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Membrane Biosynthesis in the Frog Retina: Opsin Transport in the Photoreceptor Cell †

David S. Papermaster,* Carolyn A. Converse,‡ and Julie Siu§

ABSTRACT: Rhodopsin biosynthesis and transport in the photoreceptor cell have been analyzed by subcellular fractionation of frog retinas after short periods of radioactive amino acid incorporation in vivo. Labeled membrane proteins were identified by autoradiography of sodium dodecyl sulfate-polyacrylamide gels. One of the most intensely labeled proteins in retina had a molecular weight comparable to opsin isolated from purified rod outer segments (ROS). Incorporation of label into this protein was rapid; the relative specific activity then diminished after the first 2 hr as radioactivity was transferred from retinal subcellular fractions to ROS. The kinetics of this transfer resembled rates previously observed by Hall et al. (Hall, M. O., Bok, D., and Bacharach, A. C. E. (1969), *J. Mol. Biol. 45,* 397). To identify the rapidly labeled protein as opsin we devised a new technique of two-dimensional immunoelectrophoresis of detergent solubilized membrane proteins. Antibodies were prepared against both whole ROS and opsin. After initial separation of retinal proteins on sodium dodecyl sulfate-polyacrylamide gels, a second dimension of electrophoresis in agarose, containing antisera, resulted in the formation of specific immunoprecipitates. Immunochemical analysis of all membranous and soluble retinal subcellular fractions indicated that newly synthesized opsin was membrane bound upon completion of synthesis. At no period of incorporation was a soluble form of newly synthesized opsin detectable. On this basis, we suggest that this protein is apparently transported as a water-insoluble membrane-bound molecule through the cytoplasm or along membranes of the inner segment to its assembly site near the base of the outer segment.

Cell membrane proteins are synthesized and transported to their appropriate cell surfaces by mechanisms which are still obscure. The detailed analysis of this process is hampered by the molecular heterogeneity of most membranes, the small proportion of the cell membrane mass contributed by any single protein, and the limitations on cell fractionation imposed by the similar biophysical properties of many subcellular components. Some of these obstacles have been overcome in studies of rhodopsin biosynthesis in retinal rod photoreceptor cells. The unique photoreceptor function of these cells resides in a compartment named the rod outer segment (ROS)[1] which is composed of a stack of membranous disks enveloped by a plasma membrane and connected to the remainder of the cell by a narrow connecting cilium (Cohen, 1969). A very large fraction of ROS protein (80–90%) is rhodopsin (Hall et al., 1969; Papermaster and Dreyer, 1974). In addition to synthesizing many other proteins the rod cell continuously produces rhodopsin which is transported to the base of the outer segment where assembly of disks apparently occurs (Young, 1968; Hall et al., 1969). Older disks are displaced apically, engulfed by the adjacent pigment epithelium, and finally digested in phagolysosomes (Young and Bok, 1969). Because of the molecular simplicity and continuous renewal of ROS membranes and the ease of isolation of purified ROS, we considered the photoreceptor cell to be a suitable system for studies of the synthesis and transport of membrane proteins. It is possible that the molecular processes involved in the transport of rhodopsin resemble the general processes of membrane protein transport required by other eukaryotic cells.

Prior biochemical studies of ROS protein biosynthesis have demonstrated that the major final product of in vivo or in vitro radioactive amino acid incorporation is labeled rhodopsin (Hall et al., 1969; O'Brien et al., 1972; Basinger and Hall, 1973). The in vitro studies of O'Brien et al. (1972, 1974) identified the final product of biosynthesis by incubation of the radiolabeled protein from ROS with 9-*cis*-retinal. The protein had properties of rhodopsin upon gel filtra-

† From the Department of Pathology, Yale Medical School, New Haven, Connecticut 06510. *Received July 22, 1974.* This investigation has been supported by Research Grants 382-1 from the Anna Fuller Fund, BC-129 from the American Cancer Society, and RR-05358 and EY-00845 from the United States Public Health Service.

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§ Present address: Laboratory of Reproductive Biology, Harvard Medical School, Boston, Mass.

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

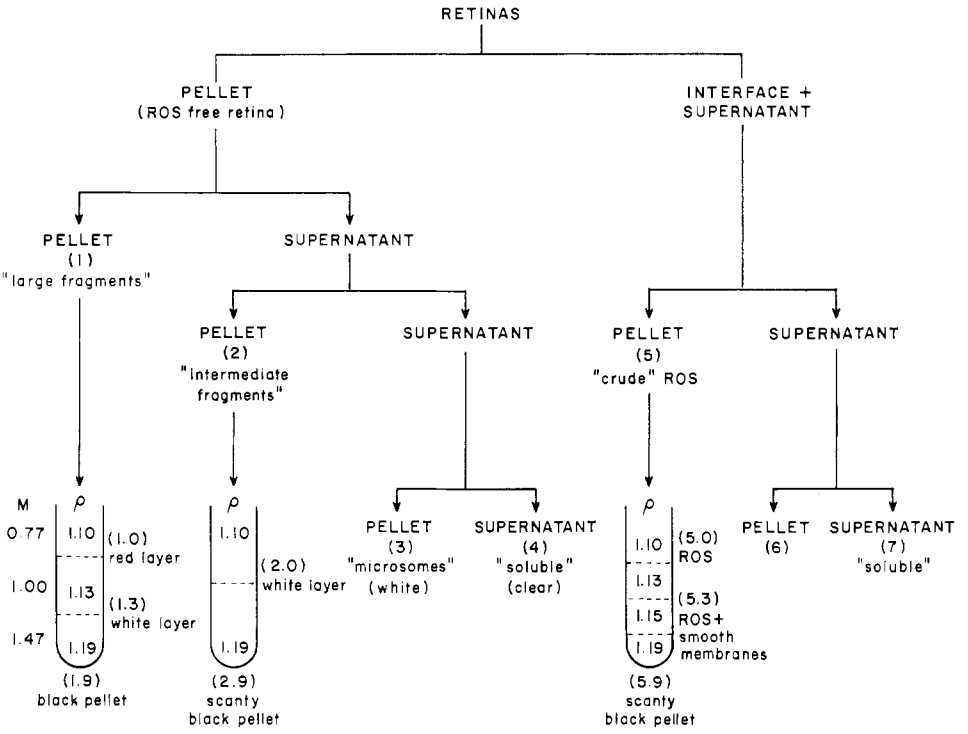

FIGURE 1: Diagram of retinal fractionation. Three pellets obtained during differential centrifugation (1, 2, and 5) are further fractionated on sucrose gradients. These gradient subfractions are numbered according to the pellet from which they originate and the sucrose density layer upon which they float (see text for details).

tion in detergents. Shortly after in vitro pulse labeling of cattle retina, radioactivity was concentrated in a "crude microsomal" fraction. After a chase with nonradioactive leucine, radioactive rhodopsin content increased in ROS while the intensity of label in the "microsomal" fraction diminished. They noted that the kinetics of transport of incorporated label from "microsomes" to ROS paralleled the time course of transport of label from the inner segment to the outer segment assessed by autoradiography of retinal rods by Young (1967) and Hall et al. (1969). Similar observations in frog retina incubated in vitro were reported by Basinger and Hall (1973).

