

# ULTRASTRUCTURAL LOCALIZATION OF RHODOPSIN IN THE VERTEBRATE RETINA

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## ABSTRACT

Early work by Dewey and collaborators has shown the distribution of rhodopsin in the frog retina. We have repeated these experiments on cow and mouse eyes using antibodies specific to rhodopsin alone. Bovine rhodopsin in emulphogene was purified on an hydroxyapatite column. The purity of this reagent was established by spectrophotometric criteria, by sodium dodecyl sulfate (SDS) gel electrophoresis, and by isoelectric focusing. This rhodopsin was used as an immunoadsorbent to isolate specific antibodies from the antisera of rabbits immunized with bovine rod outer segments solubilized in 2% digitonin. The antibody so prepared was shown by immunoelectrophoresis to be in the IgG class and did not cross-react with lipid extracts of bovine rod outer segments. Papain-digested univalent antibodies (Fab) coupled with peroxidase were used to label rhodopsin in formaldehyde-fixed bovine and murine retinas. In addition to the disk membranes, the plasma membrane of the outer segment, the connecting cilium, and part of the rod inner segment membrane were labeled. We observed staining on both sides of the rod outer segment plasma membrane and the disk membrane. Discrepancies were observed between results of immunolabeling experiments and observations of membrane particles seen in freeze-cleaved specimens. Our experiments indicate that the distribution of membrane particles in freeze cleaving experiments reflects the distribution of membrane proteins. Immunolabeling, on the other hand, can introduce several different types of artifact, unless controlled with extreme care.

## INTRODUCTION

The primary visual response of vertebrates takes place in the outer segment of the retinal rod. Light bleaches the visual pigment rhodopsin (1), the major protein constituent of the rod outer segment (ROS) (2). The initial photochemical reactions of rhodopsin result in a reduction of sodium conductance in the rod outer segment membrane and hence in a hyperpolarization of the rod membrane potential at the synaptic end (3). The events that take place between the exposure of rhodopsin to

light and the permeability change on the cell membrane are of great interest but are not fully understood. It would seem probable that, if a relatively detailed map of the distribution of rhodopsin in the photoreceptor cell was available, it would be useful in devising models for the mechanism of the primary light response.

In each rod outer segment there are several hundred retinal disks that are closely packed and oriented perpendicular to the long axis of the rod

(4). X-ray diffraction (5) and optical dichroism (6) studies indicate that the rhodopsin molecules are closely packed in the disk membranes with their chromophores lying in the plane of the membrane. However, the localization of rhodopsin relative to the lipid core of the membrane has been a rather difficult problem in X-ray diffraction studies, owing to the loss of phase information (7). Rhodopsin is also found in the inner segment and on the cell membrane of the outer segment, as suggested by a study of frog retina labeled indirectly with fluorescent antibodies (8).

The present report describes an attempt to purify rabbit antibodies specific to bovine rhodopsin and to label mouse and bovine retinas with the purified specific antibodies. We compare the distribution of marked antibodies bound to rhodopsin with that of intramembrane particles seen in freeze etching. Our experiments suggest that great caution must be exercised in the interpretation of surface labels, as the patterns observed can be profoundly influenced by the labeling procedure itself.

## MATERIALS AND METHODS

Rabbits were injected with a preparation of solubilized bovine retinal disks. Specific antibodies were isolated from the antisera by use of an immunoadsorbent prepared from highly purified rhodopsin. The specificity of the antibodies was established, and either they or their Fab fragments were coupled to peroxidase. An outline of the procedure used is presented in Table I.

### *Preparation of Bovine Rod Outer Segments*

Fresh bovine eyes were purchased at a local slaughterhouse (Quality Meat Packing, 4512 Alcoa, Vernon, Calif.). The eyes were placed in the dark on ice right after removal. A modified version (9) of the procedure of D. G. McConnell (10) was used to separate bovine ROS on a discontinuous sucrose gradient at 4°C under dim red light.

### *Immunization of Rabbits with Bovine Rhodopsin*

The ROS isolated from bovine eyes were extracted in the dark with 2% digitonin, 1/15 M phosphate buffer, pH 6.4 (11), centrifuged at 13,000 rpm for 10 min in a SS-34 rotor, and the supernate was used as the immunizing agent for rabbits. A digitonin solution (0.5 ml) containing 1 mg of bovine rhodopsin was homogenized in an equal volume of Freund's complete adjuvant and then injected into the footpads of each rabbit. After 3 wk these rabbits received first an intraperitoneal injection of 1 mg of

bovine rhodopsin dissolved in 1 ml of the 2% digitonin solution, and 2 days later the same amount of antigen injected intravenously. The rabbits were bled twice, on the 7th and the 9th day after the intravenous injection.

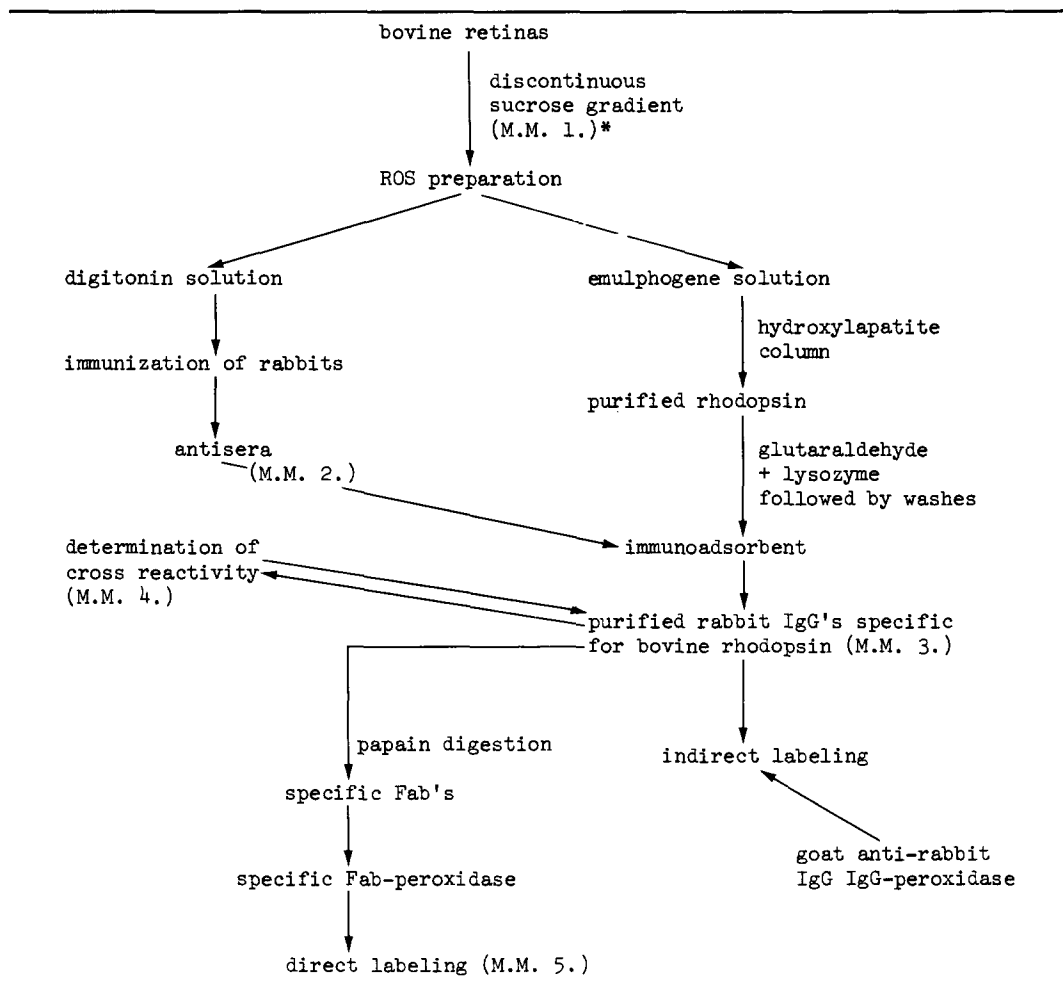
### *Purification of Specific Rabbit Antibodies with an Immunoadsorbent*

