

# Differences in the Protein Composition of Bovine Retinal Rod Outer Segment Disk and Plasma Membranes Isolated by a Ricin-Gold-Dextran Density Perturbation Method

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**Abstract.** The plasma membrane and disk membranes of bovine retinal rod outer segments (ROS) have been purified by a novel density-gradient perturbation method for analysis of their protein compositions. Purified ROS were treated with neuraminidase to expose galactose residues on plasma membrane-specific glycoproteins and labeled with ricin-gold-dextran particles. After the ROS were lysed in hypotonic buffer, the plasma membrane was dissociated from the disks by either mild trypsin digestion or prolonged exposure to low ionic strength buffer. The dense ricin-gold-dextran-labeled plasma membrane was separated from disks by sucrose gradient centrifugation. Electron microscopy was used to follow this fractionation procedure. The dense red pellet primarily consisted of inverted plasma membrane vesicles containing gold particles; the membrane fraction of density 1.13 g/cc consisted of unlabeled intact disks and vesicles. Ricin-

binding studies indicated that the plasma membrane from trypsin-treated ROS was purified between 10-15-fold.

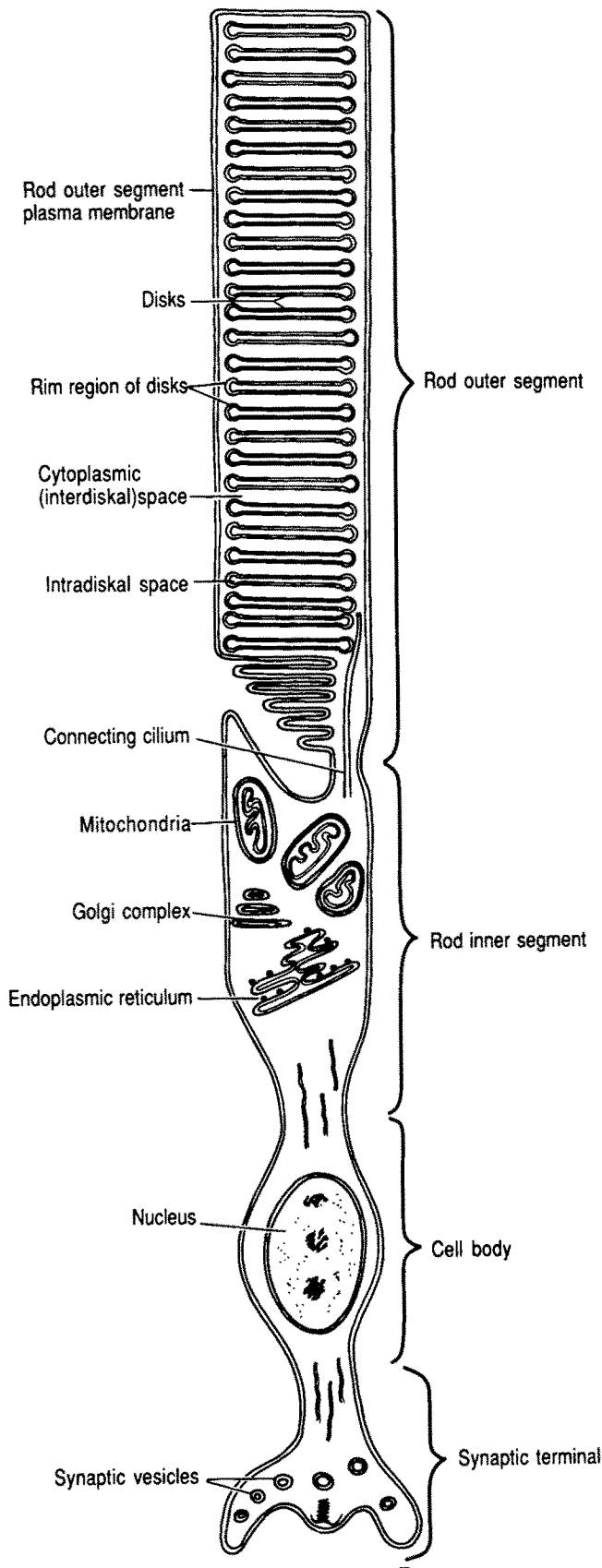
The protein composition of plasma membranes and disks was significantly different as analyzed by SDS gels and Western blots labeled with lectins and monoclonal antibodies. ROS plasma membrane exhibited three major proteins of 36 (rhodopsin), 38, and 52 kD, three ricin-binding glycoproteins of 230, 160, and 110 kD, and numerous minor proteins in the range of 14-270 kD. In disk membranes rhodopsin appeared as the only major protein. A 220-kD concanavalin A-binding glycoprotein and peripherin, a rim-specific protein, were also present along with minor proteins of 43 and 57-63 kD. Radioimmune assays indicated that the ROS plasma membrane contained about half as much rhodopsin as disk membranes.

**R**OD outer segments (ROS)<sup>1</sup> of vertebrate rod photoreceptor cells (Fig. 1) are specialized organelles which function in the transduction of light into electrical signals as part of the visual excitation process. These organelles consist of a plasma membrane that encloses a stack of over a thousand closely spaced disks. Recent electrophysiological studies indicate that the plasma membrane contains cation (sodium) channels which are modulated by 3'5' cyclic guanosine monophosphate (cGMP) (Fesenko et al., 1985; Zimmerman et al., 1985; Yau and Nakatani, 1985). In the dark high levels of cGMP preferentially maintain these channels in their open state. Photobleaching of rhodopsin in disk membranes initiates the activation of the visual enzyme cascade system (Fung et al., 1981), a reduction in the concentration of cGMP, and a closing of the sodium channels (Liebman and Pugh, 1979; Cote et al., 1984; Pugh and Cobbs, 1986; Baylor, 1987). The ROS plasma membrane also appears to contain specific components or ligands on the surface of the ROS plasma membrane that

bind to receptors on adjacent retinal pigment epithelial cells as an initial step in the process of ROS phagocytosis (Mayer-son and Hall, 1986).

Although recent biochemical studies have provided insight into the structural and functional properties of rhodopsin and proteins involved in the light-activated enzyme cascade system (Kuhn, 1980; Fung and Stryer, 1980; Baehr et al., 1979), only a few studies have been directed toward investigating the molecular properties of ROS plasma membrane proteins (Clark and Hall, 1982; Kamps et al., 1982; Witt and Bownds, 1987), and in particular the cGMP-dependent sodium channels (Cook et al., 1987; Matesic and Liebman, 1987). This is largely due to the difficulties involved in purifying the plasma membrane from ROS. These organelles are predominantly composed of disk membranes with the plasma membrane comprising only 5-6% of the total membrane of bovine ROS. Since both disk and plasma membranes contain significant amounts of rhodopsin (Jan and Revel, 1974; Nir and Papermaster, 1983; Hicks and Molday, 1986), it is possible that both these membranes may have similar, although not necessarily identical, compositions. As a result it is unlikely that an adequate separation of these membranes can be obtained by techniques that rely on differences in the phys-

1. *Abbreviations used in this paper:* AuDex, gold-dextran particles; cGMP, 3'5' cyclic guanosine monophosphate; ricin-AuDex, ricin (II)-gold-dextran conjugates; ROS, rod outer segments.



**Figure 1.** A diagram of a vertebrate rod photoreceptor cell showing its basic cellular and subcellular features.

ical properties of these membranes. More importantly, a plasma membrane-specific marker suitable for monitoring the purification of the ROS plasma membrane has not been identified until recently (Clark and Hall, 1982; Molday and Molday, 1987). Finally, recent studies indicate that the ROS plasma membrane is linked to the rim region of disks through a fibrous matrix (Usukura and Yamada, 1981; Roof and Heuser, 1982), which in part is composed of a spectrin-related protein (Wong and Molday, 1986). Disruption of this disk-plasma membrane linkage is a precondition for separation of these two membrane systems.

Due to these difficulties, to date there has been only one report describing the purification of the ROS plasma membrane (Kamps et al., 1982). In this study concanavalin A (Con A)-polystyrene beads were used to bind ROS. After hypotonic lysis a plasma membrane fraction bound to the beads was separated from an unbound disk membrane fraction by differential density sedimentation. Although some differences in protein and lipid compositions were reported for these membrane fractions, the degree of purity of the plasma membrane and disk membranes could not be determined since a specific plasma membrane marker was not available.

In this paper we describe a new procedure for separating the plasma membrane from disk membranes of bovine ROS. This procedure uses ricin-gold-dextran (ricin AuDex) particles (Molday and Molday, 1987) to label ROS plasma membrane-specific glycoproteins and to separate the labeled plasma membrane from unlabeled disk membranes by differential density centrifugation. The purity of these membranes has been confirmed by electron microscopic and biochemical analysis. Monoclonal antibodies and lectins have been used with SDS-gel electrophoresis and radioimmune assays to obtain new information about the protein composition of the isolated disk and plasma membranes.

## Materials and Methods

### Preparation of Bovine ROS

ROS were routinely prepared under dim red light from retina tissue of 80 bovine eyes obtained from Intercontinental Packers, Vancouver, British Columbia (Molday et al., 1987). Briefly, the retinas were placed in 30 ml of homogenizing buffer consisting of 20% (wt/vol) sucrose, 20 mM Tris acetate, pH 7.2, 2 mM MgCl<sub>2</sub>, 10 mM glucose, and 5 mM taurine. After gently shaking the retinas for 1 min, the suspension was filtered either through cheesecloth or through a Teflon screen and layered on six 24 ml 25–60% (wt/vol) continuous sucrose gradients containing 20 mM Tris acetate, pH 7.2, 10 mM glucose, and 5 mM taurine. Centrifugation was carried out in a rotor (SW-27; Beckman Instruments, Inc., Palo Alto, CA) at 25,000 rpm for 45 min at 4°C. The pink ROS band was collected, diluted to a final volume of 150 ml with homogenizing buffer, and centrifuged at 8,000 rpm for 10 min in a rotor (SS-34; E. I. DuPont de Nemours & Co., Inc., Sorvall Instruments Div., Newtown, CT). The pellets were resuspended in 4 ml of homogenizing buffer.

