Membrane Assembly in Retinal Photoreceptors

II. Immunocytochemical Analysis of Freeze-fractured Rod Photoreceptor Membranes Using Anti-opsin Antibodies¹

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Abstract

We have used a cytochemical technique for labeling freeze-fractured tissues (Pinto da Silva, P., C. Parkison, and N. Dwyer (1981) *Proc. Natl. Acad. Sci. U. S. A.* 78: 343-347) to examine the distribution of immunoreactive opsin in rod photoreceptor membranes. Aldehyde-fixed retinas of African clawed frogs (*Xenopus laevis*) embedded in a cross-linked protein matrix were frozen and fractured at -196°C , then thawed and labeled with biotinylated sheep anti-cow opsin IgG followed by avidin-ferritin. In thin sections of plastic-embedded retinas, rod outer segment (ROS) disc membranes exposed by fracturing bound specific antibody intensely and relatively uniformly. However, they differed from membranes of the inner segment as well as those of erythrocytes in that protoplasmic face leaflets did not assume an interrupted bilayer appearance and disc exoplasmic face leaflets were apparently lost during thawing. The disposition of opsin immunoreactivity in the cell membrane was highly asymmetric. Although ROS plasma membranes from which discs are elaborated labeled heavily with anti-opsin after cleavage, fractures passing along inner segment plasma membranes bound very little antibody. In cross-fractures exposing inner segment cytoplasm, we found specific labeling of Golgi complex elements, as well as both perimitochondrial and periciliary vesicles. The latter are presumed to be the vehicle shuttling newly synthesized membrane to the ROS for disc assembly. These results suggest that opsin-containing membrane is sorted out within the cell, being transported from synthetic sites to the immediate periciliary zone where localized insertion into the cell membrane takes place. Furthermore, the close correspondence of the present immunocytochemical analysis with the distribution of opsin deduced from prior quantitative freeze-fracture analysis (Besharse, J. C., and K. H. Pfenninger (1980) *J. Cell Biol.* 87: 451-463) offers the possibility that fracture-label may be generally

useful for study of patterned membrane topography in neuronal cells.

The mechanisms involved in the post-Golgi transfer of membrane proteins to the plasma membrane and the generation of distinctive surface membrane domains are subjects of current intense interest for neurobiologists. Vertebrate rod photoreceptors are particularly suited for studies of these mechanisms because their function as an extraordinarily sensitive photon-capturing and transducing device (Baylor et al., 1979) depends in large measure on the compartmentalized organization of their membrane components. For example, the visual pigment apoprotein (opsin) is an integral membrane protein highly localized to the rod outer segment (ROS). The ROS consists of an array of membranous discs derived from and enclosed within the plasma membrane. This organelle is separated from the cell's inner segment by a short, non-motile connecting cilium. Most of the cellular organelles are restricted to the inner segment and, thus, biosynthetic activity is the exclusive function of this compartment.

After the initial postnatal development of outer segments, elaboration of discs continues throughout life (Young, 1967). Largely from radiotracer experiments, it is now well established that newly synthesized opsin first appears in the inner segment myoid in association

Figure 1. Thick ($0.5\ \mu\text{m}$) section of an albumin-retina gel fragment after freeze-fracture and thawing. Part of the fractured edge of the gel is indicated by the arrow, and a large fissure is seen traversing the photoreceptor cell layer (asterisk). OS, outer segment layer; IS, inner segment layer; NL, nuclei of photoreceptors. Magnification $\times 520$. Bar = $2\ \mu\text{m}$.

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Figure 2. Electron micrographs of freeze-fractured native ROS discs. A conventionally prepared specimen fractured at -110°C and platinum-carbon replicated *in vacuo* is shown in A. The alternating pattern of particulate PFs and relatively particle-free EFs is characteristic of such preparations. B is from a retina fractured at -196°C and subsequently thawed to room temperature prior to plastic embedding and thin sectioning. Exposed discs appear as uninterrupted profiles disposed in a step-like arrangement and are not distinguishable morphologically from contiguous unfractured membrane. This specimen, unlike others in this study, was initially fixed in 2.5% glutaraldehyde-2.0% paraformaldehyde. The micrographs in C to E represent material fractured at liquid nitrogen temperature but then freeze-substituted in osmium-acetone. PF leaflets (PF) are now distinguishable as unilaminar profiles (C and D). The absence of definable EF leaflets is indicated by the exposure of trilaminar bilayers (D and E) which present the intradiscal membrane surface (ES). Magnification: A, $\times 100,000$; B, $\times 188,000$; C, $\times 188,000$. Bars, $0.1\ \mu\text{m}$.

