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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.
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 antiserum 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 column (Bio-Rad, bio-gel HTP Bio-Rad Laboratories, Richmond, Calif.) column (2.5 cm x 4 cm) equilibrated with 1% emulphogene in 0.01 M imidazole, pH 7. The rhodopsin peak was eluted before applying the NaCl gradient. 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 ) 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 ben egg white lysozyme (Sigma Chemical Co., St. Louis, Mo., No. L-6876). An excess of lysozyme (2:1 by weight) was...

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**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 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.

**Materials and Methods**

Rabbits were injected with a preparation of solubilized bovine retinal disks. Specific antibodies were isolated from the antiserum 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 1.

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

| bovine retinas |
|----------------|
| ROS preparation |
| digitonin solution |
| immunization of rabbits |
| antisera (M.M. 2.) |
| determination of cross reactivity (M.M. 4.) |
| purified rabbit IgG’s specific for bovine rhodopsin (M.M. 3.) |
| papain digestion |
| specific Fab’s |
| specific Fab-peroxidase |
| direct labeling (M.M. 5.) |
| emulphogene solution |
| hydroxylapatite column |
| purified rhodopsin |
| glutaraldehyde + lysozyme followed by washes |
| purified rabbit IgG’s specific for bovine rhodopsin (M.M. 3.) |
| goat anti-rabbit IgG IgG-peroxidase |

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

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used. Rhodopsin in emulphogene has a low density, and the excess lysozyme insured that the immunoadsorbent would not float. The insoluble protein gel was first washed with the eluting salt solutions (2.5 and 5 M MgCl₂) followed by 0.85% saline until the OD₂₅₀ 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 OD₂₅₀ reading of less than 0.04. The specific antibodies were then eluted using 2.5 M and 5 M MgCl₂ 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 antiserum against various substances was also tested.

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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 antisera. 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% CaCl₂), 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 N₂, 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, A₄₀₀:A₅₀₀ and A₄₅₀:A₅₀₀, thus serve as a criterion of the purity of the rhodopsin preparation. The A₄₀₀:A₅₀₀ 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 ration of A₄₀₀ and A₅₀₀, the A₂₈₀:A₃₀₀ 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-
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\(^2\)-peroxidase staining in 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

\(^2\) Rabbit antiovine rhodopsin IgG will be referred to as specific IgG, and rabbit antiovine rhodopsin Fab as specific Fab.

FIGURE 1  Tracings of OD\(_{280}\) readings along Coomassie brilliant blue stained SDS acrylamide gels. (A) Bovine rod outer segment preparation containing roughly 50 \(\mu\)g rhodopsin, (B) about 50 \(\mu\)g 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.
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 H2O2 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 H2O2 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 |
|------------|--------------|-------------------------|
| Direct labeling | Specific Fab-peroxidase, Diaminobenzidine + H$_2$O$_2$ | Specific sites: disk membranes, cell membrane of ROS and inner segments, connecting cilium |
|             | None, Same as above | Nonspecific sites: patches between disks, outmost surfaces of retina |

Control

1. None, Same as above
2. Nonspecific, Same as above
3. Peroxidase only, Same as above
4. BSA-peroxidase, Same as above
5. BSA wash, specific IgG-peroxidase (direct labeling), Same as above

Indirect labeling

| Experiment | Reagents used | Distribution of staining |
|------------|--------------|-------------------------|
| Indirect labeling | Specific IgG + goat IgG-peroxidase | Specific sites: disks and ROS membranes that are close to the surface |
| 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 membrane 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. × 90,000.

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. The rod outer segment plasma membrane will variously be referred to as ROS plasma membrane, ROS membrane, or rod plasma membrane. The membrane limiting the disks will always be described as "disk membrane" in the description of freeze-fracturing 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.).
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. × 80,000. Staining on the membrane of the rod inner segment membranes and of a cilium is evident in the inset. × 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. × 80,000.
regions of close packed particles (Fig. 8a). 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. 8b). 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 expected 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. 8c). 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

![Figure 7](image-url)
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 | 4°C, 16 h | 4°C, 16 h | – | – | – | – |
|                         | Karnovsky's fixative | 4% formaldehyde | 4% formaldehyde | – | – | – | – |
| 5-min washes | – | – | yes | – | – | – | – |
| once in 0.1 M cacodylate | – | – | – | – | – | – | – |
| 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 | 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, ½ h | 32°C, ½ h | – |
| Fixation right after incubation | – | – | 4°C, 1 h | 4°C, 1 h | 4°C, 1 h | 4°C, 1 h | 4°C, 1 h |
| Karnovsky's fixative | Karnovsky's fixative | Karnovsky's fixative | Karnovsky's fixative | Karnovsky's fixative |
| Structure of rod outer segment membrane as revealed in freeze-fracturing experiments | see Fig. 8a | same as 1. | see Fig. 8b | same as 1. | same as 1. | same as 1. | see Fig. 8c |
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 membrane 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
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.  

DISCUSSION

Dewey et al. (8) have previously reported the localization of rhodopsin antibody in the frog retina. Using highly purified rabbit antibovine 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
deterioration of the rod outer segments. In freez-
as the deficiency persists, also have lowered
of the cytoplasmic leaflet of the fractured disk
Correlation between rhodopsin and the particles
cleaved ROS plasma membrane represent either
This indicates that the particles seen in freeze-
Treating mouse retinas with specific IgG's at
have not been exposed to any antibodies.
the cytoplasmic leaflet of the freeze-fractured
brane particles found in rod outer segments in
chemically detected rhodopsin and the mem-
There is a good correlation between immuno-
periments we can make some suggestions about
periments with those of the immunolabeling ex-
results of freeze-fracturing and deep-etching ex-
core of the membrane based on the labeling ex-
periments alone. However, by combining the re-
results of freeze-fracturing and deep-etching ex-
periments we can make some suggestions about
localization of rhodopsin in membranes.
There is a good correlation between immuno-
chemically detected rhodopsin and the mem-
brane 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, re-
sulted 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 re-
sulting 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 vita-
min A-deficient diet for 9 mo, in addition to find-
ing many distended vesicles in the rod outer seg-
ments we also could see some relatively intact
disks which contained, however, small smooth
areas devoid of particles on the cytoplasmic leaf-
et 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 experi-
ments are indeed manifestations of the presence
of rhodopsin in the membrane. The observations
that protein-free lipid membranes give the ap-
pearance of smooth surfaces after freeze fractur-
ing, while liposomes containing purified rhodop-
sin showed membrane particles on the fractured
face but not on the outer surface that was exposed
after etching (19) also serve as a supporting evi-
dence that the membrane particles are correlated
to rhodopsin molecules.

When the shadowing conditions were appro-
priate, we observed pits on the inside leaflet of
the fractured disk membranes in addition to par-
ticles 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 ex-
periments 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 pen-
etrated deeply into the hydrophobic region of the
disk membrane. In the immunolabeling exper-
iments, 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 mole-
cules. However, the fact that both sides of the
membrane are labeled by the antibody conjug-
gates is clear. Combined with the observations in
freeze fracturing and etching, the assumption
that there is just one layer of rhodopsin mole-
cules 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 antirhodopsin 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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