### Neuraminidase Treatment and Ricin-AuDex Labeling

The ROS suspension in homogenizing buffer was treated with 0.1 U of *Anthrobacter ureafaciens* neuraminidase for 2 h at 4°C in the dark. The suspension was diluted with 10 ml of homogenizing buffer and the neuraminidase-treated ROS were centrifuged at 8,000 rpm for 10 min in a rotor (SS-34; Sorvall Instruments). The pellet was resuspended in 4 ml of homogenizing buffer and treated with 2 ml of ricin-AuDex having an absorbance of 3–6 at a wavelength of 520 nm. After 2 h of gentle rocking at 4°C, the ROS suspension was diluted with 20 ml of homogenizing solution, and centrifuged at 5,000 rpm for 5 min. The ROS were subjected to hypotonic lysis in 0.02 M Tris buffer, pH 7.2, for 30 min at 4°C and subsequently washed twice

in the same buffer (containing 2 mM EDTA) by centrifugation at 8,000 rpm for 10 min.

### Trypsin Treatment of ROS

The ROS pellet was resuspended in 4 ml of 0.02 M Tris buffer pH 7.2 and trypsin was added to final concentration of 0.2 µg/ml. After 30 min at 4°C the trypsin reaction was stopped by the addition of 4 µg of soybean trypsin inhibitor. The ROS suspension was washed twice with 0.02 M Tris buffer pH 7.2 by centrifugation at 15,000 rpm for 30 min. In some experiments the ROS were not treated with trypsin, but instead washed with 0.002 M Tris buffer and maintained in this buffer overnight at 4°C before density gradient centrifugation.

### Density-Gradient Centrifugation of ROS Membranes

The untreated and trypsin-treated ROS membranes were layered on sucrose gradients consisting of 9 ml of a 15–50% (wt/vol) sucrose underlayered with 1 ml of 60% (wt/vol) sucrose in 0.02 M Tris pH 7.2. Centrifugation was carried out in a rotor (SW-41; Beckman Instruments, Inc.) at 35,000 rpm for 3 h. The pellet was collected in 0.5 ml of 0.02 M Tris buffer, pH 7.2. The membrane band was diluted with 3 vol of 0.02 M Tris, pH 7.2, centrifuged at 15,000 rpm for 20 min, and resuspended in 1–2 ml of the same buffer. For most studies the samples were exposed to light after the density-gradient centrifugation step.

### Protein Reagents

Ricin communis agglutinins I and II were prepared from castor beans by the method of Nicolson and Blaustein (1972). Con A and L-1-*p*-tosylamino-2-phenylethyl chloromethyl ketone (TPCK)-trypsin were obtained from Sigma Chemical Co. (St. Louis, MO) and neuraminidase *Arthrobacter ureafaciens* was from Boehringer-Mannheim (Penzburg, Federal Republic of Germany). Goat anti-mouse Ig was purified by affinity chromatography on a mouse-Ig-Sepharose 4B column. Anti-rhodopsin monoclonal antibodies rho 1D4 against the COOH terminus (MacKenzie et al., 1984) and rho 4D2 and rho 4A2 against the NH<sub>2</sub> terminus (Hicks and Molday, 1986; Molday and MacKenzie, 1983), and anti-peripherin antibody 2B6 (Molday et al., 1987) were obtained from the corresponding hybridoma cell culture fluid.

Goat anti-mouse Ig, ricin I and II, and Con A were iodinated by the chloramine T method (Hunter and Greenwood, 1972). The reagents typically had a specific activity of 1–2 × 10<sup>6</sup> dpm/µg.

### Gold-Dextran Reagents

Colloidal gold particles (average diameter 9 nm) were prepared and coated with a glutaraldehyde-activated dextran derivative as previously described (Molday et al., 1987). Ricin II or goat anti-mouse Ig was coupled to the gold-dextran (AuDex) particles by reacting 4 ml of glutaraldehyde-activated AuDex (absorbance of 10–30 at wavelength of 520 nm) with 1 mg of protein in 0.01 M Hepes buffer, pH 7.0, for 1.5 h at 25°C. The reaction was stopped by the addition of an equal volume of TBS buffer (0.01 M Tris, 0.15 M NaCl pH 7.4) containing 1 mg/ml BSA. The protein-gold-dextran conjugate was separated from unbound protein by centrifugation through 20% (wt/vol) sucrose in TBS buffer in a rotor (SW-27; Beckman Instruments, Inc.) at 26,000 rpm for 2 h. The pellet was resuspended in 3–4 ml of TBS containing 1 mg/ml BSA and dialyzed overnight against TBS. A 1:100 dilution of the protein-gold-dextran conjugate had an absorbance of 0.1–0.3 at 520 nm.

### SDS-Gel Electrophoresis and Western Blots

For SDS-gel electrophoresis ROS membranes (2–5 mg/ml) were solubilized in an equal volume of denaturing buffer containing 5% SDS, 40% sucrose, 10% 2-mercaptoethanol, and 0.01 M Tris buffer, pH 7.4. Samples (10–15 µl) were applied to lanes of a 6.5–15% polyacrylamide gradient slab gel and electrophoresis was carried out as previously described (Molday et al., 1987). In some experiments ricin-AuDex-ROS membranes dissolved in SDS were centrifuged at 40,000 rpm to remove the gold particles before SDS-gel electrophoresis. Gel slices were either stained with Coomassie Blue or silver (Wray et al., 1981) or subjected to electrophoretic transfer onto nitrocellulose paper as previously described (Molday et al., 1987). Transfer papers were washed with buffer containing 0.01 M sodium phosphate, pH 7.4, 0.15 M NaCl, 4% BSA, 0.2% Triton X-100, and 10 mM NaN<sub>3</sub>, and labeled directly with [<sup>125</sup>I]ricin II or [<sup>125</sup>I]-Con A (sp act 1 × 10<sup>6</sup> dpm/mg), or indirectly with monoclonal antibody hybridoma culture fluid and [<sup>125</sup>I]-goat

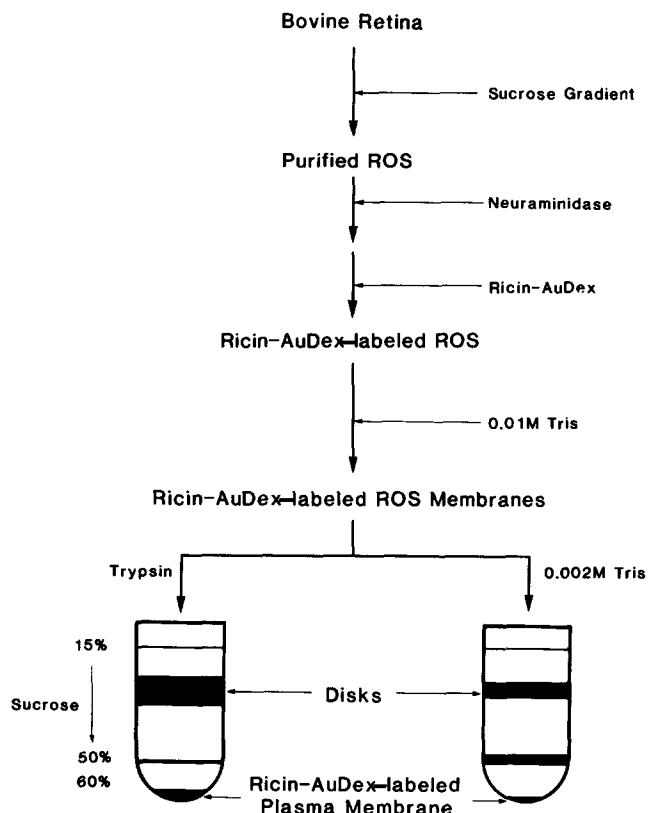


Figure 2. A flow diagram showing the main steps in the purification of disk and plasma membranes from hypotonically lysed and mild trypsin-treated bovine ROS.

anti-mouse Ig (sp act 1 × 10<sup>6</sup> dpm/mL) as previously described (Molday et al., 1987). Labeled papers were airdried and subjected to autoradiography on Kodak Royal X-Omat film for 1–3 d. For quantitative analysis, autoradiograms of stained gels were scanned with a densitometer (Ultrascan XL, LKB Instruments, Inc., Gaithersburg, MD). Alternatively, labeled protein bands were excised and counted in a gammacounter (model 8000; Beckman Instruments, Inc.). Protein concentrations were determined by the Lowry method using BSA as a standard (Lowry et al., 1951). In the case of ricin-AuDex-labeled membranes, samples were solubilized in SDS and subjected to high speed centrifugation to remove the gold before determination of protein concentrations.

### Electron Microscopy

ROS membranes at various stages of purification were fixed in 1.25% glutaraldehyde/0.1 M cacodylic buffer, pH 7.4, containing 10% (wt/vol) sucrose for 1–2 h at 23°C, washed in 0.1 M cacodylic buffer, postfixed in 1% osmium tetroxide for 1 h, dehydrated with ethanol, and embedded in epon/araldite resin (Polysciences, Inc., Warrington, PA) for thin sectioning. Samples were viewed under a JEOL 1200 EX electron microscope.