In order to determine the cellular mechanisms governing transport and localized assembly of ROS membrane proteins, we examined the intermediate steps in the transport of opsin after completion of its synthesis. New techniques of retinal subcellular fractionation were devised for isolation of membrane fractions. The radiolabeled proteins were identified by a new procedure of two-dimensional immunoelectrophoretic analysis of detergent-solubilized water-insoluble membrane proteins. The major rapidly labeled protein reacted with antibodies to ROS and opsin. Thus, by a combination of kinetic, immunochemical, and molecular weight analyses, we were able to establish that a protein with the properties of opsin may be readily followed during transport and may be identified in membranous form while still within the inner segment, prior to its assembly in its final cellular site.

Experimental Section

Materials and Methods. North American *Rana pipiens* or Mexican *Rana berlandieri forreri* (Sanders and Smith, 1971; Bagnara and Stackhouse, 1973) weighing 100 to 200 g (Connecticut Valley Corp.) were stored at temperatures between 20 and 23°. The frogs were treated twice each day for 5 days after receipt with tetracycline (10–20 mg/100 g body weight) (Gibbs et al., 1966), nitrofurantoin (1 mg/100 g), or ampicillin (1 mg/100 g) by esophageal intubation in order to prevent bacteremia, and rested without feeding at least 1 week prior to injection of isotope. Radiolabeled amino acids, L-[^{14}C]leucine (0.3 Ci/mmol), L-[^{14}C]phenylalanine (0.466 Ci/mmol), and L-[4,5-^{3}H]leucine (36.6 Ci/mmol) from New England Nuclear, Boston, Mass., were dried just prior to use under a stream of nitrogen and redissolved in 50 m*M* NaCl to achieve a tenfold greater concentration. Sodium dodecyl sulfate (Sipon-WD, Alcolac Chemical Corp., Baltimore, Md.) was recrystallized at 4° from a boiling 20% solution in 80% ethanol. Acrylamide (Eastman Organic Chemicals, Rochester, N.Y.) was recrystallized at 4° from a 50% solution in acetone at 50° after passage through a filter pad of acid washed Norit to lower the absorbance at 280 nm. *N,N*-Methylenebisacrylamide, (Bis), *N,N,N′,N′*-tetramethylethylenediamine, (TEMED) from Eastman, ammonium persulfate (Fisher), and agarose (Nutritional Biochemicals) were used without further purification. Frogs were anesthetized by immersion for 20 to 30 min in a 0.1% solution of *m*-aminobenzoic acid ethyl ester, methanesulfonate salt (MS222, Calbiochem Corp.) at 22°.

In Vivo Incorporation of Radioactive Amino Acids and Retinal Membrane Isolation. Frogs were dark-adapted for 12–15 hr. All subsequent procedures were conducted in darkness or under dim red light (>650 nm) with the aid of an infrared image converter (Varo, Inc., Garland, Tex.). After anesthetization [^{14}C]leucine or phenylalanine was injected into the vitreous chamber (2.5 μCi/eye). Retinas were prepared free from pigment epithelium after periods of incorporation of 30, 60, and 90 min, 2 and 4 hr, and at 3 days. Seven frogs were used for each period of isotope incorporation. Retinal subcellular fractions were isolated at

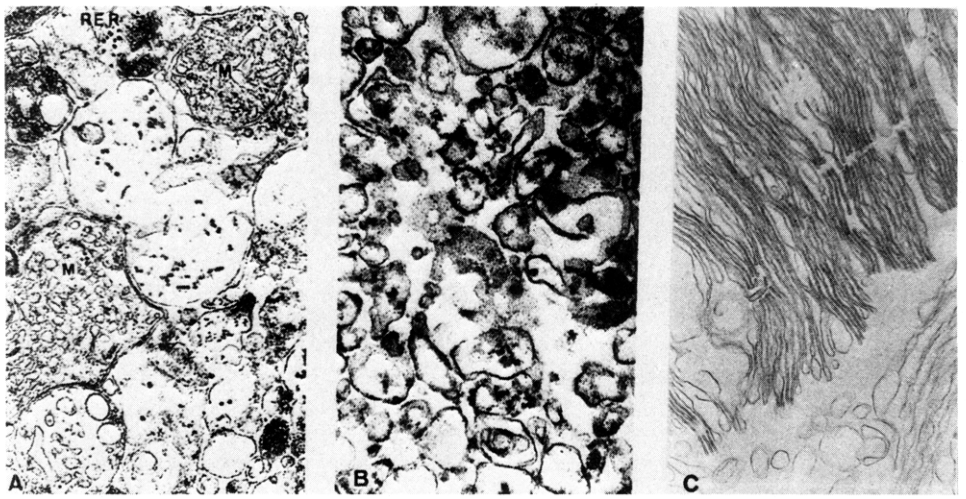

FIGURE 2: Electron micrographs of some of the important retinal subcellular fractions. (A) Fraction 2.0, "intermediate fragments", contains numerous membrane particles readily sedimented at 15,000 rpm. Some fragments are identified readily as mitochondria (M), rough endoplasmic reticulum (RER), and smooth membranes. (B) Fraction 3 contains numerous small vesicles largely derived from smooth membranes. Occasional broken mitochondria and other ribosomal bearing membranes are observed. (C) Fraction 5.0 consists of purified ROS with characteristic lamellar arrays partially broken into vesicles as a result of the rehomogenization (× 20,000).

