RHODOPSIN IN THE ROD OUTER SEGMENT PLASMA MEMBRANE

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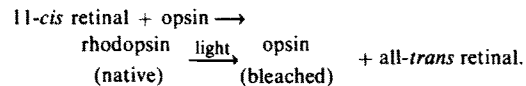
ABSTRACT

Isolated frog retinas were incubated in vitro with a 4-h pulse of [³H]leucine, then chased for 32 h with a nonradioactive amino acid mixture. At the end of the incubation, light and electron microscope autoradiograms were prepared from some of the retinas. The autoradiograms revealed: (a) intense radioactivity in the basal disks of the rod outer segments, (b) diffuse label evenly distributed throughout the rod outer segments, and (c) a high concentration of label in the entire rod outer segment plasma membrane. Incubation under identical conditions, but with puromycin added, significantly inhibited the labeling of all of these components. To identify the labeled proteins, purified outer segments from the remaining retinas were analyzed biochemically by SDS disc gel electrophoresis and gel filtration chromatography. SDS gel electrophoresis showed that about 90% of the total rod outer segment radioactivity chromatographed coincident with visual pigment, suggesting that the radiolabeled protein in the plasma membrane is visual pigment. Gel filtration chromatography demonstrated that the radiolabeled protein co-chromatographed with rhodopsin rather than opsin, and that the newly synthesized visual pigment in both the basal disks and the plasma membrane is present in the native configuration.

The outer segments of vertebrate photoreceptors contain stacks of hundreds of membranous disks bounded by a plasma membrane (Fig. 1). In the rod outer segment (ROS) these disks are separated, closed sacs throughout the outer segment, except at the base where the first few consist of continuous infoldings of the plasma membrane (10, 11). The disks in the rod are constantly renewed throughout the life of the animal, with new ones being formed at the base of the ROS from proteins and lipids synthesized in the inner segment (32, 33, 35). This process displaces the older disks toward the apex of the outer segment, where small packages of them are shed, then phagocytized and digested by the pigment epithelium. The ROS is thus maintained at a relatively uniform length (34).

The composition of ROS disk membranes has been well characterized. They consist of about 50% protein and 50% lipid with the visual pigment glycoprotein, rhodopsin,[1] comprising 80–90% of the total protein present in the disk (19, 20, 29). The lipid composition of the ROS is primarily phospholipid, and it has been shown that the

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[1] The terms rhodopsin and opsin are used to describe visual pigment in its native and bleached state, respectively. The more general term, visual pigment, is used when the bleaching state of the protein is not known. The interactions of visual pigment protein and its vitamin A chromophore are described by the following:

11-cis retinal + opsin ⟶
$$\text{rhodopsin} \xrightarrow{\text{light}} \text{opsin} + \text{all-}trans \text{ retinal.}$$
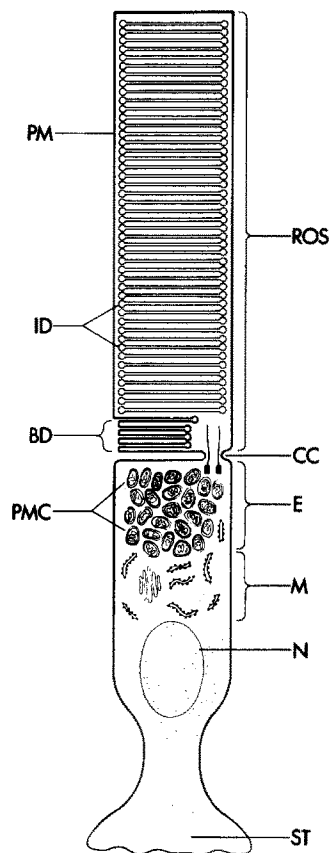(native)   (bleached)

FIGURE 1 Diagram of frog red rod photoreceptor. *ROS*, rod outer segment; *PM*, outer segment plasma membrane; *ID*, isolated outer segment disks; *BD*, basal outer segment disks which are continuous with the plasma membrane; *CC*, connecting cilium; *E*, mitochondria-rich ellipsoid; *PMC*, perimitochondrial cytoplasm; *M*, myoid region containing endoplasmic reticulum and Golgi apparatus; *N*, nucleus; *ST*, synaptic terminal.

However, because the plasma membrane has been implicated in controlling the photocurrent (16, 17), the composition, nature, and function of the ROS plasma membrane have been subjects of interest for some time. Some of the interest has centered upon the presence or absence of rhodopsin in this membrane. An early report by Dewey et al. (14) and a recent report by Jan and Revel (22) have tentatively identified opsin as a component of the plasma membrane. Additionally, the early receptor potential has been suggested to originate in the plasma membrane and/or the basal disk membranes which are continuous with the plasma membrane (25, 30).

This paper concerns the renewal and composition of the plasma membrane of the frog ROS. Using autoradiographic and biochemical techniques, we have demonstrated the presence of rhodopsin in the plasma membrane and have shown it to be in the native configuration under fully dark-adapted conditions.

MATERIALS AND METHODS

Labeling Procedure

Retinas from adult frogs (40–60 g) were labeled by the in vitro procedure of Basinger and Hall (4, 5). All procedures were carried out in complete darkness or under dim red light. Frogs were dark adapted overnight, killed by decapitation, and after enucleation the retinas were dissected free of pigment epithelium and choroid. The retinas were then incubated at 23°C in 10 ml of Ringer's-bicarbonate-glucose (RBG) containing 580 μCi/ml of [4,5-^{3}H]leucine (sp act, 55 Ci/mmol; Schwartz/Mann div., Becton, Dickinson & Co., Orangeburg, N.Y.) and gassed with 1.2 ft^3/h of O_2/CO_2 (95%/5%). After 4 h of incubation, the retinas were placed in 10 ml of nonradioactive RBG containing 10 μg/ml casamino acids (Difco Laboratories, Detroit, Mich.), and then incubation was continued for another 32 h. The medium was replaced with 10 ml of RBG + 10 μg/ml casamino acids every 4 h. Additional retinas were incubated under identical conditions, except that 10 μg/ml of puromycin (Sigma Chemical Co., St. Louis, Mo.) was added at zero time and was present throughout the entire incubation.

Autoradiography

After 36 h of incubation, retinas treated under the conditions mentioned above (with and without puromycin) were fixed overnight in 1% formaldehyde and 1% glutaraldehyde buffered with 0.085 M sodium phosphate (pH 7.2). The aldehyde-fixed retinas were cut into small pieces, fixed additionally in phosphate-buffered 1% osmium tetroxide, dehydrated, and embedded in Araldite

phospholipids contain an unusually large percentage of polyunsaturated fatty acids, particularly decosahexenoic acid (22:6) (1, 2, 3, 7, 26). This composition suggests a fluid membrane, and recent experiments have shown that the visual pigment protein can undergo both lateral and rotational diffusion within the disk membrane (9, 12, 13, 23, 28).

