INVESTIGATIONS ON RHODOPSIN AND BACTERIORHODOPSIN

I. Ultrastructural Localization of Rhodopsin in Vertebrate Retina

II. The Isomeric Configuration of the Bacteriorhodopsin Chromophore

Thesis by

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In Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1974

(Submitted May 30, 1974)

ACKNOWLEDGMENTS

First, I would like to thank the professors on my thesis committee: Dr. Max Delbrück for the excellent introduction he gave me on subjects such as membranes, nerve excitation, vision and biology in general, and for his advice over these four years; Dr. Jean-Paul Revel, for his in-depth instructions on cell biology and electron microscopy, and for his tremendous help and advice on my research; Dr. Dreyer for his help and advice on biochemistry; Dr. Owen for his generous offer of professional help on immunology; and Dr. Zweig for the many interesting discussions on physics as well as on biology.

My graduate study was made financially possible by the McCallum Fund and the Calbiochem fellowship. My work at graduate school was also made a lot easier with the very kind help of Mrs. Jeanette Navest, Mr. Pat Koen, Mrs. Phyllis Yip and Mr. Mike Rees. The task of thesis writing was less formidable than it might have been, with Miss Sue Kolden as my thesis typist. To them I am truly grateful. I also owe my thanks to Pamela Hoch, Lih-Lih Chang and Dr. Revel who joined my ambitious operation of denucleating two thousand chicken eyes, and to Libby Blankenhorn, Marcia Miller, and Sue Rosenberg who lent me their hands when I shivered at a screaming rabbit and shared my excitement over the anaphylactic shock of my rabbits. To Henry Huang and Bob Molday, I wish to express my appreciation for their consultation on rhodopsin biochemistry and for their

kindness in doing the first isoelectric focusing experiment on my rhodopsin sample. I also wish to thank Franz Hubmann and Klaus Thomson for helping me with thin layer chromatography experiments.

I am especially thankful to Dr. Meredithe Applebury in the Max-Planck Institute at Göttingen who sent me her manuscript before publication and also milligrams of detergent-free rhodopsin. I also owe a debt of gratitude to Dr. W. Stoeckenius at UC Medical School at San Francisco, who gave me the Halobacterium strains, rescued me when I killed all my bacteria with Difco peptone in Cold Spring Harbor, grew many liters of bacteria in his fermentor for the thin layer chromatography experiments, and held many interesting discussions with me on the bacteria. I also wish to thank Dr. D. Oesterhelt at the Max-Planck Institute at München, for many open communications with me on the purple membrane research and for his checking under his standard culturing conditions my mutant strains devoid of bacteriorhodopsin.

The experiments described in Chapter 2 of this thesis were done in collaboration with Dr. M. Schutz, R. G. Painter, K. T. Tokuyasu and S. J. Singer. I would like to thank Dr. Singer for providing this opportunity of collaboration and the laboratory facilities. I enjoyed very much working together with Mike, Richard and Tok in La Jolla. I would like to thank them for their hospitality and for the many discussions.

Finally, I wish to thank my family. I like to express my deep gratitude to my husband, Yuh-Nung Jan, for his understanding and

patience. Among the numerous favors he did for me are the many before-dinner discussions over the details of my experiments whenever I ran into obstacles. And I would like to thank my late father and my mother for the intensive care and education they gave me, and for their trust in me as they let me make decisions for myself during and after my high school years.

ABSTRACT (Chapter I)

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 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 disc 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 disc 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 types of artifact, unless controlled with extreme care.

ABSTRACT (Chapter II)

A method for cutting thin sections of frozen glutaraldehyde-fixed tissue developed by Tokuyasu and collaborators has made it possible to label antigens on thin sections of a tissue. Thin sections of frozen retina obtained by this method were treated first with rabbit antibodies specific for bovine rhodopsin, then with conjugates of ferritin and goat antibodies specific for rabbit antibody. Both mouse and bovine retina were labeled specifically by this method. The ferritin label on mouse retina was less heavy than that on bovine retina. Chicken retina was not labeled at all by these reagents. Ferritin label was formed on the disc membranes, on rod outer and inner segment cell membranes, on the inside of the rod inner segment, on the outer membrane of the connecting cilium and in the cytoplasmic bridge of the mouse retina.

ABSTRACT (Chapter III)

Oesterhelt and Stoeckenius in 1971 found a pigment in the cell membrane of Halobacterium halobium which they called bacteriorhodopsin because it resembled rhodopsin in many aspects. We have studied the isomeric configurations of its chromophore by thin layer chromatography of the retinal itself and of the retinal oxime derivatives. Under physiological conditions the dark-adapted bacteriorhodopsin contains 13-cis retinal, in contrast to the 11-cis retinal of rhodopsin. Upon illumination the 13-cis retinal is converted to all-trans retinal. Substantial thermal isomerization of retinal occurs if the extraction procedure is performed at room temperature. Implications of the different isomeric forms found in bacteriorhodopsin and rhodopsin are discussed.

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To my parents

GENERAL INTRODUCTION

In both vertebrate and invertebrate, highly specialized cells have evolved for the purpose of detecting light signals from the environment (1). These cells are generally elongated in shape and contain numerous membrane folds lying perpendicular to the long axis of the cell. These membranes accommodate a high density of visual pigment molecules. The chromophore, retinal (vitamin A aldehyde), is in a cis isomer form in the dark-adapted state (2). Light "bleaches" the visual pigment with a quantal efficiency of 0.6-1 (3), presumably by isomerizing the chromophore in the pigment to all-trans retinal. The photoreceptor cell responds by producing a membrane potential change upon illumination, which triggers the secondary neurons and passes the electrical signal to the brain (4).

The intriguing problems about vision at the level of the receptor cells include the mechanism by which the photochemical acts in the visual pigment, induces the membrane potential change, the mechanism of adaptation to light or darkness achieved at the level of the photoreceptor cell and the regeneration of cis-retinal-opsin.

Extensive studies have been done on one type of vertebrate photoreceptors, the retinal rod. Each rod contains a stack of several hundred isolated membrane sacks (the "discs") (5). The visual pigment, rhodopsin, has a high density in the disc membrane (6). It is free to rotate around an axis perpendicular to the membrane (7) and to diffuse laterally in the plane of the membrane (8). The vertebrate rod is specialized in such a way that more than 80% of the total

protein content of the disc membranes in the retinal rod are rhodopsin (9). All of the discs are segregated and closely packed in one portion of the cell (the rod outer segment). The other portion of the cell, the rod inner segment, contains mitochondria, endoplasmic reticulum and other organelles, and is connected to the rod outer segment by the stalk of a cilium and sometimes also a cytoplasmic bridge (10). This anatomical arrangement allows one to obtain easily a relatively pure preparation of resealed rod outer segments or a preparation of rhodopsin (11). This greatly facilitates different experimental analyses of the system, such as X-ray diffraction studies (12) and biochemical studies of rhodopsin (13).

A great deal of information on retinal rods has accumulated. At the level of the visual pigment extensive studies have been performed on various intermediates of the photochemical reactions of rhodopsin (14), and on the regeneration mechanism of rhodopsin (15). At the level of the photoreceptor cell, it has been learned that light reduces the local sodium conductance of the rod outer segment cell membrane, thus reducing the dark current flowing into the outer segment and resulting in hyperpolarization of the rod at the synaptic end (16). The amplitude of the photocurrent (reduction of the dark current) increases with flash energy and saturates when about 30 photons are absorbed by each rod. The maximum response amplitude is that just sufficient to cancel the dark current. The rate of rise of a response is proportional to the

flash energy up to the level of 10^5 photons absorbed per rod, where hyperbolic rate saturation ensues. The response continues to increase in duration with even more intense flashes until at the level of 10^7 photons absorbed per rod, they last longer than 50 min (17).

A big gap still remains in our knowledge about the mechanism by which rhodopsin induces a conductance change in the cell membrane. This question can be broken down into three: (1) How does rhodopsin in the disc work to optimize the system so that even a few photons can be detected by the organism? (2) How is the light induced signal transmitted from discs bearing rhodopsin to the separate rod cell membrane housing the "sodium channels"? (3) How does bleaching of 1% of the rhodopsin cause a very large decrease in visual sensitivity?

Various models have been suggested. With respect to the first and third step, possibilities of cooperativity among rhodopsin molecules have been discussed (18). As to the second step, models suggesting a light induced permeability change of disc membranes to certain ions have been suggested (19) and examined experimentally (20).

In the work leading to this thesis, my effort has been directed at gaining more detailed information about the distribution of rhodopsin in retinal rods. An attempt was also made to establish a model system for the visual pigment rhodopsin. I shall describe the rationales of these experiments in the following:

Extensive studies using X-ray diffraction and microspectrophotometric techniques have been performed to establish the orientation and arrangement of rhodopsin in disc membranes. The dynamic state of rotation and lateral diffusion of rhodopsin has also been investigated. Questions remain, however, as to the localization of rhodopsin relative

to the lipid core of the membrane, owing to the lack of phase information in low angle X-ray diffraction studies (21). Information about the distribution of rhodopsin in places other than the discs is also incomplete and can best be obtained in immunolabeling experiments. Immunolabeling experiments using fluorescent reagents were performed on the frog retina in 1969 (22). It was thought that immunolabeling experiments using the electron microscope could help to answer questions about the location of rhodopsin in the membrane and about the distribution of rhodopsin in the rod.

The location of rhodopsin in the membrane is of interest because of the possible function of rhodopsin of carrying light induced changes in permeability to messengers. Results of rhodopsin-localization experiments might be useful in testing another model. Rhodopsin in normal retinas has been shown to be closely packed with a nearest neighbor distance of about 70 Å by X-ray diffraction studies. In mice deficient in vitamin A, however, the level of rhodopsin and opsin drops considerably before any anatomical derangement occurs, thus presumably resulting in a much lower density of rhodopsin in the discs (23). If cooperativity among rhodopsin is essential for vision, animals with reduced levels of rhodopsin might have to keep the remaining rhodopsin molecules in clusters with small nearest neighbor distance to optimize its ability to detect light. This could be checked experimentally. It must be realized, however, that any observations on the stationary localization and distribution of rhodopsin by immunolabeling experiments can at most be taken as circumstantial evidence.

These experiments are designed to establish within the limitation of the techniques a detailed map of the rhodopsin distribution in retinal rods, so as to provide some backgrounds for the model building tasks.

Our efforts to localize rhodopsin also provided a rare chance to examine closely the techniques employed in studying the distribution of membrane constituents at the electron microscope level, to estimate their limits of resolution, and to reveal artifacts introduced by the experimental procedure. With the help of the massive amount of information accumulated about rhodopsin molecules in retinal rods, it might be relatively easy to separate experimental artifacts and to test the limitations of different techniques. Besides immunolabeling techniques, freeze-fracturing and freeze-etching techniques have been developed recently. It has been shown that the fractured surface revealed in those experiments corresponds to the inner hydrophobic face of a bilayer membrane (24). The membrane particles observed on the fractured face have been suggested to represent membrane protein molecules (25). If one can establish a correspondence between membrane particles observed in rod outer segment membranes and rhodopsin molecules, one would be able to study the distribution of rhodopsin in the membrane without the tag of antibody and labeling molecules. This correspondence might be established with the help of purified antibodies specific for rhodopsin and the substantial understanding of the system acquired from the many years of intensive vision research.

The last part of this thesis deals with a project motivated by the desire to search a model system for the light-sensing and signal-transducing mechanism of rhodopsin. Halobacterium halobium synthesizes purple membrane as part of its cell membrane when the oxygen tension in the culturing medium is low. The sole protein constituent of purple membrane, bacteriorhodopsin, resembles the visual pigment rhodopsin in many ways. Bacteriorhodopsin is closely packed in purple membrane, as rhodopsin is closely packed in disc membranes (26). Bacteriorhodopsin has a molecular weight of 26,000, as compared to 30,000 to 40,000 for the molecular weight of cattle or frog rhodopsin. Bacteriorhodopsin also has a chromophore, retinal, attached via Schiff base to a lysine residue of the protein (27). Bacteriorhodopsin absorbs maximally at 560 nm, is light-sensitive, and "bleaches" to a 412 nm form (28). Unlike vertebrate rhodopsin, the retinal moeity is not dissociated from bacteriorhodopsin by light. Bacteriorhodopsin also differs from many invertebrate rhodopsins such as squid rhodopsin by the fact that bacteriorhodopsin does not stay "bleached" after the absorption of light, rather, it regenerates spontaneously to the 560 nm form. It has been found recently that bacteriorhodopsin is used in the bacterium not only for "vision" in phototaxis but also for energy transduction, acting as a light-driven proton pump, when there is plenty of light energy but not enough oxygen or other energy sources (28).

If bacteriorhodopsin proves to be a reasonably close analog of rhodopsin, and if it is possible to find mutants of Halobacterium halobium which produce altered species of bacteriorhodopsin, it would be very interesting to study with the help of appropriate mutants the protein chemistry of bleaching and the concomitant proton translocation. Preliminary experiments indicate that nitrosoguanidine mutagenesis induces with a reasonable frequency mutations in genes related to the synthesis of bacteriorhodopsin in Halobacterium halobium. Bacteriorhodopsins from different mutants can be compared by SDS acrylamide gel electrophoresis, isoelectric focusing, and by their cross-reactivity with wild-type bacteriorhodopsin as judged by the pattern of rabbit antibody-antigen precipitate arcs in immunoelectrophoresis experiments.

A key question in building the analogies between rhodopsin and bacteriorhodopsin is whether their retinal chromophores are both in the 11-cis configuration in the dark adapted pigment and whether their chromophores are both isomerized to the all-trans form by light. Chapter III of this thesis describes thin layer chromatography experiments designed to answer this question.

References

1. "The Eye" vol. II "The visual process" 1962. Edited by H. J. A. Davson. Academic Press, New York and London.

2. G. Wald. 1968. Molecular basis of visual excitation. Science 162, 230-239.

3. H. J. A. Dartnall. 1968. The photosensitivities of visual pigments in the presence of hydroxylamine. Vision Res. 8, 339-358.

4. T. Tomita. 1970. Electrical activity of vertebrate photoreceptors. Quart. Rev. Biophys. 3, 179-222.

5. A. I. Cohen. 1968. New evidence supporting the linkage to extracellular space for outer segment saccules of frog cones but not rods. J. Cell Biol. 37, 424-444.

6. P. A. Liebman. 1962. In situ microspectrophotometric studies on the pigments of single retinal rods. Biophys. J. 2, 161-178.

7. R. A. Cone. 1972. Relaxation times of rhodopsin detected by photodichroism. Biophys. Soc. Abs. 12, 246a.

8. M. Poo, R. A. Cone. 1973. Lateral diffusion of rhodopsin in the visual receptor membrane. J. Supramol. Struct. 1, 354.

9. W. E. Roberson, A. Gordon-Walker, and D. Bownds. 1972. Molecular weight of frog rhodopsin. Nat. New Biol. 235, 112-114.

10. M. F. Moody, and J. D. Robertson. 1960. The fine structure of some retinal photoreceptors. J. Biophys. Cytol. 7, 87-106.

11. D. S. Papermaster, and W. J. Dreyer. 1974. Rhodopsin content in the outer segment membranes of bovine and frog retinal rod. Biochemistry, in press.

12. C. R. Worthington. 1973. X-ray diffraction studies on biological membranes. Curr. Topics Bioengnr. 5, 1-39.

13. R. A. Morton. 1972. The chemistry of the visual pigments. In "Handbook of sensory physiology" vol. VII, edited by H. J. A. Dartnall. Springer-Verlag, pp. 33-68.

14. E. W. Abrahamson, and J. R. Wiesenfeld. 1972. The structure, spectra and reactivity of visual pigments, ibid., pp. 69-121.

15. Ch. Baumann. 1972. The regeneration and renewal of visual pigment in vertebrates, ibid., pp. 395-416.

K. Hong, and W. L. Hubbell. 1973. Lipid requirements for rhodopsin regenerability. Biochem. 12, 4517-4523.

16. W. A. Hagins, R. D. Penn, and S. Yoshikami. 1970. Dark current and photocurrent in retinal rods. Biophys. J. 10, 380-412.

17. R. D. Penn, and W. A. Hagins. 1972. Kinetics of the photocurrent of retinal rods. Biophys. J. 12, 1073-1094.

18. G. Wald. 1954. On the mechanism of the visual threshold and visual adaptation. Science 119, 887-892.

S. Rosenberg. 1966. In "Advances in radiation biology" vol. II. Academic Press, pp. 193-241.

G. W. Robinson. 1974. Rhodopsin cooperativity in visual response. Vision Res., in press.

19. W. A. Hagins. 1972. The visual process: Excitatory mechanisms in the primary receptor cells. Ann. Rev. Biophys. Bioengnr. 1, 131-158.

20. G. Falk, and P. Fatt. 1973. An analysis of light-induced admittance changes in rod outer segments. J. Physiol. 229, 185-220.

21. C. R. Worthington, G. I. King, and T. J. McIntosh. 1973. Direct structure determination of multilayered membrane-type systems which contain fluid layers. Biophys. J. 13, 480-494.

22. M. M. Dewey, P. K. Davis, J. K. Blasie, and L. Barr. 1969. Localization of rhodopsin antibody in the retina of the frog. J. Mol. Biol. 39, 395-405.

23. J. E. Dowling, and G. Wald. 1960. The biological function of vitamin A acid. Proc. Nat. Acad. Sci. U.S.A. 46, 587-608.

24. P. Pinto da Silva, and D. Branton. 1970. Membrane splitting in freeze-etching. J. Cell Biol. 45, 598-605.

25. L. H. Engstrom. 1970. Structure in the erythrocyte membrane. Ph.D. dissertation. Univ. of California at Berkeley.

V. T. Marchesi. 1970. Isolation and characterization of a sialic acid-containing glycoprotein from red cell membranes. Fed. Proc. 29, 600.

T. W. Tillack, R. E. Scott, and V. T. Marchesi. 1970. Cell membrane ultrastructure studied by freeze-etching. Fed. Proc. 29, 489.

26. A. E. Blaurock, and W. Stoeckenius. 1971. Structure of the purple membrane. Nat. New Biol. 233, 152-155.

27. D. Oesterhelt, and W. Stoeckenius. 1971. Rhodopsin-like protein from the purple membrane of Halobacterium halobium. Nat. New Biol. 233, 149-152.

28. D. Oesterhelt, and W. Stoeckenius. 1973. Functions of a new photoreceptor membrane. Proc. Nat. Acad. Sci. U.S.A. 70, 2853-2857.

Chapter I

ULTRASTRUCTURAL LOCALIZATION OF RHODOPSIN IN THE VERTEBRATE RETINA

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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 to 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 discs 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 disc 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 discs. 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.