5° according to the protocol outlined in Figure 1. The first low shear homogenization of the 14 retinas was achieved with five passes of the suspension through a #15 trochar in 5 ml of homogenizing medium which contained 34% (w/w) sucrose, 65 m*M* NaCl, 5 m*M* Tris-acetate (pH 7.4), and 0.2 m*M* $MgCl_2$. Crude ROS were separated from the sedimented retina by flotation at 20,000 rpm (JA 20 rotor, Beckman Instrument Co.) to the 1.10/1.15 g/ml interface as described previously (Papermaster and Dreyer, 1974). The retinal pellet was rehomogenized under conditions of slightly greater shear by five passes of a moter driven Teflon-glass homogenizer (Thomas Tissue Grinder, size AA) in a solution of 0.25 *M* sucrose containing 1 m*M* $MgCl_2$, 10 m*M* Tris-acetate (pH 7.4), and 50 m*M* NaCl. The resulting suspension was mixed with sufficient 0.2 *M* disodium ethylenediaminetetraacetate to achieve a final concentration of 2 m*M* and centrifuged at 4000 rpm for 4 min (JA 20 rotor) to sediment "large fragments", designated fraction 1, which included nuclei, unbroken cells, and large membranous particles. The supernatant was centrifuged at 15,000 rpm for 15 min (JA 20 rotor) producing a pellet composed of mitochondria, synaptosomes, and other "intermediate fragments" which was termed fraction 2. The resulting supernatant was further centrifuged at 50,000 rpm for 40 min (50 Ti Rotor, L-2 ultracentrifuge, Beckman Instruments) producing a crude "microsomal" pellet, designated fraction 3, and a "soluble" cytoplasmic supernatant designated fraction 4. The crude ROS were collected from the initial flotation by dilution with 2 vol of 10 m*M* Tris-acetate (pH 7.4) and sedimentation at 15,000 rpm for 15 min (JA 20 rotor) and were designated fraction 5. Supernatant from the crude ROS was further centrifuged at 50,000 rpm for 40 min (50 Ti Rotor). The resulting pellet containing additional "microsomes" was designated fraction 6 and the final supernatant fraction 7 contained "soluble" materials released during the initial gentle homogenization and unincorporated isotope retained in the vitreous and intercellular spaces. Thus, all soluble and insoluble components of retina at each period of incorporation of isotope were fractionated on the same day.

Fractions 1, 2, and 5 representing "large" fragments, "intermediate" fragments, and "crude ROS", respectively, were resuspended in 1 ml of sucrose solution (density 1.10 g/ml) containing 0.1 m*M* $MgCl_2$ and 1 m*M* Tris-acetate (pH 7.4) and rehomogenized by repeated passage through #18 and #26 needles. These three fractions were further subfractionated on discontinuous sucrose gradients containing 0.1 m*M* $MgCl_2$ and 1 m*M* Tris-acetate (pH 7.4) at 50,000 rpm for 30 min (SW 50.1 rotor) as illustrated in Figure 1. The gradient subfractions were designated by the number of the original pellet, a decimal point, and the final numeral of their approximate bouyant density (e.g., 1.3, from fraction 1, layering at the 1.13/1.15 g/ml interface). Portions of each fraction were fixed for electron microscopy in 4% formaldehyde buffered with 0.1 *M* sodium phosphate (pH 7.4) and were post-fixed in osmium in 0.1 *M* cacodylate buffer (pH 7.4) and then dehydrated through a graded series of ethanol solutions and embedded in Epon 812 (Luft, 1961). Thin sections were stained with lead and uranyl acetate and viewed in a Philips 300 electron microscope. Some of the most intensely labeled subcellular fractions are illustrated in Figure 2. Whole retinas were fixed in phosphate buffered 4% formaldehyde after incorporation of L-[^{3}H]leucine and embedded in Epon after dehydration. Thin sections were covered by a thin film of Ilford L-4 emulsion diluted with an equal volume of water, exposed for 1 week to 1 month, and developed in Kodak D-19 (Figure 3).

Polyacrylamide Gel Electrophoresis and Autoradiography. The isolated subcellular fractions were solubilized in sodium dodecyl sulfate (2.5%) containing disodium ethylenediaminetetraacetic acid (0.5 m*M*), Tris-Cl (10 m*M*, pH 8.0), β-mercaptoethanol (2.5%, v/v), and Pyronin Y (0.5 μg/ml). Samples were treated at 37° for 3 hr if they dissolved readily or at 80° for 30 min in the case of fractions 1.9, 2.9, and 5.9, which contained nuclear fragments and were found to be resistant to solubilization at the lower temperatures.

Membranes dissolved in sodium dodecyl sulfate were electrophoresed according to the procedure of Fairbanks et al. (1971) modified for a vertical slab gel technique on an apparatus similar to that described by Reid and Bieleski (1968) and Studier (1973). The slabs were 12 cm long from the base of the slot, 16 cm wide, and 3 mm thick (60 ml). The initial electrophoretic conditions were 60 V and 75 mA

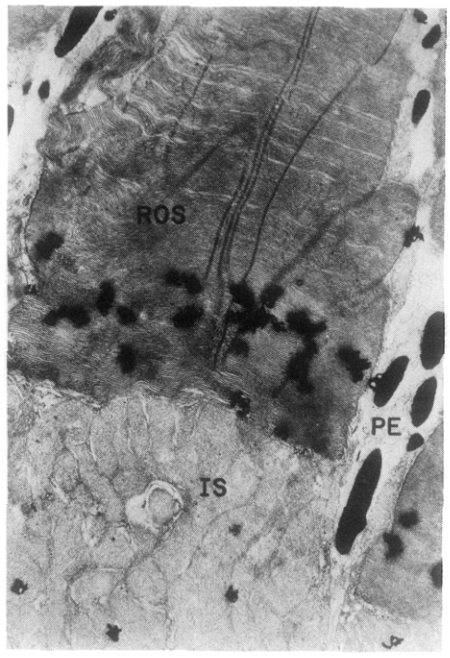

FIGURE 3: Electron micrograph of thin sectioned frog retina after 3 days of [^{14}C]leucine incorporation. Isotope was injected in the vitreous chamber. Silver grains localize newly synthesized opsin after completion of transport to the most basal disks of ROS. Pigment epithelium (PE) processes project to the junction of the ROS and inner segment (IS).

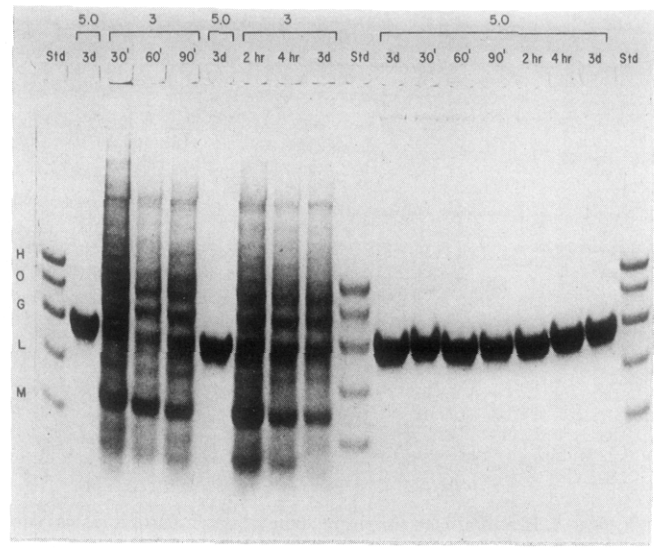

FIGURE 4: The stained protein patterns of two retinal subcellular fractions separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Fairbanks et al., 1971) on a slab gel (Studier, 1973). Membrane proteins of fractions 3 and 5.0 are compared at each interval of radioactive incorporation in this example. To determine the relative migration of [^{3}H]leucine labeled proteins in each fraction, ROS membrane proteins labeled by 3 days of incorporation are used as markers to localize the position of opsin. Gels are dried for subsequent detection of labeled proteins by autoradiography. Water-soluble proteins serving as useful indicators of molecular size include heavy chain of IgG (H), ovalbumin (O), glyceraldehyde-3-phosphate dehydrogenase (G), light chain of IgG (L), and myoglobin (M).