The plasma membrane of the ROS comprises only about 1% of the total frog ROS membrane system and about 4–5% of the rat ROS. Thus far, it has not been possible to isolate the plasma membrane free of the disk membranes, and little is known about the composition of this structure.

502 (Ciba-Geigy Corp., Ardsley, N.Y.). The retinal fragments were oriented so that longitudinal sections of the photoreceptor cells could be obtained. Sections of 0.5 μm were placed on glass microscope slides, and light microscope autoradiography was performed according to previously published methods (34). The preparations were exposed for 5 days at 4°C in the presence of a dessicant and developed in Kodak Dektol for 2 min at 17°C.

Electron microscope autoradiograms were prepared from retinas incubated without puromycin. Silver sections (~700 Å) were cut with a diamond knife on a Reichert OMU-2 ultramicrotome and deposited 2.5 cm from the ends of glass microscope slides which had previously been coated with a thin layer of celloidin. The sections were stained with uranium and lead, coated with carbon, and then dipped in Ilford L-4 emulsion. Before use, the emulsion was diluted approximately 1:5 with distilled water and maintained at 40°C. Under these conditions, the emulsion over the sections exhibited a purple interference color, and the monolayer of silver halide crystals was closely and regularly packed. The preparations were exposed in the dark in the presence of a dessicant at 4°C for 80 days and developed for 1 min at 15°C in Phenidon developer (24).

Biochemical Analysis

ROS from retinas incubated in the absence of puromycin were isolated according to a modification of the method of Papermaster (27). The retinas were added to a cellulose nitrate centrifuge tube containing 1 ml of 76% (wt/vol) sucrose buffered with 5 mM Tris-HCl, 0.2 mM $MgCl_x$, 62 mM NaCl, pH 7.4, then homogenized with six passes of a tight-fitting Teflon pestle. An additional 2 ml of 76% sucrose was added, and after vortexing, a discontinuous sucrose gradient was formed above the homogenized retinas by successively layering 4 ml of ρ = 1.15, 5 ml of ρ = 1.13, and 4 ml of ρ = 1.11 sucrose in Tris-Mg buffer. After centrifugation at 100,000 g for 1 h at 4°C, the ROS band at the 1.11/1.13 interface was recovered and washed four times with 67 mM sodium phosphate buffer containing 4 mM $MgCl_2$, pH 7.0. A small aliquot was taken for gel electrophoresis, and the remainder was solubilized at 4°C for 2 h in 1 ml of 2% Emulphogene (General Aniline and Film Corp., New York) in 0.05 M Tris-HCl, pH 8.5.

Column Chromatography

Emulphogene-solubilized ROS were chromatographed on a 150 × 0.9 cm column of Agarose A-1.5 M (100–200 mesh, Bio-Rad Laboratories, Richmond, Calif.) equilibrated with 1% Emulphogene in 0.05 M Tris-HCl buffer at pH 8.5 at a rate of 2.0 ml/h. This column procedure separates opsin from rhodopsin with respective elution volumes (V_e) of 46.5 and 51.5 ml. 1-ml fractions were collected. Each fraction was read at 280 nm and 500 nm in a Cary 118 spectrophotometer and

0.5 ml aliquots were counted in 10 ml of Aquasol (New England Nuclear, Boston, Mass.). The results were corrected for quenching by the external standard method. Specific activities are expressed as dpm/ml/absorbance at 280 or 500 nm.

Disc Gel Electrophoresis

An aliquot of ROS was dissolved in 1% sodium dodecyl sulfate (SDS; Sigma Chemical Co) and incubated at 37°C for 1 h. Electrophoresis, staining with Coomassie blue, and destaining were done according to the method of Fairbanks et al. (15), using 7.5% gels and a current of 3 mA/gel. By this procedure, frog rhodopsin has an apparent mol wt of 35,000 and always constitutes at least 70% of the ROS protein applied to the gel. Stained gels were scanned at 550 nm with a Gilford model 240 spectrophotometer. Gels were then sliced into 1-mm sections. Each slice was dissolved in 1 ml of 30% H_2O_2 at 60°C overnight (36), and radioactivity was counted in a Beckman LS-230 scintillation counter with 10 ml of Aquasol. The A_{550} scan of the gel and the cpm/mm were plotted to determine the percent of radioactivity in each protein peak on the gel.

Analysis of Electron Microscope Autoradiograms

The previously published data of Salpeter, Bachmann and Salpeter (31) were used to calculate the resolution of our autoradiographic method. These authors use the concept of half distance (HD) to calculate resolution, where one HD is defined as the distance on each side of a linear source of radiation within which one-half of the silver grains fall. They state that this value varies with the type of developer and emulsion used, the thickness of the emulsion layer, and the section thickness. In the present study, it was assumed that the resolution obtained with Phenidon developer (1-phenyl-3-pyrazolidone) more closely resembles that of paraphenylenediamine than that of Microdol X. Therefore, our calculations for resolution interpolated the data of Salpeter et al. (31) for paraphenylenediamine developer, Ilford L-4 emulsion, and a section thickness of 700 Å. An HD value of 1,350 Å was thus obtained.

Electron micrographs were enlarged to a final magnification of 13,000, and well-oriented ROS with sharply delineated plasma membranes were selected for analysis. At appropriate intervals, points were marked 3 HD's away from each side of the plasma membrane. The points were then connected so that the plasma membrane surrounding the outer segment disks was paralleled by two lines 6 HD's apart. A calibrated transparent grid was placed over each electron micrograph and the area between parallel lines surrounding the plasma membrane was measured. Likewise, the remaining area over the outer segment disks was measured, except the area occupied by intensely radioactive basal outer segment

disks. The silver grains over plasma membrane and diffusely labeled outer segment disks were then counted. It should be pointed out at this point that the methods of Salpeter et al. (31) were not used to establish the origin of the radioactive source but merely to correct for radiation spread from a source established on the basis of more qualitative evidence.

According to Salpeter et al. (31), an area extending for 3 HD's on each side of a linear source will include 78.5% of the silver grains due to radioactivity from that source. Accordingly, the data for the plasma membrane were extrapolated to 100%. On the other hand, due to the close proximity of the plasma membrane to the outer segment disk edges, some of the plasma membrane counts were really contributed by the disks and some of the disk counts were contributed by the plasma membrane. With the aid of the standard curves of Salpeter et al. (31) for a band source of infinite width, we calculated the disk contribution to plasma membrane counts and the appropriate correction was made. Corresponding corrections for plasma membrane contribution to disk counts were not made because the difference was within the statistical variation of the data.