with rough endoplasmic reticulum (RER) and migrates through the Golgi apparatus enroute to its eventual appearance in forming discs of the ROS (Young and Droz, 1968; Hall et al., 1969; see Young, 1976, for a review of early work). More problematic to explain are the latter stages of this process. Specifically, questions of where and in what form disc precursors are added to the cell membrane and how they find their way into the outer segment must be

addressed. Because of the limited resolution offered by autoradiographic techniques, immunocytochemical investigations at the electron microscopic level utilizing albumin and Lowicryl-embedded retinal sections treated with anti-opsin antibodies have been carried out. Such studies have revealed discrete labeling in areas of the inner segment corresponding to the ellipsoid periciliary region, as well as myoid RER and the Golgi apparatus (Papermaster et al.,

Figure 3. Thin section views of ROS discs after fracturing *in situ* followed by treatment with biotinylated sheep antibodies and labeling with AvF. AvF binds to disc membranes treated with biotinylated sheep anti-cow opsin IgG (A and B) but not to those treated with biotinylated sheep preimmune IgG (C). In A, the plane of fracture is very shallow, whereas a steeper angle is evident in B. Discs labeled with specific antibody in general display uniform ferritin binding, although gaps in the labeling pattern are occasionally apparent (B, arrows). Magnification $\times 172,000$. Bar, $0.1 \mu\text{m}$.

1978, 1979, 1985; Papermaster and Schneider, 1982). Furthermore, intramembranous particles (IMPs) similar to those of photoreceptor outer segments are found in freeze-fractured cytomembranes including periciliary vesicles (Besharse and Pfenninger, 1980). These findings are consistent with observations that opsin is co-translationally inserted into microsomal membrane (Goldman and Blobel, 1981) and is at all times during its transit within the cell associated with readily sedimentable particles (Papermaster et al., 1975).

Such observations have contributed to a model of disc assembly (Besharse and Pfenninger, 1980) in which ROS membrane precur-

sors in the form of periciliary vesicles fuse with the inner segment plasma membrane in the area immediately surrounding the cilium and, as individual components or patches of membrane, move via the ciliary plasmalemma into basal ROS plasma membrane evaginations, where disc morphogenesis takes place. To evaluate this model critically we have utilized the fracture-label technique pioneered by Pinto da Silva et al. (1981a, b) to localize cellular opsin determinants using specific antibody as probe. The main advantages offered by this method include ready access to cytoplasmic membranes and the fact that high resolution, positive contrast images of

Figure 4. Diagram depicting the mode of cleavage of native disc membranes upon freeze-fracturing at low temperature. Invariably, two fractures are produced per disc, one of which is an EF and one a PF.

Figure 5. Diagram summarizing the types of fractures expected to be produced in albumin gels of isolated discs as a result of crushing at low temperature. ES, exoplasmic surface.

cytochemically labeled tissues can be obtained. This combination of factors has been previously unavailable in any single technique. The results provide new data consistent with the involvement of periciliary vesicles in disc assembly and suggest ways in which cellular polarity may be maintained. This study also demonstrates the usefulness of fracture-label for immunocytochemical analysis.

Materials and Methods

Animals and tissues

Postmetamorphic *Xenopus laevis* (3.5 to 5.0 cm body length) obtained from Nasco, Inc. (Fort Atkinson, WI) were maintained at 24 to 26°C under a lighting regimen of 12 hr light:12 hr dark and were fed commercial frog pellets (Nasco, Inc.) three times weekly. Adult Fisher rats were kept on an identical lighting cycle and were fed commercial rat chow *ad libitum*. Animals were placed in the dark overnight prior to an experiment. Under dim red light (Kodak Wratten no. 2 filter), they were then decapitated and their eyes were enucleated. After removal of the cornea, iris, and lens, retinas were dissected free of sclera and pigmented layers.

