sample and running buffers, resulted in identical patterns in unattached and unsplit, or attached and unsplit or split cell and membrane monolayers.

Split, Labeled Cell Monolayers: Fluorography

Fig. 4 shows a gel of split and unsplit radioiodinated membrane polypeptides visualized by silver staining (Fig. 4, left) and its companion fluorographs (Fig. 4, center and right). Triplicate samples of unsplit WCM and paired complementary split PLG and HVG fractions are shown. The fluorograph (Fig. 4, center) of this silver-stained gel was used as a template to cut out the radioactive bands in one set of samples (far left) for solubilization and scintillation counting. Fig. 4, right, is a fluorograph of an identical but unstained gel. The stained and unstained gels were loaded with identical samples and electrophoresed simultaneously in the same chamber. Neither of these representative fluorographs shows the presence of any bands in the PLG (E-face enriched) or HVG (P-face enriched) samples that are not also present in the unsplit sample.

Fig. 5 shows a fluorograph after a 7-d exposure. Fluorographs of dried gels of split samples were routinely overexposed in an attempt to detect new bands that might indicate polypeptide cleavage. No distinct new bands have ever been observed, although occasionally a broad region of faint activity between PAS 2 and PAS 3 has been detected (Fig. 5). By densitometry, such areas represent <1% of the total radioactivity. The presence of such regions often correlates with the presence of aggregated material at both the sample well/stacker and stacker/separation gel interfaces. Before electro-

phoresis, this sample was lyophilized, a procedure that favors such aggregation.

The data from four separate radioiodination experiments are given in Table III. Data have not been corrected for sampling differences between unsplit and split preparations and thus the sums of the splits (PLG + HVG) do not match the controls (WCM). However, the complementary PLG and HVG fractions of each split were sampled from equal volumes and thus may be compared. In all unsplit samples, band 3 is most heavily labeled, followed by PAS 1 and PAS 2.

To determine if both glycosylated and band 3 polypeptides co-partition to the same side of the membrane after splitting,

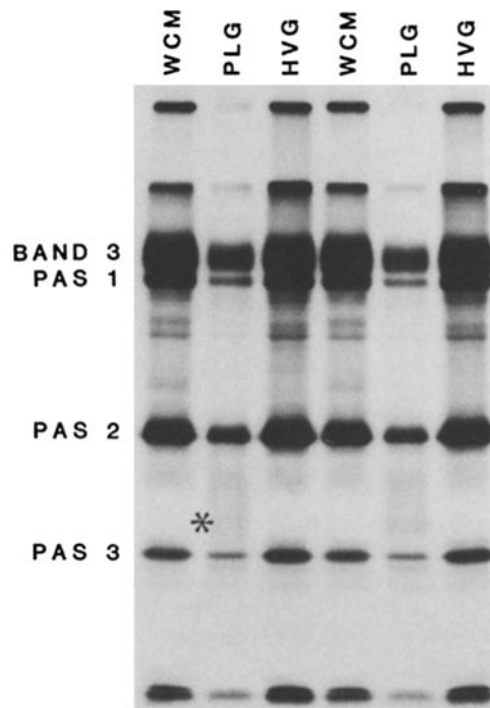


Figure 5. Looking for minor bands. Fluorograph exposed for 7 d. Note numerous minor bands in the regions between PAS 1 and 2, and 2 and 3. The broad bands between 2 and 3 (asterisk) have also been seen in PLG E-face enriched "half" membrane preparations.

Table III. Partitioning of Radioactivity between PAS Bands and Band 3

	Expt. 1			Expt. 2			Expt. 3			Expt. 4		
	Unsplit		Split	Unsplit		Split	Unsplit		Split	Unsplit		Split
	WCM	PLG		WCM	PLG		WCM	PLG		WCM	PLG	
Radioactivity (cpm)												
Band 3	521	183	585	1,400	258	1,048	ND*	187	816	148	48	144
PAS 1	151	65	211	407	83	266	ND	43	97	58	15	42
PAS 2	110	69	161	358	88	280	ND	33	169	43	13	27
PAS 3	47	20	48	231	48	74	ND	ND	ND	ND	ND	ND
Ratios												
PAS 1 + 2/Band 3	0.50	0.73	0.64	0.55	0.66	0.52	ND	0.41	0.33	0.68	0.58	0.48
PAS 3/Band 3	0.09	0.11	0.08	0.16	0.19	0.07	ND	ND	ND	ND	ND	ND
Synopsis of ratios (mean ± SD)				Unsplit		Split						
				WCM		PLG		HVG				
PAS 1 + 2/Band 3				0.58 ± 0.09		0.60 ± 0.14		0.49 ± 0.13				
PAS 3/Band 3				0.13 ± 0.05		0.15 ± 0.05		0.08 ± 0.01				