Grain counts over intensely radioactive basal outer segment disks were made in the same manner as those for the plasma membrane. Points were marked 3 HD's away from each side of the most basal disk, parallel lines were drawn and silver grains were counted with the aid of a calibrated transparent grid. The perimitochondrial cytoplasm, part of which lies adjacent to the basal disks, was quite radioactive and therefore contributed to some of the basal disk counts. With the aid of the transparent grid, the silver grain density over this cytoplasm was measured at points well away from the base of the outer segment. Since the band of perimitochondrial cytoplasm adjacent to the basal outer segment disks has an average width of about 6 HD's, its contribution of grain counts to the basal disks was calculated from the standard curves of Salpeter et al. (31) for a band source with a width of 6 HD's. Basal disk counts were also corrected for contributions from diffusely radioactive disks.

The corrected grain counts/unit grid area for plasma membrane and intensely labeled basal discs were used to calculate the radioactive density of these structures. These calculations were based on a plasma membrane area of 782 μm^2/outer segment (outer segment length = 40 μm and diameter = 6 μm) and a section thickness of 700 Å. Likewise, the radioactive density of diffusely labeled outer segment disks was calculated from the section thickness, the disk diameter (6 μm) and the number of disks/outer segment (2,000). All calculations for disk radioactivity took into account the fact that each disk is composed of a double layer of membrane.

The raw data were reduced by a method of Poisson statistics[2] in which each category of organelle (plasma

membrane, basal disks and diffusely labeled disks) was assumed to be homogeneous with respect to silver grain density. This assumption was verified by analysis of the standard deviations based upon grain counts made for each individual micrograph.

RESULTS

Autoradiography

Rod photoreceptors from retinas that were incubated with [^{3}H]leucine for 4 h and then chased for 32 h with nonradioactive amino acids showed radioactivity distributed throughout their cytoplasm with heavier concentrations of label in certain areas of the cell. This was best observed in light microscope autoradiograms in which the entire cell could be observed at one time (Fig. 2). The most striking aspect of the distribution was a concentration of silver grains at the base and around the entire periphery of the ROS. The myoid, nucleus, and synaptic terminal showed high concentrations of radioactivity, as did a portion of the cytoplasm surrounding the mitochondria (the perimitochondrial cytoplasm) (35). The portions of the cell that were least radioactive included the remainder of the outer segment and mitochondria in the ellipsoid region. Retinas that were similarly pulsed for 4 h with [^{3}H]leucine but with 10 μg/ml of puromycin present throughout the incubation showed a strikingly different distribution of radioactive label (Fig. 3). There was no concentration of radioactivity at either the outer segment base or the periphery as was seen in retinas incubated without puromycin. The most radioactive components of the cell were the myoid, nucleus and synaptic terminal, although the levels of radioactivity were much lower than those observed without puromycin. The mitochondria of the ellipsoid and the outer segments were the least radioactive cell components.

Electron microscope autoradiograms were prepared from the same tissue specimens as the light microscope autoradiograms (without puromycin only) in order to better relate levels of radioactivity to specific cell organelles. Electron microscope autoradiography of the retinas showed that the basal disk membranes of the ROS and the investing plasma membrane were much more radioactive than the rest of the membranes of the outer segment (Fig. 4). Plasma membrane labeling was evident in longitudinal sections made at right angles to the plane of that structure and was discernible at the tip of the outer segment where the plasma membrane continues around the end of
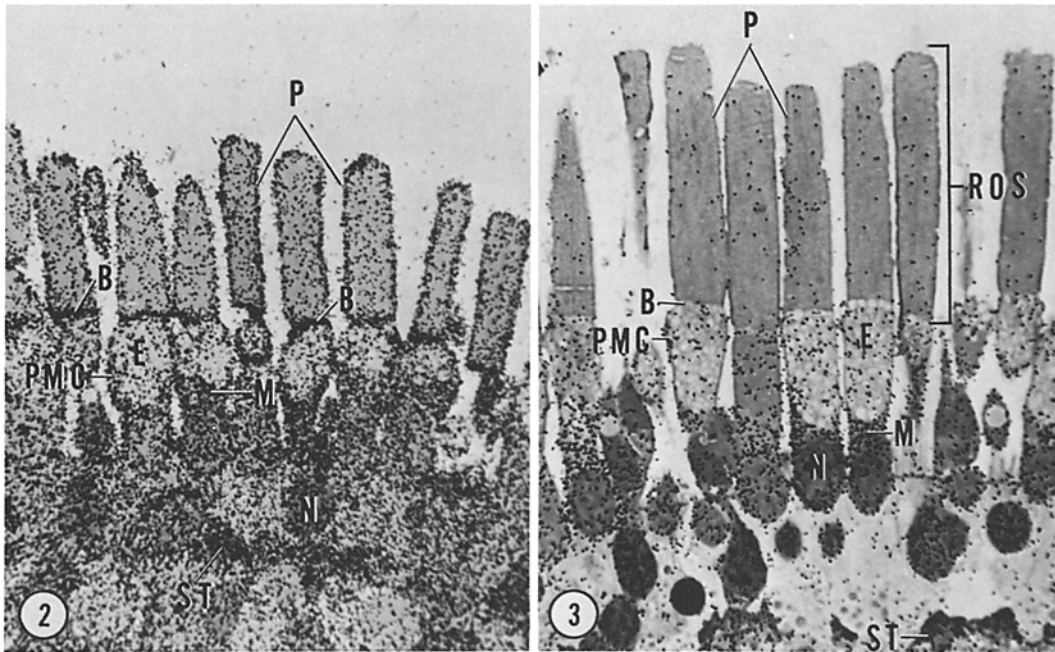
FIGURES 2–3 Light microscope autoradiograms of isolated frog retinas. 0.5-μm Araldite sections, toluidine blue. × 890.

FIGURE 2 Frog retina, incubated in [³H]leucine for 4 h and chased for 32 h in medium containing nonradioactive amino acids. The myoid (M), perimitochondrial cytoplasm (PMC), nucleus (N), and synaptic terminal (ST) are nearly obscured by silver grains. The ROS periphery (P) and base (B) are heavily radioactive, whereas the remainder of the outer segment and ellipsoid (E) are the least radioactive components of the cell.