Preparation of freeze-fractured tissues

Retinas. The methodology was essentially that described by Pinto da Silva et al. (1981a, b) with minor modifications. Retinas, obtained from dark-adapted African clawed frogs, *Xenopus laevis*, were fixed in 1.25% (v/v) glutaraldehyde in 0.1 M phosphate or cacodylate buffer (pH 7.4) for 2 hr at room temperature. For certain morphological studies, a fixative containing 2.5% glutaraldehyde and 2.0% paraformaldehyde was also used. After rinsing in the same buffer, tissues were embedded in 30% bovine serum albumin which was then gelled by addition of glutaraldehyde (1% final concentration). Because of their small size (2 to 3 mm in diameter), individual retinas were not sliced into segments but were simply trimmed of excess albumin gel. They were infiltrated gradually with 30% (v/v) glycerol in phosphate-buffered saline (PBS; 0.01 M phosphate buffer containing 0.14 M NaCl at pH 7.4) and frozen on aluminum foil supports in melting Freon 22. Frozen retinas, after transfer to and submersion in liquid nitrogen, were pulverized with a glass rod to produce multiple fractures and then thawed into 1% glutaraldehyde in 30% glycerol-PBS.

Isolated discs. Topologically closed ROS discs were prepared from rat retinas using the Ficoll density gradient procedure outlined by Smith and Litman (1982). In this method damaged discs and contaminating membranes pellet to the bottom of the tube while osmotically active discs float and are recovered in pure form. Intact, osmotically active discs obtained in this way were fixed in suspension in 1% glutaraldehyde in H₂O for 30 min. Ammonium chloride (0.1 M) was added to quench excess aldehyde groups, and the fixed discs were rinsed three times in distilled H₂O by centrifugation. The final pellet, along with an aliquot of fixed red blood cells (see below), was mixed with 30% bovine serum albumin and gelled as described above. Unlike retinas, these disc gels were sliced into small pieces (1 × 2 × 2 mm) prior to glycerination and freeze-fracturing. The inclusion of erythrocytes in the albumin matrix aided in distinguishing cut surfaces from low-temperature fractures (Pinto da Silva et al., 1981a).

Red blood cells. Blood (O positive) was drawn from healthy human donors into heparinized containers, and erythrocytes were isolated by centrifugation (three times at 6300 × g, 4°C), followed each time by resuspension in 310 mOsm of phosphate buffer (pH 7.4). Cells were fixed in 1% glutaraldehyde in the same buffer (4 hr at 4°C), rinsed several times, and, together with isolated discs, embedded in cross-linked albumin.

Immunocytochemical labeling and processing for electron microscopy

Freeze-fractured gel and tissue fragments, after thawing and deglycerination, were rinsed successively in 0.1 M NH₄Cl in PBS, PBS alone, and finally 0.1 M Tris-HCl (pH 7.4) with 1% bovine serum albumin added (Tris-albumin). They were then labeled for 90 min at room temperature with sheep anti-cow opsin IgG (anti-opsin), which was affinity purified and biotinylated (Papermaster et al., 1978), and dissolved at a concentration of 30 µg/ml in Tris-albumin. After extensive rinsing with albumin-containing buffer, specimens were stained for 60 min with avidin-ferritin (AvF) (33 or 66 µg/ml in 0.15 M NaCl-1% bovine serum albumin) (Heitzmann and Richards, 1974), then exhaustively washed with NaCl-albumin. Control treatments consisted of labeling with biotinylated sheep preimmune IgG (30 µg/ml in Tris-albumin) plus AvF, or with AvF alone.

The biotinylated antibodies were a generous gift of Dr. David S. Papermaster (Veteran's Administration Hospital, West Haven, CT). Specificity for opsin was confirmed by using immunoadsorption on nitrocellulose replicas of SDS-polyacrylamide gels. Gel-fractionated proteins from either whole retinas or isolated ROS (*Xenopus* and rat) were electrophoretically transferred to nitrocellulose (essentially according to the method of Burnette, 1981), and bound antibody was detected using a conjugate of avidin and horseradish peroxidase (Sigma Chemical Co., St. Louis, MO). Labeled bands were then visualized using the diaminobenzidine and H₂O₂ incubation procedure de-

Figure 6. Gallery of thin sections of fracture-labeled isolated discs. Concave profiles, regardless of the angle of entry of the fracture plane, resemble native membranes morphologically and bind AvF to similar extents. Convex profiles, a criterion for EF leaflets, were never observed in these preparations. Note that shallow fractures (**A** and **B**) presumably exposed PF leaflets, whereas the more nearly spherical profiles (**C** and **D**) probably represent cross-fractured membranes. Also, albumin gel is virtually unlabeled in this and other preparations in this study. Magnification $\times 165,000$. Bar, $0.1 \mu\text{m}$.

scribed by Wood and Sarinara (1975). The only protein staining specifically in any of the immunoreplicas was opsin (37,000 daltons) and its dimer, trimer, and tetramer.