for 30 min; the power was then raised to 95 V and 125 mA at constant voltage for an additional 2.5 hr until the tracking dye had run to 10 cm. In each experiment the outermost slots were filled with a mixed solution of molecular weight standards including the heavy and light chains of human IgG (50,000 and 23,500 daltons, respectively) (Lederle), ovalbumin (43,000) and glyceraldehyde-3-phosphate dehydrogenase (36,000) (Worthington), and myoglobin (17,200) (Sigma). The membranes and supernatant solutions obtained at each of the six time periods for each subcellular fraction were run simultaneously in order to avoid minor migration variations during electrophoresis. A typical electrophoretic separation of two of the subcelluar fractions is illustrated in Figure 4. In addition to the molecular weight markers of known water-soluble proteins, ROS membranes isolated after 3 days of incorporation, which contained intensely labeled opsin, were coelectrophoresed in every third slot and acted as the radiolabeled molecular weight calibration for comparison with the migration of radioactive proteins from other subcellular fractions. Aliquots of the subcellular fractions were chosen to deliver approximately 75–200 μg of protein to each slot. Following electrophoresis, the gels were marked with India ink containing [^{14}C]leucine at 0, 2, 4, and 8 cm in order to establish distance and position of the gel on the autoradiograph. Gels were fixed, stained, and destained according to the procedure of Fairbanks et al. (1971) and then dried on Whatman No. 50 filter paper under vacuum by the procedure of Fairbanks et al. (1965).

The dried gels were exposed for 3 to 12 weeks to Kodak RP/R2 X-Omat X-ray film. Autoradiographic patterns from several important fractions are shown in Figure 5. Semiquantitative analysis of the radioactivity of these various subcellular fractions was obtained by scanning the autoradiographs on a Canalco densitometer (Ueda et al., 1973) (Figures 6 and 7). Since all retinas were dissected and fractionated on the same day and all corresponding subcellular fractions denatured in the same final volume, variations in specific activity of individual proteins reflected their relative biosynthetic incorporation. The reliability of the densitometric scan was checked by comparison of the scan profile and the counts per minute profile of 1-mm slices of gel for each sample. The gel slices were rehydrated in 0.1 ml of water and 0.2 ml of Protosol (New England Nuclear) for 90 min at 60°. An additional 0.4 ml of Protosol and 1.0 ml of 0.35 *M* Tris-Cl (pH 6.9) were mixed with the sample which was then incubated in 8.8 ml of Aquasol (New England Nuclear) overnight at 20° before counting. The scan profile closely paralleled the plot of counts per minute; a ratio of 100 cpm/density unit on the densitometer was observed. The most intense bands (about 200 cpm) did not exceed the linear response range of the film during the exposure time of 1–3 months. The counts recovered in the rehydrated gel represent those components precipitated during its fixation.

The Distribution of Radioactivity among Subcellular Fractions. Five percent aliquots of each of the subcellular fractions isolated after 90 min of [^{14}C]leucine incorporation were applied to sodium dodecyl sulfate–polyacrylamide gels. Comparable aliquots dissolved in 0.5% sodium dodecyl sulfate solution were counted in Aquasol to determine the total counts in each fraction. After electrophoresis, fixation, drying, and autoradiography, the entire longitudinal strip was cut out of the slab gel. The strips, representing fixed and precipitable label, were rehydrated and digested with 7% Protosol in Aquasol and counted. The counting efficiency was 82% as determined by internal spiking and channels ratio. Table I lists the total counts present in the original aliquots and the proportion precipitated in the fixed and stained polyacrylamide gel. The ratio gives the fraction precipitable. Macromolecules are usually totally precipitated