FIGURE 3 Autoradiogram of frog retina incubated in [³H]leucine and puromycin for 4 h and chased for 32 h with a mixture of amino acids and puromycin. The myoid (M), nucleus (N), and synaptic terminals (ST) are the most radioactive cell components but their labeling is significantly reduced when compared to the retina in Fig. 2. The perimitochondrial cytoplasm (PMC), ellipsoid (E) and components of the rod outer segment (ROS) are the least radioactive. The ROS periphery (P) and base (B) are virtually devoid of label when compared to similar areas in Fig. 2.

the cell (Fig. 5). Further evidence of plasma membrane labeling was provided in autoradiograms in which the outer segment and its plasma membrane were sectioned obliquely (Fig. 6). As the plasma membrane profile became progressively smudged due to the oblique plane of the section, the concentrated pattern of silver grains overlying the membrane became more widely scattered. The strongest evidence for radioactive protein in the plasma membrane was obtained from sections where the membrane was separated from the edges of the disks (Figs. 4 and 7) for distances as great as 15,000 Å (11 HD's). In these autoradiograms, the heaviest silver grain distribution clearly followed the plasma membrane rather than the disk edges. In addition to the marked

concentration of radioactivity in basal outer segment disk and plasma membrane, there was a significant amount of diffuse label associated with the remainder of the outer segment disks.

Silver grain densities were determined for various areas of the photoreceptor cell outer segment, taking into consideration the resolution of the autoradiographic procedure (see Materials and Methods). The outer segment plasma membrane had a grain density of $375 \pm 26/\mu m^2$ whereas the diffusely labeled disks had a density of $0.805 \pm 0.019/\mu m^2$. The grain density for the basal, intensely labeled disks was more difficult to calculate because it was not known how many disks (and therefore how many μm^2 of membrane) were labeled during the radioactive pulse. The average
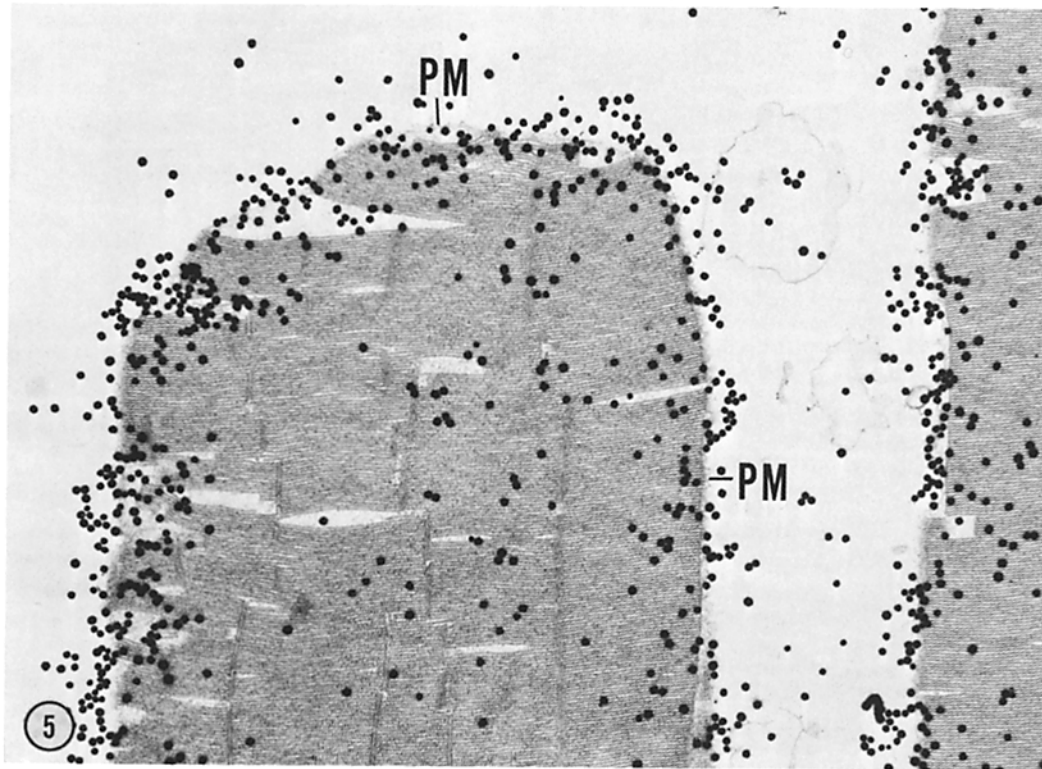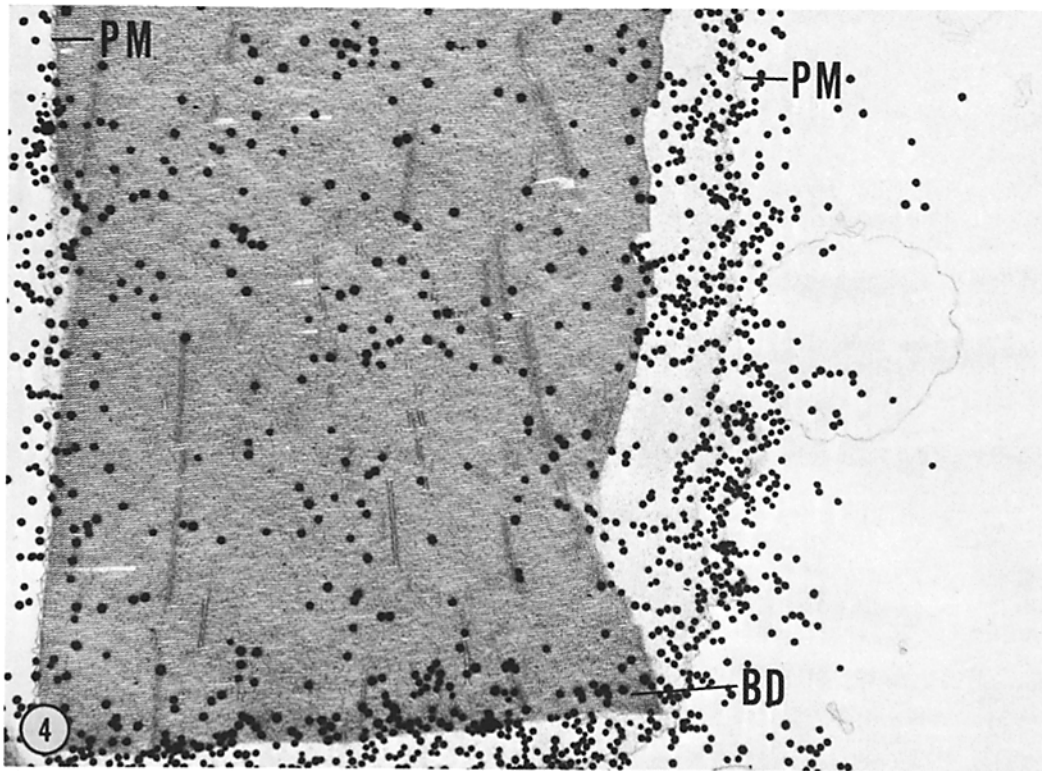
grain density across the outer segment base was about twice that over the plasma membrane. Therefore, if it is borne in mind that each disk is comprised of a double membrane, the grain density ($331 \pm 20/\mu m^2$) was compatible with the labeling of a single bimembranous disk. The assumption is made that the labeled protein was evenly distributed throughout the basal disk and plasma membrane.

