

ORIENTED ADSORPTION OF PURPLE MEMBRANE TO CATIONIC SURFACES

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ABSTRACT

We have investigated the orientation of isolated fragments of *Halobacterium halobium* purple membrane (PM) adsorbed to poly-L-lysine-treated glass (PL-glass), by quantitative electron microscopy. Three lines of evidence support the conclusion that the cytoplasmic side of the membrane is preferentially adsorbed. First, monolayer freeze-fracture reveals nonrandom orientation; more fracture faces (89%) are particulate than smooth. Second, the amount of each membrane surface present can be assayed using polycationic ferritin; 90% of all adsorbed membrane fragments are labeled. Third, it is possible to distinguish two surfaces, "cracked" (the extracellular surface) and "pitted" (the cytoplasmic surface), in slowly air-dried, platinum-carbon-shadowed membranes. When applied under standard conditions, more than 80% appear cracked. Selection for the cytoplasmic side by the cationic substrate suggests that the isolated PM, buffered at pH 7.4 and in the light, has a higher negative charge on its cytoplasmic surface than on its extracellular surface. Nevertheless, cationic ferritin (CF) preferentially adsorbs to the extracellular surface. Orientation provides a striking example of biomembrane surface asymmetry as well as the means to examine the chemical reactivity and physical properties of surfaces of a purified, nonvesicular membrane fragment.

KEY WORDS purple membrane · freeze-fracture · polylysine · cationic ferritin · membrane asymmetry

The plasma membrane of the halophilic bacterium *Halobacterium halobium* contains differentiated patches (1) that can be isolated by hypotonic cell lysis followed by density gradient centrifugation (24, 27). The membrane fragments are purple with a broad absorption maximum around 570 nm, and contain a single 26,000-dalton protein, bacteriorhodopsin (1, 3, 22), 75% by weight, and several classes of glyco-, sulfo-, and phospholipids (15, 16), 25% by weight. Two sulfated lipids (glycolipid sulfate and phosphatidyl glycerosul-

fate) are found exclusively in the purple membrane (PM) fraction whereas the dominant phospholipid (phosphatidyl glycerophosphate) is also present in the red plasma membrane fraction (15, 16). Upon absorption of light, bacteriorhodopsin undergoes a photoreaction cycle (17, 18) that involves the uptake and release of hydrogen ions (14, 17, 18, 23). There is good evidence that ion release is vectorial (14, 17, 18), generating a proton gradient that can be used as an energy source (25) consistent with the chemiosmotic mechanism for energy transduction (20, 23).

Although the bulk lipid composition of the PM has been well studied (15, 16) and the three-dimensional structure of the bacteriorhodopsin

molecule has been resolved at 7 Å by electron microscopy (12, 28), there remains a general lack of information about the distribution of lipid classes or of protein reactive sites on each side of the membrane bilayer. Conventional membrane-labeling approaches (4) that rely on reagent impermeable cells or vesicles are not applicable to the sheets of PM which in bulk solution have both sides equally in contact with the same aqueous milieu. We have adsorbed the membrane nonrandomly to a surface so that only one side is exposed to the label or probe; the membrane surface can be selectively examined using conventional biochemical or cytochemical methods.

Nonrandom and known orientation is also an advantage for physical studies. X-ray or electron diffraction, where whole cells or envelope preparations cannot be used, provide obvious examples. In freeze-fracture, too, the presence of an extended flattened monolayer of PM offers unique opportunities (10): Not only can the plane of fracture and shadowing angle be precisely adjusted, but the areas of fracture faces produced are much larger than those found in whole cells (1) or pelleted membrane preparations. In addition, membrane surfaces and their relation to fracture faces can be easily discerned.

Because polylysine-treated glass (PL-glass) will adsorb plasma membranes quite tenaciously (7, 19) and because orientation in an electric field suggests that PM possesses an asymmetric charge distribution, the properties of adsorbed PM were investigated by electron microscopy. We describe here the conditions for preparation of oriented membrane fragments, and the electron microscopy of platinum-carbon-shadowed PM.

MATERIALS AND METHODS

Preparation of PM

Halobacterium halobium R₁ was grown in complex medium under high light intensity and low oxygen tension (24). Fragments of PM were isolated by lysis in low salt essentially as previously described (24), dialyzed for 5–7 days, washed at least two times (pelleted at 20,000 rpm, 30–40 min, Sorvall RC2-B centrifuge, SS-34 head, DuPont Instruments-Sorvall, DuPont Co., Wilmington, Del.) with 20 mosmol phosphate buffer (6), pH 7.4 (20-7.4 buffer), and pellets were resuspended and again centrifuged (15,000 rpm, 10 min) to remove large aggregates. Membranes were gently resuspended in 20-7.4 buffer. The concentration of PM in buffer or water was determined by comparing light-adapted sample (17) dry weight to optical absorbance

(details will be presented elsewhere). An optical density of one at 570 nm corresponds to 0.646 ± 0.009 mg PM/ml. Membranes stored in phosphate buffer in 0.01% sodium azide, in the dark at 5°C, were centrifuged immediately before use (15,000 rpm, 10 min), to remove aggregates.

Preparation of PL-Glass

Cover glasses were prepared as previously described (7). PL-glass was usually used within 60 s, never more than 5 min, after N₂-gun drying.

Application of Membranes to Glass

Light-adapted PM in 20-7.4 buffer and at concentrations of from 0.125 to 12.5 mg/ml (specified for each experiment) was applied to freshly prepared PL-glass. The volume of buffer applied was kept constant, as were the conditions of surface area, pH and ionic strength, time, temperature, and methods of washing. PM dilutions that flowed evenly over the entire surface of the glass were gently swirled for 30 s, washed for 30 s with buffer, and processed as follows:

To produce or prevent cracked surfaces, 22 × 22 mm cover glasses were treated with <10 μg PM in 50 μl of buffer, rinsed for 10 s with glass-distilled water to remove buffer salts before shadowing, and subjected to five drying procedures: (a) slow air drying in a covered Petri dish, 20°C, 2–18 h; (b) same as (a) but over P₂O₅, 2–4 h; (c) drying with a heat gun at about 40°C, 3–5 min; (d) drying with a burst of N₂ at 20°C, 5 s; or (e) freezing in Freon-22 (–150°C) and drying by sublimation *in vacuo*.