1. Preparation of Bovine Rod Outer Segments

Fresh bovine eyes were purchased at a local slaughterhouse (Quality Meat Packing, 4512 Alcoa, Vernon, Ca.). 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.

2. 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 weeks 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.

3. 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 to 30 mg of rhodopsin were solubilized in 2% emulphogene BC-720 (General Aniline and Film Corporation), 0.01 M imidazole (CalBiochem), pH 7, to a final concentration of about 1 mg/ml rhodopsin. This emulphogene solution (20 to 30 ml) of rhodopsin was centrifuged at 13,000 rpm for 10 min and applied to a hydroxyapatite (Bio-Rad, bio-gel HTP) 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 amino acid composition analysis,[1] 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. They were cross-linked with glutaraldehyde to hen egg white lysozyme (Sigma, No. L-6876). An excess of lysozyme (2:1 by weight) was used. Rhodopsin in emulphogene has a low

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[1]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.

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 $MgCl_2$) followed by 0.85% saline until the 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 supernatant of the washes had an OD_{280} reading of less than 0.04. The specific antibodies were then eluted using 2.5 M and 5 M $MgCl_2$ 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.

4. Cross-reactivity of the Rabbit Antiserum

a) 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.

b) 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 non-specific 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.

5. 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, New Jersey) 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% (w/v) 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 Lab.). 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.

6. 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% $CaCl_2$), 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

N_2, mounted on a stage at liquid nitrogen temperature, and fractured or etched using a Balzers unit.

RESULTS

1. Specificity of the Adsorbed Antibodies

To localize rhodopsin molecules in the retina at the EM 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 experiments indicate that purified antibodies are in the IgG class (Fig. 3).

2. Localization of Rhodopsin in Mouse and Bovine Retinas

Electron-opaque precipitates of peroxidase-catalyzed reaction products were found in both mouse and bovine retina. 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 disc membranes were stained specifically by the Fab-peroxidase conjugates. The cytoplasmic face of the disc membrane was uniformly covered by the peroxidase stain, and a fainter reaction was also seen

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[2]Rabbit antibovine rhodopsin IgG will be referred to as specific IgG, and rabbit antibovine rhodopsin Fab as specific Fab.

on the intradisc face (Fig. 4). In addition, we observed heavy stain patches located between adjacent disc membranes with a fairly regular spacing of a few hundred Å 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 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.

3. 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 discs. 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 discs 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 prior to treatment with specific rabbit IgG-peroxidase conjugates, the interdisc staining was essentially blocked, but not the reaction of the discs 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 discs where the radius of curvature was small showed such

endogenous peroxidase activity. This endogenous peroxidase staining was only obvious at those discs which stayed intact with little intradisc spacing. We do not know whether the decreased level of peroxidase staining at the rim of swollen discs was due to simple geometrical factors or the fact that certain substances were lost and caused the swelling of these discs. 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. Based on these control experiments, we conclude that the staining by rabbit antibovine rhodopsin Fab-peroxidase conjugates of the cell membrane of the rod outer and inner segments, the connecting cilium, and the disc membranes represents the locations of rhodopsin molecules. The patches of staining seen between discs, however, were attributed to the presence of "sticky" material in the narrow interdisc space. The background peroxidase activity present at the rim of the discs made it difficult to judge whether rhodopsin molecules were present there.

4. 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). 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 disc membrane of the rod outer segment are indeed rhodopsin molecules or membrane structures resulting from the 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 [expt. (1), Table III], freeze fracturing showed a uniform distribution of particles with diameter of about 50 Å on the cytoplasmic leaflet of the disc membrane (the half of the membrane adjacent to the interdisc space). A somewhat lower density of similar particles was found on the cytoplasmic leaflet of the fractured rod outer segment plasma membrane.[3] Here, however, the distribution of particles was

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[3]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 discs will always be described as "disc 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 disc 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 disc (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 (27).

patchy. There were islands of membrane devoid of particles separated by regions of close packed particles (Fig. 8a). Because of this discrepancy we had to examine possible artifacts introduced by the observational techniques.

a) POSSIBLE ARTEFACTS 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 [expt. (2), Table III]. However, incubating the formaldehyde-fixed retina with specific bivalent IgG's at room temperature for 3 h before freeze fracturing [expt. (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 disc membrane was not expected nor observed. The cytoplasmic leaflets of disc 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 (MW $\sim$ 40,000) might bind one or more univalent Fab-peroxidase conjugates (MW > 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.

b) 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 prior to a 1 h fixation at 4°C in Karnovsky's fixative [expt. (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 [expt. (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 [expt. (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 [expt. (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. Based on 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 the uniform distribution seen by immunolabeling. The possibility of rearrangements of membrane constituents after fixation in glutaraldehyde during the rapid freezing of samples prior to freeze-fracturing has not been clearly ruled out, but the fact that glutaraldehyde deprives rhodopsin of its freedom of both rotational and translational motions (22, 23) seems to make that a rather remote possibility.

5. 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 disc 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 disc membrane was superimposed on a background distribution of electron-opaque material between the discs, even in control retinas which had not been exposed to any antibodies or label molecules. The density of the interdisc cytoplasm is higher than that of the intradisc space.

In freeze-fracturing experiments, we observed, in both mouse and cow retinas, many particles on the cytoplasmic leaflet of both the disc membrane and the rod outer segment plasma membrane (A face), whereas we found the intradisc leaflet of disc membrane and the outer leaflet of ROS plasma membrane (B face) to be relatively smooth (Fig. 7). The cytoplasmic leaflet of the disc membrane could be identified because the rims of individual membrane sacs could often be seen (Fig. 9). The inner surface of the disc is homologous to the outer surface of the plasma membrane and the cytoplasmic surfaces of both the disc membrane and the ROS plasma membrane are equivalent. When isolated bovine discs were deep-etched after freeze-fracturing, both true surfaces of disc 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 (24).

In retinas of healthy mice fixed either in Karnovsky's fixative or in 4% formaldehyde, we found the intradisc leaflet of disc

membranes to be smooth and the cytoplasmic leaflet closely packed with particles. We occasionally also observed pits on the intradisc leaflet of the fractured disc membrane when the shadowing conditions were appropriate (Fig. 9). Severe vitamin A deficiency has been reported to cause anatomical degeneration of rods in rats (25). When the retinas of mice that had been on a vitamin A-deficient diet for nine months were freeze fractured, many distended vesicles and tubules together with some intact discs are seen in the rods. In addition, we found islands of smooth areas on the cytoplasmic leaflet of the fractured disc 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 disc membrane, taken together with the asymmetry in the particle distribution in ROS plasma membrane and disc 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 disc membrane (5).

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 retina. 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 disc 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 discs where the radius of curvature is small, and also showed that peroxidase and nonspecific 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 discs. We can therefore conclude that there are rhodopsin molecules on the rod cell membrane as well as on the disc 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 immunochemically 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 prior to 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 disc membrane has also been suggested by freeze-fracturing of retinas of vitamin A-deficient mice. It has been reported (25) 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 nine months, in addition to finding many distended vesicles in the rod outer segments we also could see some relatively intact discs 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 disc 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 disc 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 disc 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 disc membrane, the morphology of disc membranes revealed in freeze-fracturing experiments would tend to suggest that rhodopsin molecules penetrated deeply into the hydrophobic region of the disc membrane. In the immunolabeling experiments, we observed staining on the cytoplasmic side of both the plasma membrane and the disc membrane, much heavier staining on the outside of the plasma membrane and less intense staining on the intradisc side of the disc 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 disc membrane (22, 23) 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 disc 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 molecular weight of 55,000) and small label molecules (peroxidase with molecular weight of 40,000). Furthermore, inside the rod inner segment where we expect some rhodopsin molecules being synthesized and transported (26), 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 antibovine 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.

REFERENCES

1. Wald, G., J. Durell, and R. C. C. St. George. 1950. The light reaction in the bleaching of rhodopsin. Science 111:179-181.

2. Roberson, W. E., A. Gordon-Walker, and D. Bownds. 1972. Molecular weight of frog rhodopsin. Nat. New Biol. 235:112-114.

3. Hagins, W. A. 1972. The visual process: Excitatory mechanisms in the primary receptor cells. Ann. Rev. Biophys. Bioengineering 1:131-158.

4. Sjöstrand, F. S. 1961. In The Structure of the Eye. G. K. Smelser, editor. Academic Press Inc., New York.

5. Worthington, C. R. 1971. Structure of photoreceptor membranes. Fed. Proc. 30:57-63.

6. Liebman, P. A. 1962. In situ microspectrophotometric studies on the pigments of single retinal rods. Biophys. J. 2:161-178.

7. Worthington, C. R., G. I. King, and T. J. McIntosh. 1973. Direct structure determination of multilayered membrane-type systems which contain fluid layers. Biophys. J. 13:480-494.

8. Dewey, M. M., P. K. Davis, J. K. Blasie, and L. Barr. 1969. Localization of rhodopsin antibody in the retina of the frog. J. Mol. Biol. 39:395-405.

9. Papermaster, D. S., and W. J. Dreyer. 1974. Rhodopsin content in the outer segment membranes of bovine and frog retinal rod. Biochemistry, in press.

10. McConnell, D. G. 1965. The isolation of retinal outer segments. J. Cell Biol. 27:459-473.

11. Hubbard, R., P. K. Brown, and D. Bownds. 1971. In Methods in Enzymology, vol. 18: Vitamins and Coenzymes, part C. D. E. McCormick and L. D. Wright, editors. Academic Press, Inc., New York. p. 621.

12. Fairbanks, G., Y. L. Steck, and D. F. H. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry 10:2606-2617.

13. Huang, H. V., R. S. Molday, and W. J. Dreyer. 1973. FEBS Lett. 37:285-290.

14. Avrameas, S., and T. Ternynck. 1969. The cross-linking of proteins with glutaraldehyde and its use for the preparation of immunoadsorbents. Immunochemistry 6:53-66.

15. Campbell, D. H., F. S. Garvey, N. E. Cremer, and D. H. Sussdorf. 1970. In Methods in Immunology. W. A. Benjamin, Inc., New York. pp. 260-267.

16. Campbell, D. H., F. S. Garvey, N. E. Cremer, and D. H. Sussdorf. 1970. In Methods in Immunology. W. A. Benjamin, Inc. New York. pp. 228-230.

17. Avrameas, S., and T. Ternynck. 1971. Peroxidase labeled antibody and Fab conjugates with enhanced intracellular penetration. Immunochemistry 8:1175-1179.

18. Schichi, H., and M. S. Lewis. 1969. Biochemistry of visual pigments. I. Purification and properties of bovine rhodopsin. J. Biol. Chem. 244:529-536.

19. Hong, K., and W. L. Hubbell. 1972. Preparation and properties of phospholipid bilayers containing rhodopsin. Proc. Nat. Acad. Sci. U.S.A. 69:2617-2621.

20. Engstrom, L. H. 1970. Structure in the erythrocyte membrane. Ph.D. dissertation. University of California at Berkeley.

21. Marchesi, V. T. 1970. Isolation and characterization of a sialic acid-containing glycoprotein from red cell membranes. Fed. Proc. 29:600.

Tillack, T. W., R. E. Scott, and V. T. Marchesi. 1970. Cell membrane ultrastructure studied by freeze-etching. Fed. Proc. 29:489.

22. Cone, R. A. 1972. Relaxation times of rhodopsin detected by photodichroism. Biophys. Soc. Abs. 12:246a.

Brown, P. K. 1972. Rhodopsin rotates in the visual receptor membrane. Biophys. Soc. Abs. 12:248a.

23. Poo, M., and R. A. Cone. 1973. Lateral diffusion of rhodopsin in the visual receptor membrane. J. Supramol. Struct. 1:354.

24. Pinto da Silva, P., and D. Branton. 1970. Membrane splitting in freeze-etching. J. Cell Biol. 45:598-605.

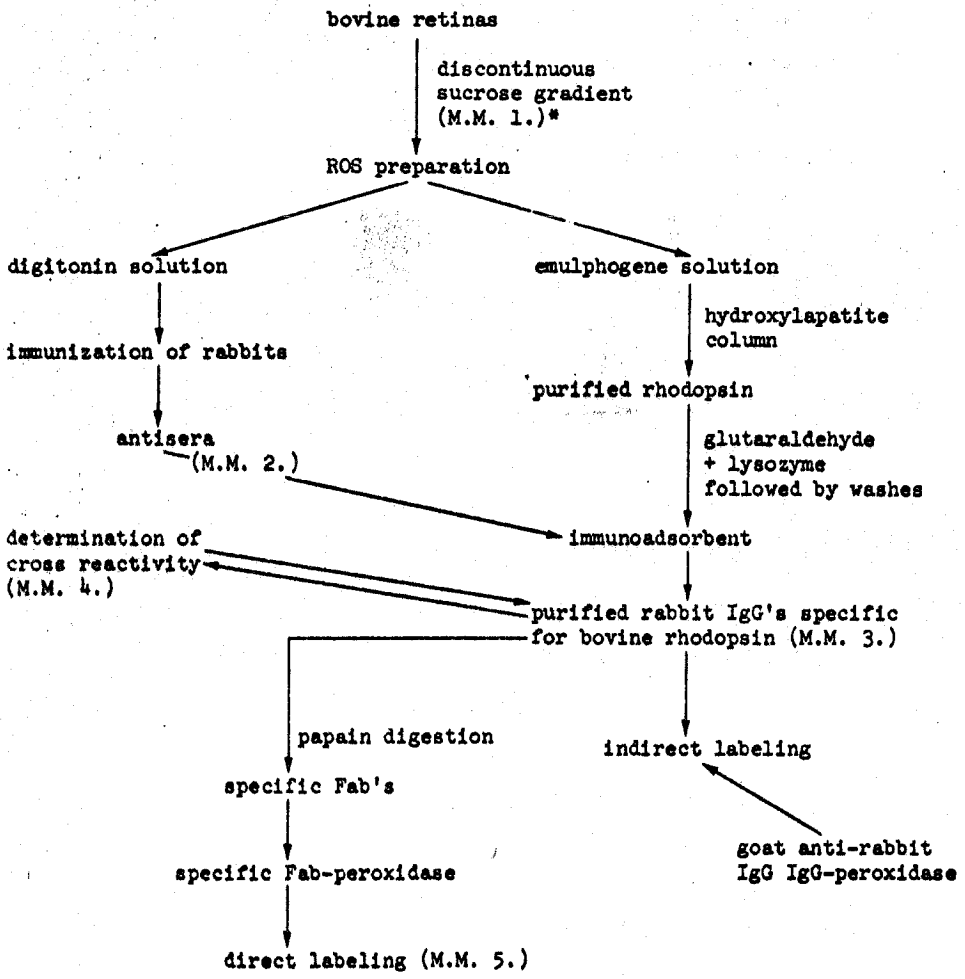
25. Dowling, J. E., and G. Wald. 1960. The biological function of vitamin A acid. Proc. Nat. Acad. Sci. U.S.A. 46:587-608.

26. Hall, M. O., D. Bok, and A. D. E. Bacharach. 1969. Biosynthesis and assembly of the rod outer segment membrane system. Formation and fate of visual pigment in the frog retina. J. Mol. Biol. 45:397-406.

27. Corless, J. M., W. H. Cobbs, III., M. J. Costello, and J. D. Robertson. 1974. On the asymmetry of retinal rod outer segment disc membranes. To be published.

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.

Table II. Summary of the Immunolabeling Experiments

Experiment[a]	Reagents used 1.	Reagents used 2.	Distribution of staining
Direct labeling	specific Fab-peroxidase	diaminobenzidine + H_2O_2	specific[b] sites: disc membranes, cell membrane of ROS and inner segments, connecting cilium nonspecific sites: patches between discs, outmost surfaces of retina
Control 1.	none	same as above	rim of discs
2.	nonspecific Fab-peroxidase	same as above	patches between discs, outmost surfaces of retina
3.	peroxidase only	same as above	outmost surfaces of retina
4.	BSA-peroxidase	same as above	patches between discs that are close to the surface[c]
5.	BSA wash, specific IgG-peroxidase (direct labeling)	same as above	specific sites: discs and ROS membranes that are close to the surface of the retina[d]
Indirect labeling[e]	specific IgG + goat IgG peroxidase	same as above	specific sites: discs and ROS membranes that are close to the surface of retina[d]
Control	same as above	diaminobenzidine only	none

LEGEND TO TABLE II

[a]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.

[b]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.

[c]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.

[d]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.

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

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 once in 0.01 M glycine in phosphate saline once in phosphate saline	-	-	yes	-	-	-	-
type of rabbit antibodies with which the retina was treated	-	-	specific	nonspecific	specific	nonspecific	specific
temperature and length of the treatment	-	-	room temp., 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	-	-	at room temp.	at 4°C	at 4°C	at 4°C	at 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. 8a	same as 1.	see Fig. 8b	same as 1.	same as 1.	same as 1.	see Fig. 8c

FIGURE 1 Tracings of OD_{600} 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.