An immunoadsorbent was built for the purification from rabbit antisera of antibodies specific to bovine rhodopsin. For this purpose the bovine ROS preparations (9) were further purified on an emulphogene-hydroxyapatite column. ROS containing 20–30 mg of rhodopsin were solubilized in 2% emulphogene BC-720 (General Aniline and Film Corporation), 0.01 M imidazole (CalBiochem, San Diego, Calif.), pH 7, to a final concentration of about 1 mg/ml rhodopsin. This emulphogene solution (20–30 ml) of rhodopsin was centrifuged at 13,000 rpm for 10 min and applied to a hydroxyapatite (Bio-Rad, bio-gel HTP Bio-Rad Laboratories, Richmond, Calif.) column (2.5 cm × 4 cm) equilibrated with 1% emulphogene in 0.01 M imidazole, pH 7. The rhodopsin peak was eluted before applying the NaCl gradient.<sup>1</sup> (The gradient was formed by gradually mixing 50 ml 0.01 M imidazole, pH 7, 1% emulphogene solution with 50 ml 1.0 M NaCl in 0.01 M imidazole, pH 7, 1% emulphogene solution.) The purity of the rhodopsin solution after the column purification was checked by SDS acrylamide gel electrophoresis (12) and by urea gel isoelectric focusing (13). The ratios of the extinction coefficients of the rhodopsin solutions at 280 nm and at 500 nm ( $A_{280}:A_{500}$ ) and at 400 nm and at 500 nm ( $A_{400}:A_{500}$ ) (18, 19) were determined. In the best preparation  $A_{280}:A_{500}$  was 2.4 and  $A_{400}:A_{500}$ , 0.22. In the worst ROS preparation used,  $A_{280}:A_{500}$  was 3.0 and  $A_{400}:A_{500}$ , 0.35. After passing through the hydroxyapatite column, the purified rhodopsin in emulphogene solution had a value between 1.8 and 2.0 for  $A_{280}:A_{500}$  and a value of about 0.26 for  $A_{400}:A_{500}$ . Emulphogene, a detergent whose molecular structure is very different from digitonin, was used in order to reduce the number of antibodies specific to digitonin molecules which would be left after immunoadsorption.

The fractions recovered under the rhodopsin peak after the hydroxyapatite column were pooled and concentrated to about 1 ml, using an Amicon PM10 membrane filter (Amicon Corp., Lexington, Mass.). They were cross-linked with glutaraldehyde to hen egg white lysozyme (Sigma Chemical Co., St. Louis, Mo., No. L-6876). An excess of lysozyme (2:1 by weight) was

<sup>1</sup> The amino acid composition of the emulphogene solution of ROS before column separation is very similar to that of the fractions under the rhodopsin peak, and very different from that of the pooled fractions eluted by the NaCl gradient after the rhodopsin peak.

TABLE I  
Summary of the Preparation of Specific IgG's and Fab's for the Immunolabeling Experiments



\* Details of this procedure are given in Materials and Methods, paragraph 1.

used. Rhodopsin in emulphogene has a low density, and the excess lysozyme insured that the immunoabsorbent would not float. The insoluble protein gel was first washed with the eluting salt solutions (2.5 and 5 M  $\text{MgCl}_2$ ) followed by 0.85% saline until the  $\text{OD}_{280}$  of the washes was 0.0. Specific antibodies from the rabbit antiserum were then adsorbed in a batchwise fashion. After adsorption the protein gel was washed in 0.85% saline until the supernate of the washes had an  $\text{OD}_{280}$  reading of less than 0.04. The specific antibodies were then eluted using 2.5 M and 5 M  $\text{MgCl}_2$  in 0.05 M Tris-HCl, pH 7.5 solutions (14). The purity of the eluted antibodies was checked by immunoelectrophoresis (15). The cross-reactivity of the whole rabbit antisera against various substances was also tested.

### Cross-reactivity of the Rabbit Antiserum

**LACK OF CROSS-REACTIVITY WITH LIPIDS:** Lipid extract in chloroform-ethanol (3:1) from bovine ROS preparations (4.3 mg rhodopsin/ml) was dried under nitrogen. This was then dissolved in 2% emulphogene, 0.1 M imidazole, pH 7, and brought to a final volume equal to that of the original ROS preparation. Seven serial dilutions by a factor of two were made. Electrophoresis of each dilution of lipid extract was carried out on agar plates along with the emulphogene solution of bovine ROS preparation of the corresponding dilution. A few components of the bovine rod outer segment preparation did precipitate with the specific antiserum even at the lowest rhodopsin concentration, but no precipitates were formed for the lipid extracts at any concentrations.

**CROSS-REACTIVITY WITH PROTEINS:** The antisera from the rabbits immunized against bovine rhodopsin were found by immunoelectrophoresis to precipitate with bleached as well as with unbleached bovine rhodopsin in 2% digitonin or in 2% sodium cholate, pH 8. The cross-reactivity of antisera against bovine rhodopsin solubilized in other detergents such as emulphogene varied from rabbit to rabbit. Possible artifacts due to nonspecific precipitation of detergent-solubilized membrane proteins during electrophoresis could be ruled out because no arcs of precipitate were found if ordinary rabbit serum was substituted for the antiserum. The antisera were also found not to cross-react with the hen egg white lysozyme which was present in the immunoadsorbent.

### *Preparation of Peroxidase-Labeled Retinas for EM Studies*

For the labeling experiments described in this report, the purified specific rabbit antibodies were digested by papain to give univalent Fab antibodies (16), and then cross-linked to horseradish peroxidase (Worthington Biochemical Co., Freehold, N. J.) via glutaraldehyde (17). This Fab-peroxidase conjugate solution was centrifuged at 20,000 rpm for 10 min right before use. The conjugate solution was incubated at room temperature for 150 min with mouse or bovine retina slices that had been fixed at 4°C for 16 h in 4% formaldehyde, 0.1 M cacodylate buffer, pH 7.2. After the incubation the retina slices were washed three times in 0.1 M cacodylate, pH 7.2, and then reacted with 75% (wt/vol) 3,3'-diaminobenzidine, 0.075% hydrogen peroxide, in 0.05 M Tris-HCl, pH 7.6, for 20 min at room temperature. At the end of the reaction the slices of retina were washed three times in 0.05 M Tris-HCl, pH 7.6, fixed in osmium tetroxide at 4°C for 1 h, dehydrated and embedded in Epon-Araldite for thin sectioning. Sections were examined, either after staining with uranyl acetate and lead citrate or without any further staining, in a Philips 301 or Philips 201 electron microscope.

Similar procedures were followed for the indirect labeling experiments. Here the tissue was washed after treatment with specific rabbit IgG's and then incubated with peroxidase-labeled goat IgG's which are specific for rabbit IgG. The goat IgG's specific for rabbit IgG were purified by use of an immunoadsorbent built from rabbit IgG's purified on a DEAE column (Miles Laboratories, Inc., Kankakee, Ill.). Glutaraldehyde was used to couple the goat IgG with peroxidase. After the coupling, goat IgG and goat IgG-peroxidase were separated from the unreacted peroxidase in the reaction mixture by ammonium sulfate precipitation.