* ND, not determined. All data as corrected cpm.

the activity in gel bands was analysed by liquid scintillation counting as previously described. Table III summarizes the results of four experiments and also provides a synopsis of the fraction of label found in PAS bands relative to band 3. Two additional experiments showed unexplainably high ratios of PAS activity relative to band 3 and/or high molecular weight aggregates and have not been included. Comparison of split PLG and HVG samples to unsplit controls revealed that, relative to band 3, the amount of PAS polypeptides for both the summed PAS 1 and PAS 2 bands and for the PAS 3 band were slightly higher on the PL-glass side of the split (E-face enriched fraction) than the unsplit control membrane. Similarly the complementary HVG side (P-face enriched fraction) shows a depletion of PAS polypeptides (lower ratio of PAS 1 + 2/band 3 and of PAS 3/band 3) relative to the intact membrane. Although these data suggest an asymmetric partitioning of the two classes of transmembrane polypeptides, statistical analyses show the precision of the data to be poor. For example, the difference in PAS 3/band 3 ratios between the PLG cytoplasmic and HVG extracellular fractions is significant only at the 80% level of confidence.

Discussion

The present report describes the effect of freeze-fracture on transmembrane polypeptides of both chemically unmodified and enzymatically labeled erythrocytes. In studies of unmodified membranes, silver-stained gels have both theoretical and practical advantages for the study of membrane glycoproteins. One can examine unlabeled polypeptides whose relative mobility on gels might otherwise be altered by chemically or enzymatically induced cleavage, aggregation, or conformational rearrangement (1, 5). In addition, silver binds not only to nonglycosylated peptides, but, as shown here, to glycoproteins, as well as to nucleic acids (2, 40) and lipopolysaccharides (42). Finally, sialoglycoprotein bands can be readily distinguished from other bands because the total amount of protein applied to the gel is small, and resolution increases with decreasing protein concentration. From a practical viewpoint, the method is simple, rapid, and sensitive (27).

We first observed staining of sialoglycoprotein by silver (17) using the techniques of Merrill et al. (27). Recently Dzandu et al. (8) published similar findings using a double-stain approach followed by Coomassie Blue. In contrast to their report (8), however, and as described in our previous (17) and present reports, we find that both Coomassie Blue and PAS-positive bands stain with the Merrill et al. (27) and the Poehling and Neuhoff (35) silver methods. Relative to the method of Merrill et al., the Poehling and Neuhoff method has several advantages. First, the background is clear and thus especially suited for densitometry. Second, the PAS-positive bands stain gray. Even in gels of whole cells containing numerous bands, they can be distinguished from the yellow-to-brown staining bands. And third, because the amount of total protein applied to the gel in the silver stain method is much smaller than that applied in the combined Coomassie Blue/silver stain method, band sharpness (resolution) is significantly improved. Disadvantages are those common to SDS PAGE. For example, membrane glycoproteins migrate anomalously on SDS gels (26, 38), and, thus, gels cannot be used to establish definitive molecular weights. A disadvantage unique to the silver stain procedure is that spurious bands appear in the band 4.5

region. Furthermore, the mechanism of silvering has not been established (35), although a chemical explanation has been proposed (21).

The silver gel patterns and fluorographs of the split membrane preparations revealed several interesting features. There were no new bands generated nor old bands lost, suggesting a lack of significant cleavage of the covalent backbones of any membrane peptide during freeze-fracture. This was true for peptides derived from integral, transmembrane proteins whether minimally (band 3) or heavily (PAS 1, 2) glycosylated; and it is similarly true, as anticipated (14), for peripheral proteins. Although all classes of RBC membrane peptides partitioned to the cytoplasmic side of the membrane after splitting, there were some indications of enrichment or depletion of certain bands relative to others. When split fractions were compared to intact controls, for example, there was a decrease in glycophorin A relative to band 3 on the cytoplasmic side and a concomitant increase on the extracellular side. These observations, however, have been inconsistent, due in part to the difficulty of accurately quantifying silver gels (35), and minimal enrichment of P-face membrane proteins on the HVG side and of E-face proteins on the PLG side of split monolayers. Furthermore, we were unable to use the double-labeled membrane splitting approach (15) to quantify fractions of extracellular or cytoplasmic "halves," since heating in Laemmli sample buffers containing mercaptoethanol significantly altered both hemoglobin absorbance and FITC fluorescence signals.