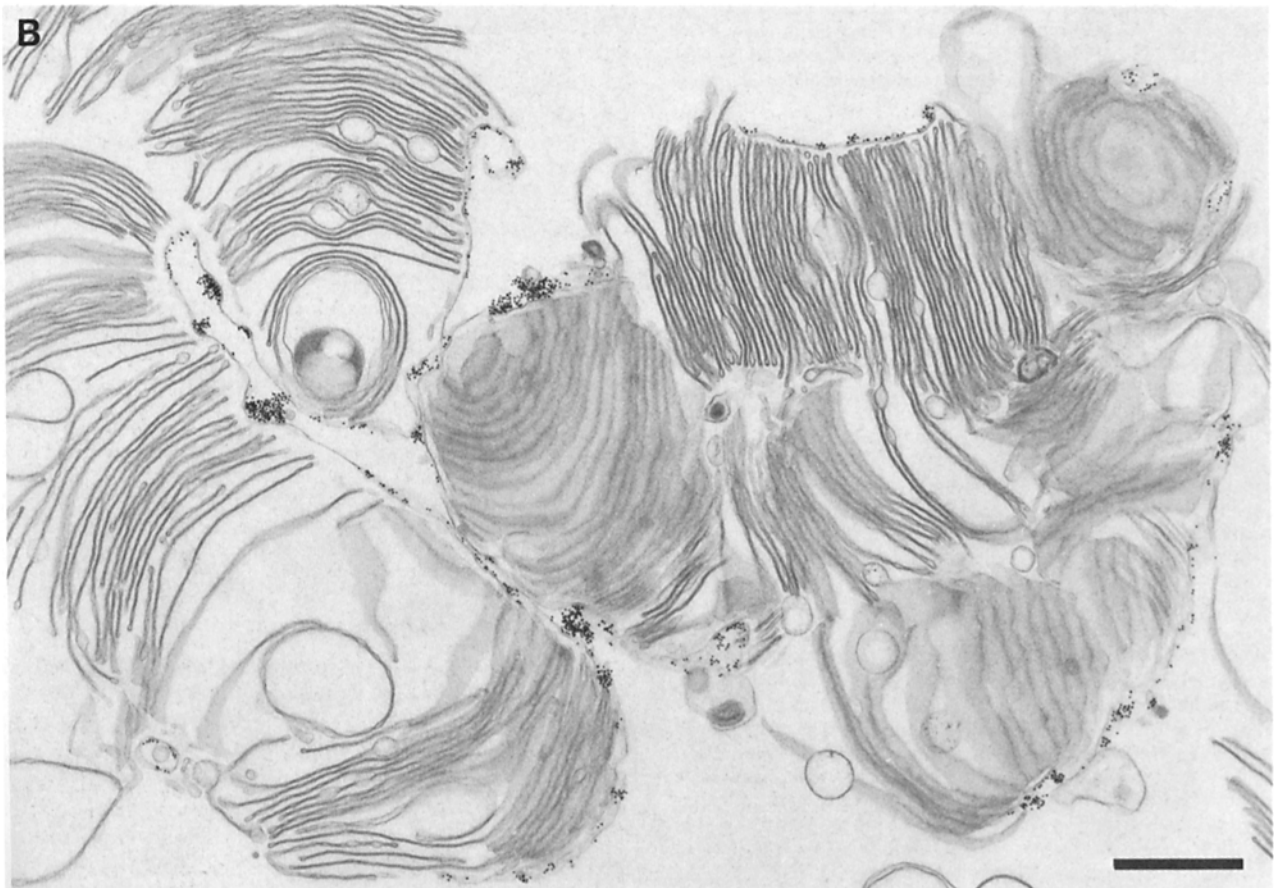
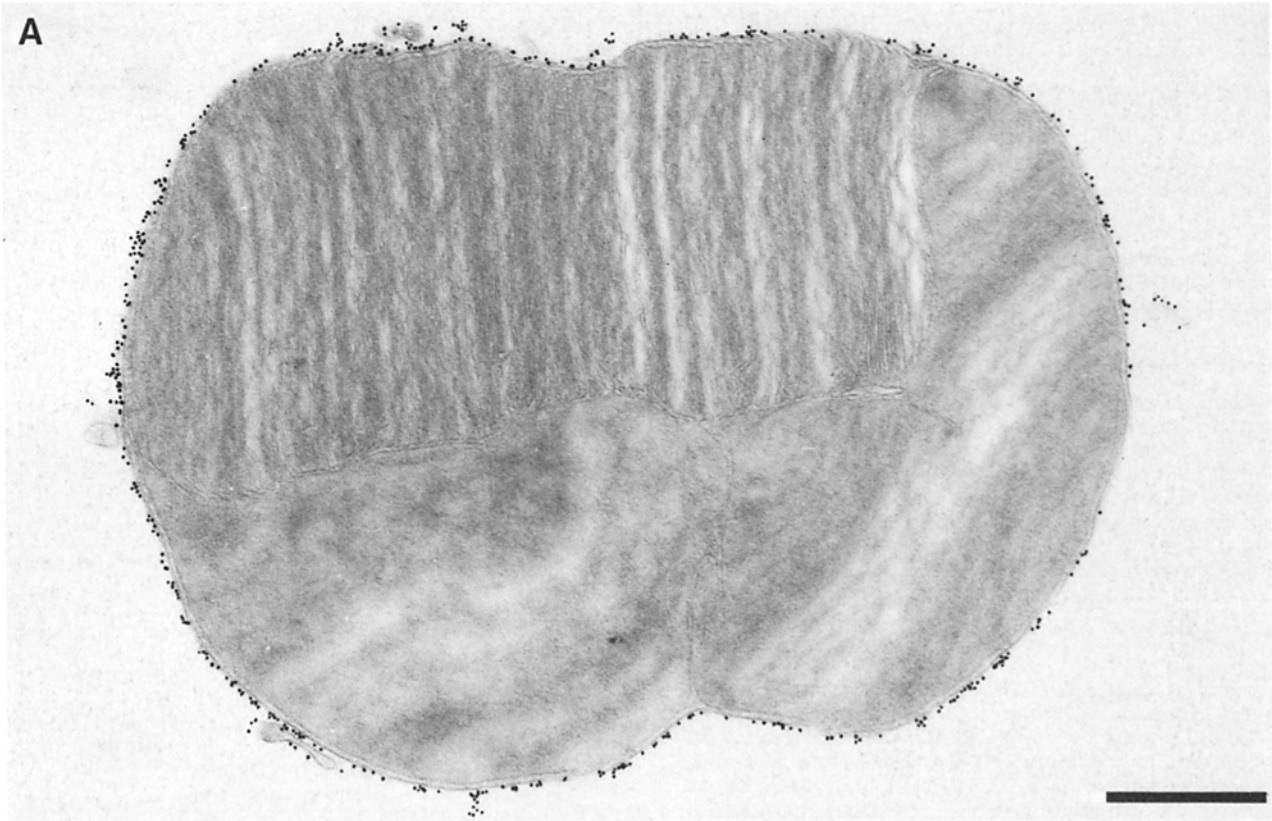
### Radioimmune Competition Assays

The binding of antirhodopsin and antiperipherin monoclonal antibodies to ROS membranes and isolated disk and plasma membrane fractions was studied using a radioimmune solid-phase competition assay as previously described (Molday et al., 1987; Molday and Mackenzie, 1983).

### Results

#### Methodology for the Purification of ROS Plasma Membranes

A flow diagram showing the important steps in the purification of the bovine ROS plasma membrane is shown (Fig. 2).



**Figure 3.** Neuraminidase-treated ROS labeled with ricin-AuDex particles (average diameter = 9 nm). (A) ROS labeled and maintained in 20% sucrose. The plasma membrane that enclosed stacks of disks is randomly labeled with gold particles. (B) ROS labeled in 20%

In this procedure ROS isolated from freshly dissected bovine retina by centrifugation on a continuous sucrose gradient were treated with neuraminidase in order to generate plasma membrane-specific markers in the form of ricin-binding glycoproteins with  $M_r$  of 230 and 110 kD (Molday and Molday, 1987). Ricin I ( $M_r = 120$  kD) or ricin II ( $M_r = 60$  kD) conjugated to AuDex particles were then used to specifically label exposed galactose sites on the surface of neuraminidase-treated ROS. In the next step the ricin-AuDex-labeled ROS were disrupted by hypotonic lysis and the disks were further dissociated from the ROS plasma membrane either by mild trypsin digestion or by prolonged incubation in low ionic strength ( $\sim 0.002$  M) buffer. In the final step the "dense" ricin-AuDex-labeled plasma membrane fraction was separated from the unlabeled disk membrane fraction by centrifugation on a sucrose gradient.

The banding pattern on this gradient was dependent on the conditions used to dissociate disks from the plasma membrane. When trypsin was used a dark red pellet and a large light-sensitive membrane band of average density 1.13 g/cc was obtained. As indicated below these fractions correspond to the gold-labeled plasma membrane and unlabeled disks, respectively. A small red band was sometimes observed at the interface of the 60% (wt/vol) sucrose solution. When trypsin was omitted and low ionic strength buffer was used to promote dissociation, a smaller disk membrane band of density 1.13 g/cc was obtained along with a moderately large red band on top of the 60% sucrose solution and a small red pellet.

The amount of disk and plasma membrane obtained from the trypsin fractionation procedure was quantitated from protein assays. In a typical experiment 30 mg (protein) of unbleached neuraminidase-treated ROS yielded 24 mg (protein) of unbleached ROS membranes after hypotonic lysis and trypsin treatment. The 20% loss is primarily due to the loss of soluble or weakly associated membrane proteins (Kuhn, 1980). After density-gradient centrifugation 15 mg of protein was recovered in the disk membrane fraction, 1.6 mg of protein from the plasma membrane pellet, and 2 mg of protein from the 60% (wt/vol) sucrose layer. Approximately 78% of the total membrane protein was recovered in these three fractions. The plasma membrane pellet constituted 9% of the recovered membrane or 7% of the total ROS membrane applied to the sucrose gradient.

### ***Electron Microscopic Analysis of Membrane Fractions***

Ricin-AuDex-labeled ROS membranes at various stages of the fractionation procedure were analyzed by transmission electron microscopy. ROS purified from sucrose gradients and sequentially treated with neuraminidase and ricin-AuDex particles in the presence of sucrose consisted largely of elongated or circular ROS organelles with the ricin-AuDex-labeled plasma membrane enclosing stacks of disk membranes (Fig. 3 A). Some disrupted ROS fragments were also seen with a discontinuous plasma membrane and exposed stacks

of disks. No labeling of the disks was observed in these fragmented ROS.

Resuspension of ricin-AuDex-labeled ROS in 0.01 M Tris buffer in the absence of sucrose resulted in lysis and fragmentation of the ROS. Structures consisting of stacks of disks associated with fragments of ricin-AuDex-labeled plasma membrane were found as illustrated in Fig. 3 B. Ricin-AuDex particles on the ROS plasma membrane were sometimes observed to have redistributed into small clusters. Structures consisting of disks radiating out from central plasma membrane vesicles were also observed in these preparations. The inner surface of these central vesicles was labeled with ricin-AuDex particles indicating that the plasma membrane was in an inverted or inside-out orientation (Molday and Molday, 1987).