### *Procedure for Freeze-fracturing or Freeze-etching Experiments*

Unless otherwise specified, the retinas were fixed at 4°C for 1 h in Karnovsky's fixative (20 parts of 10% formaldehyde, 9.8 parts of 25% glutaraldehyde, 18 parts

of 0.2 M cacodylate buffer, pH 7.2, and 2 parts of 2%  $\text{CaCl}_2$ ), and then soaked in either 25% glycerol in Ringer solution for freeze-fracturing or in distilled water for freeze-etching experiments. Tissue blocks were rapidly frozen in Freon 22 in liquid  $\text{N}_2$ , mounted on a stage at liquid nitrogen temperature, and fractured or etched using a Balzers unit (Balzers High Vacuum Corp., Santa Ana, Calif.).

## RESULTS

### *Specificity of the Adsorbed Antibodies*

To localize rhodopsin molecules in the retina at the electron microscope level, it is crucial that the antibodies used be specific to rhodopsin. The rabbit antisera we prepared were found in immunoelectrophoresis experiments not to cross-react with either lipid extracts of bovine ROS or with hen egg white lysozyme. (This lysozyme was cross-linked to the purified bovine rhodopsin-emulphogene micelles in preparing the immunoadsorbent.) Thus the specificity of our antibodies depends on the purity of the rhodopsin used in building the immunoadsorbent. In addition to the absorption maximum at 280 nm common to most proteins, the absorption spectrum of rhodopsin shows a peak at 498 nm for the dark-adapted pigment in contrast to a peak at 380 nm for the bleached pigment. The ratios of extinction coefficients,  $A_{280}:A_{500}$  and  $A_{400}:A_{500}$ , thus serve as a criterion of the purity of the rhodopsin preparation. The  $A_{400}:A_{500}$  value of about 0.26 obtained for the column purified rhodopsin indicates that a portion of the rhodopsin preparation was bleached. Since bleached rhodopsin cross-reacts with unbleached rhodopsin, the presence of some bleached form of the pigment would not do much harm. Even with this relatively high ratio of  $A_{400}$  and  $A_{500}$ , the  $A_{280}:A_{500}$  value of our purified rhodopsin is fairly close to the best values that have been reported (18, 19). Purified rhodopsin migrated as a single band in SDS acrylamide gel electrophoresis whereas the ROS preparation showed several minor impurity bands in addition to the major band of rhodopsin (Fig. 1). Isoelectric focusing, which separates a mixture of proteins according to their isoelectric points rather than their molecular weights, also suggested that only a single protein species was present in the purified rhodopsin preparation (Fig. 2). We conclude therefore that the antibodies purified by the immunoadsorption procedure are specific against bovine rhodopsin. Immunoelectrophoresis experi-



FIGURE 1 Tracings of OD<sub>600</sub> readings along Coomassie brilliant blue stained SDS acrylamide gels. (A) Bovine rod outer segment preparation containing roughly 50 µg rhodopsin, (B) about 50 µg of emulphogene-hydroxyapatite column-purified rhodopsin. All rhodopsin samples were dissolved in SDS and dithiothreitol and applied on top of the gel for electrophoresis without prior heating. Arrows indicate positions of the standard marker proteins. Gels are 8 cm in length.

ments indicate that purified antibodies are in the IgG class (Fig. 3).

#### *Localization of Rhodopsin in Mouse and Bovine Retinas*

Electron-opaque precipitates of peroxidase-catalyzed reaction products were found in both mouse and bovine retinas. A summary of the experiments carried out and their controls are given in this and the following paragraph and are summarized in Table II. Specific Fab<sup>2</sup>-peroxidase staining in

<sup>2</sup> Rabbit antibovine rhodopsin IgG will be referred to as specific IgG, and rabbit antibovine rhodopsin Fab as specific Fab.

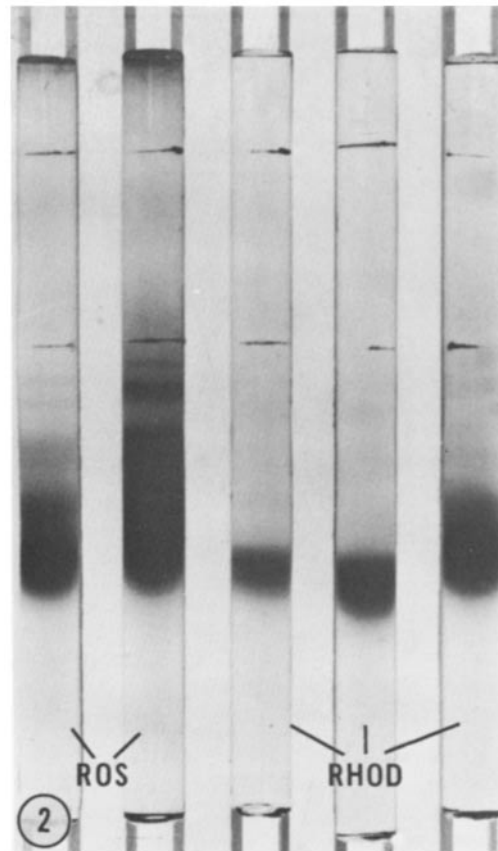


FIGURE 2 Isoelectric focusing on urea gels. The two gels on the left represent different loadings (50 µg, 100 µg) of the bovine rod outer segment preparation. The other three are different loadings (25 µg, 50 µg, 100 µg) of the column-purified rhodopsin. A 600-nm filter was used to visualize the bands on the gels. Gels are 8 cm in length.

bovine retina is much more intense than that in the mouse retina. In both cases, however, the cell membrane of the inner and outer segments of rods, the connecting cilia, as well as the disk membranes were stained specifically by the Fab-peroxidase conjugates. The cytoplasmic face of the disk membrane was uniformly covered by the peroxidase stain, and a fainter reaction was also seen on the intradisk face (Fig. 4). In addition, we observed heavy stain patches located between adjacent disk membranes with a fairly regular spacing of a few hundred angstroms between the patches (Fig. 5). This cytoplasmic staining is probably artifactual and is discussed further in the next section. The staining on the cell membrane of the rod outer segments was often heavy and uniform, whereas

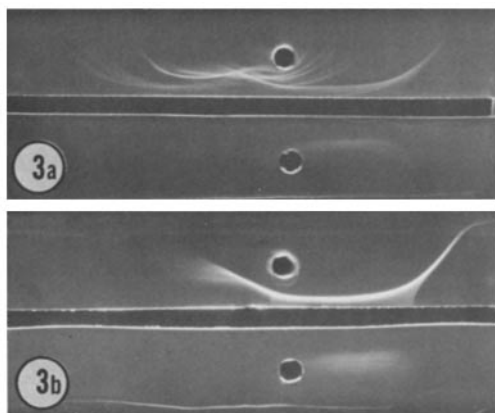


FIGURE 3 Immunoelectrophoresis: Purified specific antibody solutions of concentration 0.37 mg/ml were placed in the well below the trough and whole rabbit antiserum was in the well above the trough. After 3 h of electrophoresis (cathode was on the right-hand side of the slides) at constant current (6 mA/slide) goat antirabbit whole serum (a), or goat antirabbit IgG serum (b) was placed in the trough for the subsequent double diffusion experiment. These experiments were performed on glass slides (7.7 cm  $\times$  2.6 cm) covered with 3 ml agar gel.

the staining on the inner segment membrane was sometimes patchy and decreased as one approached the outer limiting membrane (Fig. 6). In experiments which will be presented in detail elsewhere the same specific IgG's and ferritin-labeled goat IgG's specific for rabbit IgG were used to label rhodopsin on frozen ultrathin sections of retina. In this case ferritin was found almost as abundantly on the inner segment plasma membrane both near the outer limiting membrane and distally near the connecting cilium. This suggests that the decrease in peroxidase staining of the inner segment plasma membrane might reflect a concentration gradient of Fab-peroxidase conjugates from the surface to the interior of the retina rather than the real distribution of the antigen rhodopsin. One must, however, also consider the possibility that because of possible steric hindrance, ferritin conjugates could give an underestimate of the number of rhodopsin sites where the local density of rhodopsin molecules is high, i.e. on the outer segment membranes.

