Rhodopsin Content in the Outer Segment Membranes of Bovine and Frog Retinal Rods†

David S. Papermaster*,‡ and William J. Dreyer

ABSTRACT: Retinal rod outer segment membranes (ROS) have been isolated by a simplified procedure from fresh and frozen retinas. When cattle and frog ROS membrane proteins are compared by sodium dodecyl sulfate polyacrylamide gel electrophoresis an extraordinary molecular simplicity and similarity of these membranes is evident. One component, rhodopsin, comprises over 80% of the protein in ROS and exceeds each other protein by a molar ratio of 100:1 or more. The molecular simplicity of ROS membranes has significant consequences for molecular theories of visual amplification; one-to-one molecular interactions of each rhodopsin molecule and any other protein in the ROS are not likely.

Vertebrate photoreceptor cells contain a specialized membranous region known as the outer segment where efficient photon capture and amplification of the light energy occur. It is within this region of the cell that light energy is converted into neural excitation. The neural membrane and chemical synapses transmit this excitation to the remainder of the retina and subsequently to central areas of the brain. Absorption of light by 11-*cis*-retinal in the active site of rhodopsin is the initial event in rods (Wald, 1968). Subsequent molecular and electrophysiological steps in the conversion of light energy to neural excitation may involve alterations in enzymatic activity (Miki *et al.*, 1973; Kuhn and Dreyer, 1972) and changes in permeability to cations (Hagins *et al.*, 1970) or other substances. Rhodopsin in the rod outer segment (ROS)[1] is organized into a lamellar array of membranous discs stacked within an outer enveloping plasma membrane and connected to the rest of the cell *via* a narrow connecting cilium (Dowling, 1967; Cohen, 1969). Analysis of ROS protein composition and molecular weight distribution can determine the stoichiometric relationships of rhodopsin to other membrane components in the outer segment. Consequently, we initiated quantitative studies of rhodopsin content in outer segments as a first step in the analysis of the molecular basis of photoamplification in the retina.

Materials and Methods

Fresh cattle eyes were obtained from a local slaughterhouse (Quality Meat Packing, Vernon, Calif.) and stored in darkness on ice within 0.5 hr of decapitation. Occasionally, 400 eyes were obtained and under these conditions some of the eyes had been stored for as long as 6 hr. Frozen retinas (Hormel, Austin, Minn.) were stored at −20°. North American *Rana pipiens*, weighing 50–150 g (Connecticut Valley Corp.), were treated with tetracycline for 5 days by esophageal intubation in order to prevent sepsis (Gibbs *et al.*, 1966). Frogs were dark adapted overnight prior to use. All subsequent operations were done under dim red light, occasionally using an infrared image converter.

Retinal Homogenization and Isolation of Purified Rod Outer Segments. Retinas were homogenized and ROS isolated by modifications of the procedures of Saito (1938) and McConnell (1965). The procedure is summarized in Figure 1. After removal of the anterior third of the eye, the retina was removed, carefully separated from the pigment epithelium, and suspended in a sucrose homogenizing medium containing 34% sucrose (w/w), 65 mM NaCl, 2 mM $MgCl_2$, and 5 mM Tris-acetate buffer (pH 7.4). The density of this solution was slightly greater than 1.15 g/ml. Cattle retinas were suspended in approximately 1 ml of the homogenizing medium per retina. In our largest preparations 400 retinas were homogenized in a tightly capped 500 ml screw-cap erlenmeyer flask by shaking vigorously for 1 min. This procedure sheared off most of the ROS at the junction of the inner and outer segments. The ROS were then partially separated from the remainder of the retina which sedimented at 4000 rpm for 4 min (SS-34 Rotor, Sorvall, Inc.). Suspended ROS contained in the supernatant were decanted into two volumes of 10 mM Tris-acetate (pH 7.4), and the pellet was resuspended in 15 ml of fresh homogenizing medium per 50-ml polyethylene centrifuge tube. The pellet was rehomogenized by hand with 4–5 passes of a loose Teflon homogenizer in the polyethylene centrifuge tube. This gentle shearing force released additional ROS (approximately 15%) into the supernatant after a repeated centrifugation at 4000 rpm. After diluting this supernatant with two volumes of 10 mM Tris-acetate (pH 7.4) and centrifuging combined supernatants at 4000 rpm for 4 min (GSA Rotor, Sorvall, Inc.), the resulting pellets (crude ROS) were resuspended in 60 ml of sucrose, density of 1.10 g/ml, containing 1 mM $MgCl_2$ and 10 mM Tris-acetate (pH 7.4). The resuspended crude ROS were rehomogenized by a tight glass–glass Duall tissue grinder (Kontes Glass, Vineland, N. J.), drawn up in a syringe, and rapidly ejected through a 26 needle against the wall of the homogenizer. These steps sheared the crude ROS into small fragments. The rehomogenized membranes were layered on top of a discontinuous gradient buffered with 10 mM Tris-acetate (pH 7.4) and containing 1 mM $MgCl_2$ and sucrose in incremental 8-ml density steps of 1.15, 1.13, and 1.11 g/ml. Overloading the gradients shifted purified rods to a denser position on the gradient; therefore, the homogenate was divided into ten samples, approximately 8 ml each. The gradients were centrifuged at 27,000 rpm for 30 min (SW-27

† From the Department of Pathology, Yale Medical School, New Haven, Connecticut 06510 (D. S. P.), and the Division of Biology, California Institute of Technology, Pasadena, California 91109 (W. J. D.). *Received September 14, 1973.*

‡ Recipient of Postoctoral Fellowship PF 527 from the American Cancer Society and U. S. Public Health Service Special Fellowship CA 50645. This work was supported by the U. S. Public Health Service Grants GM 06965 and RR 05358 and Anna Fuller Fund Grant 382-1.