Dissociation of the disks from each other and from the ricin-AuDex-labeled plasma membrane was obtained by treatment of the ROS fragments with a low concentration (0.1  $\mu$ g/ml) of trypsin (Fig. 4 A). Numerous unlabeled, flattened disks and sealed vesicles were observed along with ricin-AuDex-containing inverted plasma membrane vesicles and ricin-AuDex-labeled membrane fragments. Partial dissociation could also be achieved by prolonged exposure (16 h) of nontrypsinized ROS fragments to low ionic strength buffer. As shown in Fig. 4 B, numerous unlabeled, isolated vesicles and small aggregated vesicles were observed by electron microscopy. Ricin-AuDex-containing vesicles surrounded by intact disks, small truncated disks, or rimlike structures were also observed.

The ricin-AuDex-labeled plasma membrane vesicles were separated from the disks by sucrose-density gradient centrifugation. For ROS treated with trypsin the membrane band at a density of 1.13 g/cc consisted of unlabeled flattened disks and vesicles as shown in Fig. 5 A. No gold particles were evident in these sections. The intense red pellet was observed to consist mostly of vesicles containing ricin-AuDex particles on their inner surface as illustrated in Fig. 5 B. A few unlabeled vesicles were also observed in these sections. The 60% sucrose membrane fraction consisted of both ricin-AuDex-labeled and unlabeled vesicles.

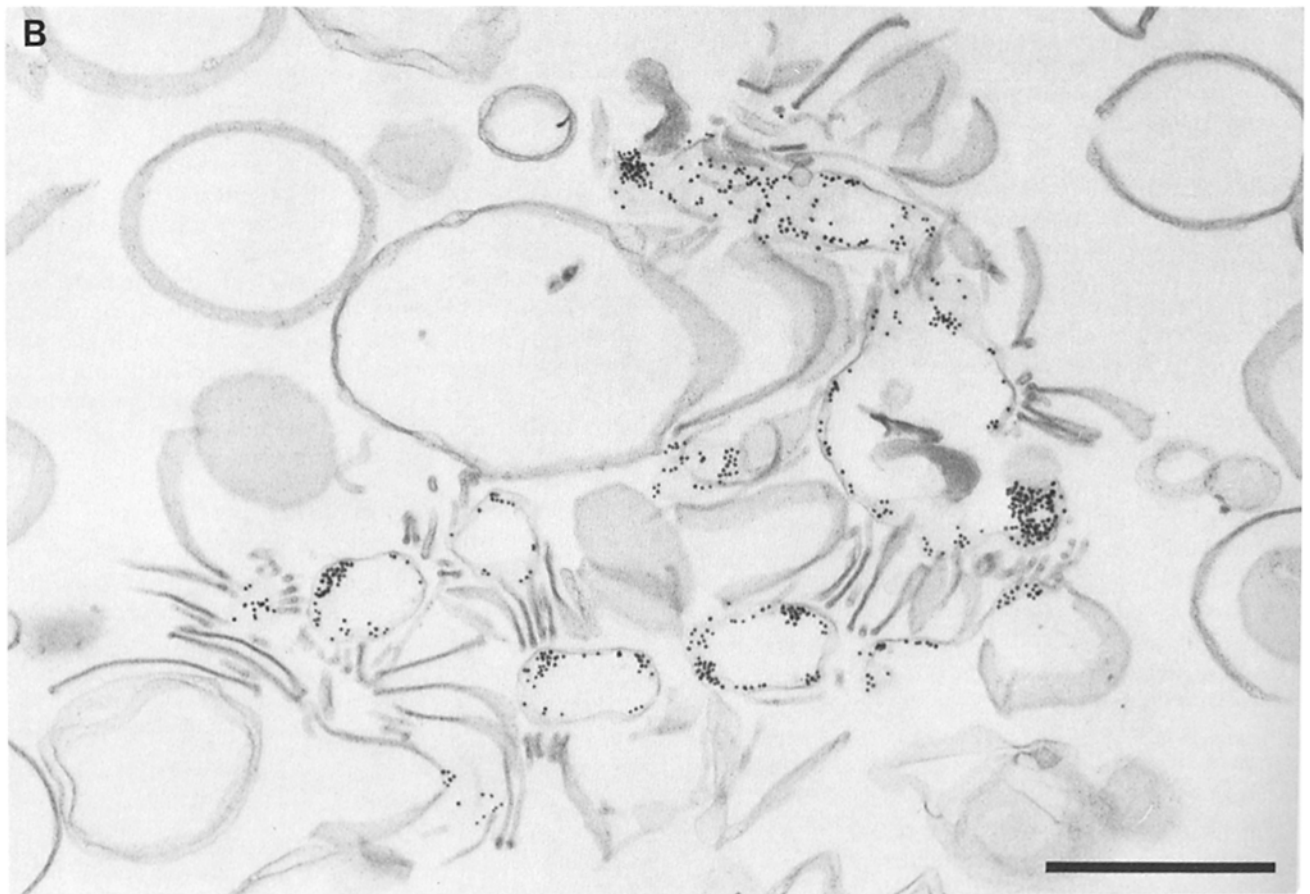
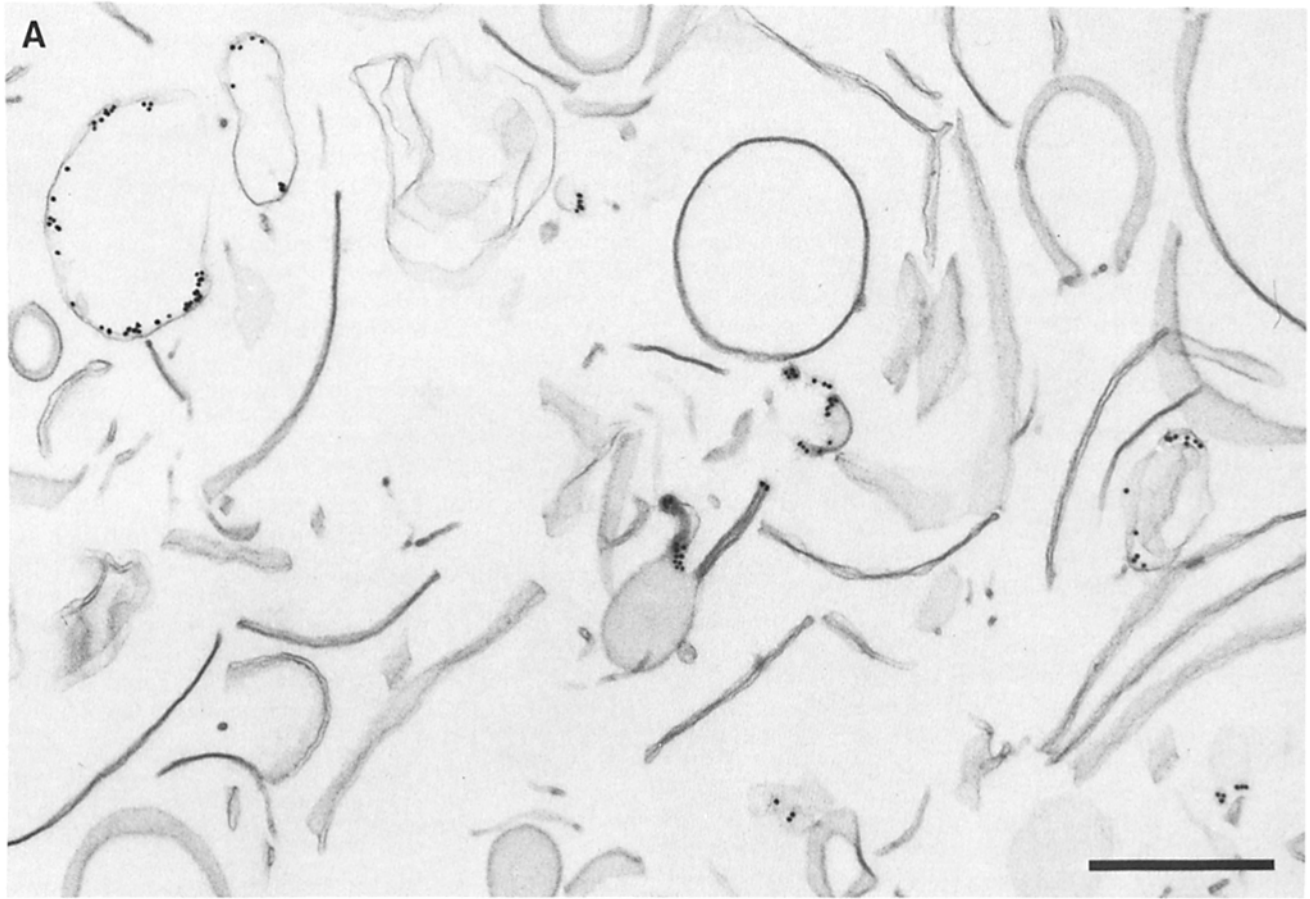
When prolonged exposure to low ionic strength buffer was used instead of trypsin to dissociate disks from the plasma membrane, the membrane band at a density of 1.13 g/cc was observed to consist of unlabeled vesicles and disk structures. The band at the 60% (wt/vol) sucrose layer and the small red pellet consisted of aggregates of ricin-AuDex-labeled and unlabeled vesicles and membrane fragments.