#### *Controls for the Fab-peroxidase Labeling Experiments*

As controls for the labeling experiments, slices of retina were treated either with nonspecific

rabbit Fab-peroxidase or with peroxidase alone instead of the specific rabbit Fab-peroxidase conjugates (Table II). In both cases one could find nonspecific staining on the outmost surfaces of the retina, i.e. the tip of the rod outer segment and the anterior surface of the internal limiting membrane. In addition, staining by nonspecific Fab-peroxidase but not by peroxidase alone was found in the rod cytoplasm in the form of dense patches spaced regularly between the closely packed disks. Since commercial horseradish peroxidase has few free amino groups, we tried to find out if the staining could be due to nonspecific binding of a protein (such as Fab) with free amino groups. Mouse retinas were treated with bovine serum albumin coupled to peroxidase. Under these conditions the same pattern of patches of staining between disks could be seen as was observed under "specific" conditions (see Fig. 5). As in the case of the experimental samples the best staining occurred in rods which had been slightly damaged and allowed easy access of the conjugates to the interior of the cell. If the retina was washed in 1 mg/ml bovine serum albumin before treatment with specific rabbit IgG-peroxidase conjugates, the interdisk staining was essentially blocked, but not the reaction of the disks themselves or that of the rod outer segment plasma membranes which were stained normally.

We tested for possible endogenous peroxidase activity in retina slices incubated with 3,3'-diaminobenzidine and  $H_2O_2$  without prior treatments with antibodies or peroxidase. Only the rim of the membrane disks where the radius of curvature was small showed such endogenous peroxidase activity. This endogenous peroxidase staining was only obvious at those disks which stayed intact with little intradisk spacing. We do not know whether the decreased level of peroxidase staining at the rim of swollen disks was due to simple geometrical factors or the fact that certain substances were lost and caused the swelling of these disks. It was further demonstrated that 3,3'-diaminobenzidine did not give any electron-opaque precipitates when  $H_2O_2$  was omitted in the reaction mixture even if the retina was specifically labeled with peroxidase-antibody conjugates. On the basis of these control experiments, we conclude that the staining by rabbit antiovine rhodopsin Fab-peroxidase conjugates of the cell membrane of the rod outer and inner segments, the connecting cilium, and the disk membranes represents the locations of rhodopsin molecules. The patches of

TABLE II  
Summary of the Immunolabeling Experiments

Experiment*	Reagents used		Distribution of staining
	1.	2.	
Direct labeling	Specific Fab-peroxidase	Diaminobenzidine + H <sub>2</sub> O <sub>2</sub>	Specific† sites: disk membranes, cell membrane of ROS and inner segments, connecting cilium Nonspecific sites: patches between disks, outmost surfaces of retina
Control			
1.	None	Same as above	rim of disks
2.	Nonspecific Fab-peroxidase	Same as above	patches between disks, outmost surfaces of retina
3.	Peroxidase only	Same as above	outmost surfaces of retina
4.	BSA-peroxidase	Same as above	patches between disks that are close to the surface§
5.	BSA wash, specific IgG-peroxidase (direct labeling)	Same as above	Specific sites: disks and ROS membranes that are close to the surface of the retina
Indirect labeling¶	Specific IgG + goat IgG-peroxidase	Same as above	Specific sites: disks and ROS membranes that are close to the surface of retina
Control	Same as above	Diaminobenzidine only	None

\* Mouse or cow retina was fixed in 4% formaldehyde at 4°C for 16 h before treatment with the reagents specified in the table. After the peroxidase reaction the tissue was washed, fixed in osmium tetroxide, and embedded in Epon-Araldite for thin sectioning.

† The staining reaction is classified as specific if it is present only in the direct staining experiment but not in any of the control experiments.

§ In the case of BSA-peroxidase staining, the specific IgG-peroxidase and the indirect staining, the penetration problem is serious. Staining can only be found in those areas of rods close to the surface of the retina.

|| The background nonspecific staining on the surface of the retina was much reduced when IgG-peroxidase instead of Fab-peroxidase was used. This is because the additional ammonium sulfate precipitation separated IgG and IgG-peroxidase from unreacted peroxidase still present in the reaction mixture.

¶ A different indirect staining technique which involves applying the specific IgG and ferritin-labeled goat IgG's that are specific for rabbit IgG to frozen thin sections of retina was tried and will be reported elsewhere.

staining seen between disks, however, were attributed to the presence of "sticky" material in the narrow interdisk space. The background peroxidase activity present at the rim of the disks made it difficult to judge whether rhodopsin molecules were present there.

#### *Distribution of Rhodopsin Molecules in the Plasma Membrane of Rod Outer Segments*

Evidence from other systems has indicated that the particles seen on the fractured face of the membrane reflect the presence of certain mem-

brane proteins (20, 21, 22). The fact that rhodopsin composes more than 80% of the protein in rod outer segments, combined with the heavy staining observed with antirhodopsin antibodies, would make it plausible to regard the distribution of the membrane particles on the fractured membranes of rod outer segments as the distribution of rhodopsin in these membranes (Fig. 7). Arguments supporting the idea that membrane particles in both the cell membrane and the disk membrane of the rod outer segment are indeed rhodopsin molecules or membrane structures resulting from the