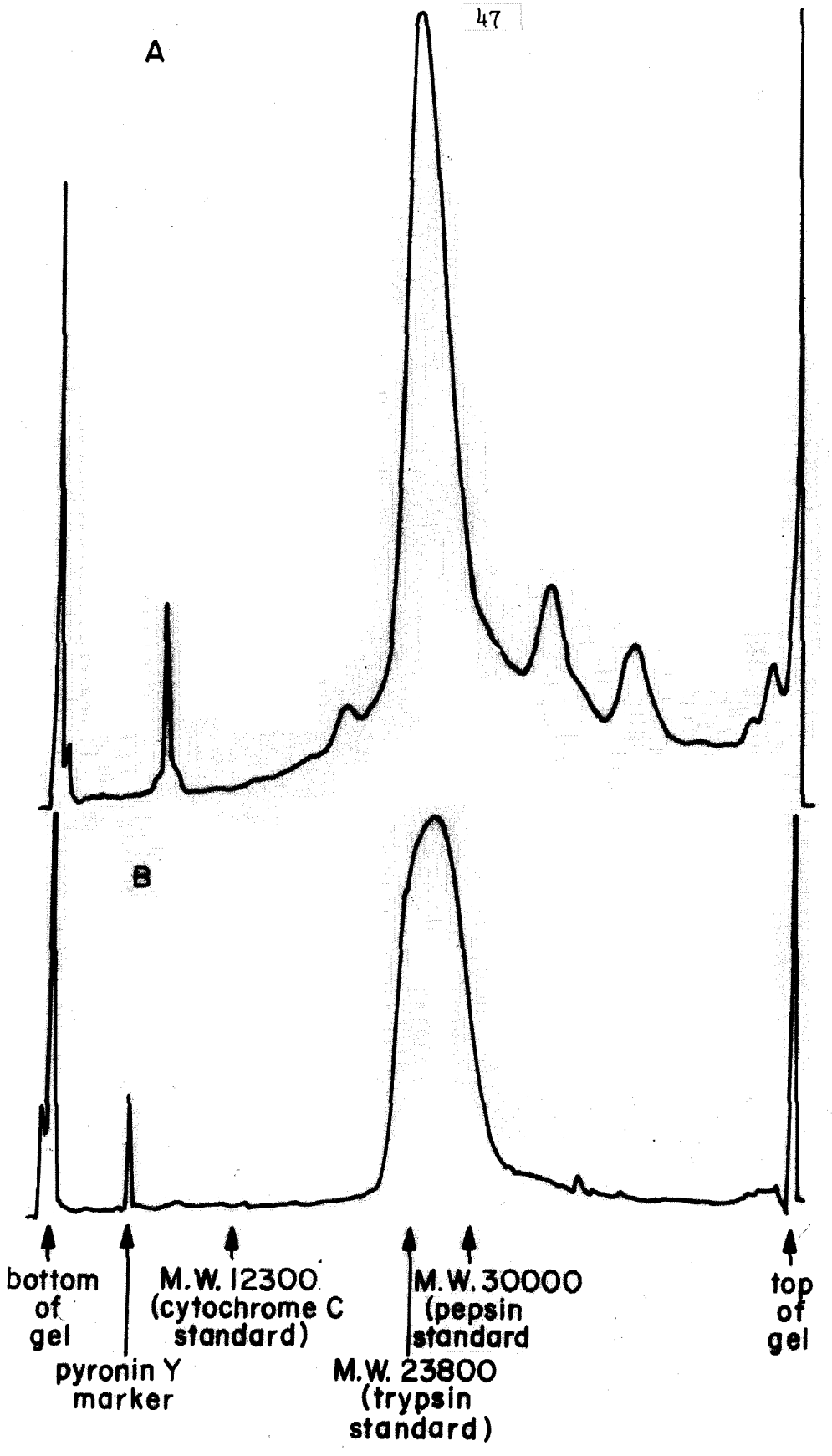

A
B
bottom of gel
pyronin Y marker
M.W. 12300 (cytochrome C standard)
M.W. 23800 (trypsin standard)
M.W. 30000 (pepsin standard
top of gel

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.


2
RHOD
ROS

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 mAmps/slide) goat anti-rabbit whole serum (a), or goat antirabbit IgG serum (b) was placed in the trough for the subsequent double diffusion experiment.

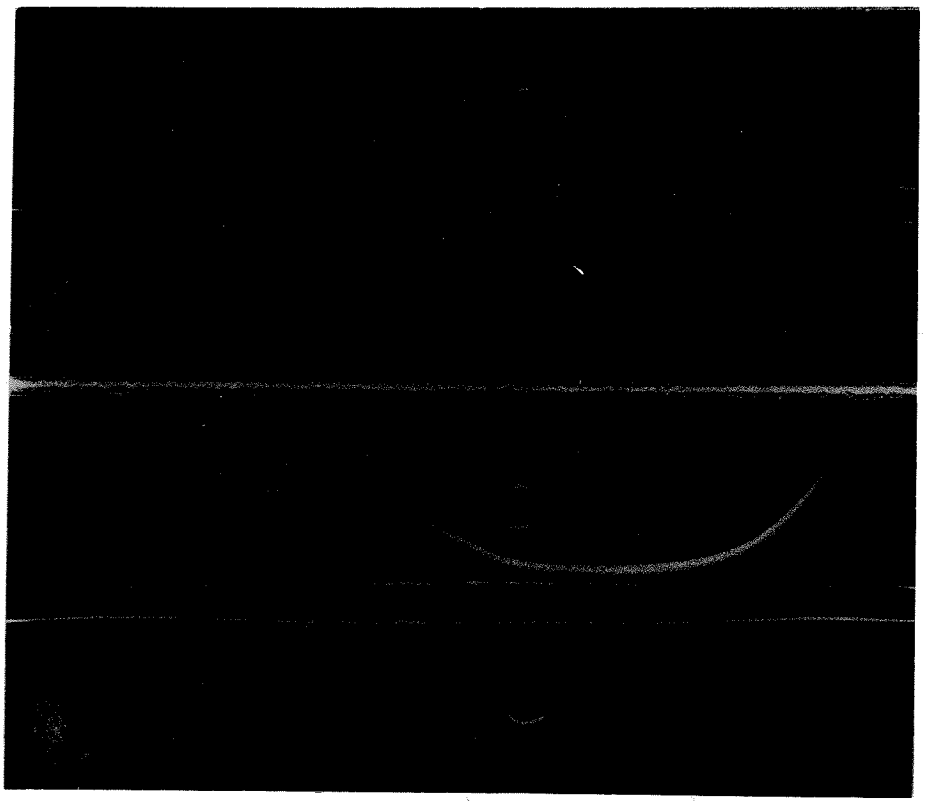


FIGURE 4 Mouse retina stained indirectly with specific IgG's and peroxidase-labeled goat IgG's specific for rabbit IgG after formaldehyde fixation. The indirect technique amplifies the intensity of the labeling. The extracellular face of the ROS membrane (ROSM) was heavily stained. The cytoplasmic side of the ROSM and both sides of the disc (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. X 90,000.

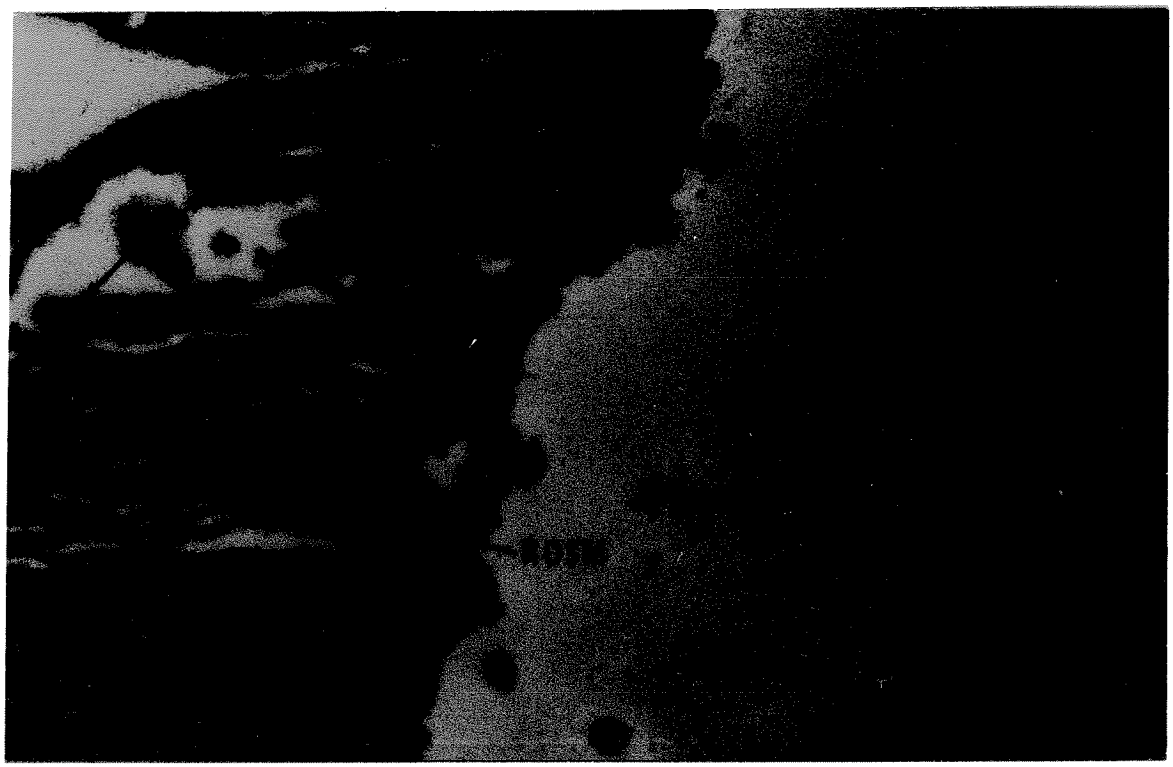


FIGURE 5 Mouse retina stained directly by specific Fab-peroxidase. The staining is less intense than in the indirect method. However, the tissue is stained much more uniformly because of the better penetration of the smaller label, i.e., Fab-peroxidase. Dense patches of staining (DP) between discs are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

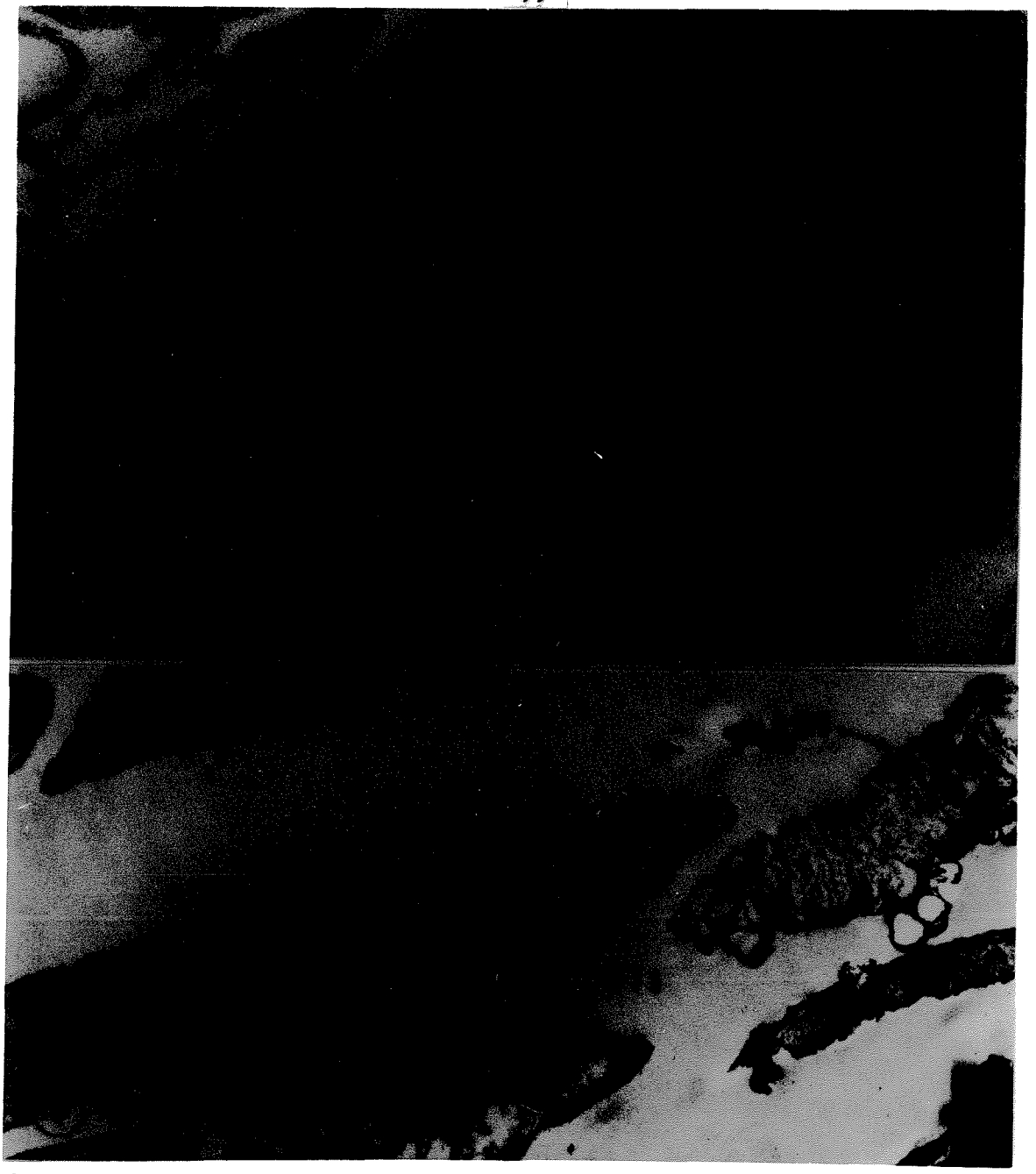


FIGURE 7 Freeze-fractured mouse retina, showing portions of inner (IS) and outer segments (ROS). The cytoplasmic (A) leaflet of the rod outer segment (ROS) plasma membrane and that of the connecting cilium (C.C.) is rich in membrane particles. The outside leaflet (B face) of the fractured rod inner segment (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 discs, 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. X 20,000. Inset X 64,000.

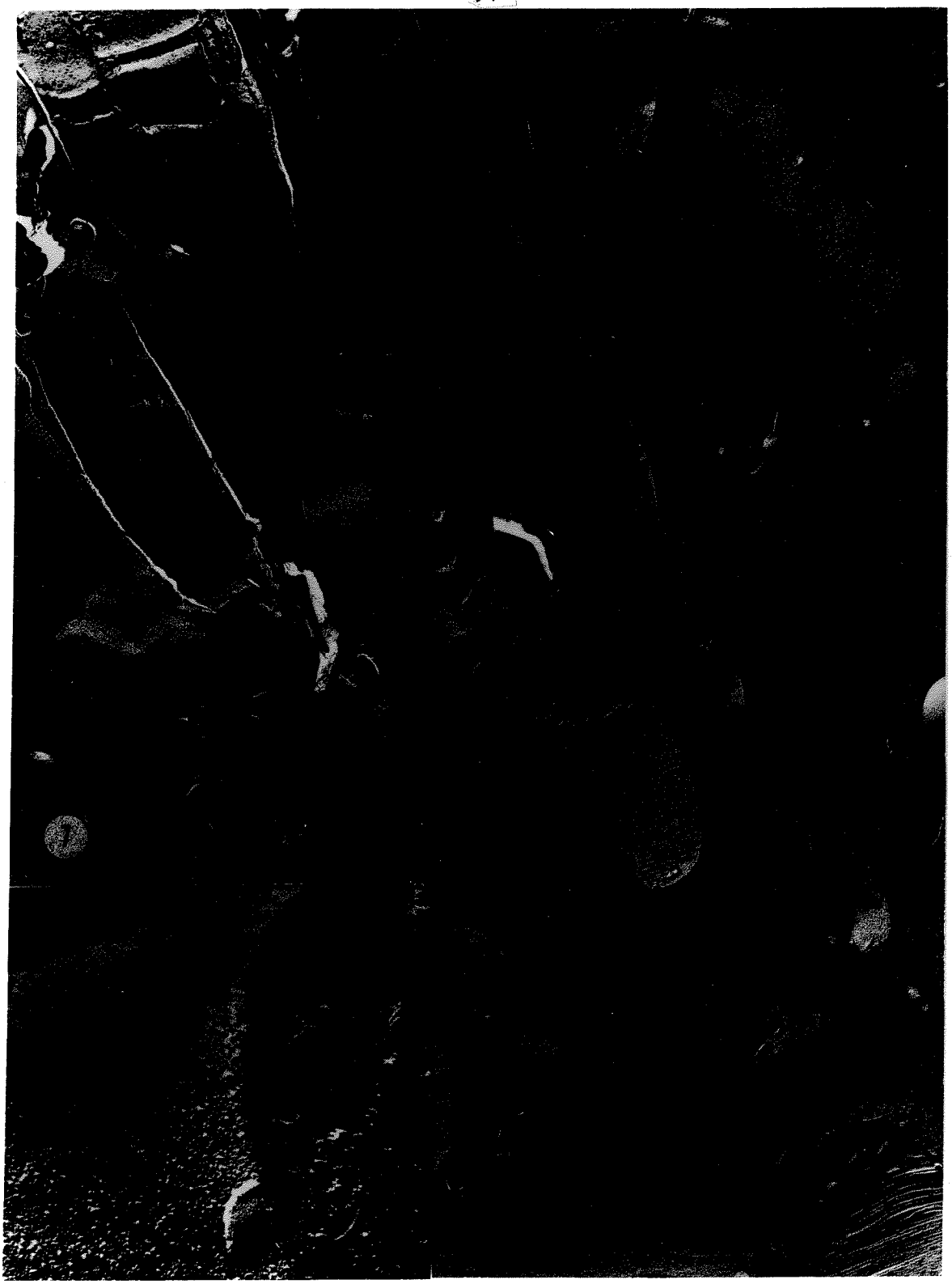


FIGURE 8 (a) Cytoplasmic leaflet (A face) of the mouse ROS plasma membrane. Mouse retina was fixed with Karnovsky's fixative containing glutaraldehyde at 4°C before freeze fracturing. Note the patchy distribution of membrane particles. X 62,500.

(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. X 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. X 62,500.