To label one surface selectively with ferritin, 8 × 22 mm cover glasses were treated with PM at concentrations of <10 μg in 20 μl, followed by washing and wet application of the cationic ferritin (CF) solution at pH 4.5, then given a salt wash (see below) followed by a 10 s-water wash and N₂-gun drying.

To freeze-fracture oriented monolayers (with or without CF labeling), glass surfaces were essentially saturated with PM (concentrations in excess of 150 μg of PM in 20 μl). After buffer and distilled water washes, samples were treated as described below.

Preparation of CF

Carbodiimide-activated ferritin carboxyl groups were covalently labeled with appropriate nucleophiles (5, 13) and for the experiments described here with 1,6-hexane diamine, HMD (no. 5932, Eastman Kodak Co., Rochester, N. Y.).

Native ferritin and CF were examined by isoelectric focussing on an LKB 8101 Ampholine column (LKB-Produkter AB, Stockholm, Sweden) using pH 3–10 carrier ampholytes. Portions were sampled for absorbance at 280 nm and pH measurements. Native ferritin ranged in pI from 3.1 to 4.8 with an absorbance peak at 4.2. CF ranged from pH 8.85 upward with a peak at the

ampholyte/cathode interface (about pH 12) indicating a $pI > 10$. A small amount of background absorbance was found over the full pH range of the column. The CF band adsorbed to the walls of the column, however, preventing accurate quantitative recovery.

Labeling Membranes with CF

Suspensions of PM (5–50 μg) were added dropwise to 20–7.4 buffer containing 50 μg of CF; final volume 2 ml. After 5-min stirring at 20°C, membranes were pelleted at 20,000 rpm for 40 min (reference tubes showed that 10–20% free CF also pelleted) and the amount of bound CF was calculated by subtracting the supernatant absorbance at 270 nm from the initial value (corrected for residual PM and pelleted free CF).

PM bound to 22 \times 22 mm cover glasses (6.8 μg in 50 μl applied) was treated with CF ranging from 0.1 to 50 μg in 50 μl and pH ranging from 4.5 to 7.4. The pH effect was separately evaluated by applying 6.8 μg of CF in 50 μl buffers ranging from pH 2 to 10 for 30 s to the bound membrane, followed by buffer washes at the same pH and finally a 10-s distilled water wash, all at 20°C, and dried with N_2 .

PM bound to PL-glass and labeled with CF was washed with 20–7.4 buffered salt solutions ranging from 0.1 to 4.0 M NaCl, 30 s, 20°C. Both the time of treatment with 1.0 M NaCl (30 s, 1.5 min, 60 min) and the buffering capacity (0.01–0.1 M phosphate buffer) were evaluated. Our standard procedure (at 20°C) is as follows: (a) Less than 10 μg of PM in 20 μl of 20–7.4 buffer is applied to 8 \times 22 mm PL-glass for 30 s; (b) washed with 20–7.4 buffer for 30 s; (c) treated with 3 μg or less of CF in 20 μl of 10 mM acetate buffer, pH 4.5, for 30 s; (d) washed with 1 M NaCl in 20–7.4 buffer for 30 s; (e) rinsed with distilled water for 10 s; and (f) dried with the N_2 -gun.

Monolayer Freeze-Fracture of

Bound Membranes

Wet glass surfaces (8 \times 22 mm) saturated with PM (150 μg in 20 μl applied) \pm CF were placed against nitric acid-cleaned 9 \times 25 \times 0.5 mm copper sheet and frozen in Freon-22 (7). They were fractured under liquid nitrogen using a cold razor blade to pry the glass from the copper (8). The glass surface shows both glass-smooth areas and "plateaus" of ice. Glasses with areas of at least 50% smooth were placed in a holder designed to insure good thermal contact, transferred to a cold stage under liquid nitrogen, and then to a Varian VE-61 vacuum evaporator (Varian Associates, Instrument Div., Palo Alto, Calif.). The stage was warmed to about -80°C , and when the pressure reached 2.7×10^{-4} Pascal (2×10^{-6} torr) or below, the stage was shadowed with Pt-C at an angle of 20° , and then with carbon at 90° . Samples were warmed to 25° – 30°C , removed from vacuum, and replicas were floated off the glass onto one part concentrated HF plus one part distilled water in

plastic Petri dishes. After three distilled water rinses, random replica fragments were transferred to grids for electron microscopy.

Electron Microscopy

Dried and/or labeled membranes attached to PL-glass were shadowed usually within 24 h after initial drying with Pt-C at an angle of 20° in the VE-61 at 9.3×10^{-5} Pascal (7×10^{-7} torr) and 20°C . Freeze-fractured replicas were produced as described above. All preparations were sampled, and examined, at random in Siemens 1 and 101 electron microscopes. Magnifications were calibrated with cross-ruled diffraction grating replicas and by monitoring objective lens current. For quantitative analysis, all micrographs were taken at the same calibrated magnification. The absolute magnification depended on the set of experiments. For example, the surface structure of slowly air dried, Pt-C-shadowed PM or CF-labeled membrane was quite distinct at low magnification and thus large areas could be measured on prints with a final magnification of 30,000. The appearance of N_2 -gun-dried or freeze-fractured membrane, however, could only be unequivocally judged at higher magnifications; thus, prints at $\times 60,000$ or more were evaluated. Membrane areas on random prints were measured with a compensating polar planimeter (model 620015, Keuffel & Esser Co., Morristown, N. J.). All micrographs included here have shadows, white, oriented from bottom to top with the Pt-C source at the bottom.