Although definitive quantitative determinations of transmembrane distributions of individual polypeptides await further studies, the limits to detecting the cleavage of covalent bonds by the methods described in this report can be estimated. Although we routinely discard "poor" splits, such a split can serve as a worse-case example of enrichment and detection of fragmented polypeptides. In a "poor" split, 75% of the hemoglobin partitions to the HVG side and 25% remains on the PLG side of the freeze-fractured monolayer. From our previous studies we know that 33% of the total surface of the cell is bound and split (13). Thus, in the PLG fraction $\sim 25\%$ ($75\% \times 33\%$) of the total membrane is split and 25% ($25\% \times 100\%$) is unsplit; in other words, given random partitioning, a maximum of 50% of all polypeptides could be derived from split membrane. However, both band 3 and the glycophorins partition preferentially to the HVG side, with the cytoplasmic side of the membrane. Assuming that 75% of split band 3 and the glycophorin bands partition to the cytoplasmic side, then 25% of those split polypeptides would remain in the PLG fraction. Thus only 12.5% ($25\% \times 50\%$) of the transmembrane peptides detected in the PLG fraction would be derived from split membrane. In this report, we determined by densitometry that the putative "new material" (asterisk, Fig. 5) is $<1\%$ of the total activity in the PLG fraction. If this "new material" indeed represented polypeptide fragments, then the amount must be $<8\%$ ($1\%/12.5\%$) of the total split polypeptides. Given a more realistic 90% HVG to 10% PLG split, where no "new material" can be detected, (i.e., the "new material" is $<1\%$ of the total), the amount of fragmented polypeptide, if it exists, must be much less than a few percent of the total split polypeptide fraction. The methods of fluorography, densitometry, and liquid scintillation counting used to quantify splitting are thus not

limiting at this level of detection. Furthermore, from previous experiments we know that our recovery of peptides in the split membrane fractions is >98%. Because all membranes and all polypeptides are electrophoresed, it is unlikely that we have lost peptide fragments. Finally, in recent experiments with sheared single-membrane monolayers, we have produced a theoretically pure fraction of cytoplasmic "half" membranes. We have found no evidence of covalent bond cleavage in these fractions. In other words, if transmembrane cleavage does occur, it is quantitatively trivial.

The observations and conclusions in this report differ from those of others. Using the planar cell monolayer technique (13), Morrison and collaborators (9, 28) and Nermut (31) examined radiolabeled RBCs, observed new bands in fluorographed gels of the split preparations, and concluded that membrane spanning glycoproteins had been cleaved (9, 10, 28). We have been unable to reproduce these observations, even though we have used similar lactoperoxidase-catalyzed radioiodination and planar cell monolayer preparation procedures (13). There were several experimental differences, however. First, we have used glass/glass preparations rather than copper/glass or copper/mica for freeze-fracture. We wished to recover and quantify both sides of the split preparation and found that copper interfered with SDS PAGE analysis. Second, we have used smaller glasses (11 × 22 mm vs. 24 × 50 mm), which allowed us to solubilize membrane polypeptides in small volumes suitable for direct sampling for electrophoresis without centrifugation, dialysis, or concentration. Third, our cells were labeled with FITC-concanavalin A in addition to ¹²⁵I for reasons not relevant to this report, but data from parallel experiments using unlabeled cells produced identical results. Finally, we have taken care to monitor distribution of radioactivity during and after labeling and during and after gel processing. With these approaches we have detected no new bands.

How can one explain the additional bands observed by others? There are at least two plausible explanations: proteolysis and/or minor band enrichment. Proteolysis would be favored, for example, by thawing the samples (9), pooling samples in dilute (0.1%) SDS followed by concentration and dialysis, or chemically labeling dilute samples after freeze-fracturing (9, 30, 31). It should be emphasized, however, that even though we have used proteolytic inhibitors in other experiments, none were used in the studies described in this report, and yet no new bands were seen. We have observed enrichment for minor lactoperoxidase radioiodinated bands, however, by pooling twelve 11 × 22-mm coverglasses that had been scraped and broken under liquid nitrogen to remove all unfractured areas (9). A broad band of activity could be detected between PAS 2 and PAS 3. Control unfrozen and unfractured cells, however, also showed faint activity at precisely the same region, suggesting proteolysis during processing.