[1] Abbreviation used is: ROS, rod outer segments.

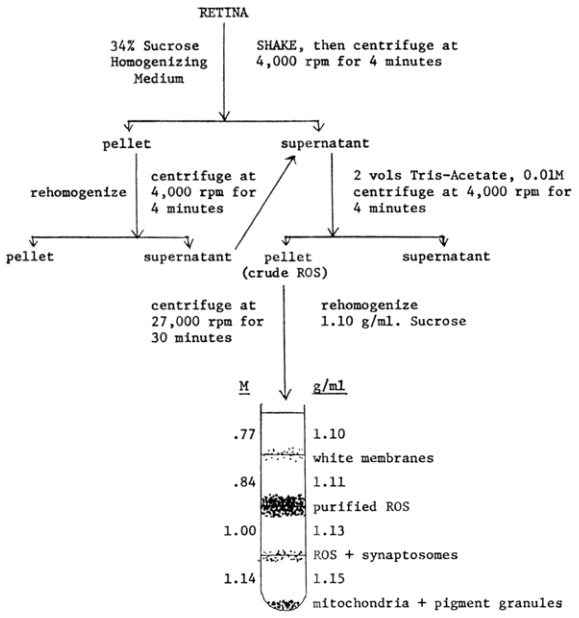

FIGURE 1: The isolation of ROS from fresh bovine retina is accomplished by gentle homogenization, differential centrifugation at low speed for short periods, and subsequent density gradient centrifugation. The composition of the solutions and minor modifications for isolation of frog ROS are detailed in the text.

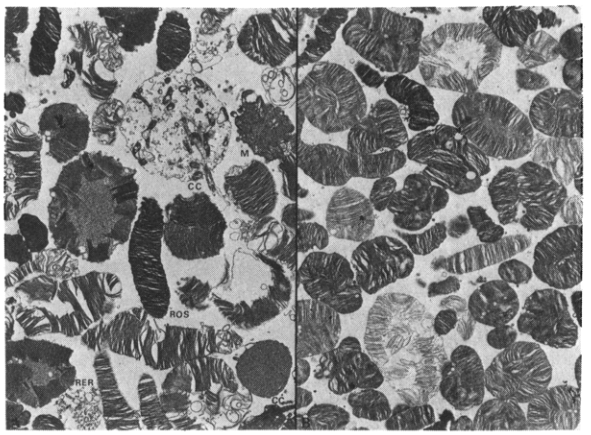

FIGURE 2: Crude bovine ROS obtained after differential centrifugation (A) contain multiple particles of mitochondria (M) and miscellaneous cellular membranes including rough endoplasmic reticulum (RER) mixed with the long cylindrical ROS fragments. Some fragments may arise from the inner segment and contain a connecting cilium (CC). After rehomogenization and gradient centrifugation (B) ROS particles have formed vesicles containing characteristic disc arrays of the ROS.

Rotor, Beckman Instruments). At the end of this period a white band appeared at the 1.10/1.11 g/ml interface. A dense red band appeared at the 1.11/1.13 g/ml interface which usually contained over 90% of the rhodopsin present in the retina if proper conditions of homogenization, loading, and darkness were maintained. The usual yield was 35 mg of ROS protein/100 fresh cattle retinas. A pink or white band appeared at the 1.13/1.15 g/ml interface and contained ROS, broken mitochondria, and synaptosomes upon ultrastructural survey of the pelleted membranes. The pellet, a combination of tan and black particles, sedimented to the bottom of the tube through the 1.15 g/ml cushion. Some of the contamination present in the crude ROS could be avoided by overlaying the initial homogenate with 1.10 g/ml of sucrose and increasing the force of the initial centrifugation from 4000 rpm at 4 min to 20,000 rpm for 20 min, which floated the crude ROS to the interace. However, for large scale preparations this prolonged centrifugation increased the time of isolation and did not result in a considerable alteration in the purification of ROS by the discontinuous gradient. A comparison of the crude ROS obtained by flotation after 20 min of centrifugation and the purified ROS after rehomogenization and gradient isolation is illustrated in Figure 2. Fractions were pelleted, fixed in buffered 4% glutaraldehyde, post fixed in OsO_4, dehydrated in alcohols, and embedded in EPON 812, and thin sections examined in a Phillips 200 electron microscope.

Frog retinas were homogenized by a similar procedure by suspending in 0.5 ml of homogenizing medium per retina. ROS were sheared free by five passes through a 18 trochar on a 10-ml syringe. The homogenate was overlaid with a sucrose solution (density 1.10 g/ml) containing 1 mM $MgCl_2$ and 10 mM Tris-acetate of pH 7.4. The ROS floated to the interface after centrifugation for 20 min at 20,000 rpm and were collected in the upper sucrose solution. This crude ROS suspension was diluted with two volumes of 10 mM Tris-acetate (pH 7.4) and pelleted at 4000 rpm for 4 min (SS-34 Rotor). After rehomogenizing the pellet as described above for cattle retina, the homogenate was overlaid on a sucrose gradient containing 1.5 ml of each of the discontinuous steps described above and in Figure 1. The tubes were centrifuged at 50,000 rpm (SW-50 Rotor, Beckman Instruments) for 40 min and the ROS collected at the 1.11/1.13 g/ml interface. If the retinal homogenates were exposed to white light briefly during isolation in the homogenizing medium in the presence of 65 mM NaCl, then the purified ROS often collected at the 1.13/1.15 g/ml interface. This was to be avoided since synaptosomes and broken mitochondira were also present at this interface and contaminated the purification.

