Journal of Supramolecular Structure 3:61–66 (1975)

HEMOCYANIN – ANTIBODY LABELING OF RHODOPSIN IN MOUSE RETINA FOR A SCANNING ELECTRON MICROSCOPE STUDY

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To reveal the presence of rhodopsin on the surface of the mouse retina, a scanning electron microscope study of the immunolabeling of rhodopsin was attempted. The glutaraldehyde-fixed mouse retina was treated first with rabbit antibodies specific against bovine rhodopsin and then with hemocyanin-labeled goat antibodies specific against rabbit antibody. The distribution of hemocyanin label on mouse retina and the technique used for labeling are discussed.

INTRODUCTION

In the course of studies on the distribution of rhodopsin in vertebrate retinal rods, we prepared rabbit IgG's specific against bovine rhodopsin ("specific rabbit antibodies") (1). Conjugates of specific rabbit antibody and peroxidase were then used to label rhodopsin in whole mouse or bovine retina. In both of these cases the disc membrane, the connecting cilium, and the cell membrane of the rod outer segment (ROS) and the rod inner segment (RIS) were stained (1). In these experiments, however, we could examine only the distribution of the label in cross-sections of the rod cell membrane. Extensive views of the true cell surface were difficult to obtain in thin Epon sections. In an attempt to map the distribution of rhodopsin in the membrane we tried to establish a correspondence between the labeled rhodopsin and the distribution of intramembrane particles on freeze-fractured ROS membranes. Freeze-fracturing revealed a pattern of membrane particles over much of the hydrophobic fractured face of the ROS cell membrane. We describe here attempts to study the distribution of rhodopsin on surface views of the outer segment membranes as seen in the scanning electron microscope. To localize rhodopsin on the surface of the retinal rod we treated glutaraldehyde-fixed mouse retina with specific rabbit antibodies and then with hemocyanin-labeled goat IgG's specific for rabbit IgG ("goat antirabbit IgG"). The presence of hemocyanin label (2, 3, 4) on the cell membrane establishes the fact that rhodopsin in the cell membrane exposes its antigenic sites to the extracellular space.

MATERIALS AND METHODS

The rabbit IgG's specific for bovine rhodopsin were purified by immunoadsorption. The detailed purification procedure and characterization of the antibodies are given in detail elsewhere (1).

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Hemocyanin was obtained from the blood of Busycon canaliculatum (5). Hemocyanin was centrifuged into a soft pellet by centrifugation for 1 hr at 35,000 rpm in a Type 40 Spinco rotor. This pellet was resuspended in a small volume of phosphate-buffered saline (0.1 M, pH 7.0, abbreviated as 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 Laboratories, Kankakee, Illinois. The rabbit IgG's are used to build an immunoadsorbent for the purification of goat anti-rabbit IgG's (6).

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 same was dialyzed against PBS at 4°C overnight. An agarose column (Bio-Gel A 1.5 m, 1.5 cm × 80 cmp) was used to separate hemocyanin and hemocyaningoat IgG conjugates from the unreacted goat IgG conjugates from the unreacted goat IgG's (7).

The labeling procedure is: (1) Fix mouse retina in the eye cup at 4°C for 1 hr in Karnovsky's fixative (1). (b) Separate the retina from the pigment epithelium. Wash in 0.1 M cacodylic buffer for 5 min at room temperature. (c) Incubate in 2 M glycine in PBS at 4°C for 1 hr. (d) Wash twice in PBS at room temperature. (e) Treat with 0.17 mg/ml specific or nonspecific rabbit IgG in PBS for 8 hr at room temperature with mild shaking. (f) Wash twice in PBS (5 min for each wash). (g) Wash extensively in PBS for 8 hr at room temperature with shaking. (h) Treat with hemocyanin-goat antirabbit IgG conjugates at room temperature for 12 hr with shaking. (i) Wash briefly in PBS twice. (j) Wash extensively in PBS for 12 hr at room temperature with shaking. (k) Fix in 0.5% glutaraldehyde in PBS for 5 min at room temperature. (l) Fix in 2% osmium tetroxide at 4°C for 1 hr. (m) Dehydrate. (n) Critical-point dry the sample from Freon 13. (o) The gold-coated samples were examined with an ETEC scanning electron microscope.