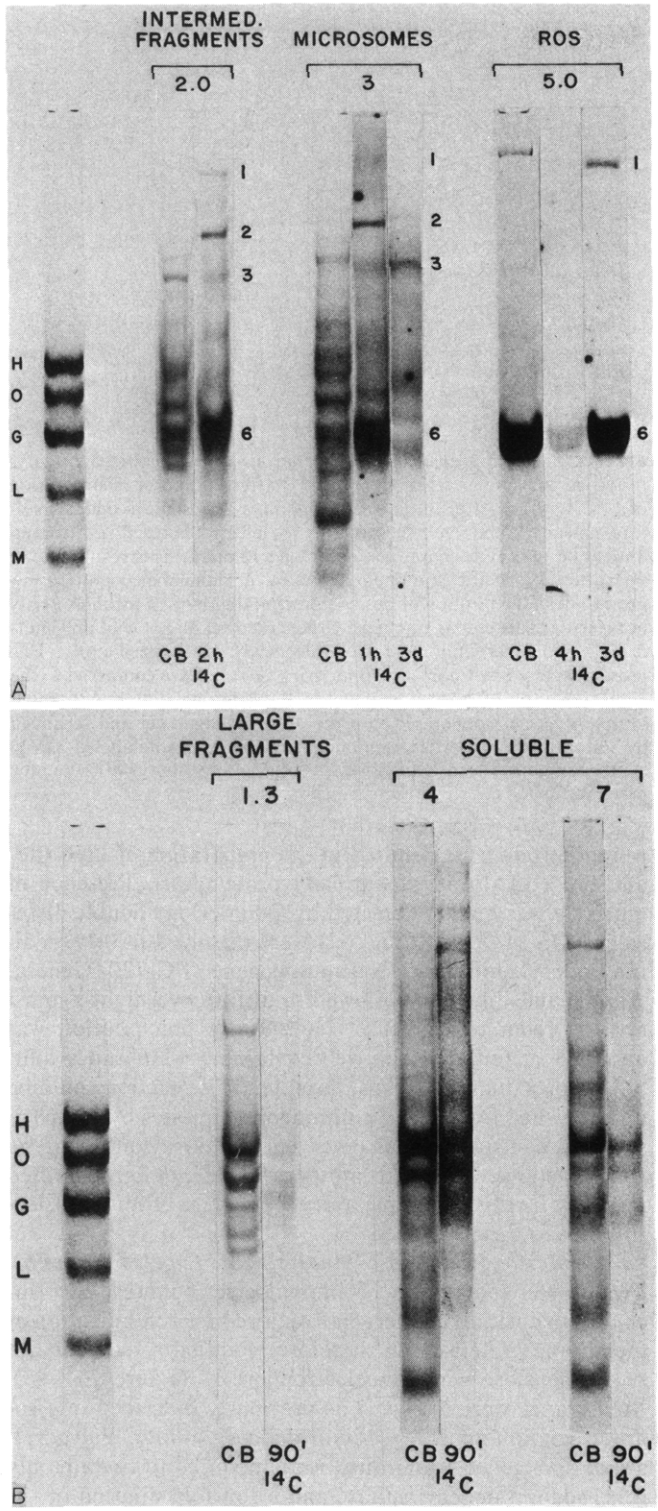

FIGURE 5: (A) Electrophoretic gel patterns of proteins from retinal subcellular fractions stained by Coomassie Blue dye (CB) and their corresponding autoradiographs after various periods of incorporation of [^{14}C]leucine. The migration distance (centimeters) is indicated by the numerals alongside the major bands. The proteins of fractions 2.0 and 3 are rapidly labeled in the region corresponding to the migration of opsin near 6 cm. Other proteins are also rapidly labeled, but are not apparently transferred to ROS (except the protein migrating near 1 cm). (B) The other major membrane fraction (1.3) and the "soluble" fractions (4 and 7) do not contain any prominently labeled proteins near the region of the opsin band.

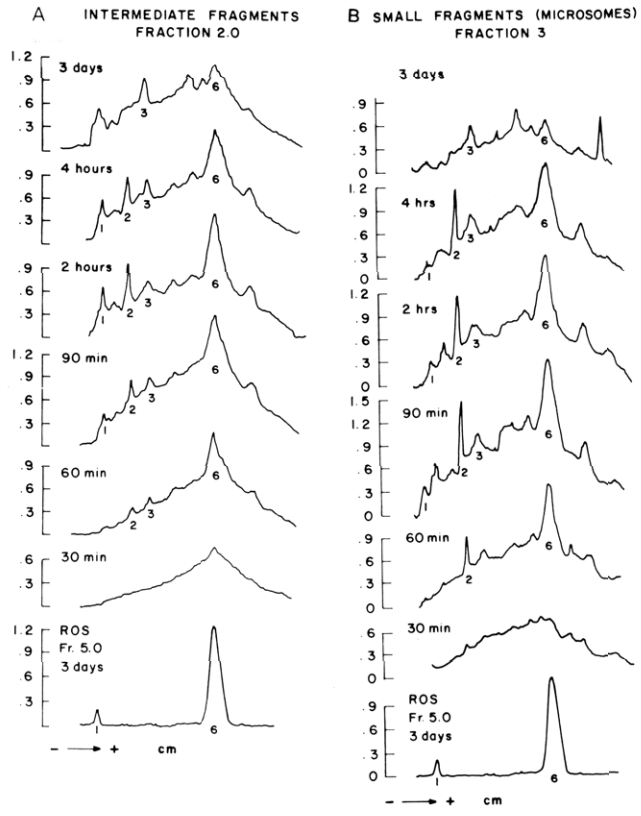

FIGURE 6: (A) Densitometric scans of autoradiographs of electrophoretically separated ^{14}C-labeled retinal proteins in fractions 2.0 and 3. The migration distance (centimeters) of major proteins is indicated by numerals beneath the peaks. The "intermediate fragments" become rapidly labeled in the region of opsin migration (6). Incorporation is apparently maximal at 90 min and 2 hr. Other proteins are also prominently labeled, but only the protein migrating at 1 cm and opsin at 6 cm eventually appear in the ROS after 3 days. (B) Small fragments (microsomes, fraction 3) are similarly labeled in the region of opsin migration. The autoradiographs of this fraction are virtually superimposable on the patterns of fraction 2.0 despite the dissimilarity of some of their stained proteins (cf. Figure 5A). The protein migrating at 1 cm is somewhat less prominently labeled in this fraction compared to fraction 2.0. Persisting label after 3 days of incorporation at 6 cm may represent reutilization of isotope or delayed transfer from the site of injection in the vitreous.

on fixed polyacrylamide gels (Ward et al., 1970). All fractions were analyzed; those relevant to opsin transport are shown in Table I. In addition to fixation, precipitation of incorporated label was accomplished immunochemically as described below and illustrated in Figure 8.

Preparation of Specific Antisera to Purified ROS and Purified Opsin. Purified frog ROS (100 μg of protein/rabbit per injection) were suspended in 0.15 *M* saline–0.005 *M* phosphate buffer (pH 7.4), mixed with an equal volume of Freund's complete adjuvant (DIFCO), and injected into multiple intradermal, subcutaneous, and intramuscular sites in female New Zealand rabbits weighing 2.5 kg. Frog opsin (2 mg of protein) was prepared electrophoretically by dissolving purified ROS in 2.5% sodium dodecyl sulfate–2.5% β-mercaptoethanol and 0.2 m*M* disodium ethylenediaminetetraacetic acid and denaturing for 30 min at 50°. The sample was electrophoresed on a 3-mm thick slab gel as described above, but in a buffer and gel prepared according to the procedure of Laemmli (1970). The position of opsin was determined by scanning the gel at 280 nm on a Gilford spectrophotometer (Papermaster and Dreyer, 1974). The opsin region was sliced from the gel; the remainder was fixed and stained to confirm that the opsin was free of the other minor ROS proteins. The gel slice containing opsin (approximately 100 μg of μg of protein/rabbit) was homog-