In all subsequent discussion, "purified ROS" will mean those membranes isolated at the 1.11/1.13 g/ml interface. Electron micrographs of isolated frog ROS revealed partially intact lamellar arrays of membranous discs and occasional vesicles. During the isolation of both cattle ROS and frog ROS, the disc membranes were often bounded by a limiting, enveloping membrane which may represent residual plasma membrane of the ROS. After isolation from the gradients, membranes were stored in sealed vials at $-196°$ after freezing in the dark at $-60°$. Membranes were occasionally diluted with two volumes of 10 mM Tris, sedimented into pellets (50,000 rpm, 40 min) and resuspended in 5% glycerol (v/v) before freezing. Dark adapted ROS were frozen in foil-wrapped vials.

Polyacrylamide Gel Electrophoresis. Gels were prepared using purified reagents (Bio-Rad Laboratories) in order to scan the gels at 280 nm. The procedures of Fairbanks *et al.* (1971), Swank and Munkres (1971), and Shapiro *et al.* (1967) and the discontinuous polyacrylamide gel techniques of Laemmli (1970) and Neville (1971) which permitted concentration of dilute samples in the stacking gel were compared (Figure 3). The conditions of electrophoresis are summarized in the figure legends. For molecular weight calibration of the gels, a mixed solution of standards was applied including the heavy and light chains of human immunoglobulin (50,000 and 22,500, respectively), ovalbumin (43,000, Worthington), glyceraldehyde-3-phosphate dehydrogenase (36,000, Worthington), myoglobin (17,500, Sigma), cytochrome *c* (12,000, Sigma), and glucagon (3,500, K & K Corp). Regardless of the analytical gel technique employed, the membranes dissolved in a modification of the Fairbanks *et al.* (1971) denaturing medium containing 2.5% sodium dodecyl sulfate, 2.5% β-

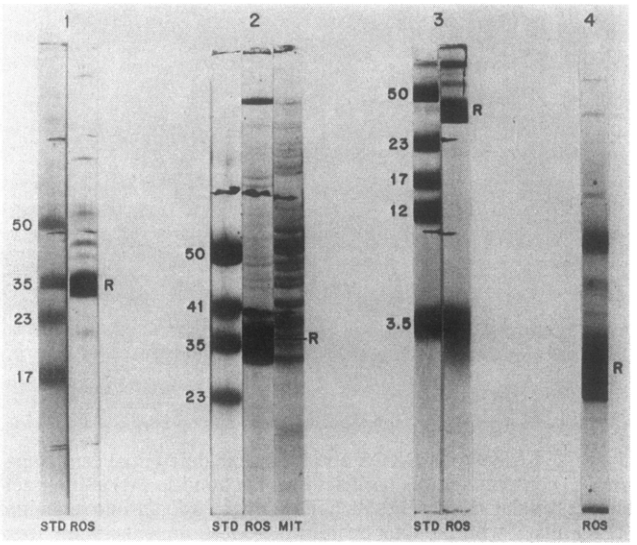

FIGURE 3: Sodium dodecyl sulfate polyacrylamide gels of bovine and frog ROS and mitochondria (MIT). Samples contained 50–100 μg of protein. Procedures illustrated are: (1) Fairbanks *et al.* (1971); (2) Neville (1971); (3) Swank and Munkres (1971); and (4) Laemmli (1970). Molecular weight standards are described in the text. R indicates the position of rhodopsin in each gel which migrates near the mobility of glyceraldehyde-3-phosphate dehydrogenase (35,000). ROS denatured at concentrations greater than 2 μg/ml often form higher molecular weight aggregates such as the dimers seen in the gel 4. No major proteins other than rhodopsin are usually seen in properly isolated and denatured membranes. Bovine ROS (1,3) and frog ROS (2,4) are not significantly different in complexity by this technique.

mercaptoethanol, 0.5 mM ethylenediaminetetraacetic acid, 10 mM Tris-Cl (pH 8.0), and 5% sucrose, and Pyronin Y, 0.5 μg/ml. Samples were denatured at 37° for 3 hr or at 50° for 1 hr and showed no alteration in electrophoretic pattern after storage for a month in tightly capped vials. If the membrane protein concentrations exceeded 1 mg/ml, higher molecular weight aggregates, dimers and trimers particularly, were formed which proved resistant to further denaturation. These artifacts were avoided when lower concentrations of protein and at least 2% sodium dodecyl sulfate were employed in the initial denaturation.

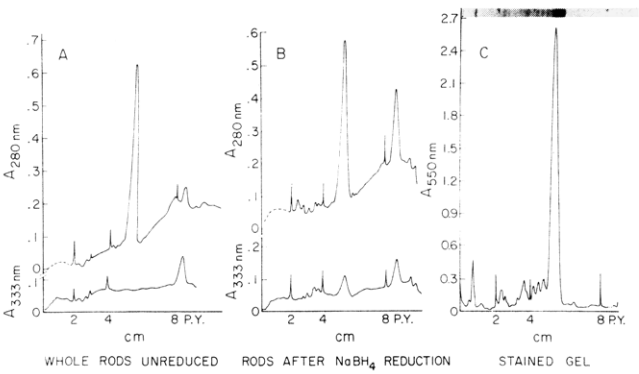

FIGURE 4: The migration of bovine ROS proteins and *N*-retinylopsin on sodium dodecyl sulfate polyacrylamide gels according to the procedure of Fairbanks *et al.* (1971). The major peak in unstained gels absorbing at 278 nm (A) is located in the same position as *N*-retinylopsin prepared by $NaBH_4$ reduction of whole ROS (B). The gels contained 80 μg of protein. The stained pattern (C) of a gel containing 40 μg of protein is compared to illustrate the minor components in ROS larger than opsin. No single peak exceeds 1 mol % the mole fraction of opsin. The peak at 9 cm is the Pyronin Y tracking dye (P.Y.). The sharp peaks at 2, 4, and 8 cm are needle tracks which provide distance calibration.