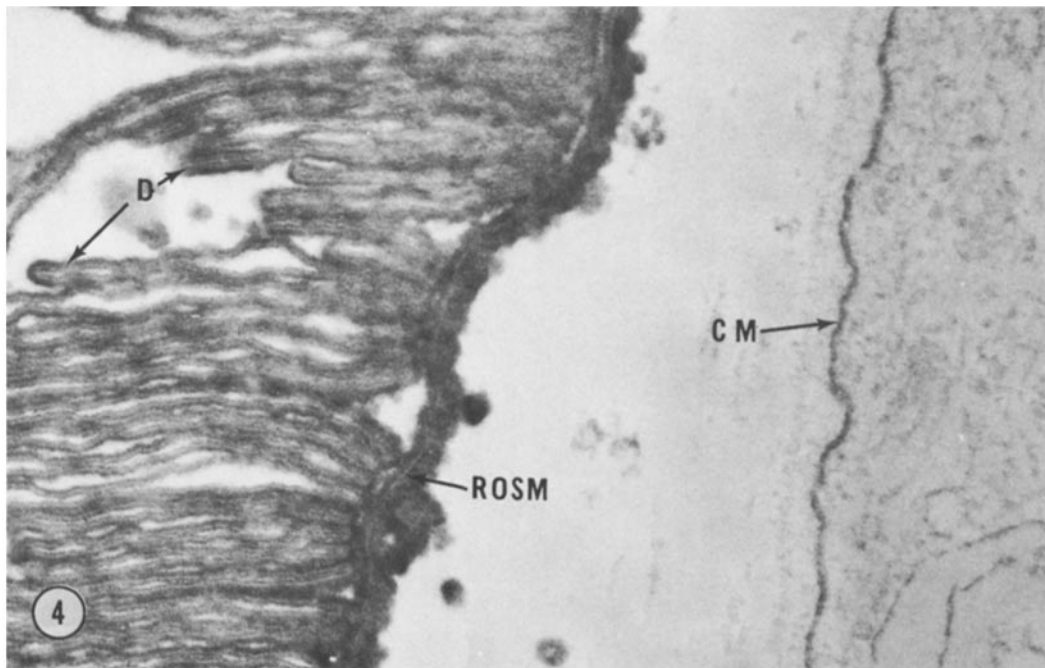


FIGURE 4 Mouse retina stained indirectly with specific IgG's and peroxidase-labeled goat IgG's specific for rabbit IgG after formaldehyde fixation. The indirect technique amplifies the intensity of the labeling. The extracellular face of the ROS membrane (*ROSM*) was heavily stained. The cytoplasmic side of the *ROSM* and both sides of the disk (*D*) membranes were also stained. The hydrophobic core of the bilayer membrane is clearly visible, as indicated by the arrow in the case of the ROS plasma membrane. This piece of retina was twisted in such a way that this ROS was in the vicinity of the internal limiting membrane on the outmost surface of the neural layers of the retina. The lack of stain on the internal limiting membrane (*CM*) serves as an internal control.  $\times 90,000$ .

presence of rhodopsin are presented in the Discussion section.

We have shown in the preceding paragraphs that the peroxidase staining on the plasma membrane of outer segments is uniform as revealed by thin sections. This seems to contradict the picture obtained in freeze-fracturing experiments. In either dark-adapted or completely bleached mouse retinas fixed in Karnovsky's fixative for 1 h at 4°C [exp (1), Table III], freeze fracturing showed a uniform distribution of particles with diameter of about 50 Å on the cytoplasmic leaflet of the disk membrane (the half of the membrane adjacent to the interdisk space). A somewhat lower density of similar particles was found on the cytoplasmic leaflet of the fractured rod outer segment plasma membrane.<sup>3</sup> Here, however, the distribu-

tion of particles was patchy. There were islands of membrane devoid of particles separated by

limiting the disks will always be described as "disk membrane" in the description of freeze-cleaving experiments. The portion of any membrane adjacent to the cytoplasm will be referred to as the "cytoplasmic leaflet." It corresponds to the so-called "A face" of the split membrane, and in the case of the plasma membrane to the convex face of the split membrane. In the disk membrane (as in other organelles) the cytoplasmic or A leaflet is the concave leaflet. The B face of the membrane is that leaflet adjacent to the lumen of the disk (or organelle) or to the extracellular space in the case of the plasma membrane. In freeze-fracture experiments the "true" surfaces of the membrane can only be seen after etching in distilled water. An extensive review and discussion of studies on the freeze-fractured membranes in rod outer segments is given by Corless, Cobbs, Costello and Robertson (1974. On the asymmetry of retinal rod outer segment disk membranes. Manuscript to be published.).

<sup>3</sup> The rod outer segment plasma membrane will variously be referred to as ROS plasma membrane, ROS membrane, or rod plasma membrane. The membrane



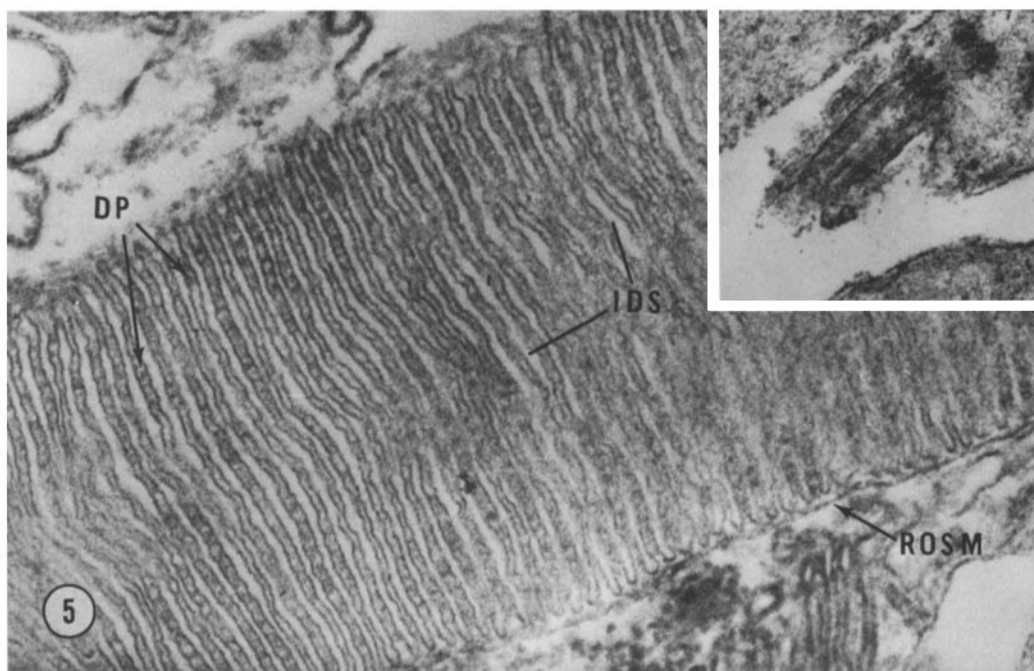
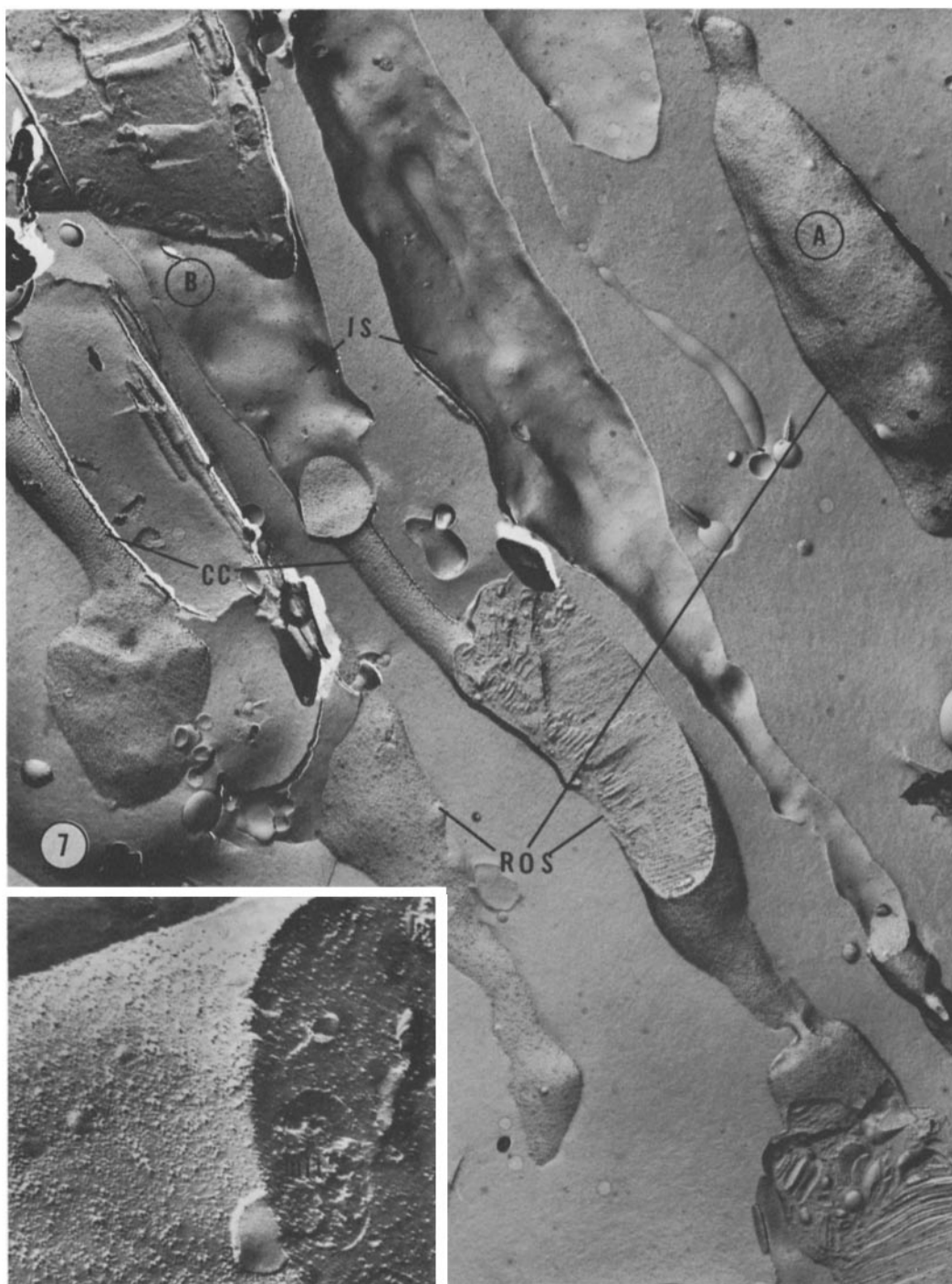