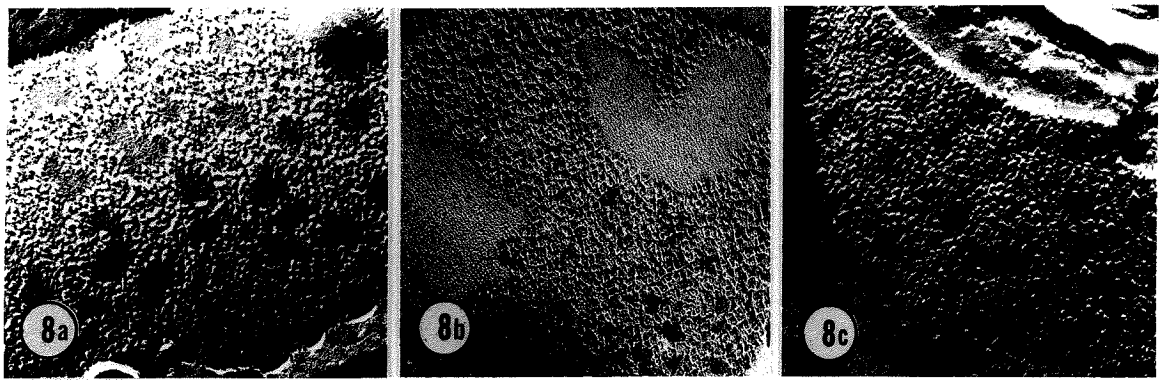
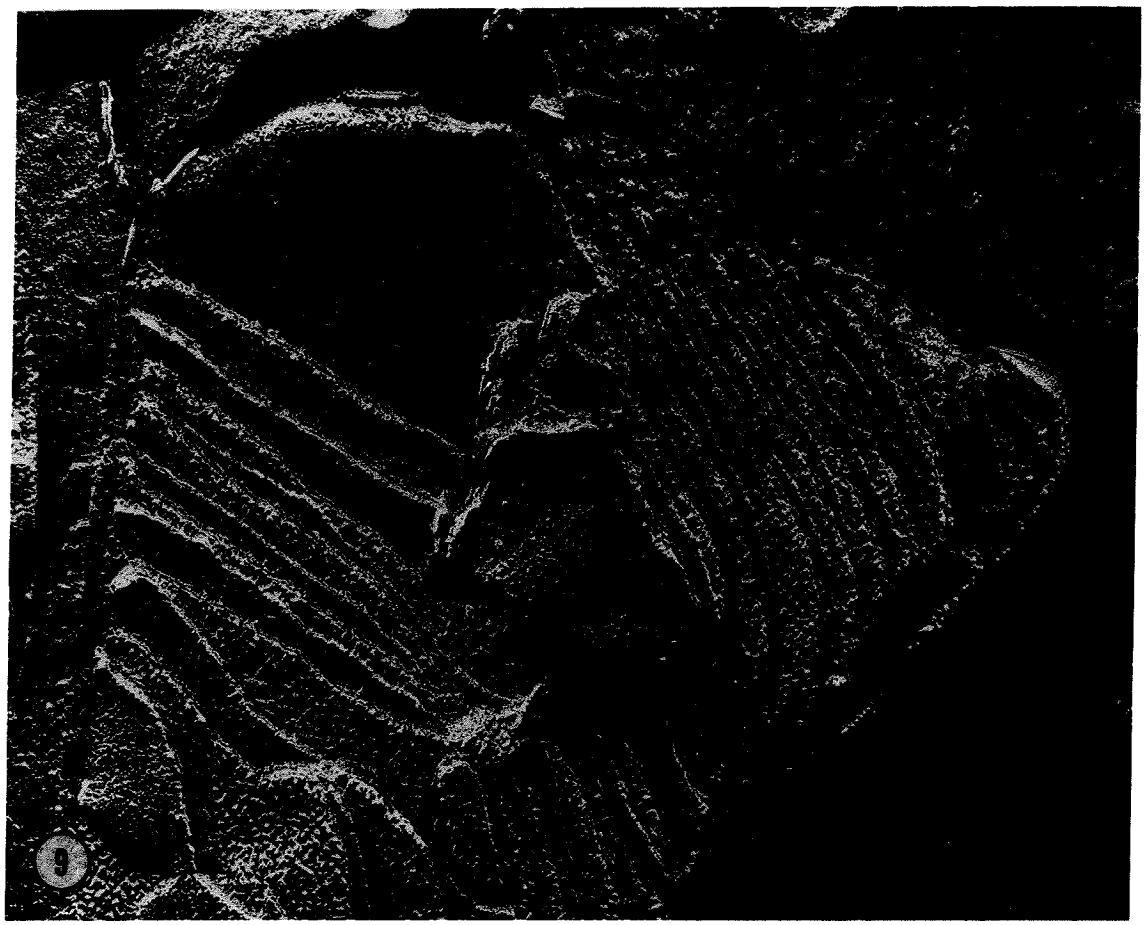

8a
8b
8c

FIGURE 9 Freeze-fractured mouse disc membranes. The leaflet adjacent to the interdisc cytoplasm (A face) contains closely packed membrane particles, whereas pits are abundant on the leaflet adjacent to the intradisc space (B face). X 81,600.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Chapter II

FERRITIN-ANTIBODY LABELING OF RHODOPSIN ON THIN SECTIONS OF FROZEN RETINA

I. INTRODUCTION

The presence of rhodopsin molecules in the disc membranes inside each rod outer segment has been indicated in X-ray diffraction, optical dichroism, as well as chemical studies (1, 2, 3). Rhodopsin is also found on the cell membrane of rod outer segment (ROS) and associated with the rod inner segment (RIS). The presence of rhodopsin on rod cell membranes is suggested in the frog by experiments using fluorescent reagents done by Dewey et al. (4) and by our own studies using specific rabbit antibodies and peroxidase on bovine and murine retinas (5). Autoradiographic studies (6) have shown that rhodopsin is synthesized in the inner segment, transported to the outer segment and later degraded in the pigment epithelial cells. The present communication reports an immunolabeling experiment different from the one done previously, designed to clarify the localization of rhodopsin in various intracellular compartments. Using conventional labeling techniques it is difficult for label molecules to reach cells located deep in the tissue and to penetrate through the cell membrane to reach loci within the cell. One way to overcome this difficulty is to section the tissue into pieces of a few hundred Angstrom's thickness before the treatment with label molecules. Conventional thin sections cannot be used in this manner because the embedding materials greatly reduce the accessibility of the tissue to any label molecules. Recently a method for cutting thin sections of unembedded but glutaraldehyde-fixed tissue frozen in sucrose solution has been developed by Tokuyasu, Singer and their collaborators (7, 8). Thus

it is possible to apply antibodies and label molecules to these thin sections, under conditions which give antibodies access to intracellular and cell surface antigens. Using this technique, we have labeled rhodopsin by the indirect method on thin sections of retina. The purified rabbit antibodies specific for bovine rhodopsin described previously, coupled to conjugates of ferritin and goat anti-rabbit antibodies (goat IgGs that are specific for rabbit IgGs) were used.

II. MATERIALS AND METHODS

A. Materials

Specific rabbit antibodies were obtained through immunoadsorption of antiserum of rabbits immunized against bovine rhodopsin. Details of the experimental procedures are given in a previous report (5). The immunoadsorbent was built from a highly purified bovine rhodopsin preparation. The antiserum was shown not to cross-react with lipid extracts from the bovine ROS preparations (5).

Goat IgGs specific for rabbit IgGs were purified on a Sepharose affinity column. Ferritin was highly purified and conjugated to goat antibodies with 2,4 toluene diisocyanate. The conjugate was separated from unconjugated reactants by gel chromatography. Details on these purification procedures have been described elsewhere (8).

B. Methods

Dark-adapted cow, mouse or chicken retinas were dissected and fixed in 2% glutaraldehyde, 0.1 M phosphate buffer pH 7.4 at 4°C

for one hour. The retinas were cut into 1 mm slices, washed three times with 0.1 M phosphate pH 7.4 and stored in the same buffer at 4°C overnight.

The retina slices were perfused with 70% sucrose (70 g sucrose dissolved in 100 ml 0.1 M phosphate pH 7.4) at 4°C for over 45 min. The sucrose-treated retina slices were mounted on a copper block, frozen in liquid nitrogen and thin-sectioned at -75°C with a Porter-Blum MT-2 ultramicrotome equipped with a cryo-kit attachment (Ivan Sorvall, Inc., Newtown, Conn.). Thin sections on the dry glass knife edge were picked up by means of a small droplet of saturated sucrose (0.5-1 mm diameter) suspended on an eyelash probe. The dry, frozen sections were picked up from the knife by touching the sucrose droplet to the sections before the droplet froze completely. After removal to room temperature, the sections were allowed to melt on the droplet surface in order to flatten them by surface tension, and mounted on carbon-coated Formvar grids by touching the droplet surface bearing the sections to the grid.

The grids were floated face down for 5 min on a large droplet of 0.1 M phosphate, 0.14 M NaCl, pH 7.4 (PBS) to remove sucrose from the sections. They were then floated on a large droplet of 5% BSA, 0.01 M glycine in PBS for 5 min to reduce nonspecific staining by blocking unreacted glutaraldehyde. Care was taken not to dry the sections during transfer. A droplet of specific rabbit antibodies or nonspecific rabbit IgGs (Miles-Pentex) (0.37 mg/ml) was applied to

each grid and allowed to incubate for 5 min. Wetting of the forceps or the backside of the grid was avoided. The grid was then extensively washed by quickly touching the grid to two large droplets of PBS followed by five successive one-minute washes on PBS droplets.

The same procedure was repeated for the treatment with ferritin-goat antibody conjugates. Namely, the grids were treated successively with (a) 5% BSA, 0.01 M glycine in PBS, (b) $\sim$ 1 mg/ml ferritin goat antibody in 0.1 M phosphate, 0.2 M NaCl pH 7.4, (c) 2 rapid washes and 5 one-minute washes on PBS droplets. Both rabbit antibody and ferritin-goat antibody solutions were centrifuged right before use.

The ferritin-labeled thin sections of retina were further treated with 2% glutaraldehyde in PBS for 5 min, washed with distilled water twice, and negatively stained with 0.2% phosphotungstic acid (PTA).

The sections were viewed with a Philips EM 301 electron microscope at an accelerating voltage of 60 Kv or 80 Kv. A goniometer stage was used on occasion to examine tilted membranes.

III. RESULTS

The indirect ferritin technique used produced very heavy staining of the homologous bovine retina or ioslated bovine ROS preparation. The heterologous mouse retina did not stain as intensely. In addition to disc membranes, the ROS and RIS cell membrane, the connecting cilium and the intracellular space of the inner segment were

stained by ferritin, thus confirming our previous results. If non-specific rabbit IgGs were substituted for the specific rabbit antibodies, none of the regions described above were stained in either bovine or mouse retina (Fig. 1b and Fig. 5). There is a low background of nonspecifically bound ferritin, consisting of small aggregates of ferritin particles scattered diffusely and randomly over the section. The staining of chicken retina was at the level of the low background even when specific rabbit antibodies were used. Thus rabbit antibodies specific for bovine rhodopsin do not bind to chicken rhodopsin.

The ultrastructural preservation achieved with mouse retina was much better than that obtained with bovine retina. The latter had to be subjected to a longer period of processing and shipping while the mouse tissue could be fixed very rapidly. Because of the lower level of staining obtained with mouse retina, it is easier to analyze the distribution of ferritin. Therefore we will concentrate on this tissue in the following description.

A. The Rod Outer Segments

Frozen thin sections of mouse retina stained with specific antibodies show ferritin molecules (diameter 200 Å, Fe core 100 Å) essentially closely packed in the region of the disc membranes. On the ROS cell membrane ferritin particles are often found in patches of 0.04 to 0.2 μ in size. The distance between patches varies from 0.03 μ to about 0.2 μ (Fig. 1a).

Because of the dimensions of the "sandwich" used in staining, it is difficult to determine if there are antigenic sites on both sides of the disc membrane. The diameter of a ferritin molecule in a negatively stained preparation is about 200 Å. The distance between two antigenic binding sites in an IgG molecule is about 100 Å (9). Therefore in the indirect technique used, the maximum distance between the center of a ferritin molecule and the antigenic site is about 300 Å (radius of ferritin + goat antirabbit IgG + rabbit antirhodopsin IgG). In intact ROS the repeat distance of discs is about 300 Å. Thus it is not possible to find out to which side of the disc membrane the ferritin molecules bind. We found however that the maximum distance between a ferritin on the outside of the ROS from the outer surface of the negatively stained ROS cell membrane is about 300 Å, suggesting that some of the label was attached to antigenic sites on the outside of the membrane. In a few cases when the ROS cell membrane was broken, isolated discs could be found far away from each other. In these discs also the distance between ferritin and the outer surface of the disc membrane was about 300 Å (Figs. 2a and 2c). In even fewer cases we found discs with significant intradisc space (greater than 300 Å). The ferritin label was then found also <u>inside</u> the disc as well as outside at about 300 Å from the inner surface of the disc membrane (Figs. 2b and 2d) suggesting that under these circumstances antigenic groups existed on the inner surface of the disc membrane. On rare occasions the ROS cell membrane was

found broken and far from the discs. Here too, ferritin molecules were found at distances up to about 300 Å from either surface of the ROS cell membrane (Fig. 2b). These observations tend to suggest that antigenic sites of rhodopsin are exposed on both sides of the membrane. One must point out that irrespective of site of binding of the antibody, ferritin might fall on either side of the membrane surface bearing antigenic sites; we cannot be sure therefore that antigenic sites are present on both sides of the membrane rather than on only one side. Since the thickness of the membrane as seen in negatively stained preparations is only about 50 Å, the resolution of the label has to be better than 50 Å, and a large number of suitable samples should be counted to obtain a statistically significant determination of the ferritin distribution. Since the resolution in our experiment is only about 300 Å (the length of the antibody sandwich coupled to ferritin) we must depend on finding isolated, swollen discs which are rare. We therefore have to emphasize the <u>symmetric</u> distributions of ferritin relative to the center of the disc membrane and the ROS cell membrane. Clearly the few cases obtained can only be suggestive of the presence of antigenic sites on both sides of the membrane.

B. The Rod Inner Segment

Ferritin molecules were found inside the rod inner segment and on the RIS cell membrane. They form small patches all along the RIS cell membrane from the connecting cilium to the outer limiting membrane. The inner segment is stained by PTA and appears darker than

the background of the Formvar film. Ferritin particles are found up to roughly 300 Å outside of the inner segment. Although ferritin molecules are always found inside the inner segment close to the boundary wherever ferritin patches are located outside the cell, we can only take that as a circumstantial evidence compatible with the idea that antigenic sites are present on both sides of the membrane, because it is not possible to tell whether those patches of ferritin located in the cytoplasm close to the RIS cell membrane are bound to antigenic sites on the cell membrane or in the cytoplasm.

Negative staining with PTA does not reveal clearly the presence of intracellular organelles other than nuclei and mitochondria. Thus it is difficult to correlate the ferritin distribution with the specific cytoplasmic structures. Ferritin molecules seem to be present in scattered patches over the entire intracellular space of the inner segment. In those cases where the mitochondria were lightly stained by PTA, ferritin could be located associated with these organelles. The density of ferritin labeling over nuclei was not significantly greater than the low background level of ferritin in nonspecifically stained retinas or in the inner nuclear layer of the specifically stained retina.

C. The Connecting Cilium and the Cytoplasmic Bridge

It has been reported (10) that, in addition to the connecting cilium, there exists a cytoplasmic connection between the inner and outer segments of mammalian visual receptors. We have observed both the ciliary and the cytoplasmic bridges between the outer and inner

segment (Fig. 3). Both were labeled specifically by antibody coupled to ferritin. The density of ferritin on the cilium varied in different rods (Figs. 3, 4, 6, and 7b). (Ferritin particles are found scattered over the whole cilium.) In the cytoplasmic bridge mitochondria are sometimes found in close contact with discs at the base of the outer segment. Ferritin particles are present on the cell membrane as well as inside the cytoplasmic bridge (Figs. 6 and 7a).

IV. DISCUSSION

Rhodopsin has been shown to be present not only in the discs but also on the ROS cell membrane and inside the rod inner segment, both by fluorescent immunolabeling and by autoradiographic studies.

We have previously utilized purified rabbit antibodies specific for bovine rhodopsin to label rhodopsin in intact retina with peroxidase-antibody conjugates. In those experiments, the staining is much heavier on the outer than on the inner surface of the ROS cell membrane or that of the disc membrane. The staining on the intradisc surface of the disc membrane is very light. We suspect that the asymmetrical distribution of staining was due to the possibility that peroxidase-antibody conjugates could not penetrate the membrane easily, thus creating an uneven distribution of the conjugates across the membrane. Furthermore, we had difficulties in establishing the presence of rhodopsin inside the inner segment, because it was difficult to recognize small amounts of peroxidase staining in the cytoplasm.

Because of these difficulties, we felt that we should check the results of the peroxidase immunolabeling experiments by labeling rhodopsin with antibodies on top of thin sections of retina.

We therefore prepared frozen sections of glutaraldehyde-fixed retina and labeled them with antibodies specific for rhodopsin and conjugates of goat-antirabbit antibodies and ferritin. We found ferritin staining on disc membranes, on the ROS and RIS cell membrane, on the connecting cilium, inside the inner segment and in and on the cytoplasmic bridge. Furthermore, we found that chicken retina, unlike bovine retina and mouse retina, was not stained by ferritin and rabbit antibodies specific for bovine rhodopsin. This finding is compatible with the observation of Dr. Bok that frog rhodopsin does not cross-react with rabbit antiserum specific for bovine rhodopsin (personal communication).

On thin sections of frozen tissue, one can resolve two spots about 20 Å apart. Hence in immunolabeling experiments the size of the label molecules determines the resolution of the technique. In our present experiments, the resolution is given by the sum of the lengths of two IgG molecules and the radius of ferritin, namely 300 Å. Significant improvement should be possible if one could negatively stain the bare antibodies. Besides resolution, the quality of staining is another important factor for the recognition of fine structures. Heavy PTA staining reveals membranes as 50 Å white lines but might bury ferritin molecules. Light PTA staining leaves ferritin clearly visible but often cannot reveal the membranes. This problem was

partially solved by the use of the goniometer stage. After light PTA staining and air-drying, membranes on thin sections are often tilted, up to 60° from the normal to the section. If we tilt the grid on the goniometer stage of the electron microscope to a corresponding angle, details of the disc membranes can be examined. Another possible solution developed by Dr. Tokuyasu is the negative staining by zinc acetate. Since zinc has a much smaller atomic number, even a heavy staining should not affect the recognition of ferritin labels. Negative staining by either PTA or zinc acetate, however, does not clearly reveal subcellular organelles, such as endoplasmic reticulum and Golgi apparatus. Positive staining usually causes damage to the tissue (7). A few trials of osmium staining on thin sections followed by critical point drying produced sections with a slightly washed and wrinkled appearance. It seems possible that a more satisfactory result might be obtained with some modifications to the technique.

One point of interest is the localization of rhodopsin relative to the lipid core of the membrane. In our present experiment the problem of penetration is eliminated. Here however we encounter the difficulty that ferritin-antibody could fall on the side of the membrane opposite to the membrane surface bearing antigenic sites. Bearing in mind the fact that the thickness of membrane is quite a bit less than the resolution of our measurement, we have to say that the symmetrical distribution of ferritin up to 300 Å from either surface of the membrane in the few observed cases is only suggestive of the

possibility that antigenic sites are present on both sides of the membrane.

Autoradiographic studies of incorporation of radioactive amino acids or radioactive sugar residues in the rod (16) have indicated that newly synthesized rhodopsin moves from endoplasmic reticulum to the Golgi apparatus, from there to the distal part of the inner segment, and finally to the discs in the ROS. We have now found ferritin particles inside the inner segment as well as patches of ferritin particles on the RIS cell membrane. From our results of the stationary distribution of ferritin staining we cannot extract information about the dynamic processes of synthesis and transport. However, we did observe cytoplasmic bridges serving as a connection between the inner and outer segment in addition to the connecting cilium. Ferritin was found both on the cilium and in the cytoplasmic bridge suggesting both may serve as a channel for transport. Cytoplasmic bridges were also found by Richardson in the retinas of rhesus monkey, albino rats, hooded and albino guinea pigs and the ground squirrel. Thus the cytoplasmic bridge should not be overlooked when one entertains models about the transport of rhodopsin from the points of synthesis in the inner segment to the points of function in the discs.

REFERENCES

1. Worthington, C. R. 1971. Structure of photoreceptor membranes. Fed. Proc. 30, 57-63.

2. Liebman, P. A. 1962. In situ microspectrophotometric studies on the pigments of single retinal rods. Biophys. J. 2, 161-178.