Optical Diffraction

The optical bench was constructed, in positional order, as follows: (a) 0.5 mW He-Ne laser (model 155, Spectra Physics, Mountain View, Calif.); (b) $\times 40$ objective, beam-expanding lens; (c) pinhole; (d) shutter; (e) Nikkor-Q $f = 135$ mm collimating lens; (f) plate holder; (g) Nikkor-Q $f = 135$ mm diffraction lens; (h) $\times 10$ objective, magnification lens; and (i) Polaroid 545 land film holder. Polarizers were used to attenuate the beam. Diffraction length was measured using a copper grid with spacings determined using a calibrated light microscope and a Joyce-Loebl Mark III CS scanning microdensitometer (Joyce-Loebl, Gateshead-on-Tyne, England). Diffraction patterns of selected negatives contact printed on 6.5 \times 9.0 cm glass electron image plates (Eastman Kodak Co., Rochester, N.Y.) were recorded on Polaroid 4 \times 5 type 55 positive/negative film.

RESULTS

Adsorption Experiments

PM fragments bind tenaciously to PL-glass. Once bound, the membranes cannot be easily dislodged by washing, except with concentrated salt solutions, e.g. 4 M NaCl. Although most bound membranes are single and flattened, a

fraction are either folded or aggregated (usually as stacked pairs). The amount of PM bound (percent of glass area covered) depends on the applied concentration (Fig. 1) when other conditions are kept equal. Given a known concentration and the standardized application conditions outlined below, the area of glass covered by PM is predictable to about $\pm 20\%$ (e.g., if one predicts 10% coverage, the measured amount will generally lie between 8 and 12%). Reproducibility of coverage is quite good when we use only totally hydrophilic glass and well-washed PM in low ionic strength buffer.

Identification of Membrane Surfaces

CRACKED OR PITTED: The appearance of air-dried membrane varies with the drying procedure. The distinct cracks and pits that appear in slowly air-dried membranes (Fig. 2*a, b, f,* and *g*) are absent when the membranes are rapidly dried with nitrogen (Fig. 2*d* and *i*) or are freeze-dried (Fig. 2*e* and *j*). Warming the sample to 40°C during drying at an intermediate rate produces yet a different structure (Fig. 2*c* and *h*). In terms of

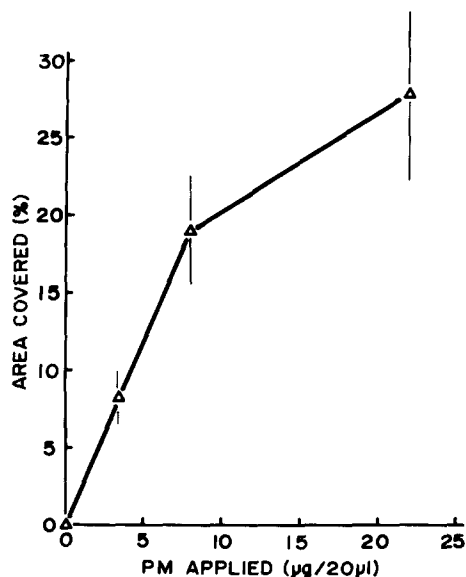


FIGURE 1 Curve showing percent area of polylysine glass (5.5×22 mm strips) covered by different dilutions of PM. Data derived from planimeter measurements of randomly sampled, Pt-C-shadowed replicas. Reproducibility about $\pm 20\%$. At 30% coverage and below, membrane pieces are evenly distributed, generally unaggregated; at 100% coverage ($\text{PM} \geq 150 \mu\text{g}$ in $20 \mu\text{l}$), there is substantial overlap.

reproducibility the slowly air-dried membrane preparations often show a wide variety of surface patterns, whereas the N_2 and freeze-dried preparations consistently show the same type of structure: generally smooth and continuous (Fig. 2*d* and *i*, and Fig. 2*e* and *j*, respectively).

Although variations are found among the slowly air-dried samples, every membrane piece and fragment can be assigned to one of two categories: "cracked" or "pitted." Intuitively, one might expect this binary distribution to correspond to one or the other surface of the membrane (24). This expectation was substantiated by the CF-labeling and freeze-fracture experiments to be described. Despite variability in surface morphology, the slowly air-dried samples (dry within 3–4 h) are easily categorized, and were used for quantitation.

CF LABELING: At pH 7.4, CF ($\text{pI} \geq 10$) adsorbs to free PM, in solution, as shown in Fig. 3. Adsorption depends on storage conditions. PM stored in basal salts for 3 wk or more bind less CF than freshly prepared membranes or membranes stored in water or 20–7.4 phosphate buffer. Experimental conditions can be adjusted so that, in both cases, a depletion level is reached where essentially all CF is removed from solution. At the point where all CF is depleted, calculations and microscopy show that both surfaces of PM are saturated with CF.

At pH 7.4, CF labels both surfaces of PM bound to PL-glass (Fig. 4*a*). The total CF bound is a function of the applied concentration. Under standard procedures, 176 mm^2 total surface area (glass, including membrane on glass) is saturated when treated with concentrations of CF in excess of $10 \mu\text{g}$ in $20 \mu\text{l}$. Solutions containing $3 \mu\text{g}$ were routinely used for membrane-labeling studies although labeling of the PL-glass (background) was high.