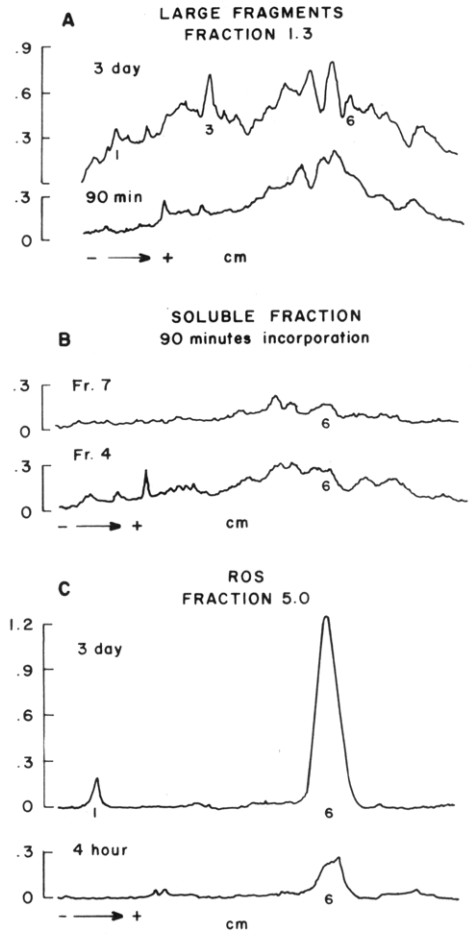

FIGURE 7: Densitometric scans of autoradiographs of electrophoretically separated ^{14}C-labeled proteins in retinal subcellular fractions 1.3, 4, 7, and 5.0. Migration distance (centimeters) is indicated by numerals beneath some peaks. (A) Several proteins in fraction 1.3 (large fragments) are labeled. In contrast to other fractions, however, the proteins do not migrate near the position of opsin. These labeled proteins are not precipitated by anti-opsin. No major bands are detected which subsequently are transported to other fractions. After 3 days of incorporation only a small amount of racioactivity is detectable in the region of opsin migration. (B) Autoradiographs of soluble fractions (4 and 7) contain no major radiolabeled proteins in the region of opsin (6). Other rapidly labeled proteins or rapidly labeled small peptides are not observed. (C) Incorporation of rapidly labeled protein into purified ROS (fraction 5.0) is not readily detectable until 2–4 hr. After 3 days of incorporation, opsin (6) is intensely labeled and a small amount of incorporation is present in the band at 1 cm.

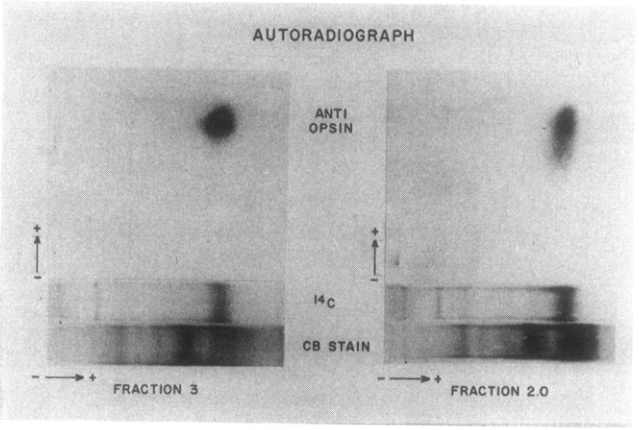

FIGURE 8: Immunoelectrophoretic analysis of radiolabeled proteins in retinal subcellular fractions 2.0 and 3 after 90 min of incorporation of [^{14}C]leucine. After initial separation on a slab sodium dodecyl sulfate–polyacrylamide gel, the proteins are electrophoresed in a second dimension into agar containing antibodies to opsin. This results in the formation of specific immunoprecipitates. A radiolabeled protein migrating near the position of opsin is precipitated by this technique only in these two subcellular fractions. Other fractions do not contain significant amounts of immunoprecipitable newly synthesized opsin. The proteins stained by Coomassie Blue dye (CB stain) are compared to the autoradiograph of labeled proteins in the same gel (^{14}C). A parallel sample was electrophoresed into the antibody–agar gel and localized by autoradiography after immunoprecipitation. Radiolabeled newly synthesized opsin is insignificantly precipitated by nonspecific immune precipitations.

enized with additional saline–phosphate by passage through a #19 needle, mixed with Freund's complete adjuvant and injected as described above. Rabbits were reinjected at intramuscular sites weekly for 4 weeks with antigen suspended in saline–phosphate buffer, rested for 2 months, then reimmunized. This resulted in the preparation of high titer antisera (0.5–1 mg of antibody protein/ml). Presence of antibody was readily detected by Ouchterlony double diffusion. ROS samples (2 mg/ml) were dissolved in 0.1% sodium dodecyl sulfate or 1% Emulphogene (BC-720, General Aniline and Film) and allowed to diffuse overnight against sera at room temperature. Nonspecific precipitation was observed surrounding the well containing membranes solubilized in sodium dodecyl sulfate, but this reaction could be distinguished from specific immunoprecipitates by comparison with controls such as detergent solutions containing no ROS proteins. Since the antigens could not be identified uniquely by this technique a new procedure of immunoelectrophoresis was developed.

Immunoelectrophoretic Analysis of Labeled Proteins. Traditional approaches of immunoelectrophoresis are incompatible with the detergents required for solubilization of membrane proteins. We therefore modified a technique of two-dimensional immunoelectrophoresis (Laurell, 1965). Membranes were dissolved as previously described in a solution containing 2.5% sodium dodecyl sulfate. Polyacrylamide slab gels 1.5 mm thick were modified to contain only 0.1% sodium dodecyl sulfate and a fourfold dilution of the

Table I: Distribution of Radioactivity among [^{14}C]Leucine Labeled Retinal Fractions at 90 Minutes.

Subfraction	Description	dpm/ Sample[a,b]	dpm Precipitated on Gel[a,c]	% dpm Precipitable
2.0	Intermediate sized particles	2,715	2624	97
3	Crude "microsomes"	1,463	1311	90
4	Soluble cytoplasm	12,390	1851	15
5.0	ROS	310	268	86
7	Soluble, vitreous, etc.	41,738	616	1

[a] Sample equivalent to one-half frog eye. [b] Aliquot in denaturing solution, counted in Aquasol. [c] Gel slice fixed, stained, and counted in 7% Protosol in Aquasol.

buffer used by Fairbanks et al. (1971). The gels were electrophoresed at 100 V and 35 mA for 1–2 hr until the tracking dye had migrated to 6 cm. Upon completion of the electrophoresis in this first dimension, individual lengthwise strips containing the separated proteins were cut from the gel and laid alongside a 3 × 6 cm slab of agarose. The agarose had been dissolved in the same buffer prepared eightfold more concentrated, then cooled to 56°, and mixed with antisera (4–12% v/v). During electrophoresis in the second dimension (1.5–2 hr) precipitin arcs formed in the regions of antigen–antibody reaction. Immunoelectrophoretic gels were prepared of each subcellular fraction. The immunochemical analysis of newly synthesized opsin is illustrated in Figure 8.