3. Robinson, W. E., A. Gordon-Walker, and D. Bownds. 1972. Molecular weight of frog rhodopsin. Nat. New Biol. 235, 112-114.

4. Dewey, M. M., P. K. Davis, J. K. Blasie, and L. Barr. 1969. Localization of rhodopsin antibody in the retina of the frog. J. Mol. Biol. 39, 395-405.

5. Jan, L. Y. and J. P. Revel. 1974. Ultrastructural localization of rhodopsin in the vertebrate retina. J. Cell Biol., in press.

6. Young, R. W. and B. Droz. 1968. The renewal of protein in retinal rods and cones. J. Cell Biol. 39, 169-184.

7. Tokuyasu, K. T. 1973. A technique for ultracryotomy of cell suspensions and tissues. J. Cell Biol. 57, 551-565.

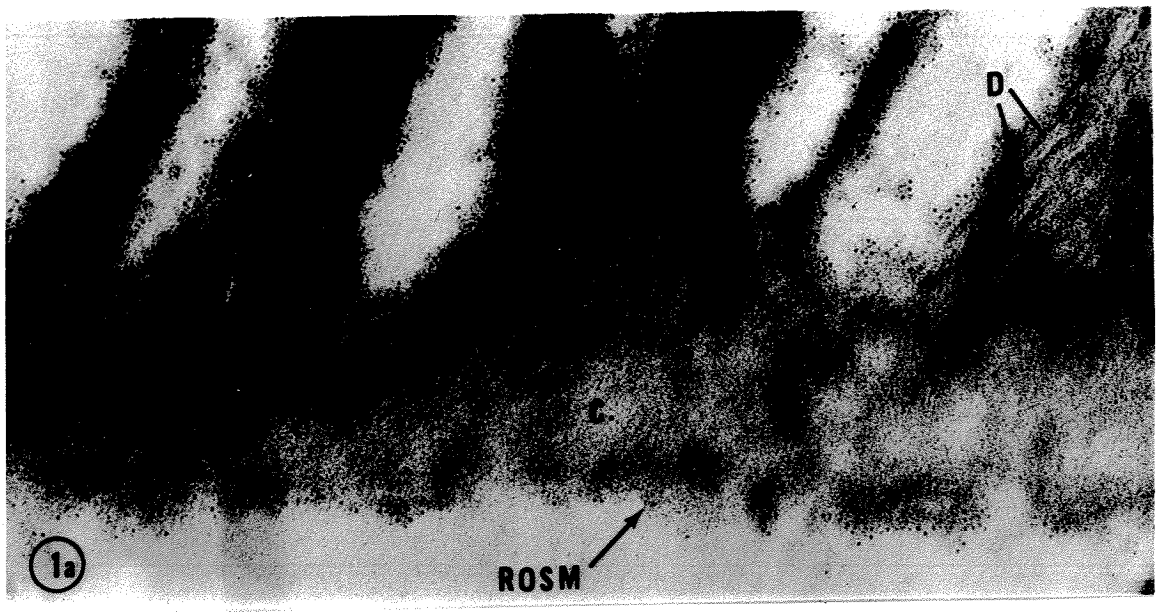
8. Painter, R. G., K. T. Tokuyasu, and S. J. Singer. 1973. Immunoferritin localization of intracellular antigens. The use of ultracryotomy to obtain ultrathin sections suitable for direct immunoferritin staining. Proc. Nat. Acad. Sci. U.S.A. 70, 1649.

9. Poljak, R. J., L. M. Amzel, H. P. Avey, B. L. Chen, R. P. Phizackerley, and F. Saul. 1973. Three-dimensional structure of the Fab' fragment of a human immunoglobulin at 2.8 Å resolution. Proc. Nat. Acad. Sci. U.S.A. 70, 3305-3310.

10. Richardson, T. M. 1969. Cytoplasmic and ciliary connections between the inner and outer segments of mammalian visual receptors. Vision Res. 9, 727-731.

FIGURE 1 (a) Mouse rod outer segment stained by the indirect method: The thin section of fixed frozen retina was first treated with rabbit antibodies specific for bovine rhodopsin, then with ferritin conjugated to goat antirabbit antibodies and negatively stained with PTA. The region shown is near the base of a rod. The clear area (C) between discs (D) and the rod outer segment cell membrane (ROSM) represents part of the cilium, which connects the rod outer segment and the rod inner segment. Many ferritin particles are found in the region of discs. On rod outer segment cell membrane the ferritin particles are often clustered. The rod outer segment cell membrane does not appear as a sharp line because it has tilted during the drying in the negative staining procedure. X108,000.

(b) Control mouse retina treated in the same way as described in (a) except that nonspecific rabbit antibodies are substituted for the rabbit antibodies specific for bovine rhodopsin. D: discs; ROSM: rod outer segment cell membrane. X108,000


D
C
ROSM
1a

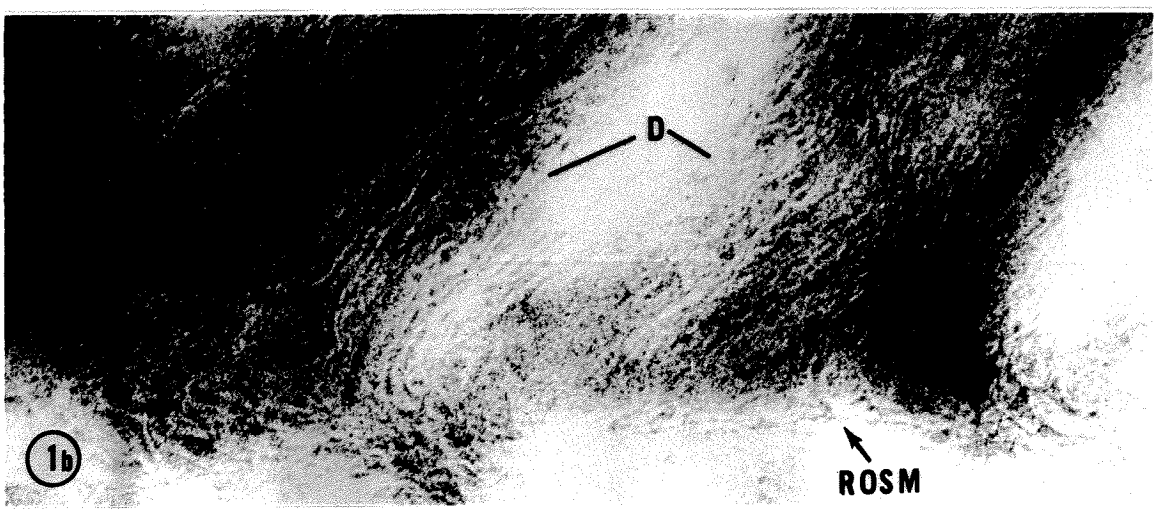
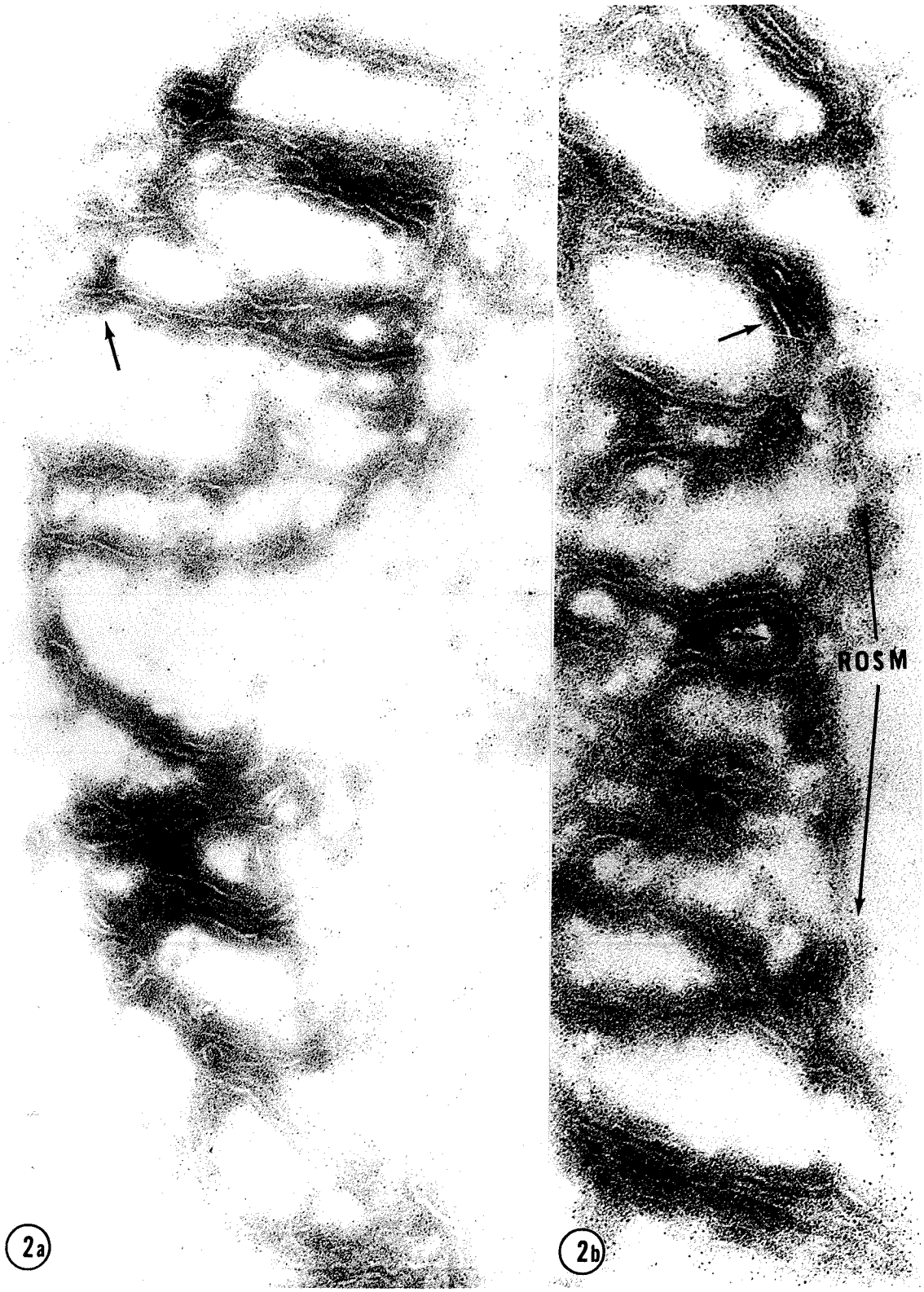

D
ROSM
1b

FIGURE 2 Mouse retina stained specifically by the procedure described in the legend for Fig. 1a. In this case the rod outer segment cell membrane (ROSM) was broken and discs became separated from each other. Isolated discs can be identified at the arrow in Fig. 2a. Some swollen discs showing a large intradisc space can be seen in Fig. 2b (arrows). Observations on disrupted outer segments such as these suggest that there are probably antigenic sites on both sides of the membrane. The greatest distance of a ferritin molecule to the membrane it is attached to is about 300 Å. X72,000

Fig. 2c and Fig. 2d are higher magnifications of Fig. 2a and Fig. 2b. X216,000



2a

2b

2c


83
300 Å
ROSM
2d

FIGURE 3 Mouse retina stained specifically with rabbit antibodies specific for rhodopsin and ferritin labeled goat antirabbit antibodies. Ferritin particles are numerous in the rod outer segment (ROS). Both connecting cilium (C.C.) and cytoplasmic bridge (C.B.) connect the rod outer segment with the rod inner segment (RIS). The outlines of mitochondria (M) in the rod inner segments are seen in this negatively stained preparation. In this picture ferritin particles in the region of the connecting cilium and the cytoplasmic bridge are more numerous than in regions nearer to the outer nuclear layer. X33,750

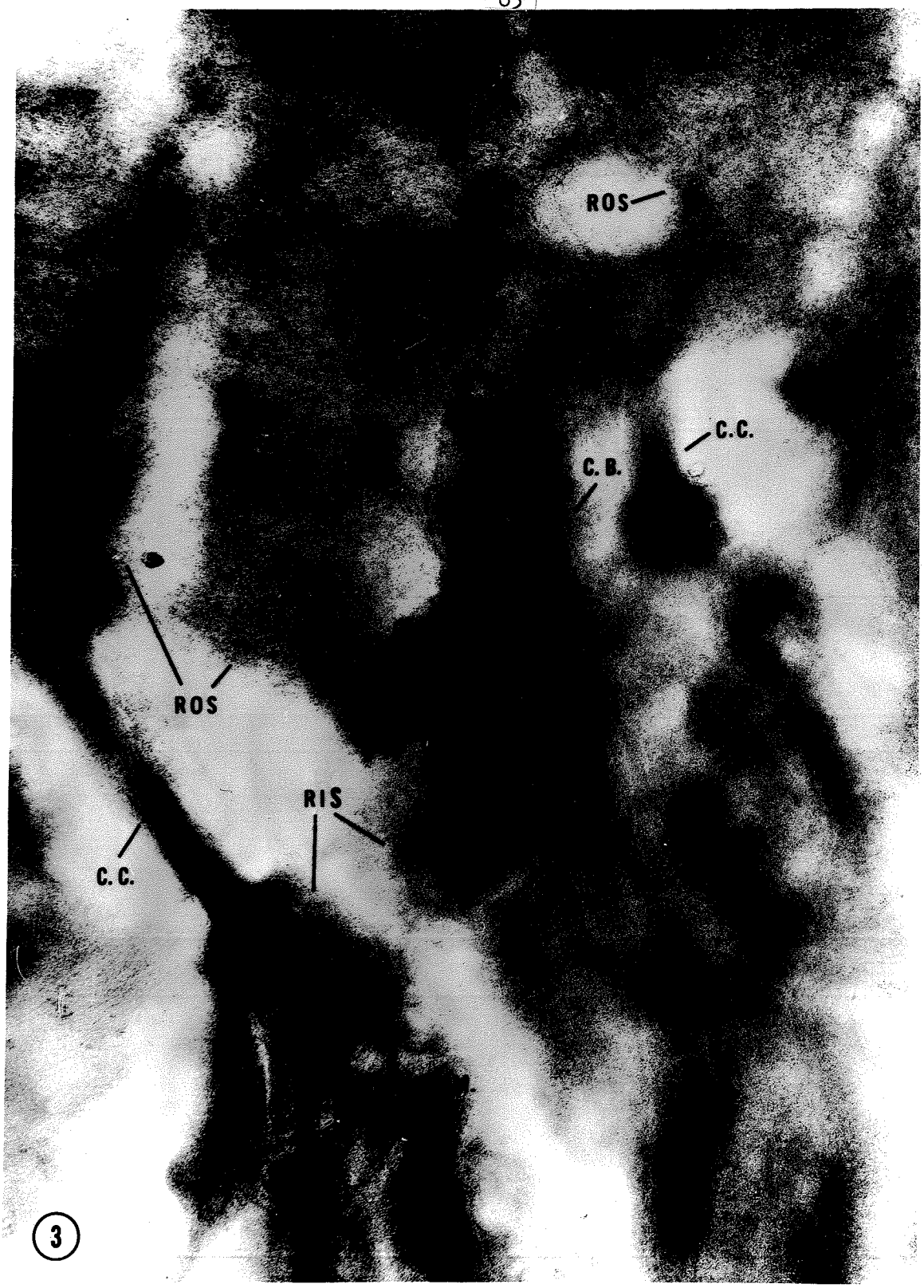

ROS
C.C.
C.B.
ROS
RIS
C.C.
3

FIGURE 4 Rod outer segment (ROS) and connecting cilium (C.C.) of a mouse retina stained specifically by the indirect method. Numerous ferritin particles at the base of the rod outer segment (arrow) can often be seen. In this example only a few ferritin particles are seen on the connecting cilium. X75,000

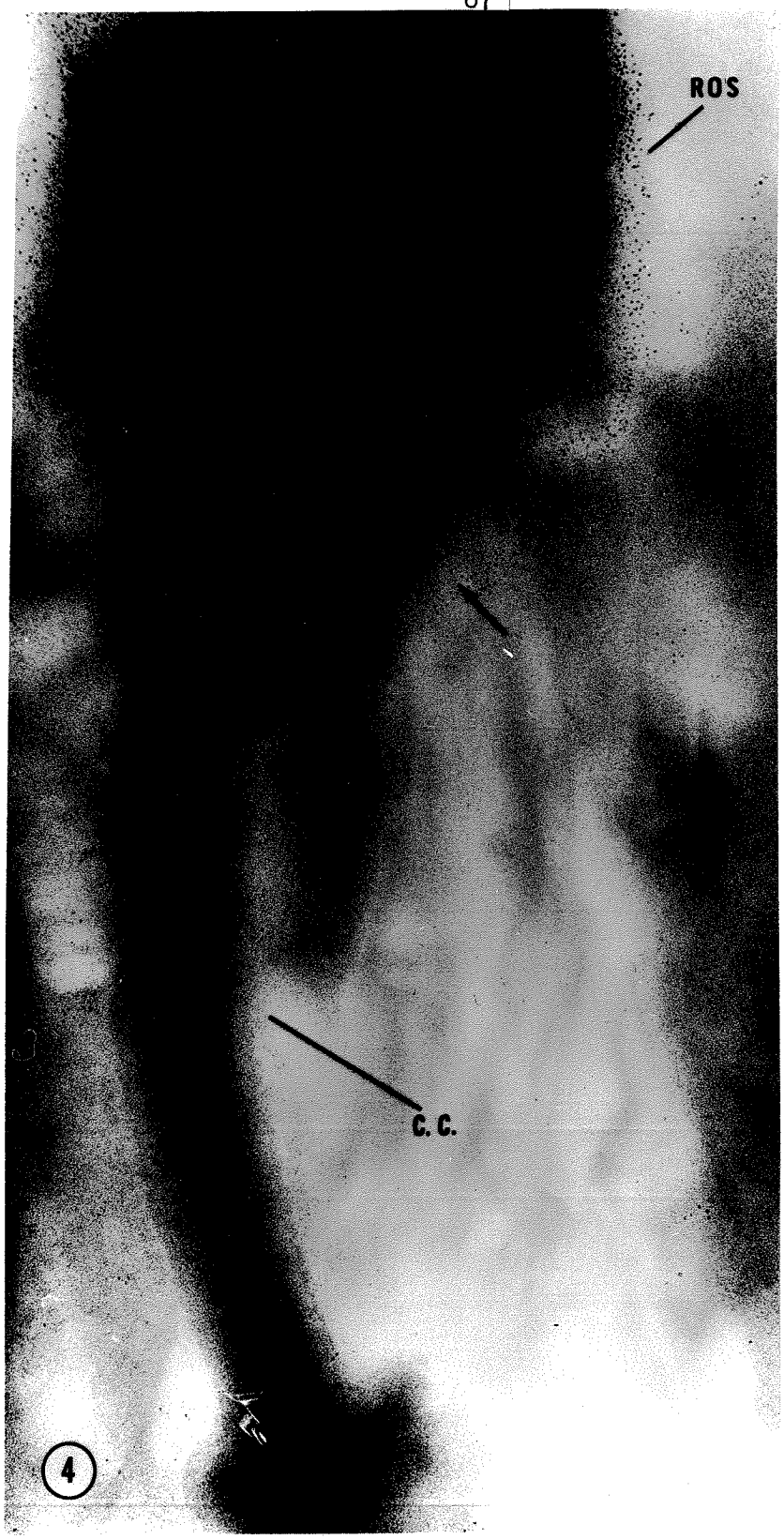

ROS
C. C.
4

FIGURE 5 Rod outer segment (ROS), connecting cilium (C.C.) and cytoplasmic bridge (C.B.) of a control mouse retina treated with non-specific rabbit antibodies and ferritin-labeled goat antirabbit antibodies. One mitochondrion can be seen in the cytoplasmic bridge. Little labeling is seen. X90,000