Washing with NaCl (Fig. 4*b–e*) selectively removes CF from a fraction (about 10%) of the membranes, leaving the rest labeled. Selectivity is especially obvious between 0.5 M NaCl (Fig. 4*c*) and 1.0 M NaCl (Fig. 4*d*). Similar nonrandom labeling patterns are seen after washes with 1 M NaCl in 0.1 or 0.01 M phosphate buffer for 30 s, 1.5, or 60 min. At 4 M NaCl, CF is effectively removed from all surfaces, membrane and glass (Fig. 4*e*). Many CF molecules bind to PL-glass ($\pm \text{PM}$) above pH 4.5; below 4.5, although binding to membrane is reduced, adsorption to PL-glass is reduced even more. Thus, CF was routinely applied at pH 4.5 to increase the ratio of

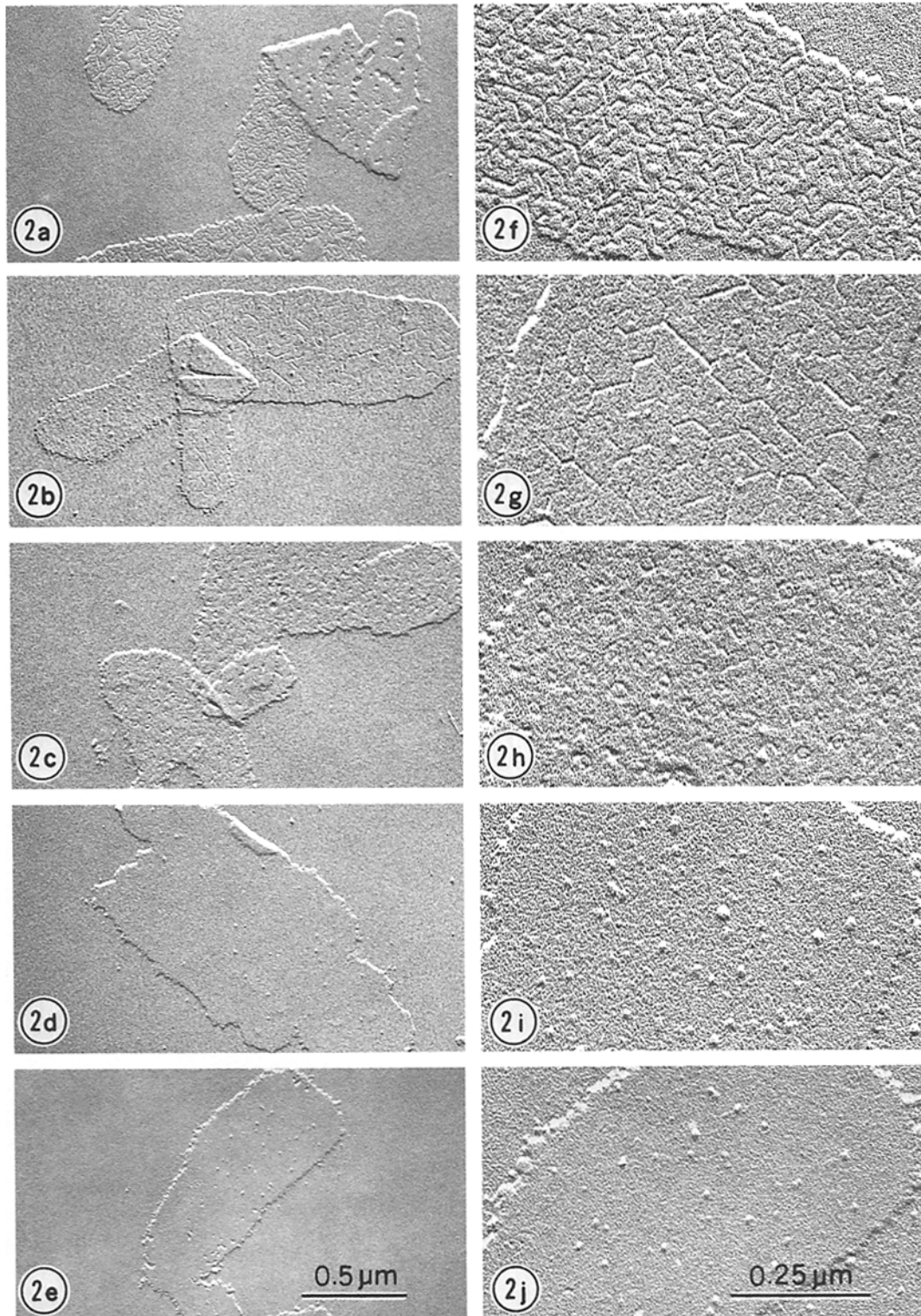


FIGURE 2 Pt-C-shadowed *H. halobium* PM adsorbed to polylysine glass. One membrane preparation used for five drying procedures. (a-e) Low magnification. $\times 31,500$. (f-j) High magnification detail of dominant surface. $\times 83,200$. (a and f) Slowly air-dried. Note folded membrane showing both pitted and cracked surfaces. (b and g) Dried over P_2O_5 . Folded membrane shows rough and cracked surfaces with angles of 60° and 120° . (c and h) Dried at $40^\circ C$. Note ring-shaped depressions. (d and i) N_2 -gun dried. No obvious surface structure. Particles and contamination on surfaces are only occasionally seen. (e and j) Freeze-dried and shadowed at low temperature. All surfaces smooth.

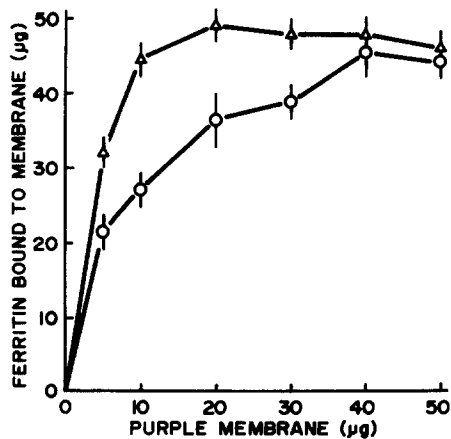


FIGURE 3 Adsorption of CF to PM in solution. Lower curve: PM >3 wk old stored in basal salts. Upper curve: PM <2 wk old stored in distilled water or dilute phosphate buffer, the type used for all subsequent experiments. Error bars represent SD of a minimum of three experiments per point.

membrane to background binding. Even higher ratios can be obtained by 10-fold dilutions of CF.