In order to test the possibility of nonspecific immunoprecipitation in this system we chose an additional antigen–antibody system involving an antigen comparable to opsin in size. Asparaginase (34,000 daltons) and rabbit anti-asparaginase (a gift of Dr. Robert Peterson) were used. Coelectrophoresis of [^{14}C]leucine labeled opsin with asparaginase and immunoelectrophoresis in the second dimension against anti-asparaginase resulted in formation of a dense asparaginase–anti-asparaginase precipitate, yet only 6% of the labeled opsin was coprecipitated by anti-asparaginase. Anti-opsin could not be shown to be reactive with any other protein in retina by these procedures. Further modifications of the method have led to increased sensitivity, so that 50 ng of antigen may be detected (C. A. Converse and D. Papermaster, 1975).

Results

Two major criteria have been used to identify newly synthesized frog opsin and to describe the subcellular components participating in its transport: (a) the kinetics of transfer from retinal subfractions to ROS should parallel the autoradiographic studies of transport in intact retinas by Young (1968) and Hall et al. (1969); (b) antigenic determinants should exist on newly synthesized opsin which are shared with opsin once it is assembled in the ROS membrane. These criteria were met by a protein identified in two of the retinal subcellular fractions. The molecular weight of newly synthesized opsin could then be determined by locating the protein sharing these criteria on sodium dodecyl sulfate–polyacrylamide gels. In this fashion, we were able to follow the transport of one membrane protein in a complex system composed of many other cells which were synthesizing other proteins.

Subcellular Fractionation of the Retina. The procedure of retinal fractionation was designed to take advantage of the anatomy of the neural retina. The long ROS were easily dislodged from the remainder of the retina by passage through a large bore needle and readily purified (Papermaster and Dreyer, 1974). Little contamination from non-ROS structures was found in purified ROS (fraction 5.0) (Figures 2 and 7). The discontinuous steps for further subfractionation of fractions 1, 2, and 5 were selected after preliminary experiments had demonstrated significnt heterogeneity in the populations of membranes obtained by differential centrifugation. Conditions were chosen to separate nuclei, pigment granules, and unbroken cells (subfractions 1.9, 2.9, and 5.9) from membrane fragments (1.0, 1.3, 2.0, and 5.0). Except for the purified ROS (subfraction 5.0), considerable heterogeneity in the crude membrane fragment fractions persisted after gradient separation. The cellular heterogeneity of the retina and similar buoyant properties of some of the fragments contributed to this mixture of cell membranes. Despite these limitations in retinal fractionation, useful separations were achieved; most of the retinal membranes (fraction 1 and its subfractions) were uninvolved in opsin transport (Figure 7) while other simple subfractions (2.0 and 3) were readily isolated which carried the proteins of interest in this study (Figures 5, 6 and 8).

Distribution of Radioactivity in Subcellular Fractions (Table I). The "large fragments" (fraction 1) contained nuclei with abundant surrounding cytoplasm, numerous neuronal processes, and segments of relatively undisrupted retina. Following 90 min of incorporation of labeled amino acids a considerable amount of radioactivity was retained in this fraction and its gradient subfractions, predominantly subfraction 1.3. Most of the radioactivity was precipitated by fixation of the sodium dodecyl sulfate–polyacrylamide gel. Little labeled opsin was detected on the gel (Figures 5B and 7A). Fraction 1.0 contained a small amount of rhodopsin which contributed an opsin band in the stained gel and a red color in the fraction. This band became intensely labeled at 3 days with kinetics resembling ROS (fraction 5.0). Thus, the source of this protein is most likely a small amount of residual ROS remaining after the initial separation of the bulk of ROS into fraction 5. The membranes of fraction 2.0 included intermediate sized particles from synaptosomes, mitochondria, endoplasmic reticulum, and other smooth membrane fragments (Figure 2A). Fraction 2.0 was white despite the isolation of these membranes from dark adapted animals. Thus, there was no detectable red rhodopsin in this subcellular fraction. These membrane fragments contained levels of radioactivity comparable to the large particle fraction (Table I). The incorporated radioactivity of the "microsomal" fraction 3 composed of small particles (Figure 2B) was mostly precipitable in the sodium dodecyl sulfate–polyacrylamide gel (Table I). Fraction 3 was usually white or faint pink when the initial homogenization incompletely sheared the ROS free. The cytoplasmic supernatant fraction 4 was composed of components which would not sediment at 50,000 rpm (150,000g max) for 40 min. Most of the radioactivity in this fraction could not be precipitated (84%) and presumably represented unincorporated [^{14}C]leucine or small peptides.

The ROS particles (fraction 5.0) were severely disrupted by the rehomogenization procedures and formed vesicles and broken lamellar arrays (Figure 2C). This fraction contained relatively little radioactive leucine after 90 min of incorporation (Table I), but subsequently contained most of the precipitable membrane-bound radioactivity in the retina after 3 days of incorporation (Figure 5A). Autoradiographic analysis of thin sections revealed intensely labeled disks after 3 days of incorporation despite the injection of only 50 μCi/eye of [^{3}H]leucine (Figure 3). The more dense fraction (5.3) was also composed of a small amount of ROS particles and was contaminated slightly by retinal membranes with a buoyant density less than 1.15 g/ml. The small amounts of material denser than 1.19 g/ml in fraction 5.9 probably arose from mitochondrial and nuclear fragments. Fraction 6, a pellet obtained from the 15,000-rpm supernatant of the isolated ROS fraction 5, contained small amounts of membranous material including fragmented ROS and microsomes whose particle diameters were too small to sediment at the low speeds used in isolating fraction 5. Once these scanty contaminating fragments were removed by high-speed centrifugation, the resulting supernatant, fraction 7, was composed of "soluble" cytoplasmic

constituents released during the initial shear as well as unutilized isotope from the contaminating vitreous fluids. Only 2% of the radioactivity of this "soluble" fraction was precipitated on the gel (Table I).

Molecular Weight Analysis of the Radiolabeled Proteins. Stained gel patterns and their corresponding autoradiographs of [^{14}C]leucine labeled proteins from the major subcellular fractions are illustrated in Figure 5. Although some of the retinal fractions have superficially similar stained protein patterns, the proportions of individual components vary greatly. The retina was not further subfractionated, however, because of the relative simplicity of the autoradiograms obtained from these fractions. The bulk of the protein was in fractions 1.3 and 1.9, which contain large membrane fragments and unbroken nuclei and retinal fragments, respectively. These particles sedimented at relatively low force fields. All of the proteins seen by protein staining were eventually labeled by the third day, but no significant amount of radioactivity was immunoprecipitable and no transport of newly synthesized opsin from these fractions to ROS was observed at times when fractions 2.0 and 3 were intensely labeled. The numerous labeled proteins present in these "large fragment" fractions may represent the relatively slow turnover of membrane proteins in other regions of retina.