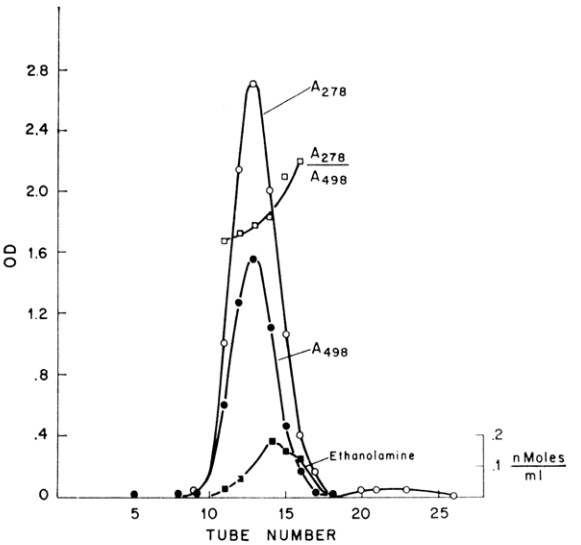

FIGURE 5: Chromatography of whole purified ROS isolated from frozen retina and dissolved in 1% Emulphogene plus 0.067 M phosphate buffer (pH 7.4). The major peak contains all A_{498} absorbing material, 75% of the A_{278} absorbing material, and 85% of the protein of the applied sample. The second peak did not contain any protein but did contain 15% of the A_{278} absorbing material. Lipid, by determination of ethanolamine, is concentrated in the trailing edge of the rhodopsin peak and may account for the increasing A_{278}/A_{498} ratio.

Preparation of N-Retinylopsin (Bownds, 1967; Akhtar et al., 1968). Purified ROS, 16 mg of protein in 4 ml, were thawed in aluminum foil wrapped vials and 4 mg of $NaBH_4$ was added to the sample during exposure to bright fluorescent light. The sample rapidly turned yellow, then white, after which the membranes were sedimented at 17,000 rpm. Reduced cattle ROS were compared electrophoretically with proteins of untreated ROS (Figure 4).

Chromatography of Rhodopsin in Emulphogene on Calcium Phosphate–Celite (Shichi et al., 1969). Frozen retinas (Hormel) were thawed and homogenized by the procedures described above, except the initial homogenization of the retinas was achieved by suspension of the tissue in sucrose–salt homogenizing medium (30% w/v) in the polyethylene 50-ml tube using a Teflon pestle (Thomas, size C). The purified ROS from 100 retinas were isolated at the 1.11/1.13 g/ml interface and suspended in 4 ml of 1% Emulphogene BC 720 in 65 mM phosphate buffer (pH 6.5). The clear solution was passed through a column of 5 g of calcium phosphate and 7 g of Celite, 2.5 × 5 cm, in the buffered 1% Emulphogene. Fractions were analyzed on a Cary 15 spectrophotometer and aliquots hydrolyzed for amino acid analysis. Results of chromatography are illustrated in Figure 5. The spectrum of rhodopsin in the leading portion of the main peak was virtually identical with the spectrum reported by Shichi *et al.* (1969).

Results

Isolation of Purified ROS. The simple procedure outlined in Figure 1 permitted the isolation of purified ROS from considerable amounts of starting material within 5 hr of the initial receipt of fresh eyes from the slaughterhouse. Our procedure was designed to minimize autolysis of neural tissue and spurious generation of small peptides. Saito (1938) had shown previously that ROS can be released by simple shaking of retinas in dense sucrose. This suggested to us that ROS could be obtained relatively free of retinal contamination by using very

gentle homogenization procedures initially. When this approach is compared to other alternatives, such as homogenization of fresh retinas with a Potter–Elvehjem homogenizer (DeGrip *et al.*, 1972) or homogenization of frozen retinas in a Waring blender as proposed by Heller (1968), the results of early gentle treatment become obvious in subsequent steps. Clearly, the more vigorously the retinas are homogenized in this early step the more subsequent procedures must be directed to the elimination of the contamination generated by the extensive fragmentation of the retinal tissue. Therefore, by keeping initial homogenization shear forces at a low level, the most labile cell constituents of the retina are released most readily and may be floated on a sucrose solution denser than the buoyant density of ROS. The second step in the isolation procedure was modified from a procedure described by McConnell (1965) who demonstrated the need for extensive rehomogenization of isolated crude ROS and gradient centrifugation to remove residual entrapped mitochondria and other retinal membranes freed during the initial homogenization. All solutions were buffered to pH 7.4 to avoid the hydrolysis of membrane proteins by acid-catalyzed proteolytic enzymes possibly present in the tissues. In addition, divalent cations were added to shift contaminating membranes from plasma membranes and endoplasmic reticulum (Figure 2) of the retinal cells from a mean buoyant density of 1.12–1.13 g/ml to a density greater than 1.15 g/ml as described by Wallach (1967) and Steck *et al.* (1970) in studies on membranes from erythrocytes and Ehrlich ascitis tumor cells. Failure to include these components in the gradients or using simpler discontinuous gradients such as a single step with a density of 1.15 g/ml resulted in contamination of the purified ROS by membranes of synaptosomes and partially fragmented mitochondria. These contaminants could only be incompletely removed by repeated centrifugation, pelleting, resuspending, and recentrifugation on simple one-step discontinuous gradients (DeGrip *et al.*, 1972; Heller, 1968).