MONOLAYER FREEZE-FRACTURE: Monolayers of polylysine-bound PM when fractured show membrane areas that appear rough or particulate, and areas that appear smooth (Fig. 5), yet bordered with particles (Fig. 5*a*). Particles outlining the edges of the PM probably represent red membrane contamination (24). Their occurrence correlates with the presence of spectroscopically detectable red membrane carotenoids. The smooth regions at higher resolution show regularly spaced fine striations, but otherwise have no distinctive features. They must be quite thin because they cast no obvious shadows.

To show that the smooth regions in fact represent the exterior "half" of the membrane or E face (2), complementary to the particulate region or P face, optical diffraction patterns were collected from adjacent rough and smooth membrane fracture faces (Fig. 5*a-c*). Whereas replicas

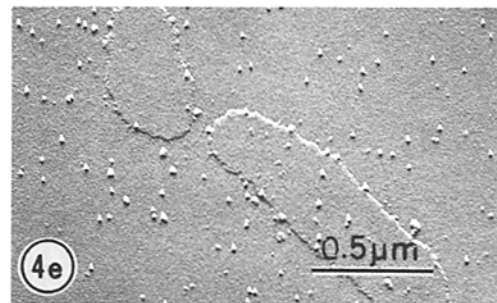


FIGURE 4 Shadowed CF-labeled, polylysine-bound PM: effect of NaCl washes. (a) Distilled water wash, 30 s, 20°C, N₂-gun dry. (b) 0.1 M NaCl, 30 s, distilled water, 10 s. (c) 0.5 M NaCl. (d) 1.0 M NaCl. (e) 4.0 M NaCl. Folded membrane in Fig. 4*a* shows heavily labeled CF on both surfaces. At 0.5 M NaCl (c) and 1.0 M NaCl (d) most membrane surfaces are heavily labeled; about 10% are sparsely labeled (PS). Background labeling is high but could be reduced by using 10-fold dilutions of CF. X 31,500.

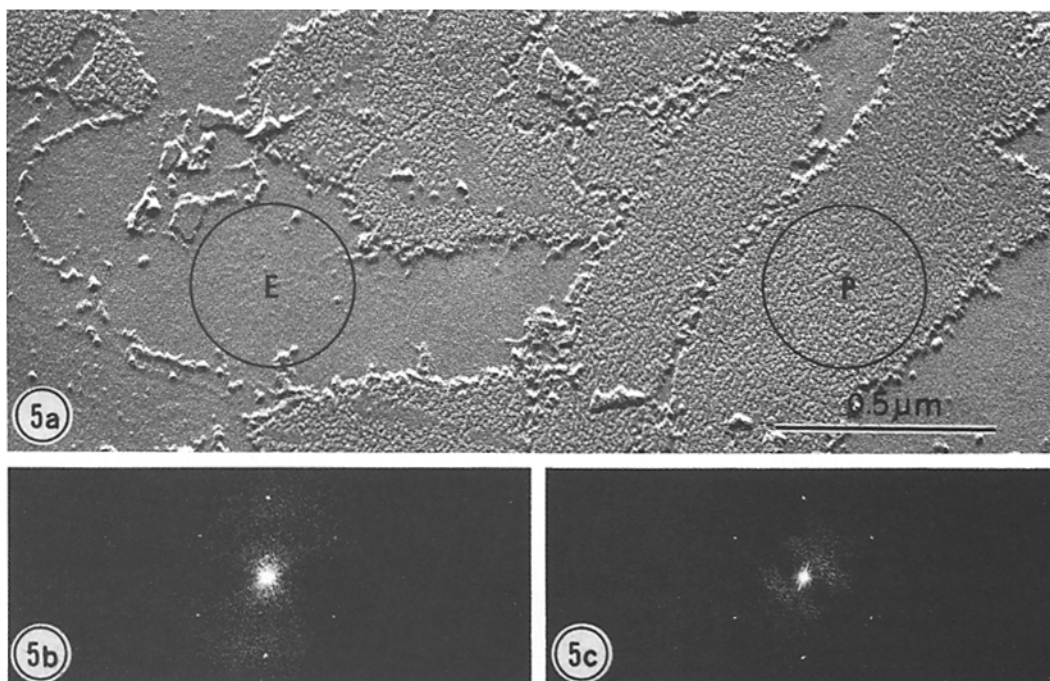


FIGURE 5 Monolayer freeze-fracture of PM bound to polylysine glass; optical diffraction patterns of adjacent complementary fracture faces show identical spacings. (a) E faces left and lower right corner; P faces right. $\times 59,000$. Circles delimit areas illuminated by laser during optical diffraction: E pattern shown in Fig. 5b; P in Fig. 5c. Shadow direction bottom to top in Fig. 5b; 8 o'clock to 2 o'clock in Fig. 5c.

of PL-glass surface produced only diffuse diffraction, the smooth areas produced first-order diffraction patterns (Fig. 5b) of a hexagonal lattice with 61–62 Å center-to-center spacings. The same lattice is obvious in diffraction from the particulate areas (Fig. 5c). Unfractured membranes have the same appearance as those shown in the freeze-dried preparations (Fig. 2e and j).

Further confirmation of membrane splitting and correlation of CF surface labeling with the two membrane “halves” was obtained by first labeling and then freeze-fracturing PM monolayers (Fig. 6). The CF-labeled preparation was washed with 1 M NaCl as described above to render labeling selective. P faces of fractured membranes are contiguous to highly labeled surfaces (Fig. 6a and c) whereas E faces, infrequently encountered, are contiguous to minimally labeled surfaces (Fig. 6a and b). Ferritin-labeled membranes provided a convenient marker system to evaluate the percentage of split membranes. In one experiment, 8,255 μm^2 of randomly selected membrane surfaces were measured (membrane only, free glass areas not included): 2,525 μm^2 were split (30.6%) and

5,730 μm^2 unsplit (69.4%); in a second experiment, of 361 μm^2 sampled, 209 μm^2 were split (58%) and 152 μm^2 unsplit (42%).