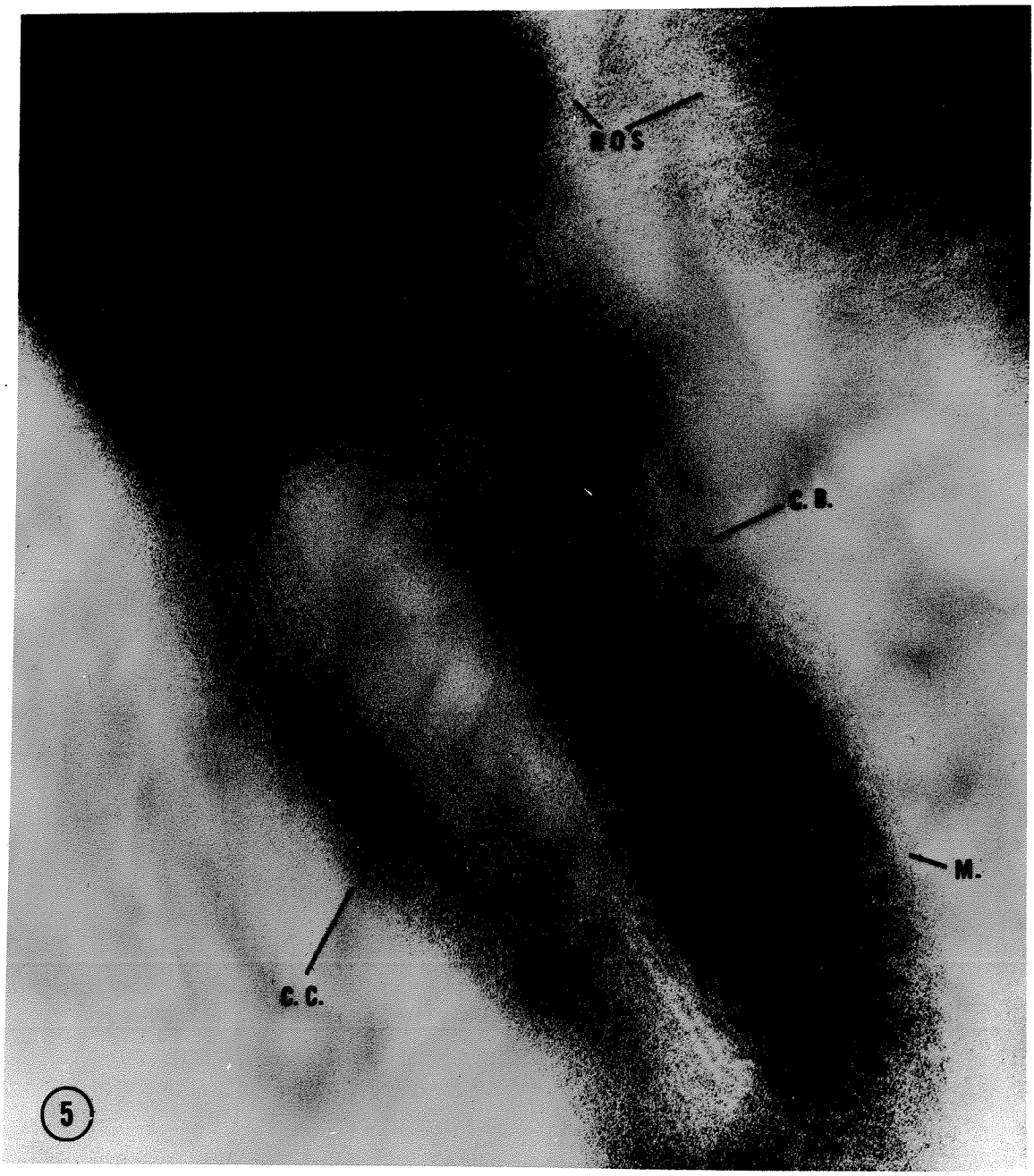


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FIGURE 6 A higher magnification of the mouse retina shown in Fig. 3. Ferritin particles are present in the rod outer segment (ROS) and the rod inner segment (RIS). Ferritin is also found over the connecting cilium (C.C.) and at the level of the cytoplasmic bridge (C.B.). A mitochondrion (M) is seen in the cytoplasmic bridge and the inner segment. X48,750

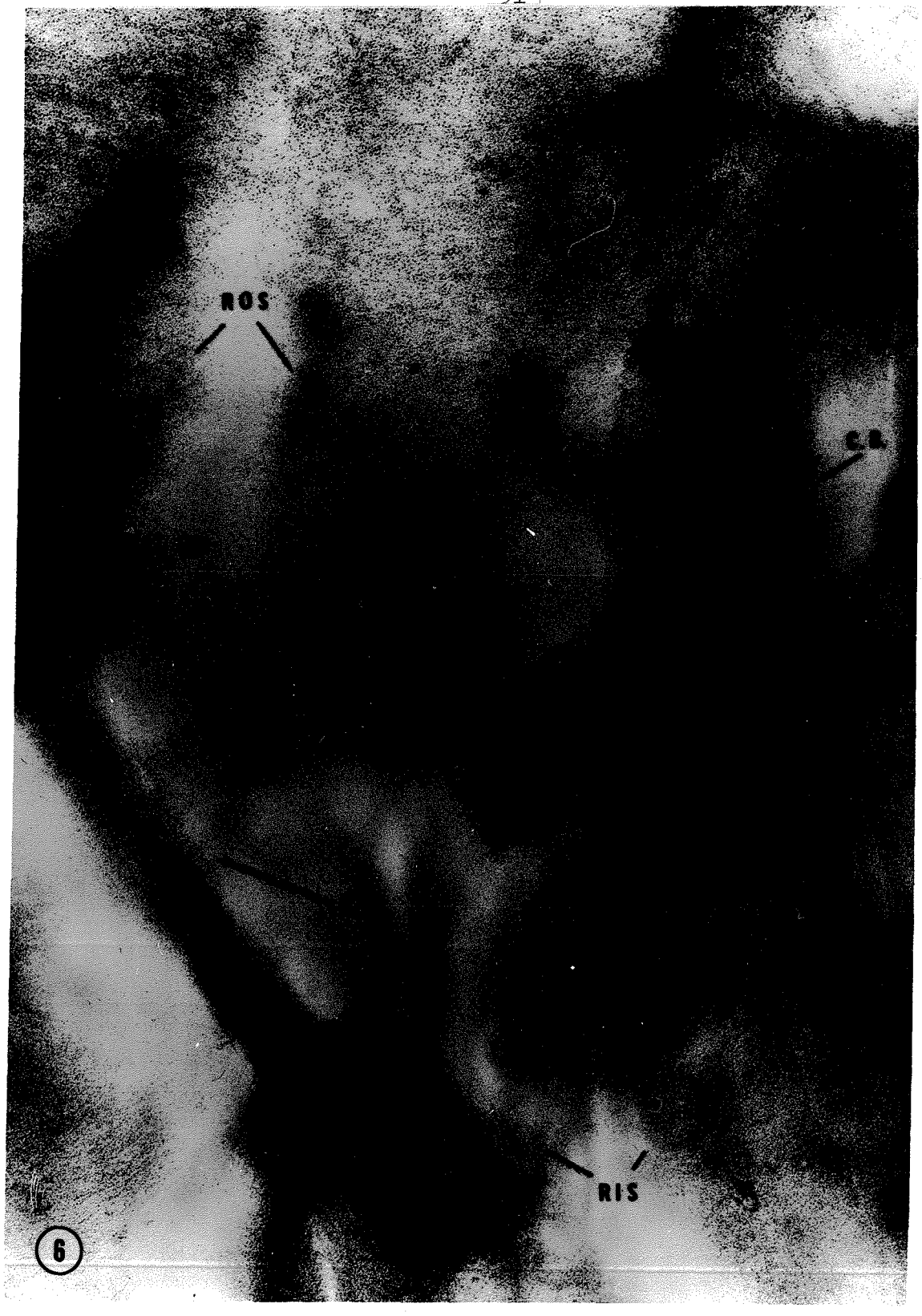
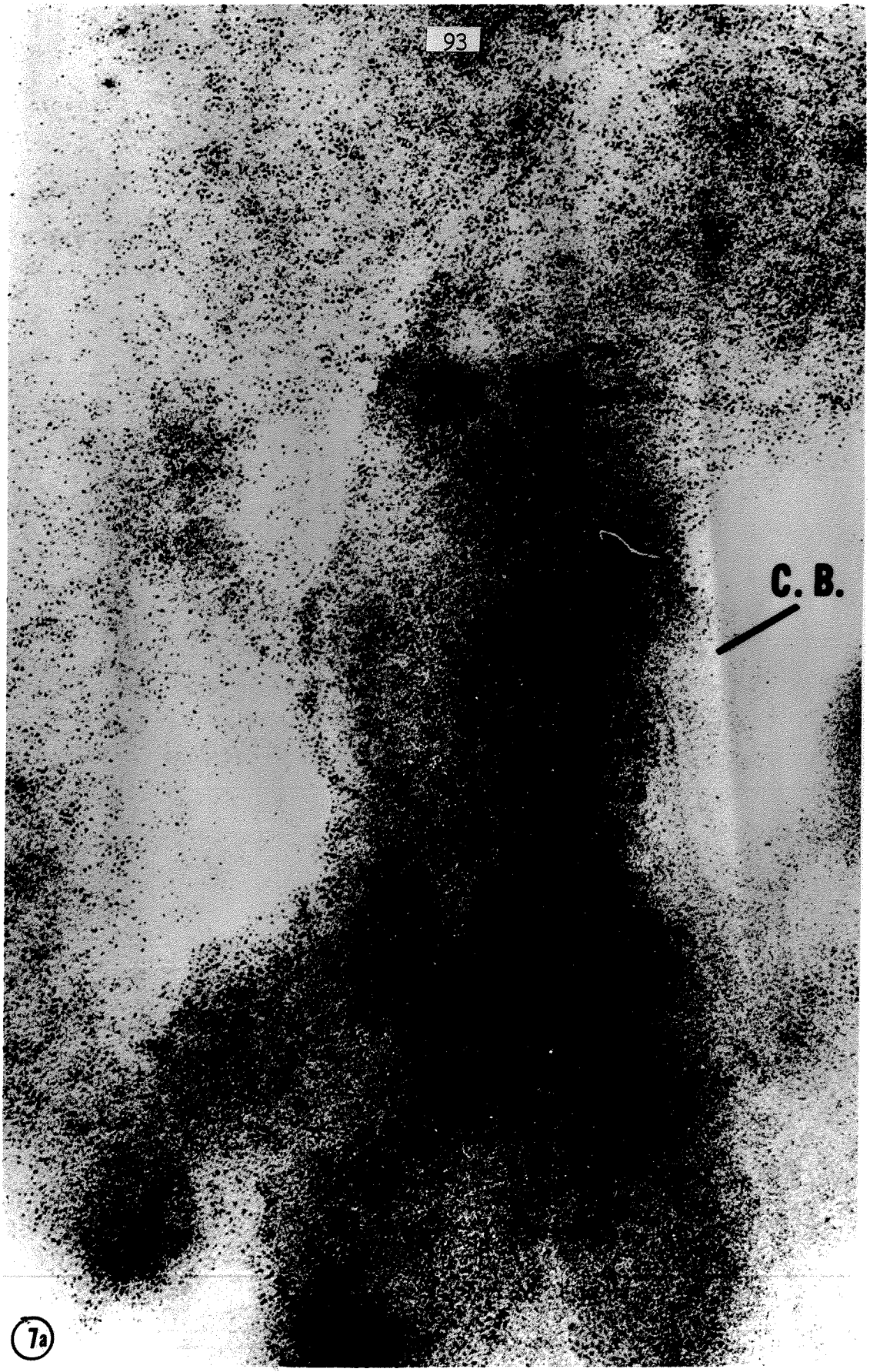
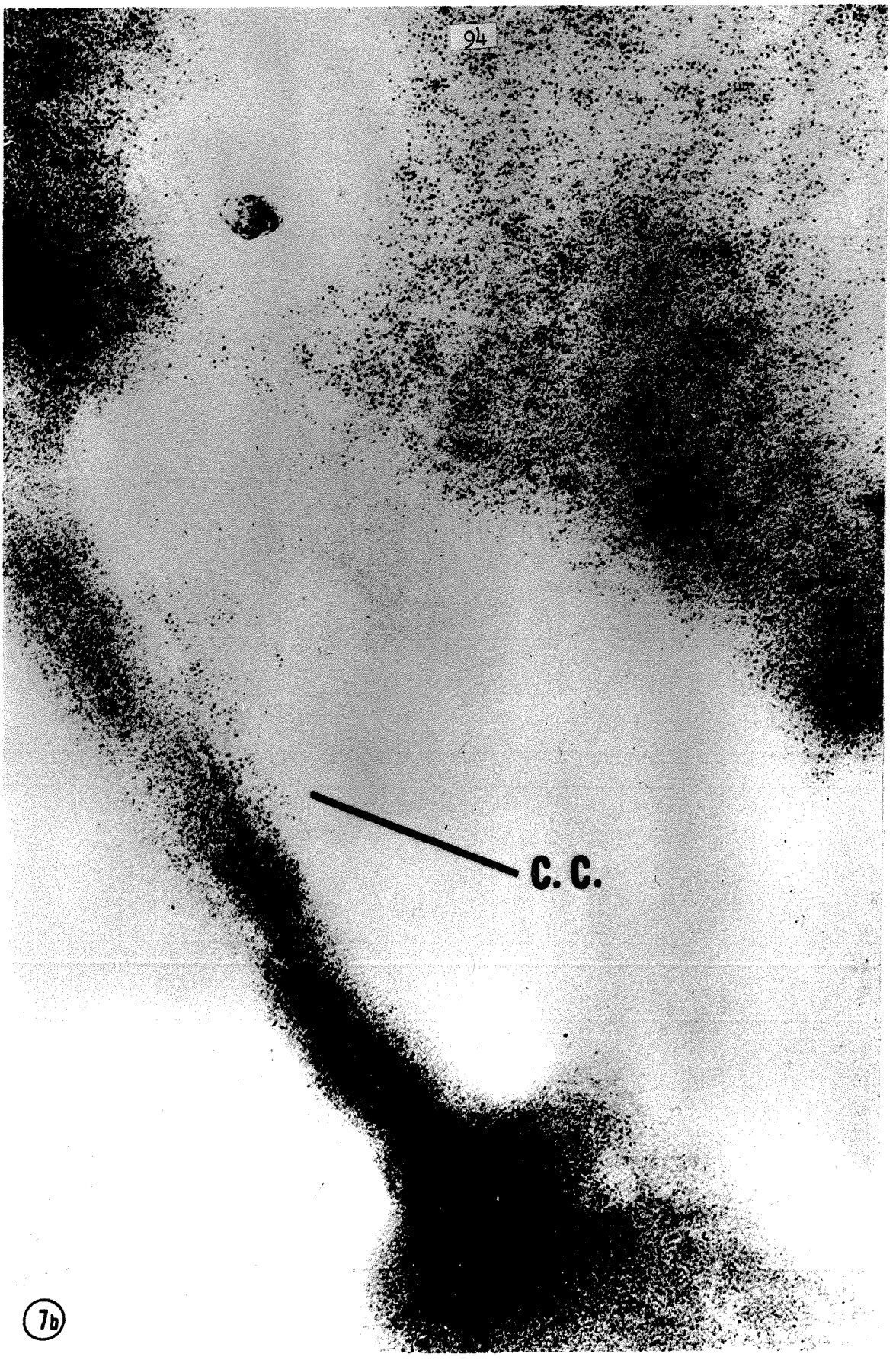

ROS
CL
RIS
6

FIGURE 7 A still higher magnification of the mouse retina shown in Fig. 3 and Fig. 6, showing the region of the cytoplasmic bridge in Fig. 7a and the connecting cilium in Fig. 7b. X97,500



7a



7b

APPENDIX

Hemocyanin-Antibody Labeling of Rhodopsin at the Level of the Scanning Electron Microscope

INTRODUCTION

In the experiments described in the first two chapters, we observed the distribution of ferritin or peroxidase staining in cross-sections of the rod outer segment (ROS) cell membrane but views of the true cell surface were hard to come by. Freeze-fracturing experiments allowed us to study the distribution of membrane particles over a big area of the hydrophobic fractured face of the ROS cell membrane but not of the cell surface itself. In the experiment reported here we have attempted to study the distribution of rhodopsin on surface views of the outer segment membranes as seen in the SEM. Glutaraldehyde-fixed mouse retina was first treated with rabbit IgG's specific for bovine rhodopsin ("specific rabbit antibody"), then with hemocyanin labeled goat IgG's specific for rabbit IgG ("goat antirabbit IgG"). Thus one could study the distribution of hemocyanin over the true surface of the retinal rod. The presence of hemocyanin label on the cell membrane would establish the fact that rhodopsin in the cell membrane exposes its antigenic sites to the extracellular space.

MATERIALS AND METHODS

Hemocyanin was obtained from the blood of Busycon canaliculatum. Hemocyanin was centrifuged into a soft pellet by centrifugation for 1 hour at 35K in a Type 40 Spinco rotor. This pellet was resuspended in a small volume of phosphate buffered saline (PBS), dialyzed against PBS at 4°C overnight, and passed through a Millipore filter (0.45 μ) before storage at 4°C.

Goat antiserum specific for rabbit IgG and DEAE column-purified rabbit IgG (nonspecific rabbit IgG) were obtained from Miles Lab, Kankakee, Illinois. The rabbit IgG's are used to build an immunoadsorbent for the purification of goat antirabbit IgG's (Avrameas, S., and T. Ternynck, 1969, Immunochemistry 6: 53-66).