Labeled tissue fragments were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4; 1 hr at room temperature), rinsed in buffer, and postfixed with 1% osmium tetroxide for 1 hr at room temperature. Most specimens were dehydrated in ethanol and embedded in Araldite 502 or Spurr's medium (Luft, 1961; Spurr, 1969). Occasionally, osmicated tissues were stained *en bloc* with 0.5% aqueous uranyl acetate for 90 min at room temperature before dehydration. Also, some frozen retinal specimens were freeze-substituted in a solution of 3% osmium tetroxide in acetone (overnight in liquid nitrogen, 3 hr at -70°C , 3 hr at -20°C , 2 hr on ice) and washed in 100% acetone at room temperature prior to embedding. Thick sections ($0.5 \mu\text{m}$) were examined by light microscopy and areas of interest were selected for thin sectioning. Silver-gold sections were stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963) and were examined at 80 kV in a Philips 400 electron microscope equipped with goniometer stage and rotating specimen holder.

Conventional freeze-fracture

To evaluate the sidedness of isolated ROS disc membranes as well as their fracturing behavior, samples of albumin gels were mounted on nickel-gold alloy specimen holders and frozen by rapid immersion in Freon 22. Frozen gels were then fractured at -115°C under a vacuum of $<1 \times 10^{-6}$ torr and etched for 30 sec before evaporation of platinum-carbon from an electron

beam gun. Replicas, reinforced with carbon, were digested in Chlorox, rinsed in distilled H_2O , and mounted on Formvar-coated grids prior to examination in the electron microscope. Additional intact *Xenopus* retinas were prepared as described previously (Besharse and Pfenninger, 1980).

Results

Characterization of freeze-fractured retinas

Light microscopy. Light microscope examination of azure II-stained thick sections allowed the preliminary localization of those retinal fractures passing through photoreceptors. Because retinas were not cut, but merely trimmed of excess albumin prior to freeze-fracture, it was not necessary to distinguish between cut and fractured interfaces as was true in previous fracture-label studies (see Pinto da Silva et al., 1981a, for a thorough discussion). Such interfaces in our material always represented fracture planes. Figure 1 shows an example of a retina prepared in this way. Abundant expanses of cleaved tissue were exposed on the surface of most retinal fragments. Occasionally, large fissures or finer cracks were seen which, because of their incomplete penetration into the tissue, offered complementary views of the fracture.

Electron microscopy. Inspection of fracture-labeled retinas in the electron microscope revealed a degree of morphological preserva-

Figure 7. Anti-opsin labeling of freeze-fractured ROS plasma membranes. In A, the original plane of cleavage can be seen where it passes successively across albumin gel (BSA), enters the plasmalemma (between arrows), and subsequently cross-fractures through disc membranes (large arrow). The fractured plasma membrane, presumably a PF leaflet, presents an uninterrupted profile similar to discs and binds anti-opsin avidly. The micrograph in B demonstrates the corresponding EF leaflet of a similar but distinct cell membrane. Unlike the protoplasmic face, this consists of short segments of unit bilayer structure (arrowheads) which are virtually unlabeled. Magnification $\times 129,000$. Bar, $0.1 \mu\text{m}$.

tion of cellular structure comparable to that of conventionally processed tissue. For example, ROS discs exposed by fracturing maintained their lamellar configuration, with little evidence of swelling or blebbing (Fig. 2B). Similarly, inner segment organelles displayed their characteristic structures and relationships (see Figs. 9 to 11). When the plane of cleavage passed obliquely through the outer segment, fracturing produced a series of step-like membrane profiles which resembled superficially that found in conventional freeze-fracture replicas (Fig. 2A). Characteristically, in the latter, highly particulate protoplasmic face (PF) leaflets alternated with relatively particle-free exoplasmic face (EF) leaflets (Fig. 2A). These fracture faces are thought to result from membrane bilayer splitting and unequal partitioning of IMPs (Clark and Branton, 1968; Leeson, 1970; Raubach et al., 1974; Corless et al., 1976; Krebs and Kühn, 1977; Andrews and Cohen, 1979; Roof and Heuser, 1982). In our fracture-labeled preparations, ROS discs were in most cases closely apposed, making delineation of individual split leaflets of the bilayer ambiguous (Figs. 2B and 3). Conspicuously absent was the interrupted bilayer appearance reported by Pinto da Silva et al. (1981a, b) and used by them as a criterion for membrane fracturing. Rather, the morphology of exposed membrane profiles was not usually