Thus, by close attention to the details of conditions of homogenization and gradient sedimentation we were able to avoid the considerable effort of prolonged centrifugation and repeated resuspension of pelleted membranes, and possible losses induced by osmotic lysis, autolysis, and handling. One measure of the purity of these ROS was obtained by dissolving the membranes at the 1.11/1.13 g/ml interface in 1% cetyltrimethylammonium bromide from dark adapted retinas of frog or from frozen cattle retinas obtained from the Hormel Corporation. Ratios of absorbance, A_{280}/A_{500} of ROS, were typically 2.2 to 2.4 after discontinuous gradient centrifugation. These values contrast with typical ratios nearer 3.0 after isolation by a variety of other techniques (Shichi *et al.*, 1969; DeGrip *et al.*, 1972; Heller, 1968). In addition, polyacrylamide gels demonstrated the simplicity of purified ROS and could detect contamination by other more complex membranes (Figure 3). It is important to note that one step in the isolation might involve osmotic lysis. After initial homogenization, supernatants of the initial centrifugation were diluted with two volumes of 10 mM Tris-acetate and crude ROS were pelleted from the suspension. In order to test whether there was considerable loss of membrane protein by this procedure, the technique was modified on a small scale to float the ROS by centrifugation at 20,000 rpm for 20 min to the 1.10 g/ml homogenizing medium interface. Crude ROS isolated at this interface were then rehomogenized in 1.10 g/ml sucrose, with which they were partially equilibrated at the interface. The rehomogenized crude ROS were again subfractionated on the discontinuous gradients and showed no significant difference in the ratios of absorbance or sodium dodecyl sulfate polyacrylamide gel pattern compared to the membranes isolated after brief osomotic shock as initially described. Such modifications might be useful, however, in the analysis of less prominent ROS components.

Many of the studies of ROS and rhodopsin start with membrane fractions as heterogeneous as the initial crude ROS obtained after one flotation and sedimentation (Figure 2A). Several groups have described insoluble membrane proteins after solubilization in detergents such as digitonin (DeGrip *et al.*, 1972) and cetyltrimethylammonium bromide (Heller, 1968). The insoluble material must then be removed by centrifugation of the detergent solution. In contrast to these observations, the purified ROS isolated after the second step of discontinuous gradient centrifugation in our procedure were completely soluble in cetyltrimethylammonium bromide and Emulphogene. This also supports our conclusion that the majority of contaminants in ROS preparations arise from excessively vigorous homogenization procedures employed too early in the isolation. Subsequent steps in the isolation are often not able to separate ROS completely from contaminants because of entrapped particles, the densities chosen, or the composition of the gradient solutions.

With a simple and rapid procedure of isolation at hand, we were prepared to examine the stoichiometric relationships of rhodopsin to other membrane proteins.

Analysis of Membrane Proteins of ROS by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis. Several procedures of sodium dodecyl sulfate polyacrylamide electrophoresis were compared in order to determine the migration of opsin and other ROS proteins in gels under varying conditions of buffer and acrylamide concentration. These gels also served as sensitive indicators of contamination during the isolation of ROS and could easily detect 5% contamination of retinal mitichondria added to purified ROS after discontinuous gradient centrifugation (Figure 3). The most striking observation is the extraordinary simplicity of the banding patterns of membranes isolated from purified ROS. Only one protein is detected in significant amounts migrating with an apparent molecular weight of 30,000–38,000 depending on the technique employed. When gels using the procedure of Fairbanks *et al.* (1971) were scanned at 280 nm, over 90% of the absorbance was present in the band migrating at approximately 5 cm (Figure 4). Even gels seriously overloaded (100–200 μg of protein) revealed only minor components other than rhodopsin, all with molecular weight larger than rhodopsin (Figure 4C). One consistent and prominent minor component migrated at an apparent molecular weight of 300,000 and was seen on all of the gel techniques employed.

The identification of the major component as rhodopsin was confirmed by coelectrophoresis of *N*-retinylopsin formed after borohydride reduction. The migration distance of this labeled protein detected by scanning at 333 nm, the characteristic peak of *N*-retinylopsin, was identical with the position of reduced opsin (Figure 4B). Examination of the stained gel pattern of an overloaded gel showed no single component exceeded 1 mol % fraction of rhodopsin. This calculation is based on the staining intensity and apparent molecular weight of these components, which were all larger than opsin. Cattle ROS and frog ROS were remarkably similar in their banding patterns; if any differences existed, the frog ROS contained fewer minor components larger than opsin. Comparing the absorbance of the Coomassie Blue stained membrane proteins, over 80% of the area of the stained protein profile lay under the opsin band. The opsin may have an unusual affinity

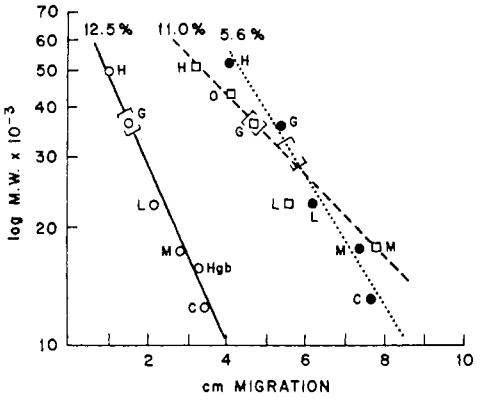

FIGURE 6: Molecular weight determination of rhodopsin by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Frog and bovine rhodopsin were not detectably different. The range of rhodopsin's relative migration [ ] is compared to standard protein mixtures containing immunoglobulin G heavy (H) and light chains (L), ovalbumin (O), glyceraldehyde-3-phosphate dehydrogenase (G), myoglobin (M), hemoglobin (Hgb), and cytochrone *c* (C) by the procedures of Swank and Munkres (1971), 12.5% (○), Neville (1971), 11% (□), and Fairbanks *et al.* (1971), 5.6% (●). The apparent molecular weight varied from 30,000 to 38,000.

for the stain; however, the estimate of rhodopsin content is probably too low since the absorbance of Coomassie Blue stained opsin was proportionally reduced with increasing concentrations of ROS proteins on the gels. Thus, the concentration of trace proteins was exaggerated. When gels were loaded with only 30–50 μg of protein, only the main opsin band and the trace band at 1 cm were detected by the staining procedure of Fairbanks *et al.* (1971) in all gel techniques employed. The less sensitive scan at 280 nm (Figure 4A) demonstrated only the opsin peak. The relative migration rate of opsin compared to several standard proteins increased by 10% when gels were overloaded. The apparent molecular weight of opsin in three polyacrylamide gel electrophoretic systems is illustrated in Figure 6. These results led us to suggest that rhodopsin is the major protein component in purified ROS and appears to exceed in concentration any other protein in the membrane by a molar ratio of 100:1 or more.