The average red ROS has 7.82×10^2 μm^2 of plasma membrane and 1.13×10^6 μm^2 of disk membrane (each of the 2,000 double membrane disks has a surface area of 56.6 μm^2), therefore the distribution of radioactivity in each component could be calculated. 73% of the silver grains could be attributed to the plasma membrane, 22% to the diffusely labeled disks, and 5% to the intensely labeled disks. As emphasized earlier, our conclusion that the plasma was a major source of radioactivity was based on a number of qualitative observations. The methods of Salpeter et al. (31) were used to correct for radiation spread from the radioactive source.

Biochemistry

The autoradiograms demonstrated the presence of newly synthesized protein in the outer segment plasma membrane of the rod photoreceptor. In order to identify these radioactively labeled proteins, a biochemical analysis was performed on ROS isolated from retinas labeled in the absence of puromycin as described above. Two analytical procedures were used: SDS-polyacrylamide gel electrophoresis to separate and quantitate the amount of radioactivity in the ROS protein, and Agarose column chromatography to determine whether the visual pigment was present as opsin or rhodopsin.

After the ROS were isolated on a discontinuous sucrose gradient, an aliquot was dissolved in SDS and the proteins were separated by SDS-disc gel electrophoresis. After staining, the gels were scanned, sliced into 1-mm sections, and counted to determine the amount of radioactivity in each ROS protein. Fig. 8 shows a graph of the distribution of both protein and radioactivity along the gel, with the position of visual pigment indicated at the top of the figure. Since this procedure bleaches rhodopsin, the visual pigment band represents the chromatographic position of the apo-protein, opsin. One major protein (opsin) and a number of minor proteins were revealed in the outer segment extract. Analysis of the amount of radioactivity in each protein band revealed that about 90% of the total radioactivity was present in the visual pigment band. This indicated that most of the newly synthesized protein in both the basal disks and the plasma membrane is electrophoretically identical with visual pigment.

Having determined that the ROS plasma membrane contained newly synthesized visual pigment, we were interested in determining whether this visual pigment was present in the native configuration (rhodopsin) or had not yet bound a vitamin A chromophore (opsin). Accordingly, another aliquot of the isolated ROS was dissolved in Emulphogene and chromatographed on a column of Agarose. The results are shown in Fig. 9. The radioactive peak, the A_{280} peak, and the A_{500} peak are coincident and have an elution volume of 51.5 ml (Fig. 9 a). These data indicate that the visual pigment in both the plasma membrane and the basal disks is native rhodopsin. The peak rhodopsin fraction (A_{280}/A_{500} = 1.97, uncorrected) was recovered, bleached with white light, and re-chromatographed on the same column of Agarose. Fig. 9 b shows that the peak of both radioactivity and A_{280} now elute at 46.5 ml, coincident with opsin. The specific activity of the visual pigment (as $dpm/ml/A_{280}$) was essentially unchanged by

FIGURES 4–7   Electron microscope autoradiograms of isolated frog retinas incubated for 4 h in [^{3}H]leucine (in the absence of puromycin) and chased for 32 h with nonradioactive amino acids. $\times$ 13,000.

FIGURE 4   Basal disks (*BD*; corresponding to the outer segment base in Fig. 2) and the plasma membrane (*PM*; corresponding to the outer segment periphery in Fig. 2) are the most radioactive components of the outer segment. Plasma membrane labeling is most clearly demonstrated when it is separated from the disk edges as on the right side of the autoradiogram. The remainder of the outer segment disks have a diffusely distributed label.