distinguishable from that of unfractured membrane. Furthermore, in our material we would frequently expect to find two fractures per disc; one which produced a PF leaflet and the other producing an EF leaflet (Fig. 4). This is particularly true when the plane of fracture is shallow relative to the orientation of discs. However, the individual steps in oblique fractures were virtually always interpretable as steps between individual disc membranes rather than between bilayers within a disc, even over a wide range of fracture angles. This pattern is distinctly different from that seen in conventional replicas (Fig. 2A and Corless et al., 1976) and suggests that membrane reorganization occurred during thawing. Although complementary aspects of fine cracks and fissures were examined, we were unable to distinguish PF and EF leaflets from the same fractured membrane.

To examine the question of post-fracture alteration more closely, identical fractured specimens were freeze-substituted in osmium-acetone at low temperature prior to thawing. As seen in Figure 2C, two distinct morphologies of fractured disc membranes result from such treatment. Surface-exposed membranes exhibited either a full-thickness, trilaminar appearance (Fig. 2, D and E) or a unilaminar, half-membrane profile (Fig. 2, C and D). Under these conditions of maximal lipid stabilization, it is likely that the continuous single layer

Figure 8. Freeze-label of rod photoreceptor inner segment plasma membranes. Very little binding of anti-opsin and AvF is present on either the extensive PF leaflet in *A* or the EF leaflet in *B*. Note that both of these fractured membranes assume an interrupted bilayer appearance (interruptions are indicated by arrowheads). The accentuated trilaminar structure of this EF leaflet, as well as that in *B*, is due to *en bloc* uranyl acetate staining. Magnification $\times 140,000$. Bar, $0.1 \mu\text{m}$.

Figure 9. Anti-opsin labeling of freeze-fractured myoid inner segment cytoplasm. Examples are shown of Golgi vesicles (*solid arrows*), whose exoplasmic aspect has been exposed and which label intensely with specific antibody. The *open arrow* in *A* points to a coated vesicle, examples of which we often find associated with Golgi regions and which we use to distinguish such membranes from those of the subellipsoidal smooth endoplasmic reticulum. Magnification $\times 115,000$. Bar, $0.1 \mu\text{m}$.

represents protoplasmic faces that are prevented from undergoing bilayer reconstruction, as proposed by Pinto da Silva et al. (1981a). We believe the tripartite membrane structures to be unfractured bilayers exposed by rearrangement or loss of overlying EF leaflets.

In interpreting these images, we have taken the view that cleavage occurs internal to the membrane, along weak points in the bilayer continuum (Branton, 1966; Pinto da Silva and Branton, 1970; Pinto da Silva et al., 1981a). The alternative view that membrane splitting does not occur (Sjöstrand and Kreman, 1978, 1979) is inconsistent with extensive analysis using conventional freeze-fracture methods (Corless et al., 1976; Roof and Heuser, 1982).

Labeling of fractured ROS discs

In situ. When retinal gels were freeze-fractured and labeled with anti-opsin and AvF, accessible disc membranes were intensely labeled (Fig. 3, A and B). Ferritin particles appeared to be uniformly distributed, although discontinuities in binding were occasionally seen. Furthermore, all labeling was present at surfaces; no evidence was found for penetration of gels or tissues by the combination of reagents (e.g., Fig. 8A). Interaction of antibody with tissues was specific since very little ferritin binding was found in controls exposed to biotinylated sheep preimmune IgG plus AvF (Fig. 3C) or with AvF

alone (data not shown). Background labeling was also very low over adjacent areas of albumin gel.

Isolated discs. Fracture-label was also performed on gels of isolated discs. One advantage of this preparation is that individual disc membranes can be viewed without interference from adjacent membranes. Furthermore, since the discs are osmotically swollen to a roughly spherical shape, there is the opportunity to make an unequivocal identification of the fracture leaflet. When samples of this preparation were fractured by conventional means and replicated at low temperature, the fracture images revealed IMP-rich PF leaflets and IMP-poor EF leaflets, as well as cross-fractures (data not shown). These observations indicate that embedding in an albumin gel does not affect fracture behavior and that the sidedness of isolated discs is similar to that of discs *in situ* (Fig. 2A).