### ***SDS-Gel Electrophoresis of Disk and Plasma Membranes from Trypsin-treated ROS***

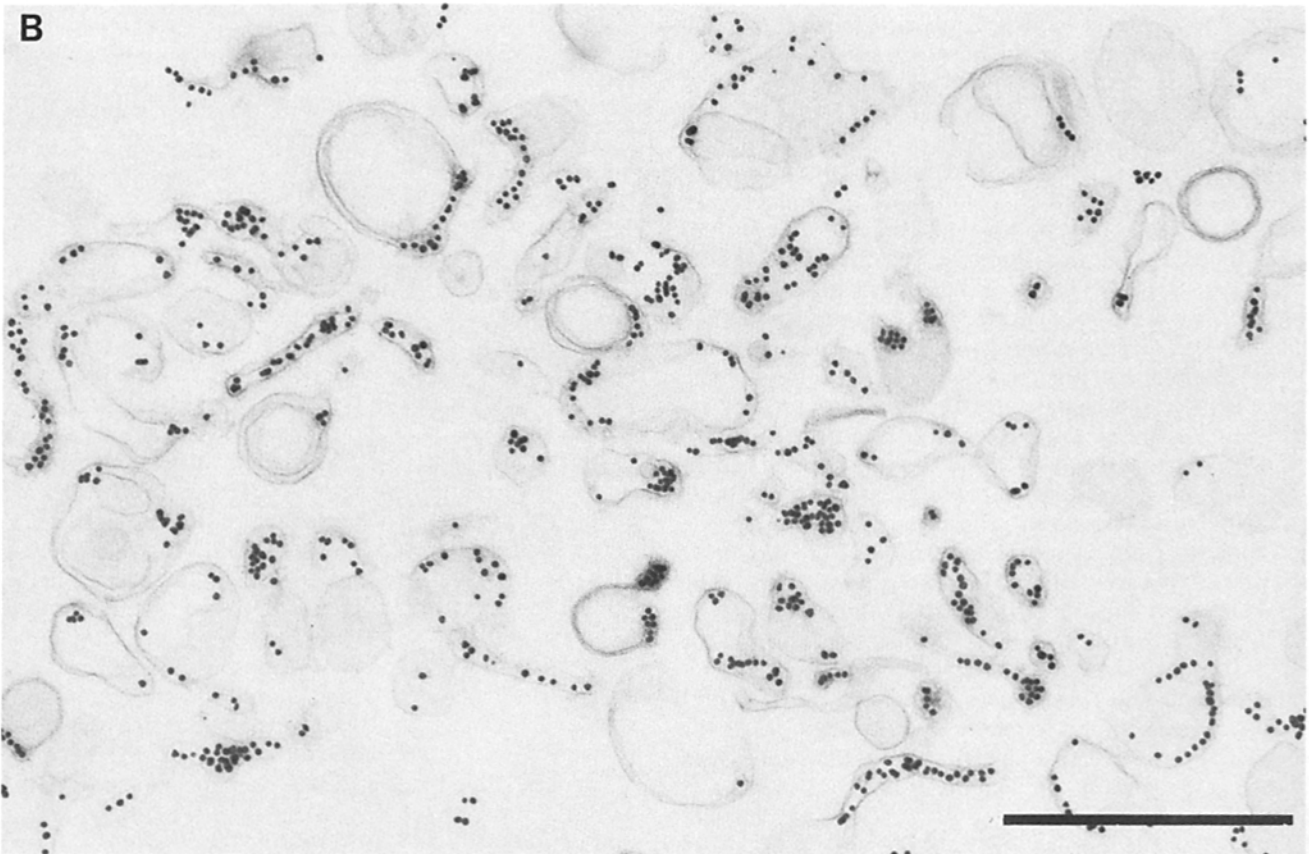
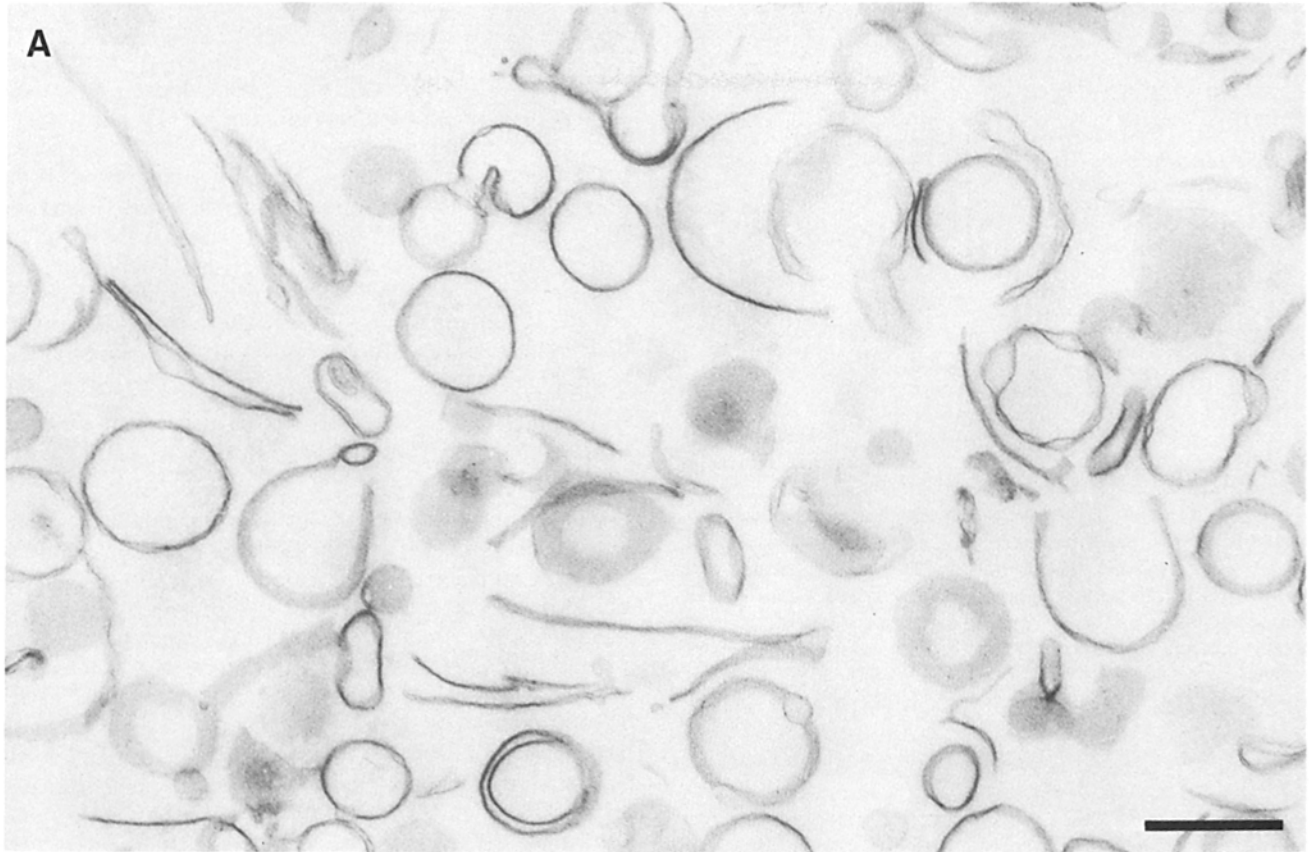
The protein composition of the sucrose gradient-purified disk and plasma membrane fractions was analyzed by SDS-gel electrophoresis and compared with intact ROS and ROS membranes treated with dilute trypsin and washed in 0.01 M Tris buffer. Fig. 6 illustrates the Coomassie Blue- and silver-

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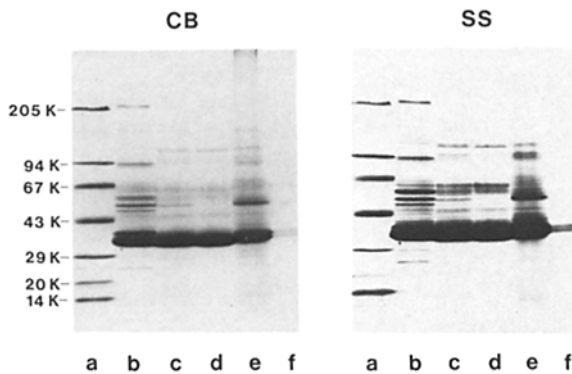
sucrose and subsequently lysed in 0.01 M Tris buffer in the absence of sucrose for 30 min. ROS fragments containing stacks of unlabeled disks and a discontinuous plasma membrane are seen along with gold-containing inverted plasma membrane vesicles with disks radiating from their surface. Bars, 0.5  $\mu$ m.



**Figure 4.** Ricin-AuDex-labeled plasma membrane vesicles and unlabeled disks. (A) Ricin-AuDex-labeled ROS subjected to dilute trypsin (0.2  $\mu\text{g/ml}$ ) for 20 min. Unlabeled, flattened disks and vesicles are dissociated from ricin-AuDex-labeled, inverted plasma membrane vesicles and membrane fragments. (B) Ricin-AuDex-labeled ROS subjected to 0.002 M Tris buffer overnight. Ricin-AuDex-labeled, inverted plasma membrane vesicles are still associated with truncated disks and structures resembling the rim region of the disks. Some isolated disks and vesicles are also observed. Bars, 0.5  $\mu\text{m}$ .



**Figure 5.** Sucrose-density fractionation of trypsin-treated ROS membranes. (A) Fraction obtained at a sucrose density of 1.13 g/cc. Only unlabeled intact disks and vesicles are observed. (B) Fraction obtained as a red pellet. Most vesicles derived from the plasma membrane contain ricin-AuDex particles. Bars, 0.5  $\mu$ m.