FIGURE 5 Mouse retina stained directly by specific Fab-peroxidase. The staining is less intense than in the indirect method. However, the tissue is stained much more uniformly because of the better penetration of the smaller label, i.e. Fab-peroxidase. Dense patches of staining (*DP*) between disks are clearly visible. The alternating clearer spaces are the intradisk spaces (*IDS*). The ROS plasma membrane (*ROSM*) is visible only on one side of the cell in this plane of section.  $\times 80,000$ . Staining on the membrane of the rod inner segment membranes and of a cilium is evident in the inset.  $\times 33,000$ .



FIGURE 6 Cow retina treated with specific Fab-peroxidase. The staining here is much heavier than that on the mouse retina. There is a gradual decrease in the intensity of staining on the rod inner segment (*RIS*) plasma membrane as one approaches the outer limiting membrane. The staining on disks is less intense relative to that on the rod outer segment (*ROS*) plasma membrane. This is probably a manifestation of the penetration problem.  $\times 80,000$ .



regions of close packed particles (Fig. 8 *a*). Because of this discrepancy we had to examine possible artifacts introduced by the observational techniques.

**POSSIBLE ARTIFACTS IN THE APPARENT RHODOPSIN DISTRIBUTION SEEN IN THE LABELING EXPERIMENT:** Freeze fracturing of retinas prepared by the same schedule used for immunolabeling (4°C for 16 h in 4% formaldehyde, 0.1 M cacodylate buffer, pH 7.2) did not introduce any obvious changes in the distribution of particles on the cell membrane [exp (2), Table III]. However, incubating the formaldehyde-fixed retina with specific bivalent IgG's at room temperature for 3 h before freeze fracturing [exp (3), Table III] caused the particles of the ROS plasma membrane to aggregate into large clusters (Fig. 8 *b*). This suggests that the formaldehyde fixation procedure used in the Fab-peroxidase labeling experiments left the ROS plasma membrane still in a fluid-like state at room temperature. (Aggregation of particles of disk membrane was not expected nor observed. The cytoplasmic leaflets of disk membranes are already closely packed with particles, presumably leaving little room for rearrangement. In addition, IgG penetrates the plasma membrane only poorly because of its size.)

In the immunolabeling experiments each rhodopsin molecule (mol wt ~ 40,000) might bind one or more univalent Fab-peroxidase conjugates (mol wt > 90,000). The increased bulk could cause a dispersion of rhodopsin molecules during the labeling at room temperature. This rearrangement would result in the uniform distribution of peroxidase staining observed in the immunolabeling experiments. The peroxidase-catalyzed reaction products are very dense and, particularly after indirect staining, form a thick layer on the surface of the ROS. The stain deposits might further obscure the patchy distribution of stain ex-

pected on the ROS plasma membrane on the basis of the freeze-cleaving data.

**POSSIBLE CHANGES IN PARTICLE DISTRIBUTION IN FREEZE-FRACTURING EXPERIMENTS:** We have done the following experiments to attempt to answer the question of whether the freeze-fracturing experiments give a better approximation of the real rhodopsin distribution on ROS plasma membrane than immunolabeling. Mouse retinas were incubated with specific IgG's at 4°C for 14 h before a 1-h fixation at 4°C in Karnovsky's fixative [exp (5), Table III]. They showed the same pattern of distribution of particles on ROS plasma membranes as that seen in retinas that were either exposed to nonspecific rabbit IgG's [exp (4), Table III] or not exposed to any antibodies at all. No rearrangements take place at 4°C, even in unfixed membranes. Raising the temperature to 32°C for 30 min after the 14-h incubation of retinas with specific rabbit IgG's and subsequent washings at 4°C, followed by a 1-h fixation in Karnovsky's fixative at 4°C [exp (7), Table I], however, resulted in the formation of very large patches of uniformly distributed particles on the ROS plasma membrane (Fig. 8*c*). This suggests that unfixed ROS membranes are fluid at 32°C. The distribution observed is quite different from the normal distribution seen in the control experiments where the retina was treated identically except that nonspecific rabbit IgG's were used [exp (6), Table III]. We conclude that the ROS plasma membrane appears to be in a relatively viscous state at 4°C. The rate of diffusion of rhodopsin in the plasma membrane at 4°C was presumably not sufficient to allow cross-linkage of rhodopsin by bivalent IgG's to occur. On the basis of these observations, we are inclined to believe that the patchy distribution of rhodopsin on the ROS plasma membrane as revealed by freeze-fracturing experiments is a better approximation of the real distribution of rhodopsin than

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**FIGURE 7** Freeze-fractured mouse retina, showing portions of inner (*IS*) and outer segments (*ROS*). The cytoplasmic (*A*) leaflet of the rod outer segment (*ROS*) plasma membrane and that of the connecting cilium (*CC*) is rich in membrane particles. The outside leaflet (*B* face) of the fractured rod inner segment (*IS*) plasma membrane appears relatively smooth. Membrane particles are typically found in high numbers on the cytoplasmic leaflet (*A* face) of fractured membranes. The inset shows the cytoplasmic leaflet (*A* face) of the rod inner segment plasma membrane, which is identifiable by the presence of mitochondria (*mit*). Rod outer segments contain disks, seen in cross-fracture in the center of the illustration. In this figure and the next two, the micrographs are so oriented that the platinum shadow always comes from the bottom.  $\times 20,000$ . Inset  $\times 64,000$ .