Figure 5 illustrates schematically these types of fractures and their expected appearance in thin section. Concave membrane profiles could, in theory, be the result of either bilayer fractures producing PF leaflets or cross-fractures in which the exoplasmic (or intradiscal) surface of the membrane would be exposed. Convex profiles protruding above the level of the fractured albumin gel should, however, be characteristic of EF leaflets. One would expect most planes of fracture entering a vesicle below its equator to produce PF leaflets

Figure 10. Anti-opsin binding of freeze-fractured ellipsoid inner segment cytoplasm. Labeled post-Golgi vesicles (arrows) are evident in the spaces between mitochondria (*M*). Note that a vesicle in *A* is labeled even though it appears to lie below the fracture surface. Such profiles are occasionally observed when the angle of thin sectioning is oblique with respect to the plane of fracture. Magnification $\times 115,000$. Bar, $0.1 \mu\text{m}$.

and those entering above its equator to produce EF leaflets (Weibel et al., 1976).

A representative sampling of thin sections of isolated ROS discs fracture-labeled with anti-opsin and AvF is shown in Figure 6. Shallow, concave membrane profiles, likely to be derived from PF leaflets, invariably displayed an uninterrupted bilayer appearance and bound AvF heavily and uniformly. These results were essentially the same as with discs presumed to have been cross-fractured at or above their equator. Examination of numerous such fractured swollen discs has failed to provide evidence for convex membrane profiles, suggesting that EF leaflets did not survive the thawing and labeling steps.

Overall, our results with isolated discs indicate that at least some of the membranes fracture-labeled *in situ* and observed in thin sections were derived from PF leaflets (see Fig. 6, *A* and *B*). They do not, however, allow us to distinguish between PF leaflets and intact membranes whose exoplasmic surfaces might also be expected to bind antibody (see Fig. 6, *C* and *D*).

Labeling of the plasma membrane

Thin section views of ROS plasma membrane PF leaflets (Fig. 7*A*) were very similar to disc fractures both in their morphological identity to native membranes and their intense reaction with labeling re-

agents. This is consistent with compositional similarities between plasma membrane and discs suggested by quantitative freeze-fracture analysis (Besharse and Pfenninger, 1980) and by previous observations demonstrating the presence of opsin in the plasma membrane (Jan and Revel, 1974, 1975; Papermaster et al., 1978; Nir and Papermaster, 1983). However, unlike the case with fractured discs in which EFs were not evident, EF leaflets of ROS plasma membranes were clearly preserved and displayed the interrupted bilayer appearance characteristic of other fracture-labeled membranes (Pinto da Silva, 1981*a, b*) (Fig. 7*B*). Their stabilization presumably results from cross-linking to adjacent albumin gel. Moreover, these EFs did not bind specific antibodies, supporting the widespread belief that a paucity of IMPs in conventional freeze-fracture replicas reflects a lack of integral protein.

In contrast to ROS membranes, fractures of inner segment plasma membranes have in all cases been found to label weakly, if at all, with anti-opsin and AvF (Fig. 8). This was true not only for EF leaflets but for PF leaflets as well. Also, these membranes exhibited interruptions in their stained profiles similar to those of fractured red blood cell membranes (Pinto da Silva et al., 1981*a, b*), but very different from ROS discs. The above findings correlate well with freeze-fracture analysis showing different IMP size distributions between inner segment and ROS plasmalemmas (Besharse and Pfen-

Figure 11. Fracture-label of the rod inner segment periciliary zone. A fine crack in the apical ellipsoid region has rendered intracellular structures accessible to anti-opsin and AvF. Fractured periciliary vesicles, two of which are indicated by open arrows, are decorated with ferritin whereas mitochondria (*M*) and interstitial cytoplasm are weakly labeled. CC, connecting cilium; OS, outer segment. The inset shows vesicles at higher magnification (ferritin particles are indicated by arrowheads). Magnification $\times 66,000$; inset, $\times 157,000$. Bar, $0.2 \mu\text{m}$.

ninger, 1980) and indicate that the different IMP classes must represent distinct integral components. As yet, images have not been found of fractured plasma membranes of the cilium or the immediate periciliary region in *Xenopus*. This is not surprising since such fractures are rare even in conventional freeze-fracture replicas.