**Figure 6.** Coomassie Blue-stained (CB) and silver-stained (SS) SDS gels of disk and plasma membrane fractions obtained by dilute trypsin treatment of hypotonically lysed ROS. Lanes *a*, molecular mass standards (lactalbumin, soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin, phosphorylase, myosin); lanes *b*, intact, neuraminidase-treated ROS isolated on a sucrose gradient; lanes *c*, hypotonically lysed, trypsin-treated ROS membranes before sucrose gradient centrifugation; lanes *d*, disk fraction (density 1.13 g/cc) from the sucrose gradient; lanes *e*, plasma membrane (pellet) from the sucrose gradient; lanes *f*, same as lanes *e* except at a 1:10 dilution. For lanes *a-e*, 20  $\mu$ g of protein was applied per lane to a 5–16% polyacrylamide gradient slab gel.

stained patterns of these membrane fractions. Similar patterns were observed in over 15 different preparations. In neuraminidase-treated intact ROS (Fig. 6, lanes *b*) rhodopsin migrated as a characteristic broad intense band with an apparent molecular mass of 34–36 kD. Intense bands corresponding to the subunits of phosphodiesterase ( $M_r = 90$ –92 kD) and transducin ( $M_r = 37$ –39 kD), the 48 kD or S-antigen ( $M_r = 48$ –52 kD) and the high molecular glycoprotein ( $M_r = 220$  kD) could be identified on the basis of their mobility and staining intensity (Kuhn, 1980; Fung and Stryer, 1980; Baehr et al., 1979; Molday and Molday, 1979). Numerous additional bands were also observed over the molecular mass range of 14–270 kD. ROS membranes treated with dilute trypsin and washed in hypotonic buffer exhibited a more simplified banding pattern (Fig. 6, lanes *c*). The major rhodopsin band was still observed, but the bands corresponding to the soluble and membrane-associated ROS proteins including phosphodiesterase, transducin, and the 48-kD protein were either absent or present in greatly reduced quantities. High molecular mass proteins including the 220-kD glycoprotein were degraded as previously reported (Wong and Molday, 1986). A new doublet band of  $M_r = 120$  kD was present; this band appears to be a tryptic fragment of the 220-kD Con A-binding glycoprotein. Minor bands of  $M_r$  34–160 kD were also observed. The disk fraction from the sucrose gradient showed a similar, although even more simplified, band pattern (Fig. 6, lanes *d*). Rhodopsin, the 120 kD molecular mass tryptic fragment and a band of  $M_r$  43 kD coincided with bands of similar intensity in trypsin-treated ROS (Fig. 6, lanes *c*). In addition, bands in the range of 57–63 kD were visible in silver-stained gels of both trypsin-treated ROS and purified disks. Other minor bands of trypsin-treated ROS were absent in the disk membrane fraction. The ROS plasma membrane fraction (Fig. 6, lanes *e* and *f*), in contrast, exhibited a different and more complex

protein-banding pattern. Two closely spaced intense bands of  $M_r = 36$  and 38 kD were observed (Fig. 6, lanes *e* and *f*; also Figs. 9 and 10) in the region of rhodopsin. An intense band of  $M_r = 52$  kD was also prominent. This band, which appeared as a minor component in ROS fractions, was absent in the disk membrane fraction. Numerous minor bands in the  $M_r$  range of 35–160 kD were also visible in the Coomassie Blue- and silver-stained gels. When ricin II or ricin-AuDex conjugates were subjected to SDS-gel electrophoresis, two faint ricin bands at 29 and 34 kD were observed as previously shown (Maher and Molday, 1977). These bands did not line up with the major bands observed in the ROS plasma membrane preparations.

### **Lectin-binding Glycoproteins of Disk and Plasma Membranes**

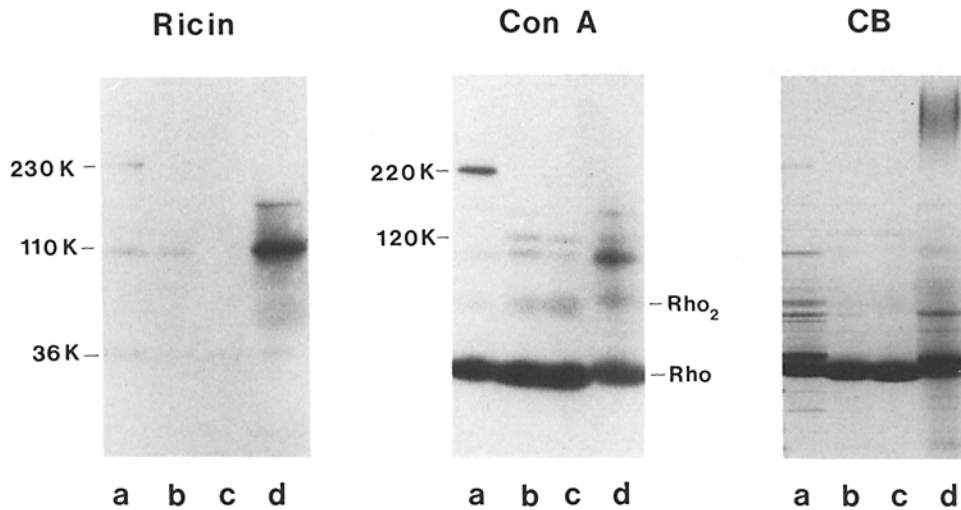
Lectin-binding glycoproteins in ROS membrane fractions were detected on Western blots using [ $^{125}$ I]ricin and [ $^{125}$ I]-Con A as probes (Fig. 7). As previously reported (Molday and Molday, 1987), ricin labeled two protein bands of 230 and 110 kD in neuraminidase-treated ROS membranes (Fig. 7, lane *a*). Bands at 160 and 36 kD were also labeled but much less intensely. Mild trypsin treatment resulted in the disappearance of the 230-kD band but only a slight decrease in intensity of the 110-kD band (Fig. 7, lane *b*). Additional ricin binding just above the 110-kD band was sometimes observed. This staining possibly arises from partial degradation of the 230-kD protein. The disk membrane fraction showed no significant ricin binding except for a faintly stained band at 36 kD (Fig. 7, lane *c*). No additional ricin-binding proteins were observed even if the disk membrane was solubilized in Triton X-100 and exposed to neuraminidase before electrophoresis. In the plasma membrane fraction (Fig. 7, lane *d*), however, a very large increase in staining intensity of the 110-kD protein band was observed. In addition, a sharp band at 160 kD was also labeled. This band appears to be the additional ricin-binding glycoprotein observed in neuraminidase-treated Triton X-100-solubilized ROS (Molday and Molday, 1987).

A different pattern of labeling with Con A was observed as shown in Fig. 7. The 220-kD glycoprotein and rhodopsin and its dimeric and trimeric forms were strongly labeled in neuraminidase-treated ROS membranes (Fig. 7, lane *a*) as well as untreated ROS in agreement with previous studies (Molday and Molday, 1979). Trypsin treatment of ROS membranes resulted in the loss of the 220 kD band and the appearance of two new Con A-labeled bands at 110 and 120 kD (Fig. 7, lane *b*). These bands were also observed in the disk membrane fraction (Fig. 7, lane *c*). In the ROS plasma membrane fraction Con A labeled both rhodopsin and its dimer as well as a band with a  $M_r$  of 110 kD (Fig. 7, lane *d*). A small decrease in mobility of rhodopsin in the plasma membrane relative to rhodopsin in disk membranes was consistently observed. This was particularly evident for the dimer of rhodopsin. Con A also labeled less intensely protein bands at 120 and 160 kD in the ROS plasma membrane fraction.

### **Anti-Rhodopsin and Anti-Peripherin Binding to Disk and Plasma Membranes**

The presence of rhodopsin and peripherin, a protein localized along the rim region of ROS disks, in isolated disk and

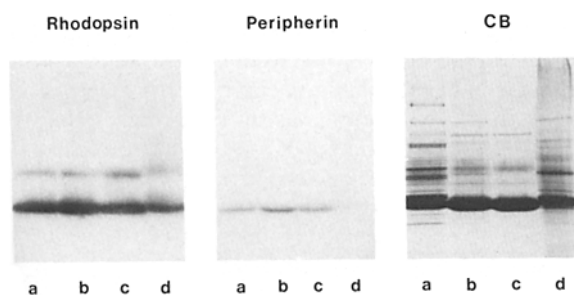




**Figure 7.** [ $^{125}$ I]Ricin II- and [ $^{125}$ I]-Con A-labeled Western blots of ROS membrane fractions obtained by the dilute trypsin procedure. Coomassie Blue-stained gel (CB) of these membrane fractions is shown for comparison. Lanes *a*, neuraminidase-treated, intact ROS; lanes *b*, ROS membrane treated with dilute trypsin before sucrose gradient centrifugation; lanes *c*, disk fraction from the sucrose gradient; lanes *d*, plasma membrane fraction (pellet) from the sucrose gradient. Each lane contains 20  $\mu$ g protein.

plasma membrane fractions was examined using previously characterized anti-rhodopsin and anti-peripherin monoclonal antibodies (Hicks and Molday, 1986; Molday et al., 1987) in conjunction with Western blotting. As illustrated in Fig. 8, the COOH-terminal-specific rho 1D4 rhodopsin antibody labeled rhodopsin and its dimer in neuraminidase-treated ROS, trypsin-treated ROS membranes, disk membranes, and ROS plasma membranes. In the plasma membrane fraction, however, rhodopsin and its dimer were observed to migrate to a slightly higher position relative to rhodopsin in disk membranes as had been observed in Con A-labeled Western Blots. This was the case even if the gold particles in the ROS plasma membrane fractions were removed by centrifugation in SDS solution before electrophoresis. Similar results were obtained using the NH<sub>2</sub>-terminal-specific rho 4D2 anti-rhodopsin monoclonal antibody.

Labeling of the membrane fractions with the 2B6 anti-peripherin monoclonal antibody is also illustrated in Fig. 8. As previously reported, a band at 33 kD was routinely observed in Western blots of ROS and disk membranes. The intensity of staining, however, was greatly reduced in the ROS



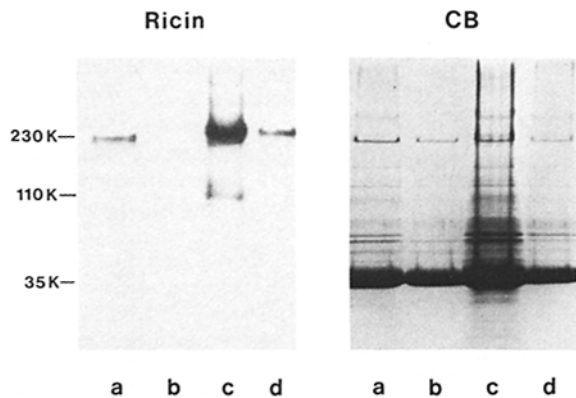
**Figure 8.** Western blots of ROS membrane fractions labeled for rhodopsin with monoclonal antibody rho 1D4 and for peripherin with monoclonal antibody 2B6. Coomassie Blue-stained gel (CB) of these membrane fractions is shown for comparison. Lanes *a*, neuraminidase-treated, intact ROS; lanes *b*, ROS membranes treated with dilute trypsin before sucrose-gradient centrifugation; lanes *c*, disk fraction from the sucrose gradient; lanes *d*, plasma membrane (pellet) from the sucrose gradient. Each lane contains 20  $\mu$ g protein.

plasma membrane fractions. In some ROS membrane fractionation studies an additional band at 35 kD and a doublet in the region of 66–68 kD was observed. The doublet nature of peripherin has been previously shown and the 66–68-kD protein may be a result of incomplete disulfide bond cleavage (Molday et al., 1987). Western blots of disk and plasma membrane labeled with specific monoclonal or polyclonal antibodies indicated that the 48-kD protein or S-antigen was present in very low quantities in the disk and plasma membrane fractions, whereas the  $\alpha$  subunit of the G-protein (Hamm and Bownds, 1984) was absent in the disks but present in the plasma membrane.