TABLE III  
Summary of Experimental Conditions and Results of the Freeze-Fracturing Experiments

Experimental conditions	Number of experiments						
	(1)	(2) Control	(3) Experimental	(4) Control	(5) Experimental	(6) Control	(7) Control
Fixation right after dissection of retina	4°C, 1 h Karnovsky's fixative	4°C, 16 h 4% formaldehyde	4°C, 16 h 4% formaldehyde	-	-	-	-
5-min washes once in 0.1 M cacodylate	-	-	yes	-	-	-	-
once in 0.01 M glycine in phosphate saline	-	-	-	-	-	-	-
once in phosphate saline	-	-	-	-	-	-	-
Type of rabbit antibodies with which the retina was treated	-	-	Specific	Nonspecific	Specific	Nonspecific	Specific
Temperature and length of the treatment	-	-	Room temperature 3 h	4°C, 14 h	4°C, 14 h	4°C, 14 h	4°C, 14 h
Three 5-min washes in phosphate saline	-	-	Room temperature	4°C	4°C	4°C	4°C
Incubation of retina in Ringer solution	-	-	-	-	-	32°C, 1/2 h	32°C, 1/2 h
Fixation right after incubation	-	-	4°C, 1 h Karnovsky's fixative	4°C, 1 h Karnovsky's fixative	4°C, 1 h Karnovsky's fixative	4°C, 1 h Karnovsky's fixative	4°C, 1 h Karnovsky's fixative
Structure of rod outer segment membrane as revealed in freeze-fracturing experiments	see Fig. 8 a	same as 1.	see Fig. 8 b	same as 1.	same as 1.	same as 1.	see Fig. 8 c

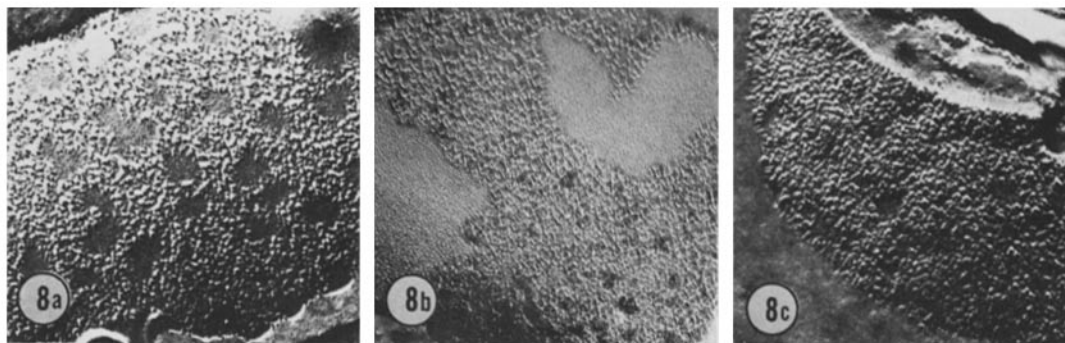


FIGURE 8 (a) Cytoplasmic leaflet (A face) of the mouse ROS plasma membrane. Mouse retina was fixed with Karnovsky's fixative containing glutaraldehyde at 4°C before freeze fracturing. Note the patchy distribution of membrane particles.  $\times 62,500$ . (b) Cytoplasmic leaflet of the mouse ROS plasma membrane. Freeze fractured after 16-h formaldehyde treatment at 4°C and 3-h treatment with specific IgG's at room temperature.  $\times 62,500$ . (c) Cytoplasmic leaflet of the mouse ROS plasma membrane, freeze fractured after 14-h incubation with specific IgG's at 4°C, 30-min incubation at 32°C in Ringer's solution and 1-h fixation at 4°C in Karnovsky's fixative.  $\times 62,500$ .

the uniform distribution seen by immunolabeling. The possibility of rearrangements of membrane constituents after fixation in glutaraldehyde during the rapid freezing of samples before freeze-fracturing has not been clearly ruled out, but the fact that glutaraldehyde deprives rhodopsin of its freedom of both rotational and translational motions (23, 24, 25) seems to make that a rather remote possibility.

#### *Localization of Rhodopsin with Respect to the Lipid Core of the Membrane*

Although the staining seemed to be much heavier on the outside of the rod plasma membrane and on the cytoplasmic side of the disk membrane, the possibility of nonuniform distribution of antibody-peroxidase conjugates across membrane barriers prohibits us from drawing any conclusions about the location of rhodopsin relative to the lipid core based on the data from these labeling experiments alone. We have already noted that the staining on disk membrane was superimposed on a background distribution of electron-opaque material between the disks, even in control retinas which had not been exposed to any antibodies or label molecules. The density of the interdisk cytoplasm is higher than that of the intradisk space.

In freeze-fracturing experiments, we observed, in both mouse and cow retinas, many particles on the cytoplasmic leaflet of both the disk mem-

brane and the rod outer segment plasma membrane and the outer leaflet of ROS plasma membrane (B face) to be relatively smooth (Fig. 7). The cytoplasmic leaflet of the disk membrane could be identified because the rims of individual membrane sacs could often be seen (Fig. 9). The inner surface of the disk is homologous to the outer surface of the plasma membrane and the cytoplasmic surfaces of both the disk membrane and the ROS plasma membrane are equivalent. When isolated bovine disks were deep-etched after freeze fracturing, both true surfaces of disk membranes were exposed and shown to be smooth. The rough A faces and smooth B faces revealed in freeze-fracturing experiments were always found beneath the etched true surfaces. These observations support the interpretation that the A and B faces revealed in both freeze-fracturing and freeze-etching experiments represent the hydrophobic inner faces of the bilayer (26).

In retinas of healthy mice fixed either in Karnovsky's fixative or in 4% formaldehyde, we found the intradisk leaflet of disk membranes to be smooth and the cytoplasmic leaflet closely packed with particles. We occasionally also observed pits on the intradisk leaflet of the fractured disk membrane when the shadowing conditions were appropriate (Fig. 9). Severe vitamin A deficiency has been reported to cause anatomical degeneration of rods in rats (27). When the retinas of mice that had been on a vitamin A-deficient

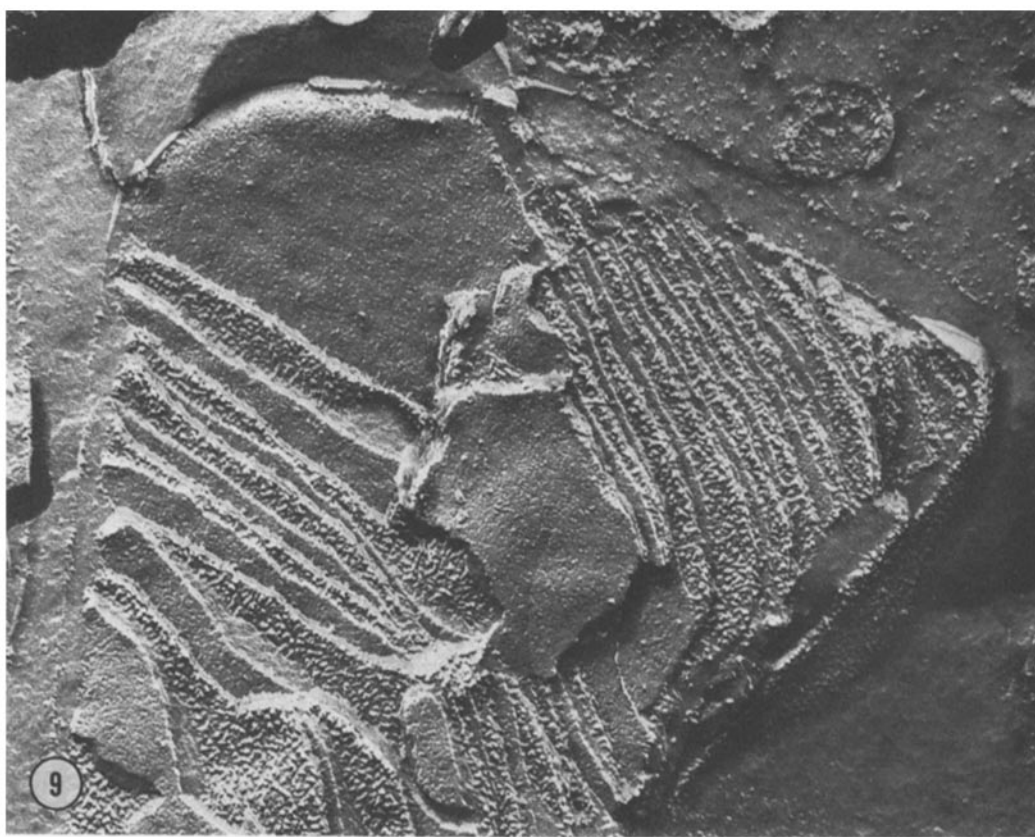


FIGURE 9 Freeze-fractured mouse disk membranes. The leaflet adjacent to the interdisk cytoplasm (A face) contains closely packed membrane particles, whereas pits are abundant on the leaflet adjacent to the intradisk space (B face).  $\times 81,600$ .

diet for 9 mo were freeze fractured, many distended vesicles and tubules together with some intact disks were seen in the rods. In addition, we found islands of smooth areas on the cytoplasmic leaflet of the fractured disk membranes. The significance of the disappearance of some of the membrane particles and of the distribution of the remaining ones will be discussed later.