FIGURE 5   Plasma membrane (*PM*) labeling is observed along the sides of the rod outer segment as well as at the apex where the membrane continues around the cell.
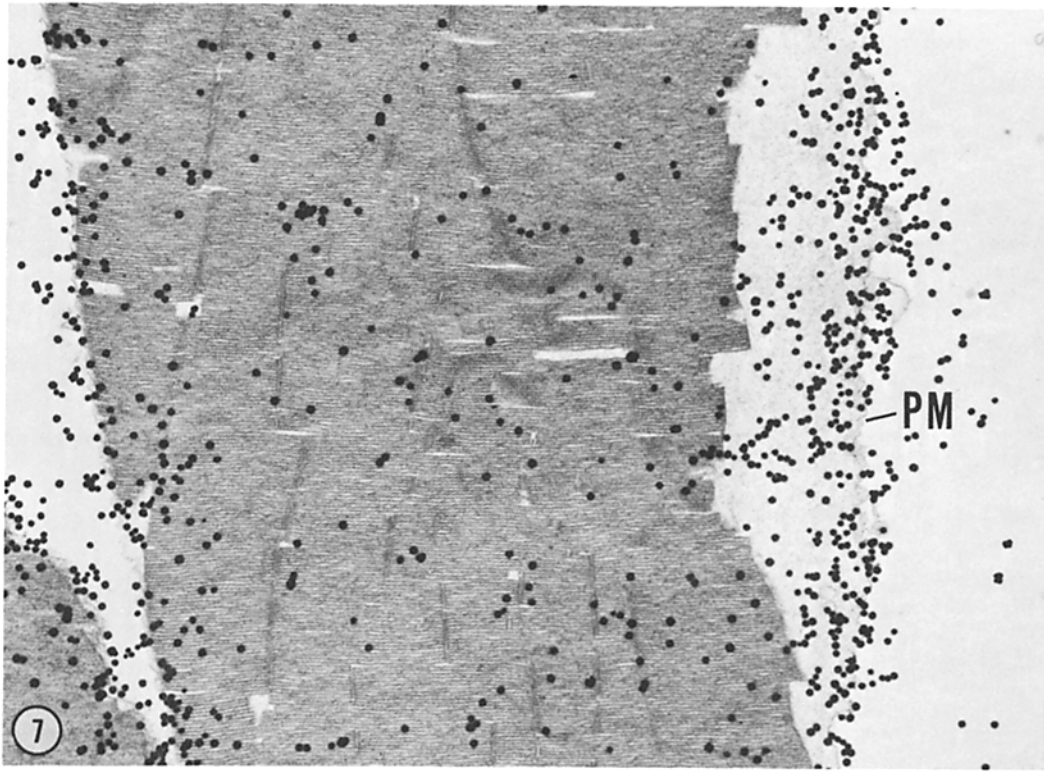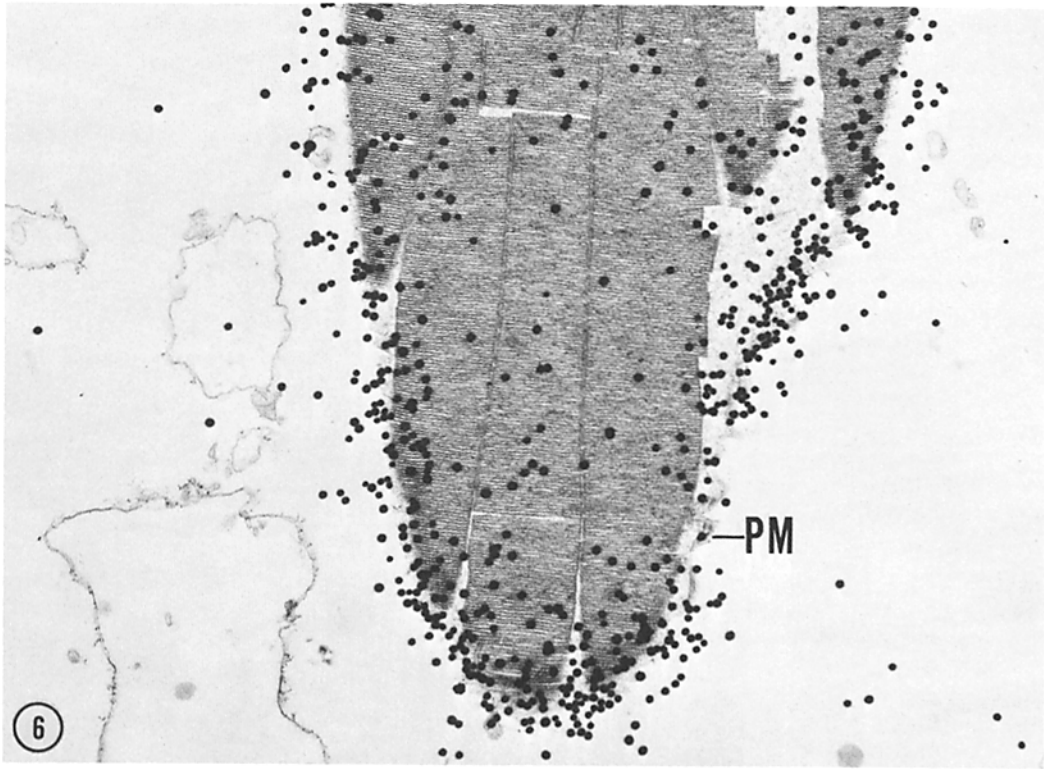
rechromatography (6.9 × 10⁶ dpm/ml/A₂₈₀ vs. 6.1 × 10⁶ dpm/ml/A₂₈₀).

DISCUSSION

Autoradiography

On the basis of both autoradiographic and radiobiochemical methods, it has been extensively documented that the rhodopsin-rich disk membranes of vertebrate rod photoreceptors are renewed under both in vivo (19, 32, 35) and in vitro (4, 5) conditions. The general sequence of events is known to involve biosynthesis of new polypeptides on ribosomes (35) in the myoid region of the photoreceptor, and partial glycosylation is believed to occur at or near the same site (5). The incomplete glycoprotein is then thought to migrate to the Golgi apparatus where further glycosylation occurs. Ultimately, the glycoprotein passes through the perimitochondrial cytoplasm, through the connecting cilium, and is inserted into the growing and continually invaginating membrane at the base of the outer segment. It is not yet known whether the addition of the chromophore (vitamin A aldehyde) occurs before or after insertion of the glycoprotein into the growing membrane.

After an in vitro incubation for 36-h (4-h pulse, 32-h chase), the entire photoreceptor cell is labeled (Fig. 2). Every class of protein is presumably undergoing renewal during the time of the [³H]leucine pulse and therefore the general distribution of label throughout the cell is not surprising. The myoid region, a primary synthetic center, remains heavily radioactive as does the nucleus and synaptic terminal. Young and Droz have shown that the majority of labeled proteins in the nucleus and synaptic terminal migrate there from sites of synthesis in the myoid (35). The perimitochondrial cytoplasm is heavily radioactive (Fig. 2) because it serves as a thoroughfare for newly synthesized protein that is making its way to the outer segment. The labeling patterns in the

myoid, nucleus, and synaptic terminal are not qualitatively affected by puromycin, as expected, but are affected in a quantiative sense, showing a reduction in the amount of labeled protein. The periphery of the outer segment, however, is not labeled when a protein synthesis inhibitor (puromycin) is present during incubation (Fig. 3). The attention of this study is focused on events that occur in the basal disks and plasma membrane of the outer segment. The remainder of this discussion, therefore, will be devoted to the outer segment membrane system.

The present autoradiographic study suggests to us that newly synthesized protein gains access into the plasma membrane as well as into basal outer segment disks. Our autoradiographic analysis deals with the quantitation of the radioactivity rather than with the proof that the radioactivity is in the plasma membrane. What is the proof for plasma membrane labeling? As can be observed from the diagram in Fig. 1, the plasma membrane extends around the distal end of the photoreceptor and is isolated from the bimembranous outer segment disks. The autoradiograms in Figs. 2 and 5 show an intense concentration of silver grains in association with the periphery of the outer segment which continues uninterrupted around the distal end of the cell. There is no other cellular structure that could exhibit a labeling pattern of this type. We believe that the strongest evidence for plasma membrane labeling is provided in numerous autoradiograms where the plasma membrane is separated from the outer segment disk edges by approximately 15,000 Å (Figs. 4 and 7). In spite of the relatively low resolution of the autoradiographic method, there is a sufficient separation to allow easy recognition of the fact that the label is associated with the plasma membrane. It should be pointed out here that this peripheral labeling of the outer segment does not represent some sort of edge effect artifact in the autoradiogram. The fact that puromycin-treated retinas do not show this type of labeling eliminates this possibility. In addition, we

FIGURE 6 Obliquely sectioned outer segment and plasma membrane. The plasma membrane profile (*PM*) becomes progressively smudged near the lower end of the section, and correspondingly, the silver grains become more scattered.