#### **SDS-Gel Electrophoresis of Disk and Plasma Membrane Fractions from Nontrypsinized ROS**

SDS-gel electrophoresis and Western blots labeled with [ $^{125}$ I]ricin are shown in Fig. 9 for hypotonically lysed and washed, neuraminidase-treated ROS membranes and membrane fractions obtained after density-gradient centrifugation. The 230-kD ricin-binding glycoprotein is clearly visible in the neuraminidase-treated ROS membranes (Fig. 9, lane *a*), the red plasma membrane-enriched pellet (lane *c*), and the red band which layers on top of 60% (wt/vol) sucrose (lane *d*). A large increase in labeling is evident in the plasma membrane-enriched pellet. The 110-kD ricin-binding glycoprotein is also visible in this fraction. This protein can also be seen in neuraminidase-treated ROS and the 60% (wt/vol) sucrose fraction when longer autoradiographic times are used. The disk fraction in contrast is devoid of these ricin-binding glycoproteins (Fig. 9, lane *b*).

Coomassie Blue-staining of the membrane fractions were dominated by the intense rhodopsin band at 34–36 kD. The 220-kD glycoprotein was also present in all the membrane fractions. A similar pattern of minor bands was observed for the washed ROS membranes, the plasma membrane-enriched pellet, and the 60% sucrose fraction. Several protein bands, however, were not present in the disk membrane fraction. This included a high molecular mass protein ( $M_r$  ~270 kD previously referred to as ROS 1.1 [Molday and Molday, 1979]) and two bands in the  $M_r$  range of 50–54 kD.

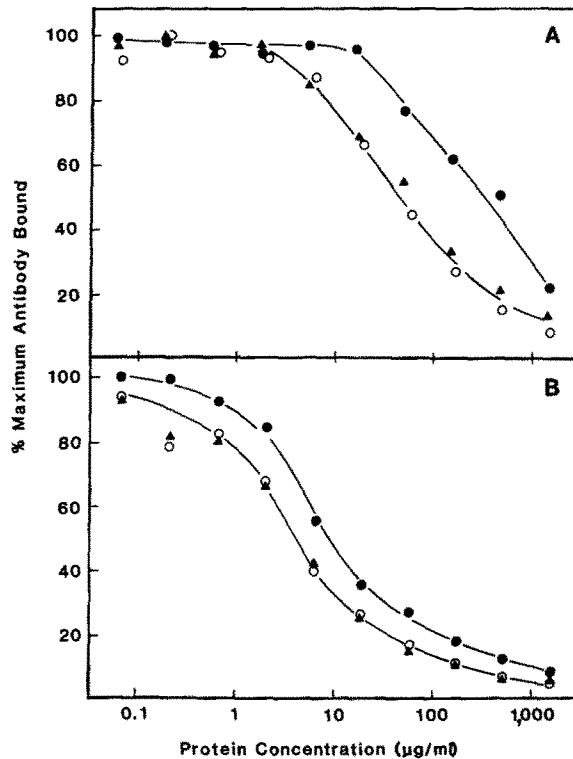


**Figure 9.** [<sup>125</sup>I]Ricin-labeled Western blot and Coomassie Blue-stained (CB) gel of ROS membrane fractions obtained by low ionic strength dissociation in the absence of trypsin. Lanes *a*, unbleached, hypotonically lysed ROS membranes (25 μg protein); lanes *b*, disk fraction from the sucrose density gradient (20 μg); lanes *c*, plasma membrane (pellet) from the sucrose gradient (35 μg); lanes *d*, membrane fraction layered on the 60% sucrose after density gradient centrifugation (20 μg).

### Relative Amounts of Ricin-binding Glycoproteins, Rhodopsin, and Peripherin in Disk and Plasma Membranes

The relative quantities of the major ricin-binding glycoproteins in the various membrane fractions were determined from radioactivity measurements of the bands excised from [<sup>125</sup>I]ricin-labeled Western blots or from densitometer scans of the autoradiograms (see Figs. 7 and 9). For the membrane fractionation procedure using trypsin the ROS plasma membrane had between 10–15 times more 110-kD ricin-binding glycoprotein than the ROS membrane fraction that had been applied to the gradient. Thus, using this plasma membrane-specific marker, on average a 12-fold increase in purification of the ROS plasma membrane relative to total ROS membranes was obtained in this one-step centrifugation method. When low ionic strength buffer was used to promote dissociation of the disks from the plasma membrane, a three- to fourfold increase in the 230-kD glycoproteins was routinely obtained in the plasma membrane-enriched pellet relative to pregradient ROS membranes. No ricin-binding glycoproteins were detectable in the disk membrane fraction obtained from either trypsin-treated ROS or low salt-treated ROS.

The relative quantity of peripherin and rhodopsin in ROS membrane before and after sucrose gradient centrifugation was measured by solid-phase radioimmune competition assays. In this assay, the concentration at which Triton X-100-solubilized trypsin-treated (pregradient) ROS membranes, purified disks, and purified plasma membrane produced half-maximum inhibition (*I*<sub>50</sub>) of binding of anti-peripherin or anti-rhodopsin monoclonal antibodies to immobilized ROS membranes was compared. As illustrated in Fig. 10 *A*, pregradient ROS membranes and disks were equally effective in inhibiting the binding of the antiperipherin 2B6 antibody to immobilized ROS membranes and had an *I*<sub>50</sub> value 50 ± 10 μg (protein)/ml. The inhibition curves for the isolated ROS plasma membrane fraction, however, was shifted to higher concentrations and the *I*<sub>50</sub> value was 310 ± 60 μg (protein)/ml. In four preparations that were analyzed the



**Figure 10.** Solid-phase competitive inhibition of (*A*) anti-peripherin 2B6 monoclonal antibody and (*B*) anti-rhodopsin NH<sub>2</sub>-terminal-specific (rho 4A2) monoclonal antibody binding to immobilized, detergent-solubilized ROS membranes by dilute trypsin-treated ROS membranes before sucrose gradient centrifugation (*solid triangles*), disk membranes from the sucrose gradient (*open circles*), and plasma membrane pellet from the sucrose gradient (*solid circles*).

amount of peripherin in the ROS plasma membrane fraction was 15–25%, the amount found in disk membranes. The low content of peripherin in the ROS plasma membrane observed by this radioimmune assay was in agreement with the low content observed by Western blotting analysis (Fig. 8). The competition curves for the NH<sub>2</sub>-terminal-specific rho 4A2 anti-rhodopsin antibody are also shown in Fig. 10 *B*. Pregradient detergent-solubilized ROS membranes and the disk membrane fraction showed a *I*<sub>50</sub> value of 4.5 ± 0.8 μg (protein)/ml indicating that the rhodopsin content in these samples were similar. The inhibition curve for the ROS plasma membrane fraction, however, was shifted to higher concentration and had an *I*<sub>50</sub> value of 8.5 ± 1.6 μg (protein)/ml. This result indicates that rhodopsin in the ROS plasma membrane is on the order of 50% that found in disk membranes. Similar results were obtained with the COOH-terminal-specific rho 1D4 monoclonal antibody.

### Discussion

Difficulties in isolating the plasma membrane of bovine ROS have been largely overcome in this study through (*a*) the generation of ROS plasma membrane-specific markers, (*b*) the determination of conditions which disrupt the disk-plasma membrane interactions, and (*c*) development of a sensitive and efficient density-gradient fractionation procedure.

dures based on the specific labeling of the plasma membrane with dense ricin-AuDex particles.

Plasma membrane-specific markers in the form of ricin-binding glycoproteins were generated by removal of sialic acid residues and exposure of galactose residues by treatment of intact ROS with neuraminidase. The plasma membrane-specific glycoproteins were labeled either with ricin-AuDex particles for membrane fractionation studies or with [<sup>125</sup>I]ricin for biochemical analysis of the membranes by SDS-gel electrophoresis and Western blots. The ricin-AuDex particles served two important functions. Firstly, the membrane-bound gold particles served as electron microscopic markers to identify the plasma membrane during the fractionation procedure for ultrastructural analysis by electron microscopy. Thus, when ricin-AuDex-labeled ROS were lysed in hypotonic buffer the plasma membrane with attached disks was found to fragment and preferentially adopt an inverted or inside-out orientation. Trypsin treatment of these ROS fragments was observed to effectively dissociate the disks from the plasma membrane vesicles. Electron microscopic analysis of ricin-AuDex-labeled membranes was also useful in confirming the purity of the disk and plasma membrane fractions. Secondly, the ricin-AuDex particles served as density perturbation reagents. Labeling of the ROS plasma membrane with ricin-AuDex particles resulted in a significant increase in density of this membrane and facilitated its separation from the more abundant unlabeled disk membranes by sucrose density gradient centrifugation.