RESULTS AND DISCUSSION

A few hemocyanin molecules were found on the ROS surface of the control retina treated with nonspecific rabbit IgG and hemocyanin goat IgG conjugates (Fig. 1). Numerous hemocyanin particles were present on the ROS cell membrane of the mouse retina treated with rabbit IgG's 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-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 is at best about 550 Å. The poor 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 (1) which could probably not be clearly detected with the resolution obtained.

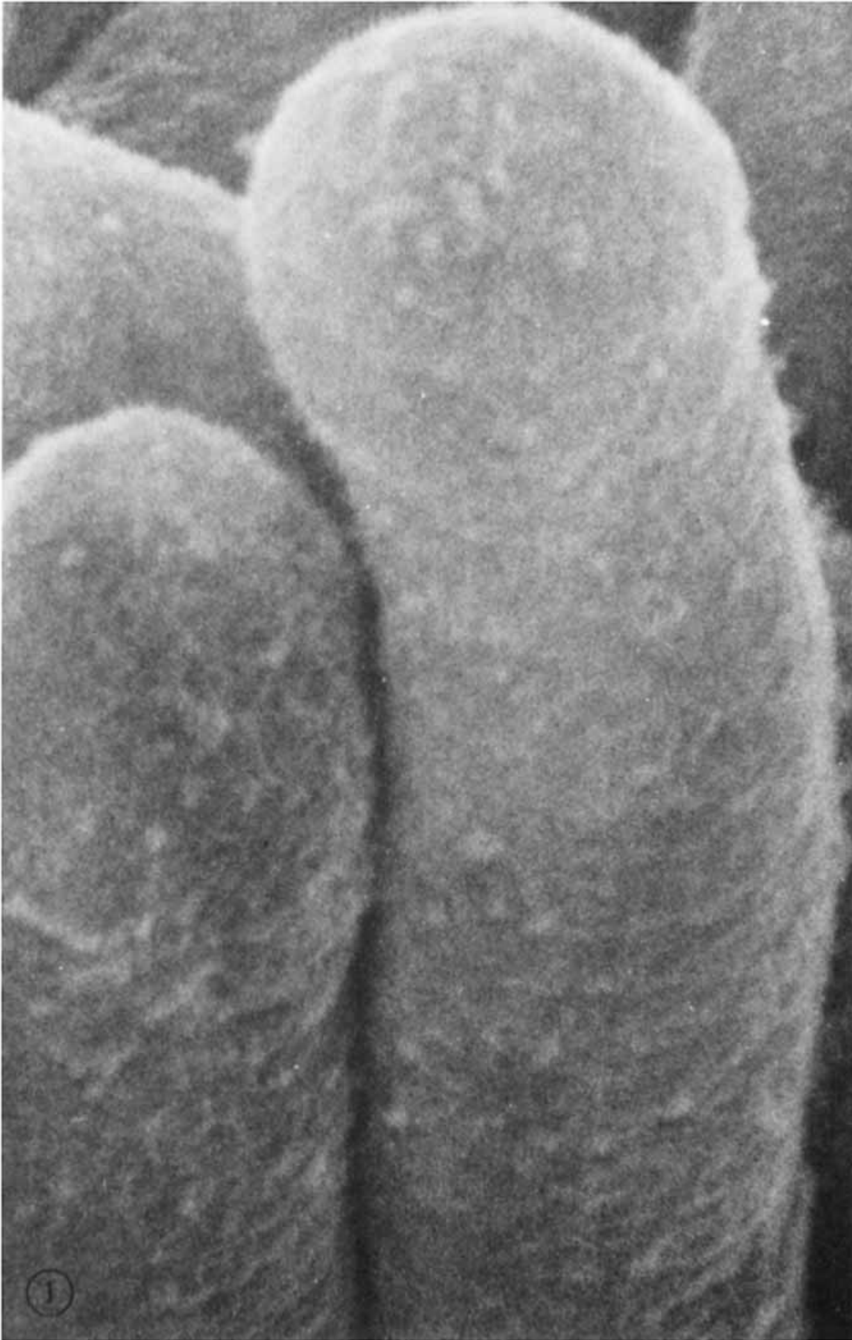

Fig. 1. Control mouse retina, treated first with nonspecific rabbit IgG and then with conjugates of hemocyanin and goat antirabbit IgG. A few hemocyanin particles are present on the surface of these rod outer segments. (× 62,500)