FIGURE 7 Autoradiogram in which the plasma membrane (*PM*) of the outer segment is widely separated from the outer segment disk. The correspondence of intense silver grain localization with the plasma membrane is clearly demonstrated.
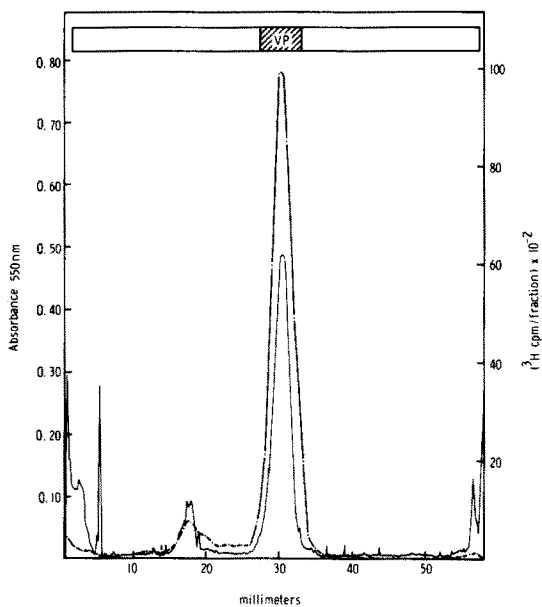
FIGURE 8   SDS acrylamide gel electrophoresis showing the distribution of radioactive protein in rod outer segments isolated from retinas pulse-labeled for 4 h with [³H]leucine, then chased for 32 h with a mixture of nonradioactive amino acids. The isolated ROS were dissolved in 1% SDS and digested at 37°C for 1 h. Electrophoresis was performed on 7.5% gels at a constant current of 3.0 mA/gel. After staining with Coomassie blue, the gels were scanned at 550 nm to determine the protein profile (solid line), then sliced into 1-mm sections. Each section was dissolved overnight at 60°C in 1 ml of 30% H_2O_2, then counted for radioactivity with 10 ml of Aquasol (broken line). The electrophoretic position of purified visual pigment is indicated at the top of the figure. Four radioactive protein bands are present, but at least 90% of the radioactivity is present in the visual pigment (opsin) band.

observed that a few of the most basal outer segment disk membranes are continuous with one another and with the plasma membrane. Thus, it is likely that, after the new protein is inserted into the growing basal membranes, it is able to diffuse throughout the entire plasma membrane. The viscosity of the disk membrane at 21°C is thought to resemble that of castor oil (28), offering the possibility for considerable mobility of proteins
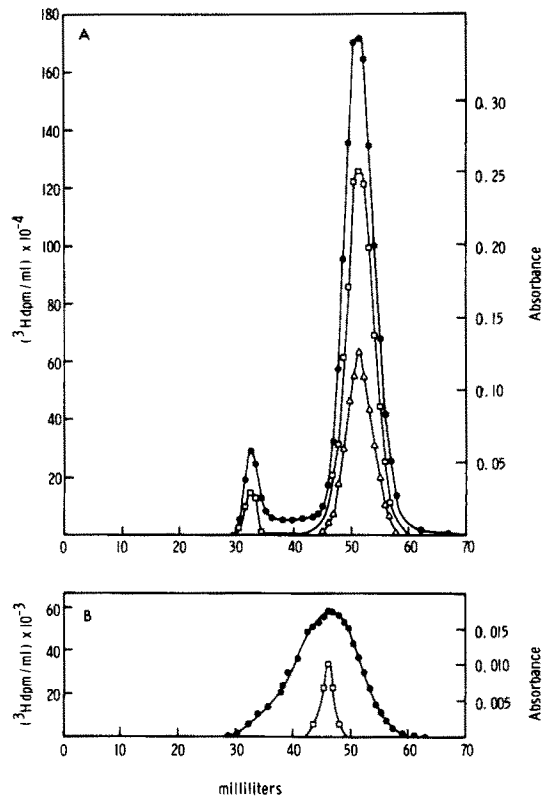

FIGURE 9   Column chromatography of detergent-solubilized ROS isolated from retinas pulse-labeled for 4 h with [³H]leucine, then chased for 32 h with a mixture of nonradioactive amino acids. The isolated ROS were dissolved in 1% Emulphogene in 0.05 M Tris-HCl buffer at pH 8.5, and chromatographed on a 150 × 0.9 cm column of Agarose A-1.5 M (100–200 mesh). (A) Chromatographic profile of Emulphogene-solubilized ROS isolated and chromatographed under dim red light (unbleached). The V_e of the peak visual pigment fraction is 51.5 ml, coincident with rhodopsin. (B) Chromatographic profile of the peak visual pigment fraction from (A) after bleaching with broad-spectrum white light. The V_e of the peak visual pigment fraction is 46.5 ml, coincident with opsin. (A_{500}; △—△) (A_{280}; □—□) (dpm; ●—●).

have never observed labeling patterns of this sort in nonradioactive retinal sections. The plasma membrane labeling is observed only when rhodopsin precursors are employed with long pulses (1–4 h) and when the radioactive products are subsequently chased for long periods (12–32 h). In addition to the present experiment, the phenomenon has been observed when mannose (Young, unpublished observations) and glucosamine (Young and Bok, unpublished observations) are used in the incubation medium. Rhodopsin is a glycoprotein which contains residues of both of these sugars.

How, then, does the plasma membrane get labeled? Referring to Fig. 1 once again, it can be

within the disk membrane. Indeed, it has been demonstrated recently that rhodopsin can undergo both rotational (9, 12) and translational (13, 23, 28) diffusion within the disk membrane. It is likely that the viscosity of the plasma membrane is similar to that of the disk membranes, and thus a similar freedom of movement of its membrane proteins would be expected. Our autoradiographic evidence suggests that this is so. Presumably, radioactive proteins are able to diffuse freely within the plane of the basal disk membranes and within the plasma membrane as well. This free diffusion of protein throughout the disk and plasma membrane is altered, however, as individual disk membranes are displaced apically and become isolated from the plasma membrane, thus confining the protein to lateral diffusion within the newly isolated disk membrane. The ultimate displacement of these heavily labeled disks from the plasma membrane accounts for the now familiar migrating band of radioactive protein observed in all vertebrate ROS that have been studied to date (33).