An important step in the fractionation procedure is the dissociation of the plasma membrane from disk membranes. Electron microscopic studies have indicated that the plasma membrane is connected to the rim region of disks through a fibrous cytoskeletal system (Usukura and Yamada, 1981; Roof and Heuser, 1982). Biochemical studies suggest that a spectrin-related protein may constitute part of this cytoskeletal system (Wong and Molday, 1986). Its disruption is required for subsequent fractionation of the disk and plasma membrane by density-gradient centrifugation. In the present study prolonged treatment in low ionic strength buffer and mild digestion with trypsin were used. With the former treatment a highly purified disk membrane fraction was obtained along with an enriched plasma membrane fraction after density-gradient centrifugation. The absence of ricin-binding glycoproteins as observed by Western blots and the absence of ricin-AuDex particles as seen by electron microscopy confirmed the absence of plasma membrane contamination in the disk fraction. By comparing the content of the 230-kD ricin-binding glycoprotein in the plasma membrane-enriched pellet and in the ROS membranes prior to density-gradient centrifugation, a three- to fourfold increase in purity of the plasma membrane was obtained. Since the ROS plasma membrane constitutes 5–6% of the total membrane, this one step procedure results in a fraction containing 15–24% plasma membrane. Electron microscopic analysis confirmed the presence of ricin-AuDex-labeled membranes and unlabeled disks and rim structures in this enriched plasma membrane fraction. In another procedure dilute trypsin in low ionic strength buffer was used to dissociate disks from each other (Molday et al., 1987) and from the plasma membrane. High molecular mass proteins including the 220-kD Con A-binding protein which is thought to be involved in disk-disk interaction (Roof and Heuser, 1982; Corless et al., 1987) and a spectrin-like

protein were degraded along with the 230-kD ricin binding glycoprotein. Density gradient centrifugation of these trypsin-treated ROS membranes yielded a pure disk fraction and a highly pure plasma membrane pellet. Based on ricin-binding to the 110-kD plasma membrane-specific glycoprotein, a 10–15-fold purification of the plasma membrane was obtained by density gradient centrifugation. Electron microscopic analysis revealed predominantly gold-containing membrane vesicles and confirmed biochemical studies indicating that the ROS plasma membrane was 50–75% pure. A membrane fraction collected on top of the 60% sucrose was found to be enriched in plasma membrane, but also contained a significant amount of unlabeled disk membranes. Higher concentrations of trypsin or prolonged digestion further dissociated the disks from the plasma membrane and reduced the amount of membrane collected at the 60% sucrose gradient. However, such treatment has the disadvantage of causing degradation of other membrane proteins including rhodopsin and peripherin.

Analysis of the pure disk membrane fractions and the enriched plasma membrane fractions by SDS-gel electrophoresis and Western blotting using lectins and monoclonal antibodies indicate that the protein compositions of these membrane fractions are significantly different (Table I). The protein composition of disk membranes is relatively simple. Rhodopsin and the 220-kD Con A-binding glycoprotein are the major proteins as detected by Coomassie Blue staining. Dilute trypsin cleaves the 220-kD protein to a 120-kD Con A-binding fragment as previously reported (Molday and Molday, 1979). The 220-kD protein is probably identical to the high molecular "rim-protein" reported by Papermaster et al. (1978a) in frog ROS. Silver-stained SDS-gels of disk membranes reveal additional bands of 43 and 57–63 kD. In addition to these proteins, peripherin, a protein of *M<sub>r</sub>* 33–35 kD, is present in significant quantities in disk membranes.

Table I. Proteins of ROS Disk and Plasma Membranes

<i>M<sub>r</sub></i>	Membrane	Detection	Properties
<i>kD</i>			
34–36 (rhodopsin)	Disks and PM	CB/SS/Con A WGA/mAb	Major protein of disks + PM
33–35 (peripherin)	Disks	mAb	Rim region of disks (Molday et al., 1987)
38	PM	CB/SS	Major PM protein
43	Disks	CB/SS	
52	PM	CB/SS	Major PM protein
57–63	Disks	SS	
110	PM	SS/Ricin/Con A	NMA sensitive
160	PM	CB/Ricin/Con A	NMA sensitive
220 (120-kD trypsin) fragment	Disks	CB/SS/Con A WGA	Rim protein of frog ROS (Papermaster et al., 1978a) Trypsin sensitive
230	PM	Ricin	NMA sensitive Trypsin sensitive
270	PM	CB	Trypsin sensitive

PM, plasma membrane; NMA, neuraminidase; CB, Coomassie Blue; SS, silver stained; WGA, wheat germ agglutinin.

This protein, which recently has been localized along the rim region of disks (Molday et al., 1987), is masked in Coomassie Blue- and silver-stained gels by the dominant rhodopsin band. Notably absent in disk membranes are the sialoglycoproteins that bind ricin after neuraminidase treatment. The protein composition of plasma membrane is more complex as summarized in Table I. In addition to rhodopsin, the plasma membrane contains significant quantities of a 38-kD protein and a 52-kD protein. A protein of 39-kD recently has been reported by Matesic and Liebman (1987) and proteins in the range of 54–57 kD have been observed by Kamps et al. (1982) in enriched ROS plasma membranes isolated on Con A Sepharose or Con A-polystyrene beads. In addition to these proteins, Western blots labeled with ricin confirm previous experiments that the 230- and 110-kD glycoproteins are present in ROS plasma membrane and are not found in disk membranes (Molday and Molday, 1987). It is possible that these proteins are the same as the 226- and 110-kD proteins which become labeled with  $^{125}\text{I}$  when ROS are subjected to lactoperoxidase-catalyzed iodination as described by Clark and Hall (1982). Kamps et al. (1982) have also reported proteins of similar relative molecular mass in their purification procedure. Ricin-labeling studies also indicate that the previously reported 160-kD glycoprotein (Molday and Molday, 1987) is also a plasma membrane-specific glycoprotein. Coomassie Blue- and silver-staining of gels further indicate that the ROS plasma membranes contain numerous minor proteins in the range of 14–270 kD. Peripherin and the 220-kD “rim” glycoprotein of disks appear to be absent or present in significantly reduced quantities in the more highly purified ROS plasma membrane fraction from trypsinized ROS. It is possible that the low content of peripherin detected in the plasma membrane fraction by Western blots and radioimmune assays represents residual disk contamination. Comparison of the protein composition of the ROS plasma membrane preparation obtained by treatment of ROS with low ionic strength buffer with the protein composition obtained by the Con A-polystyrene method of Kamps et al. (1982) suggests that these preparations are similar. Significant contamination with disk membranes is apparent by electron microscopy and analysis of SDS gels. These preparations, however, have the advantage that the proteins, in particular the high molecular mass proteins, are not degraded as is the case with the more highly pure plasma membrane preparations obtained from trypsinized ROS membranes.

The functions of these ROS plasma membrane proteins are not generally known. Matesic and Liebman (1987), however, have shown that the 39-kD protein present in both disk and plasma membranes labels with an 8-azido cGMP photoaffinity reagent and mediates cGMP cation fluxes in reconstituted systems. On the basis of these studies they have suggested that this protein may serve as the cGMP-dependent cation channel inferred from electrophysiological studies. Cook et al. (1987) have purified a 63-kD protein from ROS membranes. In reconstituted liposomes they have shown that this protein acts as a cGMP-activated channel. It is possible that this protein is the 52-kD protein observed in our plasma membrane fractions. The difference in apparent molecular mass may result from the different SDS polyacrylamide gel systems used in these studies. Other plasma membrane-specific proteins observed in this study may function as

ligands for interaction with retinal pigment epithelial cells during phagocytosis or as proteins involved in  $\text{Na}^+$ - $\text{Ca}^+$  exchange (Schnetkamp, 1986) or other regulatory processes.

Immunocytochemical-labeling studies (Papermaster et al., 1978b; Jan and Revel, 1974; Hicks and Molday, 1986) and autoradiographic analysis (Basinger et al., 1976) by electron microscopy have shown that rhodopsin is localized in both the disk and plasma membrane of ROS. Some uncertainty, however, exists concerning the relative content of rhodopsin in these membranes. In freeze-fracture studies similar densities of intramembrane particles, believed to represent rhodopsin aggregates, were reported by Besharse and Pfenninger (1980) to be present in disk and plasma membranes. Kamps et al. (1982), however, estimated that the content of unbleached rhodopsin in the plasma membrane is ~50% that found in disk membranes. In this study we have estimated the relative amount of rhodopsin in disk and plasma membranes by radioimmune assays using anti-rhodopsin monoclonal antibodies. The results indicate that rhodopsin content in the ROS plasma membrane is roughly 50–60% that of the disk membranes. On the basis of Coomassie Blue staining the 38- and 52-kD proteins appear to be the other major proteins in the ROS plasma membrane. Rhodopsin in the plasma membrane fractions is similar to rhodopsin in disk membranes in terms of its ability to bind to COOH-terminal- and  $\text{NH}_2$ -terminal-specific monoclonal antibodies and the lectin Con A. Some differences, however, are apparent as revealed by SDS-gel electrophoresis and Western blots labeled with either anti-rhodopsin monoclonal antibodies or Con A. Rhodopsin in the ROS plasma membrane preparations exhibit a diffuse band having a slightly lower average mobility than rhodopsin in disk membranes. This is particularly evident in the dimeric form of rhodopsin. Furthermore, the diffuse labeling of rhodopsin does not correspond to the relatively sharp band at 36 kD in Coomassie Blue-stained gels. One interpretation is that the 36-kD band represents contaminating rhodopsin from disk membranes and that rhodopsin from the plasma membrane stains poorly with Coomassie Blue but labels strongly with anti-rhodopsin antibodies and Con-A. If this is the case, a form of rhodopsin having perhaps altered carbohydrate chains or other modifications may exist in the ROS plasma membrane and be responsible for its translocation to the plasma membrane. Alternatively, the difference in mobility and staining may reflect an artifact induced by higher susceptibility of rhodopsin to oxidation in the plasma membrane preparations. In this regard we have noted that the 36K Coomassie Blue-stained band disappears when the ricin-AuDex-labeled plasma membranes are maintained for several days at 4°C. Under these conditions rhodopsin, as analyzed by Western blots, shows the presence of more aggregated species. The 38-kD band is not affected and supports the view that this band is not rhodopsin. It is also possible that the 36-kD band is not rhodopsin but represents another major component of the ROS plasma membrane. Studies are now in progress to further characterize these plasma membrane proteins.

In summary, these studies provide conclusive evidence that the protein composition of disk and plasma membranes is different. Rhodopsin is the only protein which to date has been conclusively shown to be present in both membranes. With the capability of obtaining highly pure disk and plasma membranes it should now be possible to isolate and charac-

terize the disk and plasma membrane-specific proteins and gain further insight into their structure and function. The striking difference in protein composition of disk and plasma membrane also raises interesting questions concerning how specific membrane proteins are directed to their final destination within the ROS organelle.

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