To make conjugate of goat IgG and hemocyanin, 1 ml of 70 mg/ml hemocyanin and 1 ml of 10 mg/ml goat antirabbit IgG's in PBS were mixed and vortexed, while 0.22 ml of 0.5% glutaraldehyde in PBS was added in a dropwise fashion. The cross-linking reaction of glutaraldehyde was allowed to proceed at room temperature for 45 min. Then 0.2 ml of 2 M glycine in PBS was added to terminate the reaction. After 15 min at room temperature, the sample was dialyzed against PBS at 4°C overnight. An agarose column (Bio-Gel A 1.5 m, 1.5 cm x 80 cm) was used to separate hemocyanin and hemocyanin-goat IgG conjugates from the unreacted goat IgG's (S. O. Rosenberg, personal communication).

The labeling procedure is: (1) Fix mouse retina in the eye cup at 4°C for 1 h in the Karnovsky's fixative. (2) Separate the retina from the pigment epithelium. Wash in 0.1 M cacodylic buffer for 5 min at room temperature. (3) Incubate in 2 M glycine in PBS at 4°C for 1 h. (4) Wash twice in PBS at room temperature. (5) Treat with 0.18 mg/ml specific or nonspecific rabbit IgG in PBS for 8 h at room temperature with mild shaking. (6) Wash twice in PBS (5 min for each wash). (7) Wash extensively in PBS for 8 h at room temperature with shaking. (8) Treat with hemocyanin-goat antirabbit IgG conjugates at room temperature for 12 h with shaking. (9) Wash briefly in PBS

twice. (10) Wash extensively in PBS for 12 h at room temperature with shaking. (11) Fix in 0.5% glutaraldehyde in PBS for 5 min at room temperature. (12) Fix in 2% osmium tetroxide at 4°C for 1 h. (13) Dehydrate. (14) Critical-point dry the sample. (15) Mount on stage with silver paint. (16) Coat with gold. (17) Examine under an ETEC scanning electron microscope.

RESULTS AND DISCUSSION

A few hemocyanin molecules were found on the ROS surface of the control retina, as shown in Fig. 1. Numerous hemocyanin particles were present on the ROS cell membrane of the mouse retina treated with rabbit IgGs specific for bovine rhodopsin and hemocyanin-goat IgG conjugates (Figs. 2 and 3). This observation establishes the fact that rhodopsin is present at a high density on the ROS cell membrane of the mouse retina, confirming the results obtained on sections with peroxidase or ferritin labeled antibodies.

Since the size of a hemocyanin molecule is 350 Å and the size of a rabbit or goat IgG about 100 Å, the resolution in this experiment is about 550 Å. This resolution makes it difficult to resolve fine details of the rhodopsin distribution on the ROS cell membrane. The observed distribution of intramembrane particles in freeze-fracturing experiments suggests patterns with a periodicity which could probably not be clearly detected with the resolution obtained.

Prolonged treatments with antibodies and hemocyanin-antibody conjugates had to be used to allow the label molecules to reach

rhodopsin antigen on the ROS membrane which is located deep in the retina. Even with such prolonged treatments hemocyanin particles are scarce near the base of the ROS. It would thus be necessary to separate individual cells after the glutaraldehyde-fixation, if one wished to label antigens on cell membrane seated deeply inside the tissue. This will clearly have to be done to study the distribution of rhodopsin on the inner segment membranes.

To summarize the experiments described in the first two chapters and in this appendix, we have tried to study the rhodopsin distribution in the retina by treating the formaldehyde-fixed retina with Fab-peroxidase conjugate, by treating thin sections of frozen retina with specific rabbit IgG and ferritin-goat antirabbit IgG conjugates, by treating the surface of glutaraldehyde-fixed retina with specific rabbit IgG and hemocyanin-goat antirabbit IgG conjugates (for the scanning electron microscope), and by studying the distribution of membrane particles in freeze-fracturing experiments and establishing the correlation between those membrane particles and rhodopsin. Each technique has its own limitations. By comparing the results of experiments using different techniques, we learned that rhodopsin is present on the disc membranes, the cell mebbrane of the inner and outer segment, on the cilium, and inside the cytoplasmic bridge and the inner segment, and that rhodopsin in the cell membrane exposes its antigenic sites to the extracellular space and probably also to the cytoplasm of the rod.

FIGURE 1 Control mouse retina, treated first with nonspecific rabbit IgG, then with conjugates of hemocyanin and goat antirabbit IgG. A few hemocyanin particles are present on the surface of these rod outer segments. X62,500

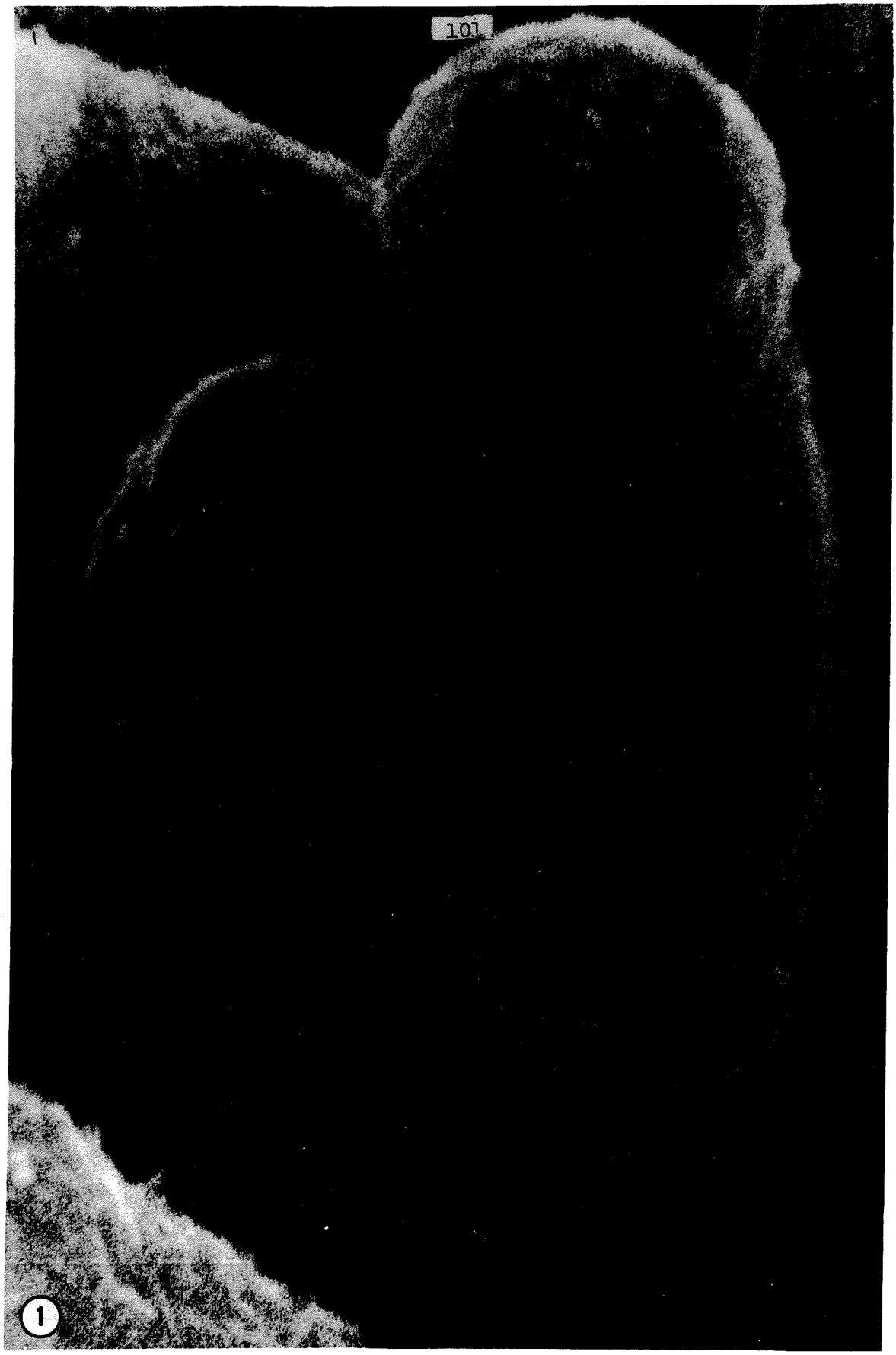

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FIGURE 2 Mouse retina treated with rabbit antibodies specific for bovine rhodopsin and hemocyanin-goat antirabbit IgG conjugates. Numerous hemocyanin molecules are seen on the cell membrane of the rod outer segments. X67,500

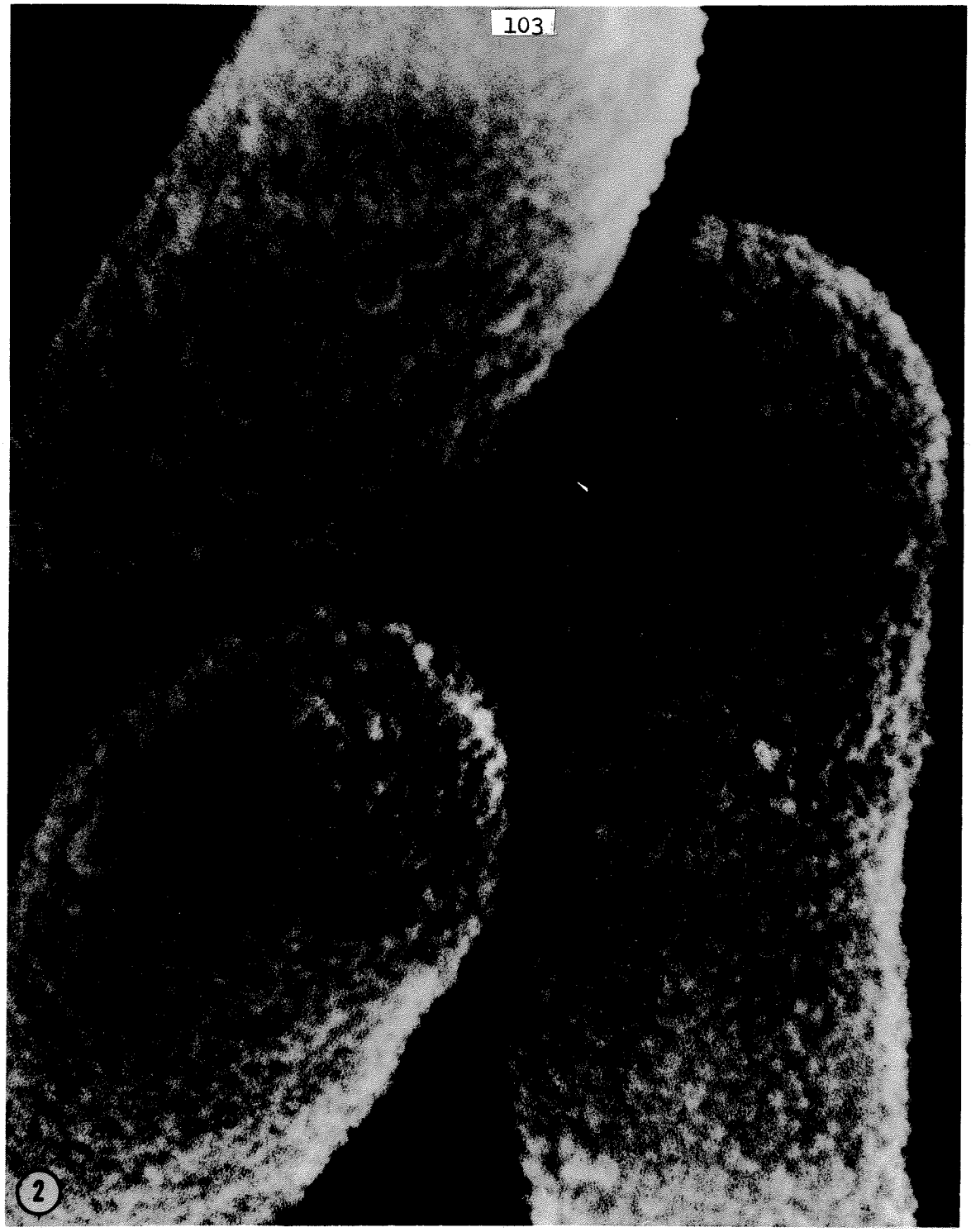
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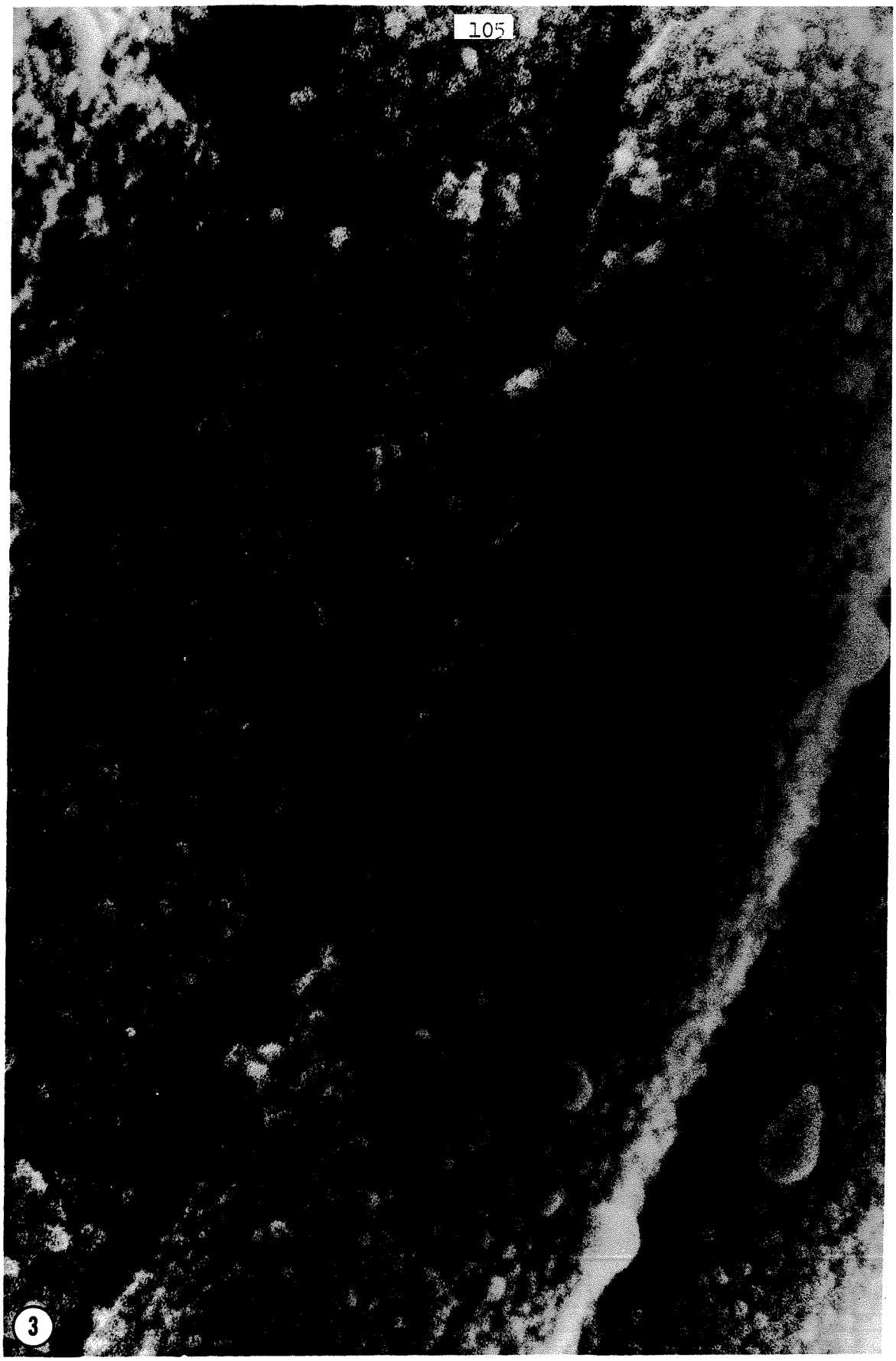
FIGURE 3 Mouse retina treated with rabbit antibodies specific for bovine rhodopsin and hemocyanin-goat antirabbit IgG conjugates. Hemocyanin molecules have the shape of a hollow cylinder, and appear as rectangles or circles on the surface of the rod outer segments. X67,500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Chapter III

THE ISOMERIC CONFIGURATION OF THE BACTERIORHODOPSIN CHROMOPHORE

INTRODUCTION

A rhodopsin-like protein was found in the cell membrane of Halobacterium halobium in 1971 (1). The striking similarities between the visual pigment of animals, rhodopsin, and this bacterium-borne protein include the light sensitivity, the general features of the two absorption spectra before and after the absorption of a photon, the presence of retinal as the chromophore, the Schiff's base linkage between retinal and a lysine residue, the segregation and close packing of the pigment in the membrane, the hydrophobic nature of the protein, the amino acid composition, and the sequences of the photochemical reactions (3, 4, 5). Therefore this rhodopsin-like protein in Halobacterium halobium was given the name bacteriorhodopsin. The pigment occurs in the cell membrane in patches containing as their only protein bacteriorhodopsin, and appearing purple due to the 560 nm absorption maximum of bacteriorhodopsin. These patches are therefore called the purple membrane.

A distinct advantage of the study of bacteriorhodopsin over that of vertebrate or invertebrate rhodopsin is the ease to generate large quantities of the protein and to obtain a purple membrane fraction free of other membranous contaminants. This is manifested by the great pace at which progress has been made in the understanding of bacteriorhodopsin. There have been indications that bacteriorhodopsin is involved in phototactic reactions of the bacterium (6). It has also been shown that the purple membrane is elaborated by the bacterium

in response to anoxic conditions and may function as a light-driven proton pump so as to utilize light energy for the synthesis of ATP (4, 7).

Another advantage of the bacteriorhodopsin system has not been fully realized in present research, namely, the possibility of genetic manipulations. Preliminary experiments indicate that nitrosoguanidine and EMS (6) can induce with a high frequency mutations in Halobacterium halobium, and various mutants with altered bacteriorhodopsin through nitrosoguanidine mutagenesis can be obtained. Different techniques for the analysis of bacteriorhodopsin are available, e.g., SDS acrylamide gel electrophoresis, isoelectric focusing, immunoelectrophoresis, etc. Thus bacteriorhodopsin may turn out to be an extremely useful model system for the study of its analog, the visual pigment rhodopsin.