Comparison of Quantitative Data

Morphometric analysis of PM bound to PL-glass and processed in four different ways is summarized in Fig. 7. An obvious numerical correspondence exists among those membranes that appear cracked (air-dried), rough (nitrogen-dried), CF-labeled, and the amount of P-fracture face. They comprise 80–95% of all membranes present. A similar relationship exists among membranes that are pitted (air-dried), smooth (nitrogen-dried), unlabeled, and E-fracture faces, i.e., they account for the complementary 20–5%. Because the particulate fracture face of the membrane is known to be closest to the cytoplasm in intact cells before isolation (1), the cytoplasmic surface is thus preferentially adsorbed to the PL-glass. This assumes, of course, that the fracture plane through isolated and PL-bound membrane is the same as that through the plasma membrane of whole cells.

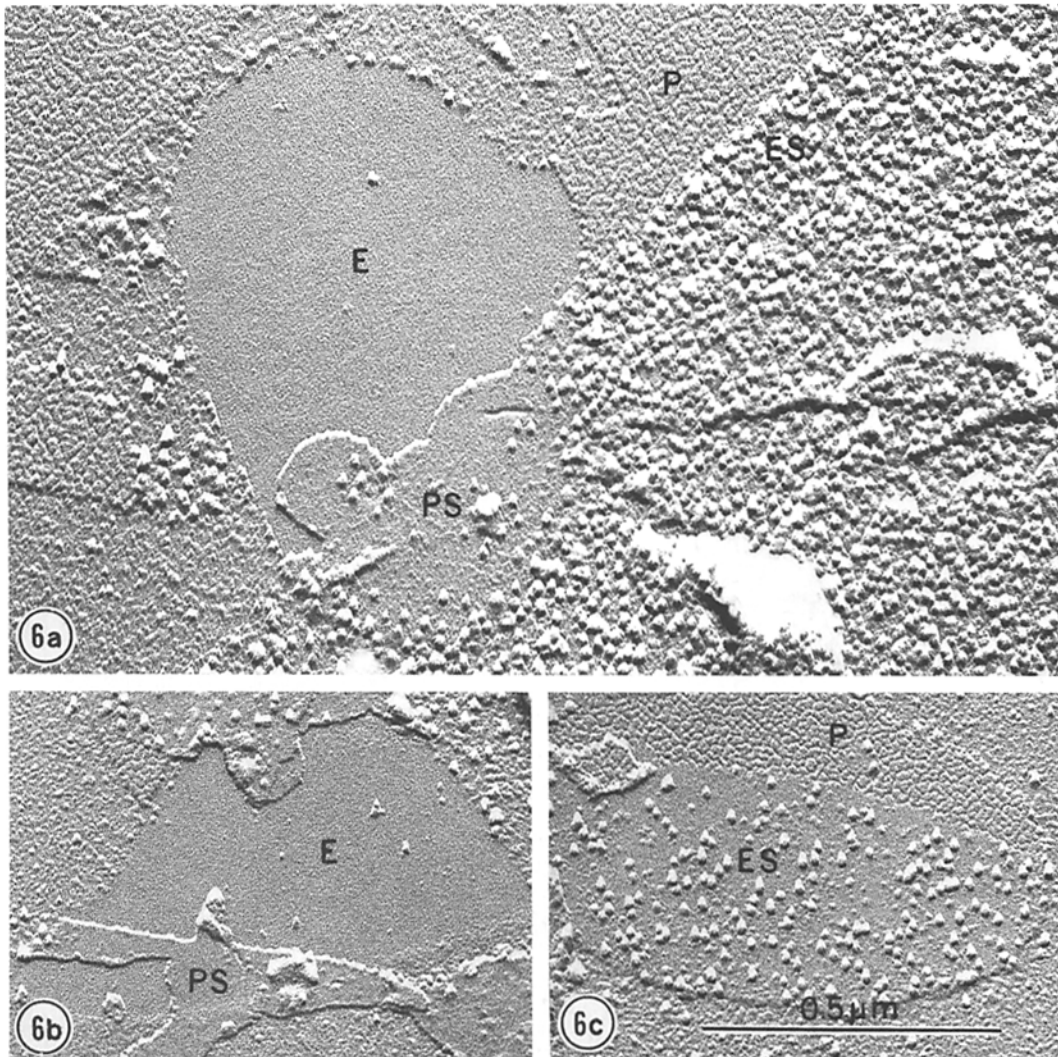


FIGURE 6 Monolayer freeze-fracture of CF-labeled PM. Regions between unfractured (lower right) and fractured portions (upper left). P surfaces, contiguous to smooth E fracture faces, are minimally labeled (*a* and *b*) whereas E surfaces, contiguous to particulate P-fracture faces, are heavily labeled (*a* and *c*). All $\times 78,800$.

DISCUSSION

Our quantitative electron microscope examination of PM applied to PL-glass reveals a nonrandom distribution of exposed extracellular surfaces. Surface structure is quite variable and depends especially upon the duration and temperature of drying. Structural variation occurs not only among different drying methods but within a single method. Nevertheless, the variety of structure tended to fall into two classes, either cracked or pitted. Quantitative evaluation revealed an 80–

90% enrichment for the cracked category. Because of the variation in surface morphology, however, we sought an additional means to verify the nonhomogeneous distribution of membrane surfaces.