The Amino Acid Analysis of Purified ROS. Whole ROS and various subfractions isolated after washing in Mg^{2+}-free buffer or after sodium dodecyl sulfate gel permeation chromatography (Dreyer *et al.*, 1972) were hydrolyzed in 5.7 N HCl. Amino acid analysis of purified ROS demonstrated a composition similar to the many published reports of the composition of rhodopsin isolated by various procedures of detergent gel permeation chromatography (Shichi *et al.*, 1969; Heller, 1968; Shields *et al.*, 1967).

Isolation of Rhodopsin from Purified ROS of Frozen Retinas. Wald's early and important studies on the rhodopsin of cattle retina established the convenience and feasibility of using frozen retinas as a starting material for the isolation of rhodopsin. Since our initial studies were designed to analyze the stoichiometric relationships of the ROS membrane proteins, we were reluctant to conduct them on membranes that might become fragmented by the freezing procedure in the absence of cryoprotective agents. It seemed to us much safer to isolate the membranes in highly purified form prior to freezing. Because of the convenience of the commercially frozen retinas, however, and their wide use as the source for the spectrophotometric studies of rhodopsin structure by numerous laboratories, we quantitatively examined the proteins in ROS membranes isolated from these frozen tissues. Our homogenization procedures could not be directly employed on the frozen tissues because of the significant alterations in tissue structure generated by freezing and thawing. For frozen retinas the initial homogenization medium was modified to contain only 30% sucrose (w/w) and the salts described above. Homogenization by a Teflon pestle in a polyethylene 50-ml centrifuge tube resulted in low shear fragmentation of the retina and released the vast majority of the rhodopsin-containing membranes. These did not sediment readily at 4000 rpm in the homogenizing medium but could subsequently be harvested by dilution, sedimentation at 20,000 rpm, and rehomogenization before sedimentation on the sucrose gradients described above for fresh membranes. Under these circumstances, purified ROS from frozen retinas were isolated with A_{278}/A_{498} ratios of 2.4 to 2.6. Ratios nearer 3 are usually reported (DeGrip *et al.*, 1972; Heller, 1968). Membranes from frozen tissues tended to sediment at greater density, however, and up to 50% of the ROS were obtained in the 1.13/1.15 g/ml interface and had an A_{278}/A_{498} ratio of 2.95. Based on an extinction coefficient of 40,000 at 498 nm, as described by Shichi *et al.* (1969), a yield of 0.7 μmol was obtained in the combined bands which was in the reported range of his isolation using a considerably different procedure. Thus, in our technique for homogenization, sedimentation, and gradient purification of rods, the rhodopsin yield was undiminished compared to techniques using far more vigorous homogenization and the contamination was considerably less. An aliquot of these ROS from the 1.11/1.13 g/ml interface was dissolved in Emulphogene buffered with potassium phosphate at pH 6.5 and chromatographed over a calcium phosphate–Celite column by the procedure of Shichi *et al.* (1969) in the dark. Comparison of the A_{278}/A_{498} ratios of the various fractions (Figure 5) demonstrated that the leading edge of the main chromatographic peak (tubes 8–17) had a ratio of 1.65 and increased to 1.8 toward the trailing edge of the peak probably because of cochromatographing lipid. Each of the fractions was hydrolyzed and analyzed for amino acid content. This technique permitted us to determine the protein content and composition and to readily follow the fractionation of the lipid as represented by the ethanolamine peak on the analyzer. From the published results of the amino acid analysis of rhodopsin (Shichi *et al.*, 1969; Heller, 1968; Shields *et al.*, 1967) and the lipid composition of ROS by Nielsen *et al.* (1970), corrections in the serine content could be made. The protein content in each fraction was estimated by summing the content of all amino acids present in the analyzed fraction. As shown in Figure 5, 75% of the absorbance at 278 nm and 100% of the absorbance at 498 nm was obtained in a single chromatographic peak shortly after the void volume of the column. A second small peak of absorbance at 278 nm (tubes 19–26) followed this main peak but did not contain any amino acids after hydrolysis; 15% of the absorbance at 278 nm was present in this second peak and probably represents nonamino acid aromatic groups (Shields *et al.*, 1967) or possibly tocophorol as reported by Dilley and McConnell (1970). By this quantitative analysis 85% of the A_{278} and 100% of the A_{498} material were recovered from the column without any evidence of other major proteins being present in the Emulphogene extract of whole ROS. The reduction in A_{278}/A_{498} ratio achieved by calcium phosphate–Celite chromatography of purified ROS in phosphate-buffered Emulphogene resulted primarily from removal of nonprotein components absorbing at 278 nm rather than selective adsorption or protein contaminants. This further supports the results of analytical

polyacrylamide gel electrophoresis and peptide analysis that suggest rhodopsin is the major protein in ROS.

Discussion

In order to determine the quantitative relationships of the membrane proteins in rod outer segment membranes, we developed a modification of previously established techniques for isolation of these membranes. This procedure enabled us to obtain ROS rapidly, simply, and in high yield from large amounts of starting material with minimal contamination from the other elements of the retina. Our procedure entailed two brief steps of differential centrifugation and a single sucrose gradient. Purified ROS had spectral properties consistent with a major reduction in non-ROS material absorbing at 278 nm.