Our observations are generally consistent with those of Pinto da Silva et al. (33), who have shown that band 3 partitions to the cytoplasmic side of the split RBC membrane. Considering the numerous technical differences between fracture-label (multiple chemical fixation and freeze-thaw cycles) and planar membrane splitting (cationic adsorption and sandwiching), it is perhaps surprising to find similar results in the partitioning of band 3 (33) to the cytoplasmic "half" and

glycophorin to the extracellular "half" of the membrane (34). Although we do find weak evidence for enrichment of glycophorin A on the extracellular "half" of the membrane, it also partitions intact with the cytoplasmic leaflet. Because the split membrane polypeptides retain their oligosaccharides and migrate on SDS gels with unchanged relative mobility, it is likely that the carbohydrate contributes to the structure of the intramembranous particle. The partitioning pattern of PAS 3 relative to band 3 suggests that it preferentially partitions to the extracellular leaflet of the split membrane.

In summary, both qualitative and quantitative analyses of membrane proteins have revealed that the polypeptide backbones of both glycosylated and nonglycosylated membrane proteins, whether peripheral or integral, remain intact during the process of membrane splitting. Thus planar monolayer freeze-fracture, a physical fractionation approach, can provide a unique new tool for investigations of the molecular mechanisms of protein-mediated transmembrane phenomena.

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References

1. Abraham, G., and P. S. Low. 1980. Covalent labelling of specific membrane carbohydrate residues with fluorescent probes. *Biochem. Biophys. Acta.* 597:285-291.
2. Berry, M. J., and C. E. Samuel. 1982. Detection of subnanogram amounts of RNA in polyacrylamide gels in the presence and absence of protein by staining with silver. *Anal. Biochem.* 124:180-184.
3. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37:911-917.
4. Branton, D., S. Bullivant, N. B. Gilula, M. J. Karnovsky, H. Moor, K. Muhlethaler, D. H. Northcote, L. Packer, B. Satir, P. Satir, V. Speth, L. A. Staehelin, R. L. Steere, and R. S. Weinstein. 1975. Freeze-etching nomenclature. *Science (Wash. DC)*. 190:54-56.
5. Carraway, K. L. 1975. Covalent labeling of membranes. *Biochim. Biophys. Acta.* 415:379-410.
6. Dailey, H. A., and P. Strittmatter. 1982. Orientation of the carboxyl and NH₂ termini of the membrane-binding segment of cytochrome b5 on the same side of phospholipid bilayers. *J. Biol. Chem.* 256:3951-3955.
7. Dodge, J. T., C. Mitchell, and D. J. Hanahan. 1963. The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Arch. Biochem. Biophys.* 100:119-130.
8. Dzandu, J. K., M. E. Deh, D. L. Barratt, and G. E. Wise. 1984. Detection of erythrocyte membrane proteins, sialoglycoproteins, and lipids in the same polyacrylamide gel using a double staining technique. *Proc. Natl. Acad. Sci. USA.* 81:1733-1737.
9. Edwards, H. H., T. J. Mueller, and M. Morrison. 1979. Distribution of transmembrane polypeptides in freeze fracture. *Science (Wash. DC)*. 203:1343-1345.
10. Edwards, H. H., H. T. Smith, and M. Morrison. 1984. Monolayer freeze-fracture of erythrocytes utilizing a glass-glass system. *J. Cell Biol.* 99(4, Pt. 2):287a. (Abstr.)
11. Eylar, E. H., M. A. Madoff, O. V. Brody, and J. L. Oncley. 1962. The contribution of sialic acid to the surface change of the erythrocyte. *J. Biol. Chem.* 237:1992-2000.
12. Fairbanks, G., T. L. Steck, and D. F. H. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry.* 10:2606-2617.
13. Fisher, K. A. 1976. Analysis of membrane halves: cholesterol. *Proc. Natl. Acad. Sci. USA.* 73:173-177.
14. Fisher, K. A. 1980. Split membrane analysis. *Annu. Rev. Physiol.* 42:261-273.
15. Fisher, K. A. 1982. Spectroscopic assays for measuring quantities of erythrocyte membrane "halves." *J. Cell Biol.* 92:44-52.
16. Fisher, K. A. 1982. Preparation of planar membrane monolayers for spectroscopy and electron microscopy. *Methods Enzymol.* 88:230-235.
17. Fisher, K. A. 1982. Freeze-fractured RBC membrane peptides and