When CF is applied at pH 4.5 to PM on PL-glass, it binds to all membrane surfaces, suggesting that both the cytoplasmic and extracellular sides of the membrane have accessible negative sites. The removal of CF by NaCl washes confirms electrostatic interaction and provides a surface-

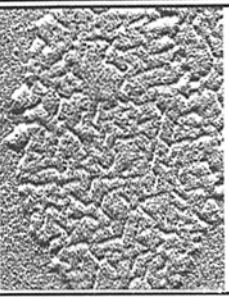


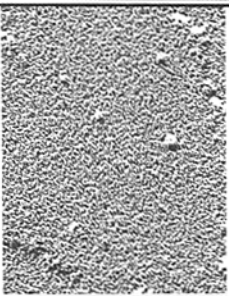
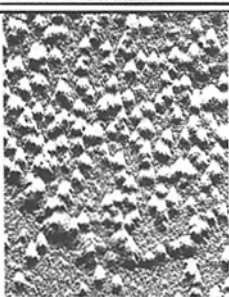
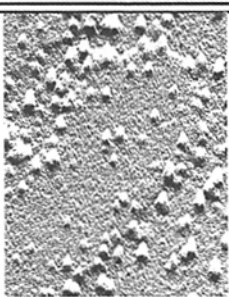
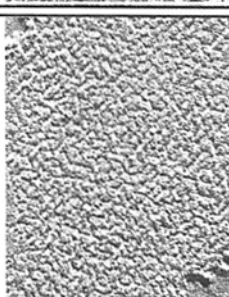
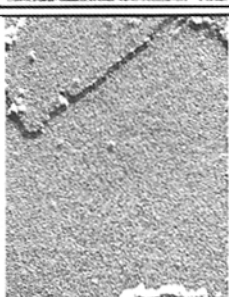
AIR DRY 11 % COVERAGE (4333 μm^2) MEASURED		CRACKED 80 % (367 μm^2)		PITTED 20 % (93 μm^2)
N ₂ DRY 6 % (145 μm^2)		ROUGH 95 % (8 μm^2)		SMOOTH 5 % (0.4 μm^2)
CATIONIC FERRITIN 11 % (4364 μm^2)		LABELED 89 % (422 μm^2)		UNLABELED 11 % (52 μm^2)
FREEZE ETCH 51 % SPLIT (90 μm^2)		P FACE 94 % (43 μm^2)		E FACE 6 % (3 μm^2)

FIGURE 7 Summary of morphometric analysis of dried, labeled, and freeze-fractured membrane. Each preparation could be divided into two categories. An example of each category and the percentage of total membrane area are given. Coverage or total replica area measured (membrane plus glass), in parentheses. Note percentage correlations among the categories "cracked," "rough," "labeled," and "P face."

specific marker for sidedness quantification. Labeling with CF provides a second line of evidence for the nonrandom orientation of the membrane, and combined with freeze-fracture electron microscopy, identifies the free and bound surfaces.

Freeze-fracturing was used to identify PM orientation. From freeze-fracture of whole cells (1),

it is known that PM particles partition with the cytoplasmic side. It is also known that both erythrocyte membranes (7, 8) and PM (9, 10) bound to PL-glass will fracture. Fracturing of PM labeled with CF shows that a significant portion of the membrane is split, that the cytoplasmic, particulate side accounts for 90% of all membranes, and

that the extracellular surfaces are heavily labeled with CF. The plane of fracture appears asymmetric with the extracellular portion being much thinner than the cytoplasmic portion.

Adsorption of PM to PL-glass is apparently due to electrostatic interaction. Because the cytoplasmic surface of the membrane preferentially binds to PL-glass at pH 7.4, one might expect that it is more electronegative than the extracellular surface. Whether or not this is due to fixed anionic sites, their number, accessibility, or distribution, or to the functioning photoreaction cycle and membrane surface potential (26) at steady state, or to permanent or induced dipoles, is not known. However, while this report was in press, we investigated the effect of pH and light-dark adaptation on membrane adsorption. At pH 3, the extracellular surface adsorbs to an enrichment of 95% or more (11), suggesting a dominant role of fixed charges (with asymmetric, surface-specific, and different pKs). Details will be presented elsewhere. We should also mention that control preparations where cleaned glass is not treated with polylysine show no adsorbed membranes at pH 7.4.

If electrostatic charge is responsible for the preferential adsorption of the cytoplasmic surface of PM to PL-glass, why does CF selectively label the extracellular surface? The difference must be due to the type of probe, CF or PL-glass, and/or to the properties of the membrane surface after adsorption to PL-glass. The epsilon amino residues of polylysine adsorbed to glass are probably more spatially constrained (21) than the free primary amino groups of the six carbon diamine covalently bound to ferritin, especially since the polylysine is of very low molecular weight. The surface charge of PM may be altered by binding to PL-glass and/or the negative sites on the extracellular surface may be sterically more accessible to CF or more favorably distributed laterally (e.g., clustered?) than the negative sites on the cytoplasmic side. Obviously, further experimentation is necessary to provide a definitive molecular interpretation of our data. Nevertheless, our interim goal, to orient and quantify bound membrane, has been satisfied by the experiments described here.

Large areas of oriented membrane can be produced simply, by adsorption to polylysine glass. The membrane fragments orient with respect to the plane of the membrane (parallel to the plane of the glass) and to the membrane surface that is exposed. Such preparations can be used to deter-

mine the side-specific location of reactive groups (4) by conventional protein modification reactions, or can be used in surface labeling (scanning electron microscopy, fluorescence light microscopy, etc.), spectroscopic studies (microspectrophotometry, x-ray photoelectron spectroscopy, etc.), or photoelectron microscopy.

Whereas freeze-fracture of PM suspensions does not produce large areas of fractured membrane, such areas are produced in monolayer freeze-fracture and are suitable for quantitative analysis. They are additionally useful for the comparison of complementary portions of membrane by optical diffraction. Since all fracture faces are similarly oriented, the diffraction patterns can easily be compared. Chemical analysis of the fractured halves (8) has not yet been attempted. A variable, unpredictable fraction of the total membranes fracture, thus requiring special methods to measure the amount of splitting at a preparative level. Such methods are currently being developed.