The initial homogenization medium contained NaCl (65 mM) and divalent cations (1–2 mM) in order to reduce Donnan effects and maintain nuclear structure during the initial fracture of the retinal membranes and sufficient sucrose (34% w/v) to float ROS. These conditions were a modification of the procedure developed by Wallach and his colleagues in studies of Ehrlich ascites tumor cell and lymphocytes (Wallach, 1967; Ozer and Wallach, 1967). Subsequent steps of analysis demonstrated the high degree of purity of the ROS after gradient centrifugation. Addition of divalent cation to the density gradient was necessary for purification of the ROS. In the absence of divalent cations additional bands appeared in the acrylamide gels of purified ROS which were also present in preparations of retinal mitochondria (Figure 3). While it is possible that ROS purified on gradients free of divalent cations might contain more protein originally present in the ROS particle, experience with other membranes has shown that salts such as Mg^{2+} tend to retain the entrapped components more successfully than gradients free of such salts. McConnell *et al.* (1969) observed that several enzymes were released from ROS when isolated in the presence of magnesium. They concluded that entrapped soluble molecules were released by the divalent cation. Alternatively, however, these enzyme losses could result from the redistribution of contaminating membranes from the zone of ROS purification to other positions in the gradients. Such shifts of buoyant density in sucrose and Ficoll gradients of homogenized endoplasmic reticulum and plasma membrane have been shown in other cells (Steck *et al.*, 1970).

It is unlikely that soluble proteins as a class within the ROS were lost by this isolation procedure since subsequent studies by Kuhn *et al.* (Kuhn and Dreyer, 1972; Kuhn *et al.*, 1973) have demonstrated that a soluble protein kinase is retained in ROS isolated by this procedure. This enzyme may be an example of proteins represented by the minor bands on polyacrylamide electrophoresis and are probably too low in concentration to be detected by N-terminal analysis. These ROS preparations might be more suitable for other studies of enzymatic functions in the outer segment than membranes that have been repeatedly washed in distilled water or solutions not containing divalent cations.

The ROS could be examined by suitable analytical procedures to determine the molecular relationships within the membrane particles. We initially explored the molecular weight distribution of ROS proteins. The determination of the molecular weight of opsin by sodium dodecyl sulfate polyacrylamide gel electrophoresis is not as reliable as the usual limitations of the procedures because of the considerable variation in migration distance of rhodopsin compared to a wide variety of water soluble nonmembrane proteins as standards. Water soluble proteins of known molecular weight ran reliably in each of the techniques except that devised by Laemmli (1970). Despite this relative constancy of behavior of some of the water-soluble proteins, the rhodopsin ranged in molecular weight from 30,000 by the procedure of Fairbanks *et al.* (1971) to 38,000 by the procedure of Swank and Munkres (1971), yet each procedure demonstrated the remarkable molecular simplicity of both cattle and frog ROS. When more complex discontinuous gel systems such as that devised by Neville (1971) were employed, however, overloaded gels occasionally contained doubled bands at 1 and 6 cm, the characteristic positions of the two most prominent proteins in ROS. Comparison of these ROS patterns with isolated rat and frog liver mitochondria, which exactly duplicated the published illustrations by Neville, demonstrated that the procedure was repeated properly. Thus, the extraordinary complexity of mitochondrial membranes demonstrated by this procedure may represent an artificially generated multiplicity. Until other independent means are available for identifying the various proteins in these more complex membranes, the highly complex banding patterns of mitochondrial and erythrocyte membranes on discontinuous gel systems must be viewed with caution compared to the simpler patterns usually seen in the procedure of Fairbanks *et al.* (1971). Regardless of the electrophoretic procedure used, however, the ROS membrane protein patterns were always simple: a major rhodopsin band and minor higher molecular weight components less than 1% the mole fraction of rhodopsin.

In contrast to the simplicity demonstrated in these studies, purification of rhodopsin from other "ROS" proteins has usually been the major first step in studies of its structure. Considerable attention has been devoted to the chromatographic purification of rhodopsin from other proteins in ROS (Shichi *et al.*, 1969; Heller, 1968). Heller (1968) examined the separation of ROS membrane proteins by gel permeation in the cationic detergent cetyltrimethylammonium bromide and described a high molecular weight protein fraction appearing in the void volume. Since the ROS in his procedure were initially isolated after vigorous homogenization in a Waring blender, it is likely that these other components are insoluble proteins in this detergent eluting as aggregates or high molecular weight proteins and probably do not represent intrinsic ROS components. His report and others (Shichi *et al.*, 1969; Shields *et al.*, 1967) have emphasized the considerable reduction in the A_{278}/A_{498} ratios as evidence of purification of rhodopsin from other protein components in purified ROS. By adsorption chromatography of 1% Emulphogene solutions of purified ROS on the calcium phosphate–Celite procedure described by Shichi *et al.* (1969), we were able to determine that most of the reduction in absorbance at 278 nm results from the removal of non-amino acid containing components that absorb at this spectral region. Thus, when purified ROS with an A_{278}/A_{498} ratio of 2.4 were chromatographed on the calcium phosphate–Celite column, all of the rhodopsin and 85% of the A_{278} absorbing material could be accounted for, and only rhodopsin could be detected in the single chromatographic peak. Many of the column chromatographic rhodopsin "purifications" that have been employed may represent either the removal of contaminating proteins resulting from too vigorous a homogenization in the initial stages of isolation or the separation of rhodospin from nonprotein components in ROS that absorb at 278 nm. Other laboratories have also concluded that rhodopsin is the predominant protein in ROS on the basis of analytical acrylamide gels by a single technique

(Bownds *et al.*, 1971) and by gel electrophoresis and amino acid analysis of progressively washed and gradient purified ROS (Daemen *et al.*, 1972).