Labeling of inner segment cytomembranes

Cross-fractures of rod inner segment cytoplasm have afforded access to immunochemical reagents of the major intracellular membrane compartments. In such preparations, we have detected binding of anti-opsin to vesicular elements of the Golgi complex in myoid regions (Fig. 9), as well as ellipsoid post-Golgi vesicles (Fig. 10). The relative uniformity of size of these labeled inner segment vesicles argues that they may all be part of a single population and thus represent intermediate stages in the intracellular transport of membrane to the ROS. At this time, we have no unequivocal evidence for anti-opsin binding to RER and Golgi lamellae above background levels. This is clearly not a result of limited accessibility of probe molecules to these membranes, however, and may reflect antigenic differences between opsins located in these versus other organellar

sites. Nevertheless, it should be noted that specific labeling of RER has previously been reported (Papermaster and Schneider, 1982). Moreover, in fortuitous fractures through apical inner segments of rod photoreceptors it has been possible to demonstrate that periciliary vesicles also bind antibodies specifically (Fig. 11). The density of labeling was found to be high, confirming previous speculation that these elements contain abundant opsin immunoreactivity.

Discussion

ROS disc structure and fracture-label. A principal finding of the present analysis is that ROS disc membranes behave differently during fracture-labeling from membranes of photoreceptor inner segments and from those of several other cell types examined, including erythrocytes (Pinto da Silva et al., 1981a, b, c, d). Disc membranes fractured *in situ* and thawed before labeling and processing for electron microscopy displayed exposed membrane profiles which, although superficially resembling conventional freeze-fracture step fracturing, gave no indication on morphological grounds that they were derived from PFs or EFs. Furthermore, virtually all membrane fractures were labeled after treatment with anti-opsin and

BEFORE THAWING

AFTER THAWING

Figure 12. Diagram illustrating the proposed alteration of freeze-fractured ROS discs which results upon thawing to room temperature. PFs and EFs generated by fracturing at -196°C are present initially in relatively equal amounts. Thawing results in preferential loss of delicate EF leaflets leaving co-extensive PF leaflets and the exoplasmic surfaces (ES) of unfractured membrane exposed at the tissue margin.

AvF. Two observations from the present investigation, however, indicate that remnants of actual split membranes were being observed. First, isolated swollen discs, in which individual bilayers can be unambiguously visualized, displayed a pattern of IMP distribution in freeze-fracture replicas and fracture-label appearance in thin sections that was identical to that of native discs. Second, clear-cut differences in membrane structure were noted after freeze-substitution of cleaved retinas in osmium-acetone (cf. Fig. 2B and Fig. 2, C to E). PFs, because of their unilaminar appearance were distinguishable from unfractured disc membrane (trilaminar). The lack of identifiable EFs in all preparations indicates that even substitution fixation is inadequate to preserve these delicate leaflets. One reason may be that, unlike pancreatic zymogen granules (Pinto da Silva et al., 1981d), discs contain very little material which might serve as an underlying support. Also, it is possible that, because EFs possess a very low IMP density and thus presumably small amounts of protein, they are not sufficiently stabilized by glutaraldehyde cross-linking. Outer segment membrane lipids, when extracted free of protein and reconstituted, have been reported to form non-bilayer configurations (DeGrip et al., 1979; but see Deese et al., 1981). Thus, the lipids of these EF leaflets may be unable to reconstruct a bilayer upon thawing and instead may be converted to micelles and lost.

Exoplasmic leaflets of both outer and inner segment plasmalemmas and protoplasmic leaflets of outer segment plasmalemmas were apparent as segments of trilaminar membrane adjacent to albumin gel and contiguous with unfractured membrane. Therefore, the absence of the interrupted bilayer in fracture-labeled discs appears to be a characteristic of PF leaflets of ROS membranes. The reason presumably lies in the fact that discs differ from many other membranes in having a very high protein content (50%) (Daemen, 1973). Because of this, a significant proportion of the staining in electron micrographs may be due to protein rather than phospholipid. The continuity of these membrane profiles probably reflects the fact that their component proteins do not reorganize

after thawing (due to glutaraldehyde cross-linking) and may actually disguise the fact that lipids are mobile. That the protein (opsin) oligosaccharide expressed at the exoplasmic surface is in fact dragged across the exoplasmic half of the membrane has been shown by experiments in which concanavalin A- and wheat germ agglutinin-binding sites partition with putative PF leaflets (D. M. Defoe and J. C. Besharse, unpublished data). Such evidence is consistent with previous fracture-label data (Pinto da Silva et al., 1981a) and theoretical analysis (Kell, 1984) indicating that peptide bonds are not normally broken in the course of freeze-fracturing.