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REFERENCES

1. BLAUROCK, A., and W. STOECKENIUS. 1971. Structure of the purple membrane. *Nat. New Biol.* **233**:152-155.
2. BRANTON, D., S. BULLIVANT, N. B. GILULA, M. J. KARNOVSKY, H. MOOR, K. MÜHLEHALER, 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. D. C.)*. **190**:54-56.
3. BRIDGEN, J., and I. D. WALKER. 1976. Photoreceptor protein from the purple membrane of *Halobacterium halobium*. Molecular weight and retinal binding site. *Biochemistry*. **15**:792-798.
4. CARRAWAY, K. L. 1975. Covalent labeling of membranes. *Biochim. Biophys. Acta*. **415**:379-410.
5. DANON, D., L. GOLDSTEIN, Y. MARIKOVSKY, and E. SKUTELSKY. 1972. Use of cationized ferritin as a label of negative charges on cell surfaces. *J. Ultrastructure Res.* **38**:500-510.
6. 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.
7. FISHER, K. A. 1975. "Half" membrane enrichment: verification by electron microscopy. *Science (Wash. D. C.)*. **190**:983-985.
 8. FISHER, K. A. 1976. Analysis of membrane halves: cholesterol. *Proc. Natl. Acad. Sci. U. S. A.* **73**: 173-177.
 9. FISHER, K. A. 1977. Protein content of a freeze-fractured membrane particle. *J. Electron Microsc.* **26**:171.
 10. FISHER, K. A., and W. STOECKENIUS. 1977. Freeze-fractured purple membrane particles: protein content. *Science (Wash. D. C.)*. **197**:72-74.
 11. FISHER, K. A., K. YANAGIMOTO, and W. STOECKENIUS. 1977. Purple membrane bound to polylysine glass: effects of pH and light. *J. Cell Biol.* **75**(2, Pt. 2):220a (Abstr.).
 12. HENDERSON, R., and P. N. T. UNWIN. 1975. Three-dimensional model of purple membrane obtained by electron microscopy. *Nature (Lond.)*. **257**:28-32.
 13. HOARE, D. G., and D. E. KOSHLAND, JR. 1967. A method for the quantitative modification and estimation of carboxylic acid groups in proteins. *J. Biol. Chem.* **242**:2447-2454.
 14. HWANG, S.-B., and W. STOECKENIUS. 1977. Purple membrane vesicles: morphology and proton translocation. *J. Membr. Biol.* **33**:325-350.
 15. KUSHWAHA, S., M. KATES, and W. G. MARTIN. 1975. Characterization and composition of the purple and red membrane from *Halobacterium cutirubrum*. *Can. J. Biochem.* **53**:284-292.
 16. KUSHWAHA, S., M. KATES, and W. STOECKENIUS. 1976. Comparison of purple membrane from *Halobacterium cutirubrum* and *Halobacterium halobium*. *Biochim. Biophys. Acta.* **426**:703-710.
 17. LOZIER, R., R. A. BOGOMOLNI, and W. STOECKENIUS. 1975. Bacteriorhodopsin: a light-driven proton pump in *Halobacterium halobium*. *Biophys. J.* **15**:955-962.
 18. LOZIER, R. H., W. NIEDERBERGER, R. A. BOGOMOLNI, S.-B. HWANG, and W. STOECKENIUS. 1976. Kinetics and stoichiometry of light-induced proton release and uptake from purple membrane fragments, *Halobacterium halobium* cell envelopes, and phospholipid vesicles containing oriented purple membrane. *Biochim. Biophys. Acta.* **440**:545-556.
 19. MAZIA, D., G. SCHATTEN, and W. SALE. 1975. Adhesion of cells to surfaces coated with polylysine. Applications to electron microscopy. *J. Cell Biol.* **66**:198-200.
 20. MITCHELL, P. 1961. Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. *Nature (Lond.)*. **191**:144-148.
 21. NEVO, A., A. DE VRIES, and A. KATCHALSKY. 1955. Interaction of basic polyamino acids with the red blood cell. I. Combination of polylysine with single cells. *Biochim. Biophys. Acta.* **17**:536-547.
 22. OESTERHELT, D., and W. STOECKENIUS. 1971. Rhodopsin-like protein from the purple membrane of *Halobacterium halobium*. *Nat. New Biol.* **233**:149-152.
 23. OESTERHELT, D., and W. STOECKENIUS. 1973. Functions of a new photoreceptor membrane. *Proc. Natl. Acad. Sci. U. S. A.* **70**:2853-2857.
 24. OESTERHELT, D., and W. STOECKENIUS. 1974. Isolation of the cell membrane of *Halobacterium halobium* and its fractionation into red and purple membrane. *Methods Enzymol.* **31**:667-678.
 25. RACKER, E., and W. STOECKENIUS. 1974. Reconstitution of purple membrane vesicles catalyzing light-driven proton uptake and adenosine triphosphate formation. *J. Biol. Chem.* **249**:662-663.
 26. RENTHAL, R., and J. K. LANYI. 1976. Light-induced membrane potential and pH gradient in *Halobacterium halobium* envelope vesicles. *Biochemistry.* **15**:2136-2143.
 27. STOECKENIUS, W., and W. H. KUNAU. 1968. Further characterization of particulate fractions from lysed cell envelopes of *Halobacterium halobium* and isolation of gas vacuole membranes. *J. Cell Biol.* **38**:337-357.
 28. UNWIN, P. N. T., and R. HENDERSON. 1975. Molecular structure determination by electron microscopy of unstained crystalline specimens. *J. Mol. Biol.* **94**:425-440.