The fact that specific Fab-peroxidase stained the outside as well as the cytoplasmic side of the ROS plasma membrane and the disk membrane, taken together with the asymmetry in the particle distribution in ROS plasma membrane and disk membranes as revealed by freeze-fracturing experiments, suggested to us the possibility that a rhodopsin molecule has antigenic sites recognized by Fab-peroxidase conjugates on both sides of the membrane. This conjecture was further encouraged by the fact that the concentration of

rhodopsin as determined in photodichroism studies agreed well with that calculated from the X-ray diffraction data if one assumed that there was only one layer of rhodopsin in each disk membrane (5).

## DISCUSSION

Dewey et al. (8) have previously reported the localization of rhodopsin antibody in the frog retina. Using highly purified rabbit antiovine rhodopsin antibodies, we now show the distribution of rhodopsin in cow and mouse retinas. In both cases we find staining of the specific univalent Fab-peroxidase conjugates on the plasma membrane of the rod outer and inner segments, the connecting cilia, as well as on the disk membranes. Control experiments exclude the possibility of nonspecific precipitation of diaminobenzidine in the absence of the peroxidase-catalyzed

reaction. Other controls revealed that endogenous peroxidase activity is found only at the rim of the disks where the radius of curvature is small, and also showed that peroxidase and non-specific rabbit Fab-peroxidase conjugates adhere nonspecifically only to the outmost surfaces of the retina and to a "sticky" material spaced regularly in the narrow space between disks. We can therefore conclude that there are rhodopsin molecules on the rod cell membrane as well as on the disk membranes. The gradient of staining we observed on the rod cell membrane might not faithfully reflect the local distribution of rhodopsin because of the existence of possible difficulty for the Fab-peroxidase conjugates to penetrate into the tissue.

It is not possible to draw any conclusions about the location of rhodopsin relative to the lipid core of the membrane based on the labeling experiments alone. However, by combining the results of freeze-fracturing and deep-etching experiments with those of the immunolabeling experiments we can make some suggestions about the localization of rhodopsin in membranes. There is a good correlation between immunohistochemically detected rhodopsin and the membrane particles found in rod outer segments in freeze-fracturing experiments. The particles on the cytoplasmic leaflet of the freeze-fractured rod outer segment cell membrane were normally found closely packed, interspersed with small smooth areas in retinas treated with specific IgG's at 4°C before fixation, as well as in retinas that have not been exposed to any antibodies. Treating mouse retinas with specific IgG's at room and at higher temperature, however, resulted in a coarse clustering of these particles on the plasma membrane of rod outer segments. This indicates that the particles seen in freeze-cleaved ROS plasma membrane represent either rhodopsin molecules or membrane structures resulting from the presence of rhodopsin molecules. Correlation between rhodopsin and the particles of the cytoplasmic leaflet of the fractured disk membrane has also been suggested by freeze fracturing of retinas of vitamin A-deficient mice. It has been reported (27) that rats on a vitamin A-deficient diet first start to lose rhodopsin and, as the deficiency persists, also have lowered amounts of opsin, with a concomitant anatomical deterioration of the rod outer segments. In freeze-fractured retina of mice that had been on a vitamin A-deficient diet for 9 mo, in addition to find-

ing many distended vesicles in the rod outer segments we also could see some relatively intact disks which contained, however, small smooth areas devoid of particles on the cytoplasmic leaflet of the fractured membrane. Thus the fall of opsin level is accompanied by the disappearance of some of the membrane particles on the disk membrane, an indication that the membrane particles observed in freeze-fracturing experiments are indeed manifestations of the presence of rhodopsin in the membrane. The observations that protein-free lipid membranes give the appearance of smooth surfaces after freeze fracturing, while liposomes containing purified rhodopsin showed membrane particles on the fractured face but not on the outer surface that was exposed after etching (19) also serve as a supporting evidence that the membrane particles are correlated to rhodopsin molecules.

When the shadowing conditions were appropriate, we observed pits on the inside leaflet of the fractured disk membranes in addition to particles on the outside leaflet of these membranes. In freeze-etching experiments we also observed that the deep-etched outer surfaces of the disk membranes were smooth. Since the concentration of rhodopsin as measured in photodichroism experiments agreed well with that calculated from the X-ray diffraction data if one assumed that there was only one layer of rhodopsin in each disk membrane, the morphology of disk membranes revealed in freeze-fracturing experiments would tend to suggest that rhodopsin molecules penetrated deeply into the hydrophobic region of the disk membrane. In the immunolabeling experiments, we observed staining on the cytoplasmic side of both the plasma membrane and the disk membrane, much heavier staining on the outside of the plasma membrane and less intense staining on the intradisk side of the disk membrane. The different degrees of labeling could be attributed to difficulties in the penetration of the label molecules. However, the fact that both sides of the membrane are labeled by the antibody conjugates is clear. Combined with the observations in freeze fracturing and etching, the assumption that there is just one layer of rhodopsin molecules per membrane would tend to suggest that rhodopsin molecules may in fact extend through the entire thickness of the membrane.

We would like to bring to attention one last point of interest here. It has been shown that rhodopsin can rotate and diffuse rather freely in

the disk membrane (23, 25) and that the rod outer segment plasma membrane at room temperature is in such a fluid state that cross-linkage of rhodopsin by bivalent antibodies could happen. However, the membrane particles revealed in freeze-fracturing experiments remained in patches that formed a certain pattern in the rod outer segment cell membrane. In disk membranes of mice on vitamin A-deficient diet which had fewer membrane particles, the nearest-neighbor distance between the particles remained small and constant and these membrane particles formed patches. The fact that when the concentration of rhodopsin was low in a membrane the membrane particles stayed in patches rather than being dispersed uniformly over the whole surface of the membrane is interesting both from the point of view of the membrane architecture and from the point of view of the study on the primary visual responses. Such observations encourage speculation about the interactions between and cooperativity among rhodopsin molecules in the membrane.

As illustrated in the previous discussions, the poor penetration of tissues by conjugates of antibodies and label molecules imposes a serious limitation on this labeling technique. The problem is not sufficiently alleviated by fixation of the tissues in formaldehyde instead of glutaraldehyde or by use of conjugates of smaller antibodies (Fab with mol wt of 55,000) and small label molecules (peroxidase with mol wt of 40,000). Furthermore, inside the rod inner segment where we expect that some rhodopsin molecules are being synthesized and transported (28), it became very difficult to identify small quantities of Fab-peroxidase conjugates scattered diffusely inside the cell. To counter these shortcomings an attempt has been made to label rhodopsin with specific rabbit anti-bovine rhodopsin IgG's and ferritin-labeled goat IgG's specific for rabbit IgG on frozen ultrathin sections of retina. These results will be reported *in extenso* elsewhere.

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