Figure 12 summarizes what we believe occurs when ROS discs are fractured *in situ*. Upon crushing at liquid nitrogen temperature, both PFs and EFs are generated. Then, after thawing, EF leaflets are selectively lost, leaving PF leaflets as well as exoplasmic surfaces underlying regions where EFs existed before. Since opsin is normally accessible at the interior of the disc, exoplasmic surfaces as well as protoplasmic faces would be expected to bind anti-opsin antibodies and thus virtually all exposed membranes would be labeled.

Implications for ROS membrane turnover. Results from previous autoradiographic, radiobiochemical, and immunocytochemical studies suggest that the flow of newly synthesized protein from the inner to the outer segment during ROS renewal is vectorial and involves RER, Golgi apparatus, and periciliary vesicles (Young and Droz, 1968; Hall et al., 1969; Papermaster et al., 1975). A principal weakness of the evidence derives from the fact that the resolution of electron microscope autoradiography is not sufficient to identify the finer steps in the membrane pathway, especially those at the inner segment-ROS boundary. For this reason, a number of immunocytochemical investigations employing albumin and Lowicryl-embedded retinal sections labeled with anti-opsin antibodies have also been conducted to specifically identify cytoplasmic membranes involved in disc biogenesis (Papermaster et al., 1978, 1979, 1985; Papermaster and Schneider, 1982). Images obtained with this technique have not resolved individual membranes clearly, and thus the precise origin of opsin determinants has remained ill-defined. A parallel approach has involved the use of freeze-fracture analysis which relies on IMP size distributions and densities to indirectly infer data about membrane composition. This technique has the virtue of allowing access to integral membrane components and provides rigorous quantitative information. Although intriguing results have been reported which suggest the interrelationship of certain photoreceptor membrane compartments (Besharse and Pfenninger, 1980), particle measurements are rather nonspecific parameters and discriminate only relatively large differences. Our application of the fracture-label technique to an immunocytochemical analysis of vertebrate rod cells has been an attempt to utilize the respective advantages of both freeze-fracture and immunocytochemistry.

The present data confirm earlier reports indicating specific anti-opsin labeling of outer segment but not inner segment plasma membranes (Papermaster et al., 1978; Nir and Papermaster, 1983), and are entirely consistent with the previous freeze-fracture results (Besharse and Pfenninger, 1980). Furthermore, this study has yielded unequivocal evidence for opsin immunoreactivity in inner segment photoreceptor membrane systems. Previous autoradiographic and immunocytochemical studies had implicated myoid RER and Golgi apparatus in the synthesis and processing of opsin-containing membrane (Papermaster et al., 1978; Papermaster and Schneider, 1982). Therefore, the present finding that specific anti-opsin antibodies bind to Golgi vesicular elements was expected. What is perhaps more significant is the definitive localization of opsin determinants in fractured membranes of ellipsoid perimitochondrial and periciliary vesicles. These vesicles are similar structurally to labeled Golgi vesicles and are roughly twice the size of coated vesicles consistently associated with Golgi cisternae (D. M. Defoe and J. C. Besharse, unpublished observations). Because the periciliary elements bear a close relationship to the ROS-cilium region and are especially prominent in *Xenopus* rods undergoing very high rates of membrane turnover, they have been obvious candidates for

shuttling membrane precursors to the outer segment. When coupled with our finding that fracture-labeled inner segment plasma membranes bind anti-opsin weakly, if at all, such data are consistent with hypotheses which propose that the initial site of membrane insertion is the inner segment plasma membrane in the region immediately adjacent to the cilium (Besharse and Pfenniger, 1980). In this scheme, either the patch of membrane destined for the ROS or individual components such as opsin would flow along the ciliary plasma membrane into outer segment plasma membrane and forming discs. Presumably, segregation of opsin within the cell membrane is maintained by a vectorial mechanism or by the existence of a barrier to diffusion of visual pigment.

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