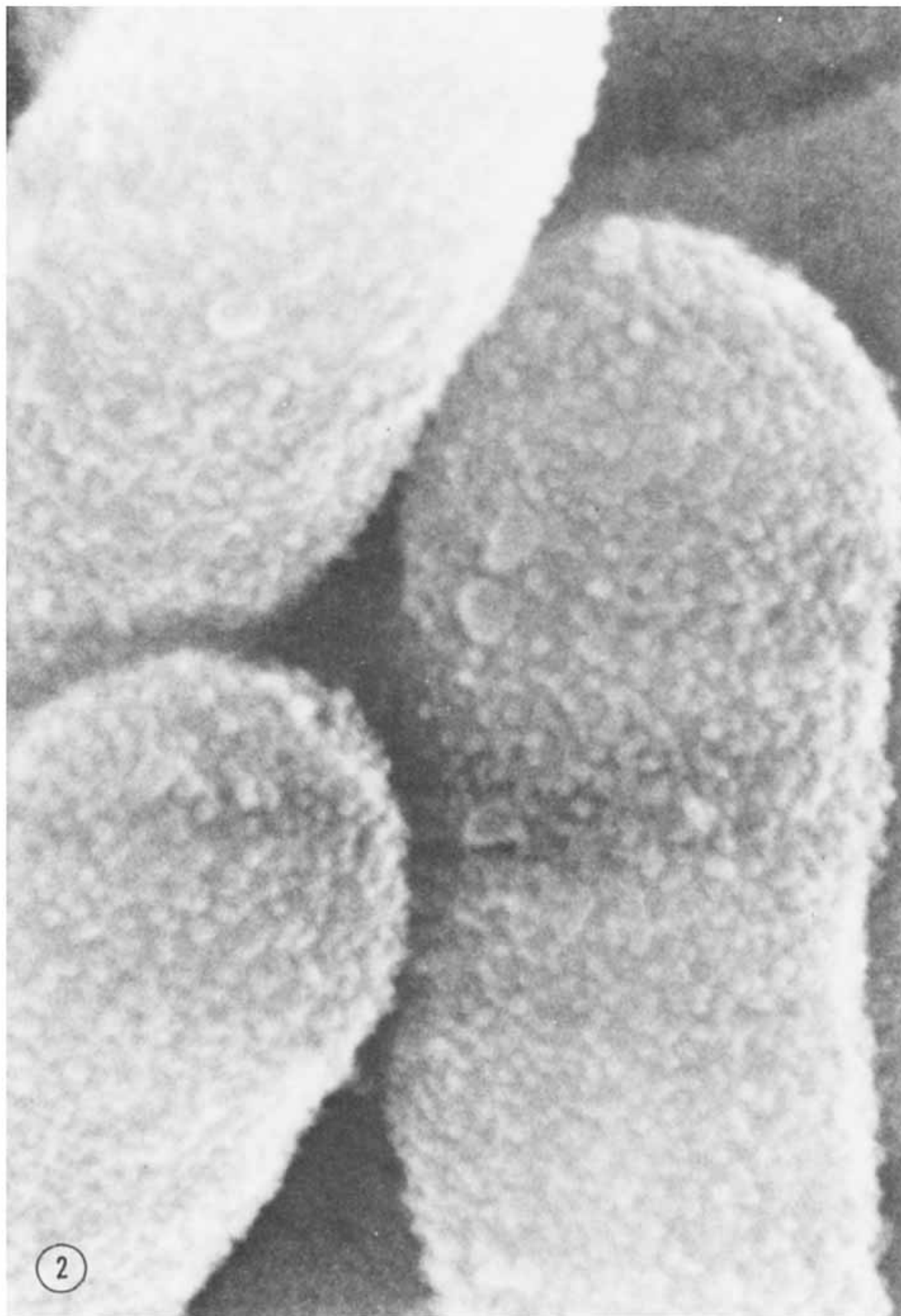

Fig. 2. Mouse retina treated with rabbit antibodies specific for bovine rhodopsin and hemocyanin-goat antirabbit IgG conjugates. Numerous hemocyanin molecules are seen on the cell membrane of the rod outer segments. (× 67,500)