The simplicity of rod outer segment membranes raises several interesting theoretical consequences for theories of membrane structure and models of photoexcitation. The nearest neighbor to a rhodopsin molecule is another rhodopsin molecule or a lipid molecule. It is therefore not feasible to establish any one-to-one molecular interaction between each rhodopsin molecule and some other protein component such as an enzyme. Yet the sensitivity of the rod cell is so high that each cell may respond to a single captured photon (Hecht *et al.*, 1942). Several functions in ROS have been claimed to be coupled to light exposure and possibly involved in photoamplification including the activity of phosphodiesterase (Miki *et al.*, 1973), the availability of rhodopsin (opsin) as a substrate for an ROS kinase (Kuhn and Dreyer, 1972; Kuhn *et al.*, 1973), and changes in the permeability of the enveloping plasma membrane to sodium (Hagins *et al.*, 1970; Hagins, 1972). It is possible that conformational changes in the structure of rhodopsin alter the permeability or interactions of some region of the outer segment and release a transmitter substance to amplify the effect of the captured photon (Hagins, 1972; Cone, 1973). These hypotheses may be tested by further analysis of the contents of discs isolated from ROS by the procedures described in this study.

Acknowledgments

We wish to express our gratitude to Mr. Ron Siemens, Mr. David Schemberger, and Ms. Julie Siu for their expert technical assistance. We also thank Dr. Max Delbrück, Dr. Paul Hargrave, and Dr. Hermann Kuhn for their helpful discussions.

References

Akhtar, M., Blosse, P. T., and Dewhurst, P. B. (1968), *Biochem. J. 110*, 693–702.

Bownds, D. (1967), *Nature (London) 216*, 1178–1181.

Bownds, D., Gordon-Walker, A., Gaide-Huguenin, A. C., and Robinson, W. (1971), *J. Gen. Physiol. 58*, 225–237.

Cohen, A. I. (1969), *in* The Retina, Morphology, Function and Clinical Characteristics, Straatsma, B. R., *et al.*, Ed., Los Angeles, Calif., University of California, pp 31–62.

Cone, R. A. (1973), *in* Biochemistry and Physiology of Visual Pigments, Langer, H., Ed., West Berlin, Springer-Verlag, pp 275–282.

Daemen, F. J. M., DeGrip, W. J., and Jansen, P. A. A. (1972), *Biochim. Biophys. Acta 271*, 419–428.

DeGrip, W. J., Daemen, F. J. M., and Bonting, S. L. (1972), *Vision Res. 12*, 1697–1707.

Dilley, R. A., and McConnell, D. G. (1970), *J. Memb. Biol. 2*, 317–323.

Dowling, J. E. (1967), *in* Molecular Organization and Biological Function, Allen, J. M., Ed., New York, N. Y., Harper and Row, pp 186–210.

Dreyer, W. J., Papermaster, D. S., and Kuhn, H. (1972), *Ann. N. Y. Acad. Sci. 195*, 61–74.

Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971), *Biochemistry 10*, 2606–2617.

Gibbs, E. L., Gibbs, T. J., and Van Dyck, P. C. (1966), *Lab. Anim. Care 10*, 142–160.

Hagins, W. A. (1972), *Annu. Rev. Biophys. Bioeng. 1*, 131–158.

Hagins, W. A., Penn, R. D., and Yoshikami, S. (1970), *Biophys. J. 10*, 380–412.

Hecht, S., Shlaer, S., and Pirenne, M. H. (1942), *J. Gen. Physiol. 25*, 819–840.

Heller, J. (1968), *Biochemistry 7*, 2906–2913.

Kuhn, H., Cook, J. H., and Dreyer, W. J. (1973), *Biochemistry 12*, 2495–2502.

Kuhn, H., and Dreyer, W. J. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 17*, 1–6.

Laemmli, U. K. (1970), *Nature (London) 277*, 680–685.

McConnell, D. G. (1965), *J. Cell Biol. 27*, 459–473.

McConnell, D. G., Ozga, G. W., and Solze, D. A. (1969), *Biochim. Biophys. Acta 184*, 11–28.

Miki, N., Keirns, J. J., Marcus, F. R., Freeman, J., and Bitensky, M. W. (1973), *Proc. Nat. Acad. Sci. U. S. 70*, 3820.

Neville, D. (1971), *J. Biol. Chem. 246*, 6328–6334.

Nielsen, N. C., Fleischer, S., and McConnell, D. G. (1970), *Biochim. Biophys. Acta 211*, 10–19.

Ozer, J., and Wallach, D. F. H. (1967), *Transplantation 5*, 652–667.

Saito, Z. (1938), *Tohoku J. Exp. Med. 32*, 432–446.

Shapiro, A. L., Vinuela, E., and Maizel, J. V., Jr. (1967), *Biochem. Biophys. Res. Commun. 28*, 815–820.

Shichi, H., Lewis, M. S., Irreverre, F., and Stone, A. L. (1969), *J. Biol. Chem. 244*, 529–536.

Shields, J. E., Dinovo, E. C., Henriksen, R. A., Kimbel, R. L., Jr., and Millar, P. G. (1967), *Biochim. Biophys. Acta 147*, 238–251.

Steck, T. L., Straus, J. H., and Wallach, D. F. H. (1970), *Biochim. Biophys. Acta 203*, 385–393.

Swank, R. T., and Munkres, K. D. (1971), *Anal. Biochem. 39*, 462–467.

Wald, G. (1968), *Science 162*, 230–239.

Wallach, D. F. H. (1967), *in* Specificity of Cell Surfaces, Davis, B. D., and Warren, L., Ed., New York, N. Y., Academic Press, pp 129–163.