Why has plasma membrane labeling not been observed in previous in vivo experiments? The period of time during which the photoreceptors were in continuous contact with [³H]leucine (4-h pulse) was particularly long in the present experiment. Such a pulse of radioactivity cannot be accomplished in vivo without multiple injections. Therefore, the specific activity of the protein in the plasma membrane achieved an unusually high level in these experiments. Of greater importance was the long chase period during which no [³H]leucine was available. The diffusely distributed proteins present in the outer segment turned over to a considerable extent in this 32-h period, hence increasing the contrast of the plasma membrane label. Previous studies have indicated that the diffusely distributed proteins do not include rhodopsin and that, unlike rhodopsin, they are unstable and have a rather high rate of turnover (6). Rhodopsin, on the other hand, is stable once it is inserted into the disk membrane (19), and only through shedding of old disks from the apex of the outer segment is the visual pigment finally removed from the cell.

An analysis of the autoradiographic data suggests that an average of one complete disk was assembled during the course of the experiment. Evidence for this was obtained from silver grain densities as described in the Results section. Fur-

ther evidence was given by the fact that the band of silver grains was positioned at the very base of the outer segment. If a significant number of new, less radioactive disks had been assembled during the extensive chase period, then the band of silver grains would have been displaced from the outer segment base by these new disks. Earlier evidence (4) has indicated that rhodopsin biosynthesis is linear for 4 h under in vitro conditions. It is clear, however, that rhodopsin biosynthesis and disk assembly were not linear throughout the entire chase period of this experiment.

Biochemistry

Autoradiographic analysis of plasma membrane radioactivity shows a striking level of newly synthesized protein in this structure. Only the newly assembled basal outer segment disks appear to have a comparable level of radioactivity. We know from earlier biochemical evidence (19) that the predominant radioactive species in the basal disks is rhodopsin. Using SDS gel electrophoresis and column chromatography, we have demonstrated that much of the newly synthesized radioactive protein in the ROS plasma membrane is also rhodopsin.

When the proteins from an aliquot of isolated ROS are separated by SDS gel electrophoresis, about 90% of the radioactivity is found in the visual pigment band (Fig. 8). Since most of the labeled protein present in the basal ROS disks is visual pigment, we would expect at least some of the label to migrate coincident with this molecule. However, the autoradiographic analysis shows that 73% of the radioactivity in the remainder of the ROS (excluding the basal disks) is associated with the plasma membrane. If the plasma membrane protein were not visual pigment, the radioactive species would easily have been detected at some other position on the gel. Thus, the majority of the radioactive protein in both the basal disks and plasma membrane is electrophoretically identical with visual pigment. The paucity of other radioactive proteins in the ROS extract reflects both the extensive chase time (32 h) and the inherent stability of visual pigment once it becomes incorporated into the ROS membrane system. The specific activity of other ROS proteins has fallen off considerably due to turnover, leaving visual pigment as the predominant radioactive protein in the ROS.

We have taken advantage of the different elution

volumes of opsin and rhodopsin during Agarose chromatography to show that the visual pigment in the ROS plasma membrane is present in the native configuration (rhodopsin). When a visual pigment molecule is bleached in detergent, there is an increase in its Stokes radius of approximately 10% (21). This means that bleached visual pigment (opsin) chromatographs as if it were a slightly larger molecule than unbleached visual pigment (rhodopsin), and thus it will be eluted slightly ahead (5.0 ml) of native rhodopsin during Agarose chromatography. Since the entire incubation was carried out in the dark with dark-adapted retinas, all of the disk membrane visual pigment existing at the start of the incubation should be present as rhodopsin. Thus, by comparing the position of the newly synthesized radioactive visual pigment with the known Agarose column elution volumes of opsin (46.5 ml) and rhodopsin (51.5 ml), we could determine whether the newly synthesized visual pigment was opsin or rhodopsin. When a detergent-solubilized aliquot of ROS isolated from fully dark-adapted retinas is applied to a column of Agarose, virtually all of the protein and radioactivity chromatograph coincident with rhodopsin (Fig. 9 a). When the peak fraction from this column is recovered, bleached with broad spectrum white light, and rechromatographed on the same column of Agarose, the peak of radioactivity and protein now chromatograph coincident with the V_e of opsin (Fig. 9 b). If the radioactive protein present in the plasma membrane and basal disks was not light-sensitive rhodopsin, all of the radioactive protein would not have eluted at 46.5 ml (V_e opsin) after light exposure. Rather, two peaks of radioactivity would be seen, one eluting at 46.5 ml (opsin) and the other remaining at 51.5 ml, and it is likely that the specific activity would have changed after rechromatography rather than remaining the same.

Taken together, our data suggest that the newly synthesized visual pigment in both the new disks and the plasma membrane is present as rhodopsin. This implies that the opsin molecule has acquired its vitamin A chromophore either before, upon, or just after insertion into the basal disk membranes, as opposed to after the disk has become isolated from the plasma membrane. One might assume, since incubation was carried out in the dark, that the chromophore was added to the newly synthesized opsin through the process of regeneration. However, it has been well established that rhodop-

sin in isolated pigment epithelium-free retinas does not regenerate (4, 8). Thus, the mechanism for chromophore addition during new disk synthesis must be different from the regeneration process, as has been previously suggested (18). This might mean that a separate pool of 11-cis retinal exists within rod photoreceptors which is used exclusively for chromophore addition to newly synthesized visual pigment molecules and not for regeneration.

Our demonstration that visual pigment is present in the plasma membrane raises another question: what role does this molecule play in the membrane? One possibility is that it may serve only a structural role, its presence being only a consequence of the special way in which new disks are made. However, as a structural entity of the plasma membrane, it would directly affect a variety of membrane properties, such as surface charge, metabolite and ion transport, and antigenic character. For example, the latter would affect events which are controlled by membrane surface markers, such as phagocytosis of ROS pieces by the pigment epithelium. Further, since the plasma membrane is the suggested cable for the transduction current as well as the site at which the dark current is regulated (17), the visual pigment may have a role in these functions, or at least must be accounted for in any model for plasma membrane function. Alternately, the visual pigment in the plasma membrane may play a role in light reception, although its low concentration relative to the total amount of rhodopsin in the entire outer segment and the fact that its chromophore would be disoriented by 90°C from the incoming photons make this possibility seem unlikely. It has been suggested that the early receptor potential might originate from rhodopsin molecules present in the continuous basal membranes and the plasma membrane (25, 30), and our experiments would lend support to this theory.

In summary, it appears that new ROS disk membranes are made by continuous insertion of newly synthesized rhodopsin molecules into those basal membranes of the ROS which are continuous with the plasma membrane. The molecules of rhodopsin are then distributed throughout the entire plasma membrane by diffusion. Some of the molecules eventually become trapped in a newly forming disk membrane as it is pinched off from the uppermost fold of the continuous membrane, thus confining the visual pigment molecule to lateral diffusion within that disk.

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