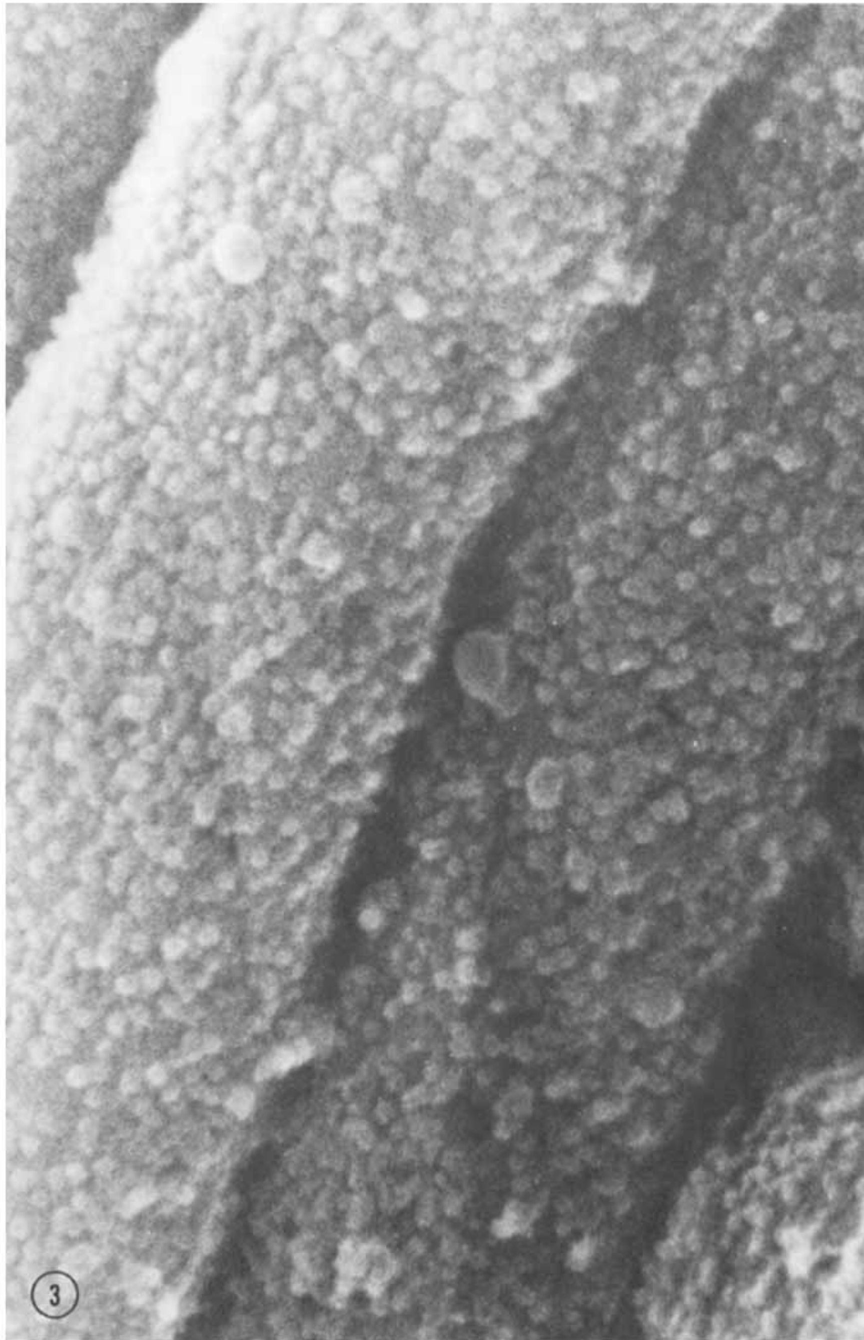

Fig. 3. Mouse retina treated with rabbit antibodies specific for bovine rhodopsin and hemocyanin-goat antirabbit IgG conjugates. Hemocyanin molecules have the shape of a hollow cylinder, and appear as rectangles or circles on the surface of the rod outer segments. (× 67,500)

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 (8) 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.

The presence of a high density of hemocyanin on the surface of the rod outer segment also confirms our observations on thin sections of immuno-labeled retina. Rhodopsin in the ROS cell membrane exposes its antigenic sites to the extracellular space. Similar techniques, using antibodies purified on an immunoadsorbent which is made of the short glycopeptide chains of rhodopsin, (9) could be used to find out whether the carbohydrate chain of rhodopsin is exposed to the extracellular space.

ACKNOWLEDGMENTS

We would like to thank Dr. Suzanne O. Rosenberg and Dr. Charles Birdwell for the discussions on the preparation of the goat antibody-hemocyanin conjugates. This work was supported in part by grants GM 06965 and GM 19224 of the U. S. Public Health Service and partly by the California Foundation for Biochemical Research.

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