- glycopeptides: analysis by SDS gel electrophoresis and silver-staining. *J. Cell Biol.* 95(2, Pt. 2):250a. (Abstr.)
18. Fisher, K. A. 1982. Monolayer freeze-fracture autoradiography: quantitative analysis of the transmembrane distribution of radioiodinated concanavalin A. *J. Cell Biol.* 93:155-163.
 19. Fisher, K. A., and K. C. Yanagimoto. 1984. Planar freeze-fracture of radioiodinated erythrocyte membranes: effects of splitting on transmembrane polypeptides. *J. Cell Biol.* 99(4, Pt. 2):286a. (Abstr.)
 20. Furthmayr, H. 1983. Glycophorin A, B, and C: a family of sialoglycoproteins: isolation and preliminary characterization of trypsin derived peptides. *J. Supramol. Struct.* 9:79-95.
 21. Guevara, J., D. A. Johnston, L. S. Ramagali, B. A. Martin, S. Capetillo, and L. V. Rodriguez. 1982. Quantitative aspects of silver deposition in proteins resolved in complex polyacrylamide gels. *Electrophoresis.* 3:197-205.
 22. Jones, M. N., and J. K. Nickson. 1981. Monosaccharide transport proteins of the human erythrocyte membrane. *Biochim. Biophys. Acta.* 650:1-20.
 23. Laemmli, U. K. 1970. Cleavage of structure proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680-685.
 24. Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265-275.
 25. Marchesi, V. T. 1979. Functional proteins of the human red cell membrane. *Semin. Hematol.* 16:3-20.
 26. Marchesi, V. T., H. Furthmayr, and M. Tomita. 1976. The red cell membrane. *Annu. Rev. Biochem.* 45:667-698.
 27. Merrill, C. R., D. Goldman, S. A. Sedman, and M. H. Ebert. 1981. Ultrasensitive stain for proteins in polyacrylamide gel shows regional variation in cerebrospinal fluid proteins. *Science (Wash. DC)* 211:1437-1438.
 28. Morrison, M., T. J. Mueller, and H. H. Edwards. 1981. Protein architecture of the erythrocyte membrane. In *The Function of Red Blood Cells: Erythrocyte Pathobiology*. Alan R. Liss, Inc, New York. 17-34.
 29. Mueller, T. J., and M. Morrison. 1974. The transmembrane proteins in the plasma membrane of normal human erythrocytes. *J. Biol. Chem.* 249:7568-7573.
 30. Nermut, M. V. 1982. The "cell monolayer technique" in membrane research. *Eur. J. Cell Biol.* 28:160-172.
 31. Nermut, M. V. 1983. The cell monolayer technique—an application of solid-phase biochemistry in ultrastructural research. *Trends Biochem. Sci.* 8:303-306.
 32. Ochs, D. 1983. Protein contaminants of sodium dodecyl sulfate-polyacrylamide gels. *Anal. Biochem.* 135:470-474.
 33. 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. USA.* 78:343-347.
 34. Pinto da Silva, P., and M. R. Torrissi. 1982. Freeze-fracture cytochemistry-partition of glycophorin in freeze fractured human erythrocyte membranes. *J. Cell Biol.* 93:463-469.
 35. Poehling, H. M., and V. Neuhoff. 1981. Visualization of proteins with a silver "stain": a critical analysis. *Electrophoresis.* 2:141-147.
 36. Ramjeesingh, M., A. Gaarn, and A. Rothstein. 1984. Pepsin cleavage of band 3 produces its membrane-crossing domains. *Biochim. Biophys. Acta.* 769:381-389.
 37. Sammons, D. W., L. D. Adams, and E. E. Nishizawa. 1981. Ultrasensitive silver-based color staining of polypeptides in polyacrylamide gels. *Electrophoresis.* 2:135-141.
 38. Silverberg, M., and V. T. Marchesi. 1978. The anomalous electrophoretic behavior of the major sialoglycoprotein from the human erythrocyte. *J. Biol. Chem.* 253:95-98.
 39. Sogin, D. C., and P. C. Hinkle. 1978. Characterization of the glucose transporter from human erythrocytes. *J. Supramol. Struct.* 8:447-453.
 40. Somerville, L. L., and K. Wang. 1981. The ultrasensitive silver "protein" stain also detects nanograms of nucleic acids. *Biochem. Biophys. Res. Commun.* 102:53-58.
 41. Steck, T. L. 1974. The organization of proteins in the human red blood cell membrane. *J. Cell Biol.* 62:1-19.
 42. Tsai, C. M., and C. E. Frasch. 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal. Biochem.* 119:115-119.