A factor of great importance to the degree of analogy between bacteriorhodopsin and rhodopsin is the isomeric configurations of the chromophore, retinal, before and after the absorption of light by the pigment. In dark adapted rhodopsin, the chromophore has been shown to be in the 11-cis configuration in the case of cattle (8), chick (9), fish (10), squid (11), octopus (12), and probably lobster (13), euphausiid (14), and honey-bee (15). During bleaching, the chromophore in rhodopsin is isomerized to all-trans retinal (16). The isomeric configuration of retinal in bacteriorhodopsin has been studied with purple membranes suspended in buffer of low salt concentration and in dimethylsulfoxide solution (17). The present report

describes an attempt to examine the isomeric configuration of the bacteriorhodopsin chromophore under physiological conditions, i.e., purple membranes suspended in basal salt solutions containing 5 M NaCl.

MATERIALS AND METHODS

11-cis retinal was a kind gift of Hoffman-La Roche Inc., Nutley, New Jersey. All-trans retinal and 13-cis retinal were purchased from Sigma. Silica gel chromatogram sheets (20 cm x 20 cm without fluorescent indicator) were obtained from Eastman Kodak Co. Petroleum ether (b.p. 20-40°C) was a product of J. T. Baker Co., Phillipsburg, N. J. 2-methyl-2-hapten-6-one was purchased from K. & K. Laboratory, Cleveland, Ohio.

To obtain chromophore from dark adapted bacteriorhodopsin, 12 ml of purple membrane (~1 mg bacteriorhodopsin/ml) in basal salt (containing 2 g KCl, 20 g $MgSO_4 \cdot 7H_2O$, 3 g Na_3 citrate, 250 g NaCl per liter) was kept at 4°C in darkness for at least 12 hours, then chemically bleached by the addition of 4 ml of 0.1 M cetyltrimethylammonium bromide (CTAB). If 2 ml of 1 N HCl was then added to hydrolyze the Schiff's base between retinal and the protein, retinal was released and could be extracted in chloroform. If 4 ml of 2 M hydroxylamine in 50% ethanol was added to the purple membrane - CTAB mixture, retinal oxime was formed and could be extracted. All operations were done under dim red light.

To obtain retinal from light bleached bacteriorhodopsin, 12 ml of 1 mg bacteriorhodopsin/ml purple membrane in ether saturated basal salt was exposed to light of wavelength greater than 530 nm (Corning glass color filters), and then rapidly homogenized in chloroform under dim red light to extract retinal. To get retinal oxime from light bleached bacteriorhodopsin, the same amount of purple membrane in ether saturated basal salt was mixed with 4 ml of 2 M hydroxylamine in 50% ethanol under dim red light, exposed to light of wavelength greater than 530 nm for 10 min on ice, then extracted in chloroform under dim red light. Anhydrous ethyl ether was used to saturate the basal salt suspension of purple membrane in order to slow down the spontaneous regeneration process (3).

5 λ of 2-3 mg/ml 11-cis, 13-cis and all-trans retinal in 95% ethanol were applied on the same silica gel sheet with the retinal samples as standards. For retinal oxime standards, 25 λ of 11-cis, 13-cis or all-trans retinal in 95% ethanol was reacted with 25 λ of 2 M hydroxylamine in 50% ethanol at room temperature for 30 min. The reaction mixtures were evaporated under nitrogen, then resuspended in petroleum ether (b.p. 20-40°C). One third or two thirds of each petroleum ether solution of retinal oxime was applied at each spot as standards. The application of samples to silica gel sheets and the development of thin layer chromatograms were also performed under dim red light.

Separation of retinal isomers was achieved by developing silica gel sheets at 4°C in petroleum ether (b.p. 20-40°C)-acetone

(100:3, v/v) (solvent A) (18). Separation of different isomers of retinal oxime was performed by developing silica gel plates at 4°C in petroleum ether (b.p. 20-40°C)-2-methyl-2-hapten-6-one (11:2, v/v) (solvent B) (17, 19). Retinals were detected by their yellow color on silica gel sheets. Retinal oximes were detected by their fluorescence under long-wavelength ultraviolet irradiation.

RESULTS

Separation of all-trans, 11-cis and 13-cis retinals and retinal oximes

For the separation of isomers of retinal, silica gel sheets were developed in solvent A three times. All-trans retinal migrated upward for 10 to 11 cm, 11-cis retinal and 13-cis retinal are both 1.5 to 2.2 cm ahead of all-trans retinal. Thus it is easy to distinguish between all-trans retinal and 11-cis or 13-cis retinal, but 11-cis and 13-cis retinal are not well separated in this system.

For the separation of retinal oximes, silica gel sheets were developed once in solvent B at 4°C. Each isomer of retinal oxime gave two spots after chromatography, presumably corresponding to the syn and anti form (17). Typical values of distances traveled by different isomers are: 3.3 cm and 5.2 cm for all-trans, 4.2 cm and 5.5 cm for 11-cis, and 4.4 cm and 5.0 cm for 13-cis retinal oxime (or 7 and 9.3 cm for all-trans, 8.9 and 10.7 cm for 11-cis, and 7.8 and 8.4 cm for 13-cis retinal oxime, if longer periods of development were used). It was found that the distance between the two spots of each isomer was relatively constant in different runs of similar

length of development, and was very characteristic of the isomer. Thus separation of all-trans, 11-cis and 13-cis retinal oximes is possible. (Under these conditions all-trans, 11-cis and 13-cis retinals migrate much faster than the retinal oximes. Therefore any unreacted retinal in the retinal oxime preparation will not obscure the result.)

The isomeric configuration of the chromophore in dark-adapted bacteriorhodopsin

Artefacts of all-trans retinal introduced through thermal isomerization during preparation

If the CTAB treated purple membrane in basal salt or in distilled water was allowed to react with hydroxylamine at room temperature for more than 30 min, all-trans retinal oxime was the major product as identified on thin layer chromatogram sheets. If chloroform extraction was repeated every 10 min from the beginning of the reaction at room temperature for 90 min, all-trans retinal oxime was still the major component. However, if the reaction was allowed to proceed on ice for only 10 min before chloroform extraction was performed on ice (see details in next section), only 13-cis retinal oxime was found. Thus the appearance of all-trans retinal oxime derived from dark-adapted purple membrane appears to be an artefact of the experimental procedure.

As a control, 11-cis or 13-cis retinal was introduced at the time when CTAB and hydroxylamine were added to the purple membrane in basal salt. After 90 min at room temperature, the chloroform extract

of the mixture was obtained, dried under nitrogen, lyophilized at liquid nitrogen temperature in a light-tight container to remove traces of water, resuspended in petroleum ether, and checked by chromatography. No thermal isomerization of either 11-cis or 13-cis retinal oxime to all-trans retinal oxime took place, although not 100% of the retinal introduced was recovered in this procedure. In these control experiments the procedure used was the same as that described in the following section for the experiments. In these tests the molar ratio of retinal to bacteriorhodopsin was 10 to 1 to make sure that the amount of native retinal oxime originating from bacteriorhodopsin would be too small to be detected. The fact that thermal isomerization did not occur to added retinal in these control experiments but apparently occurred to the retinal chromophore in bacteriorhodopsin can be attributed to one or both of the following two factors: (1) The observed thermal isomerization of bacteriorhodopsin in basal salt or in water is caused by the presence of purple membrane. The lack of thermal isomerization in the control experiments is due to the molar excess of retinal in the control experiments. (2) Hubbard investigated thermal isomerization of the rhodopsin chromophore when incubating 11-cis retinal with an equivalent amount of denatured opsin (20). Denatured opsin induced thermal isomerization of 11-cis retinal at a temperature not high enough to cause isomerization of pure 11-cis retinal in digitonin solution. The isomerization of 11-cis retinal by added denatured opsin was less extensive compared to that occurring when rhodopsin was thermally denatured at the same temperature.

She concluded that the physical contact between 11-cis retinal and opsin in rhodopsin promoted isomerization during denaturation. This might also be true in the case of bacteriorhodopsin.

The isomeric configuration of the chormophore in dark-adapted bacteriorhodopsin

Dark-adapted purple membrane in basal salt was mixed with cold CTAB and hydroxylamine and let stand for 10 min on ice before the chloroform extraction was performed on ice. The chloroform extract was dried on ice under nitrogen, lyophilized at liquid nitrogen temperature in a light-tight container, resuspended in petroleum ether, and applied on silica gel sheets for thin layer chromatography. Less than 50% of the chromophore was extracted in this manner. In three experiments, fluorescence was detected only at the position corresponding to the two 13-cis retinal oxime spots. The resolution into two spots was not very good in these experiments or in cases where less than 20 μg of 13-cis retinal oxime was applied.

If the same amount of dark-adapted purple membrane in basal salt was mixed with cold CTAB and HCl, and let stand on ice for 10 min, the amount of retinal extracted in the separated chloroform phase was even less mostly because of the emulsification of chloroform in acid solutions. (Lyophilization of the entire sample might be a solution to this problem. However, thermal isomerization might take place during the long period of lyophilization required for the large amount of sample, if the low temperature of the sample was not maintained carefully.) Nevertheless, in one experiment about 20-30 μg of

retinal was extracted and was found to migrate as a single spot with an R_F corresponding to that of 11-cis or 13-cis retinal. A similar result was obtained when concentrated purple membrane in water suspension was dark-adapted, chemically bleached by CTAB, hydrolyzed by HCl and extracted by chloroform.

Thus the results of experiments involving two systems of thin layer chromatography indicate that the chromophore of the dark-adapted bacteriorhodopsin is 13-cis retinal.

The isomeric configuration of the chromophore in light bleached bacteriorhodopsin

It was shown in control experiments that the chromophore of dark-adapted purple membrane in basal salt, in ether saturated basal salt, or in water suspension could not be extracted directly by chloroform nor could retinal react with hydroxylamine under these conditions. Thus the procedure described in Materials and Methods isolates chromophores only from the light bleached bacteriorhodopsin. The same procedure for processing chloroform extracts described previously was used here as a precaution to avoid thermal isomerization of the extracted chromophore. Under these conditions only all-trans retinal (2 experiments) or all-trans retinal oxime (one experiment) was found by thin layer chromatography. It is concluded that the chromophore of light bleached bacteriorhodopsin in basal salt is all-trans retinal.

DISCUSSION

In this study, the chromophore of bacteriorhodopsin was found to be 13-cis retinal in the dark-adapted pigment (the 560 nm form, or R_{560}) and all-trans retinal in the light-bleached pigment (the 412 nm form, or M_{412}).

In the dark-adapted bacteriorhodopsin, as in the similar case of dark-adapted rhodopsin, the chromophore is protected from mild acid hydrolysis or the attack of hydroxylamine. Therefore one has to denature the pigment thermally or chemically in the dark to release the chromophore in its native form. Thermal denaturation of rhodopsin was attempted by Hubbard (20) and was found to result in substantial isomerization of the chromophore in the dark. Chemical isomerization of rhodopsin by acetic acid, ethanol, acetone, etc., at temperatures higher than 4°C also resulted in isomerization (8). It has been reported that denaturation of rhodopsin in presence of silver nitrate does not involve isomerization of the chromophore (21). Unfortunately bacteriorhodopsin is not denatured by silver nitrate. And the chromophore in dark-adapted bacteriorhodopsin cannot be extracted by chloroform. Therefore CTAB was chosen to induce the chemical denaturation (1). In these experiments thermal isomerization did take place at room temperature if one follows the procedure of Oesterhelt *et al.* (17). However, if a procedure similar to that of Rotmans *et al.* (8) was adapted for the extraction of retinal at 0°C, thermal isomerization was much reduced, and 13-cis retinal was identified as the major component in the extract.

It has been shown in the case of rhodopsin that the 11-cis configuration of the chromophore released from thermally or chemically denatured rhodopsin represents the true isomeric configuration of retinal in dark-adapted rhodopsin. The indirect evidence is the fact that only 11-cis retinal can recombine with opsin to form the light sensitive pigment with the same action spectrum as that of rhodopsin (22). Corresponding experiments with bacteriorhodopsin have not been reported and may not be feasible because the chromophore is not detached from the pigment unless the protein is denatured. More direct evidence comes from the resonance Raman spectroscopy, comparing the spectrum of retinal in intact rhodopsin with that of different retinal isomers covalently linked to peptides via a Schiff's base (23). It should be possible to examine bacteriorhodopsin by the same method.

Bacteriorhodopsin in basal salt absorbing maximally at 560 nm (R_{560}) is bleached readily by light to a form absorbing maximally at 412 nm (M_{412}). The light-bleached 412 nm form, however, is less stable, and is thermally converted to a 570 nm form (R'_{570}) with a half-life of about 5 msec at room temperature. The 570 nm form ("light-adapted bacteriorhodopsin") is converted to the 560 form in the dark with a half-life of about 20 min at room temperature (5, 17). This differs significantly from the behavior of vertebrate or invertebrate rhodopsins that have been studied. In vertebrate rhodopsin, 11-cis retinal is isomerized by light to all-trans retinal which dissociates from the visual pigment. A series of enzymes then convert retinal to

retinol, transport retinol or retinol esters to and from the pigment epithelium, isomerize the all-trans form to 11-cis form, etc., in order to reproduce 11-cis retinal and to regenerate rhodopsin (24). The whole process of regeneration takes tenths of minutes. And the reduction of rhodopsin concentration by light due to the slow regeneration processes is closely correlated with the state of light-dark adaptation of the retina (25). In certain invertebrates such as squid, all-trans retinal is not detached from the visual pigments after bleaching. The light-bleached pigment, metarhodopsin, is stable below 15°C. The machinery for the regeneration of squid rhodopsin has not been fully understood (26).

The fact that 13-cis retinal is the chromophore of bacteriorhodopsin, while 11-cis retinal is found universally in the animal kingdom, might be a consequence of different requirements imposed on the pigment. The 11-cis retinal is a sterically hindered form. The methyl group attached to the 13th carbon has to be pushed out of the plane of the conjugated double bonds due to steric hindrance (27). Thus it is thermodynamically impossible for a pigment containing 11-cis retinal to regenerate spontaneously from the all-trans form. In contrast, 13-cis retinal might be formed readily from all-trans retinal if the protein environment of the chromophore favors the 13-cis configuration.

Since bacteriorhodopsin is used as a proton pump to utilize light energy in the synthesis of ATP in the bacterium, the efficiency of this system would be greatly improved if bacteriorhodopsin can

regenerate spontaneously and quickly. While in the animal kingdom, rhodopsin is used as a reliable photon sensor, the energy used in the signal transduction processes is provided by the organism. A slower, more evolved rhodopsin regeneration mechanism might be helpful for the organism to cope with light varying over a wide range of intensities.

REFERENCES

1. D. Oesterhelt and W. Stoeckenius. 1971. Rhodopsin-like protein from the purple membrane of Halobacterium halobium. Nat. New Biol. 233, 149-152.

2. A. E. Blaurock, and W. Stoeckenius. 1971. Structure of the purple membranes. Nat. New Biol. 233, 152-155.

3. D. Oesterhelt and Benno Hess. 1973. Reversible photolysis of the purple complex in the purple membrane of Halobacterium halobium. Eur. J. Biochem. 40, 453-463.

4. D. Oesterhelt and W. Stoeckenius. 1973. Functions of a new photoreceptor membrane. Proc. Nat. Acad. Sci. U.S.A. 70 2853-2857.

5. W. Stoeckenius, personal communication.

6. K. Foster, personal communication.

7. E. Racker and W. Stoeckenius. 1974. Reconstitution of purple membrane vesicles catalyzing light-driven proton uptake and adenosine triphosphate formation. J. Biol. Chem. 249, 662-663.

8. J. P. Rotmans, S. L. Bonting, and F. J. M. Daemen. 1972. On the chromophoric group of rhodopsin. Vision Res. 12, 337-341.

9. T. Yoshizawa and G. Wald. 1967. Photochemistry of iodopsin. Nature 214, 566-571.

10. G. Wald. 1953. Vision. Fed. Proc. 12, 606-611.

11. R. Hubbard, R. C. C. St. George. 1957-58. The rhodopsin system of the squid. J. Gen. Physiol. 41, 501-528.

12. T. Hara, R. Hara, and J. Takeuchi. 1967. Vision in octopus and squid. Nature 214, 572-575.

13. G. Wald and S. P. Burg. 1957. The vitamin A of the lobster. J. Gen. Physiol. 40, 609-625.

14. G. Wald and P. K. Brown. 1957. The vitamin A of a euphausiid crustacean. J. Gen. Physiol. 40, 627-634.

15. T. H. Goldsmith. 1972. The natural history of invertebrate visual pigments. In "Handbook of Sensory Physiology" vol. VII, ed. H. J. A. Dartnall, Springer-Verlag. pp. 711.

16. G. Wald. 1968. Molecular basis of visual excitation. Science 162, 230-239.

17. D. Oesterhelt, M. Muntzen and L. Schuhmann. 1973. Reversible dissociation of the purple complex in bacteriorhodopsin and identification of 13-cis and all-trans retinal as its chromophore. Eur. J. Biochem. 40, 453-463.

18. M. Akhtar, P. T. Blosse and P. B. Dewhurst. 1968. Studies on vision: The nature of the retinal-opsin linkage. Biochem. J. 110, 693-702.

19. C. v. Planta, U. Schwieter, L. Chopard-dit-Jean, R. Ruegg, M. Kofler, and O. Isler. 1962. Synthesen in der Vitamin-A_2-reihe. 4. Physikalische eigenschaften von isomeren vitamin-A-und vitamin-A_2-verbindungen. Helv. Chim. Acta 45, 548-561.

20. R. Hubbard. 1958. The thermal stability of rhodopsin and opsin. J. Gen. Physiol. 42, 259-280.

21. R. Hubbard, P. K. Brown, and D. Bownds. 1971. Methodology of vitamin A and visual pigments. In "Methods in Enzymology" vol. 18, ed. D. B. McCormick and L. D. Wright, Academic Press, N. Y. pp. 622-623.

22. R. Hubbard, and G. Wald. 1952. Cis-trans isomers of vitamin A and retinene in the rhodopsin system. J. Gen. Physiol. 36, 269-315.

23. A. Lewis. 1974. Tunable laser resonance Raman spectroscopy - a selective probe of the visual process. 1974 ARVO Spring Meeting Abstract, p. 37.

24. Ch. Baumann. 1972. The regeneration and renewal of visual pigment in vertebrates. In "Handbook of Sensory Physiology" ed. H. J. A. Dartnall, Springer-Verlag, N. Y. pp. 395-416.

25. J. E. Dowling. 1960. Chemistry of visual adaptation in the rat. Nature 168, 114-118.

26. T. Hara and K. Hara. 1972. Cephalopod retinochrome. In "Handbook of Sensory Physiology" ed. H. J. A. Dartnall, Springer-Verlag, N. Y. pp. 720-746.

27. C. D. B. Bridges. 1970. Biochemistry of vision. In "Biochemistry of the Eye" ed. C. N. Graymore, Academic Press, New York. pp. 563-644.