The smaller membrane fragments isolated in subfraction 2.0 and "microsomal" fragments in fraction 3 were intensely labeled after brief incorporation periods. At 30 to 60 min of [^{14}C]leucine incorporation, proteins migrating as rapidly or slightly slower (possibly higher molecular weight) than outer segment opsin were readily detected in the autoradiograms (Figures 5 and 6). Faint bands were also present at 1, 2, and 3 cm. Although the protein staining patterns of fractions 2.0 and 3 differed significantly, the autoradiographic patterns were virtually superimposable (Figure 6). The major band in the opsin region near 6 cm became most intense at 90 min of incorporation, diminished progressively thereafter, and was only faintly visible in fractions 2.0 and 3 isolated 3 days after [^{14}C]leucine injection. By that time ROS opsin (fraction 5.0) was most intensely labeled. Because of its kinetics of labeling, its molecular weight resemblance to ROS opsin, and its apparent transport to ROS, we tentatively termed this protein in fractions 2.0 and 3 "newly synthesized" opsin.

The only other labeled protein in the intermediate subfraction 2.0 that eventually appeared in rod outer segments had an apparent molecular weight of 300,000 and was also detected by staining near 1 cm. Fraction 3, the crude microsomal fraction, contained a similar prominent radioactive band after 60 min of incorporation.

In contrast to this transfer of labeled protein to ROS from fractions 2.0 and 3, the protein migrating at 3 cm was rapidly labeled by 60 min of incorporation and became progressively more prominent by the third day of incorporation. A similar band was also observed in subcellular fraction 1.3 at that time. The protein band at 2 cm in fractions 2.0 and 3 was prominent at 90 min and at 2 hr and declined in intensity after 4 hr. A similarly sized protein was prominently labeled in the soluble fraction 4 after 3 days of incorporation. These proteins have not been identified further. None of these bands were precipitated by immunoelectrophoresis against anti-opsin.

The two "soluble" fractions were obtained after considerable dilution by homogenizing media. Aliquots of these dilute solutions and additional samples after tenfold concentration by ultrafiltration were examined. Although several proteins were readily labeled, none showed the transient incorporation pattern observed in the opsin region of the membranous fractions 2.0 and 3. Most of the proteins in the soluble fractions had molecular weights differing from opsin (Figure 5B). No significant radioactivity was immunoprecipitated in these fractions.

Immunoelectrophoretic Analysis of the Radiolabeled Proteins. Because of the presence of other rapidly labeled proteins in retina, we needed an approach which would recognize some unique molecular feature common to both opsin isolated from ROS and newly synthesized opsin which was not dependent on an 11-*cis*-retinal binding site or molecular weight. Antisera prepared against purified ROS and against opsin isolated from sodium dodecyl sulfate–polyacrylamide gels were used as immunochemical reagents for this purpose. Antigenic proteins were rapidly and totally precipitated by the antibodies and formed arcs and streaks in the region corresponding to their initial position on the polyacrylamide gel (Figure 8). These reaction patterns resemble somewhat the original "rocket" electrophoretic patterns of Laurell (1965) whose procedure was extensively modified in this study. Coelectrophoresis of [^{14}C]leucine labeled opsin with asparaginase in the first dimension (the two bands coincide), followed by electrophoresis into anti-asparaginase–agarose in the second dimension, formed a strong asparaginase–anti-asparaginase precipitin arc; subsequent autoradiography revealed that the precipitate contained less than 6% of the [^{14}C]opsin that is precipitated by anti-opsin. Despite the presence of considerable amounts of radioactivity in many proteins in each retinal fraction, only two fractions, 2.0 and 3, contain an immunoprecipitable protein in significant amounts. In each of these two fractions the anti-opsin immunoprecipitated protein migrated in the opsin region (Figure 8). These represent the first results reported, to our knowledge, of successful direct coupling of high-resolution electrophoresis in sodium dodecyl sulfate–polyacrylamide gels and immunoelectrophoretic analysis.

Discussion

This study was designed to investigate the transport of a membrane protein from its site of synthesis in the rough endoplasmic reticulum to its final assembly point on a specific cell surface. The rod photoreceptor cell offers several unique advantages for the analysis of the transport of membrane proteins such as opsin. The extraordinary compartmentalization of the rod cell separates the regions of biosynthetic activity in the inner segment from the organelle specialized for photoreception in the outer segment. Moreover, since only one major protein (rhodopsin) and one minor protein (300,000 molecular weight) were transported to ROS, the proteins could be analyzed by simple techniques and useful antisera were readily prepared against them.

A new two-dimensional immunoelectrophoretic method was introduced to identify membrane proteins using specific antibodies. This parameter was needed since the molecular weight of newly synthesized opsin could have differed from its final size in the assembled membranes of ROS. Separation in the first dimension retained the high-resolution and molecular sieving properties of sodium dodecyl sulfate–polyacrylamide gels. Immunoelectrophoresis in the second dimension served to identify labeled protein sharing determinants with opsin. In this way, a rapidly synthesized protein similar to opsin in molecular weight and with appropri-

ate kinetics of labeling and transport was shown to be reactive with antibodies to opsin.

Because the protein does not have the characteristic spectral properties of rhodopsin until it is assembled in the outer segment, and may not contain bound retinal, we must consider the evidence that the incorporated radioactivity we have observed truly represents the biosynthesis of rhodopsin. The migration rate of opsin from ROS on the acrylamide gels has been previously established by localization of *N*-retinylopsin which has a characteristic absorption maximum at 333 nm (Bownds, 1967). This product is formed by borohydride reduction of ROS prior to solubilization and migrates in the same position on the acrylamide gels as the major protein in ROS (Papermaster and Dreyer, 1974). The major radioactively labeled proteins in ROS also migrated in this position after 3 days of [^{14}C]leucine incorporation (Figures 5A and 6C). The molecular weight of newly synthesized opsin during transit from inner segment to outer segment is of considerable interest since it is possible that molecular weight modification might be one of the mechanisms used by the visual cell in the assembly of the outer segment membrane. The technique of polyacrylamide gel electrophoresis chosen has the capacity of separating molecules differing by a few thousand daltons in molecular weight from one another. The ROS gels (subfraction 5.0) were relatively overloaded with opsin since most of the protein was confined to one band. The apparent migration of opsin is slightly accelerated by overloading (Papermaster and Dreyer, 1974). Since the steady-state level of newly synthesized opsin in fractions 2.0 and 3 is low, the slightly slower migration may reflect a gel loading effect. In addition, macromolecular components may interfere with entrance of opsin into the gels in other fractions. Despite this uncertainty, however, newly synthesized opsin migrated nearly as rapidly as ROS opsin. No small radiolabeled peptides were observed after 2 and 4 hr of incorporation as labeled new disks were formed. Thus, opsin is apparently transported with little or no change in primary protein structure. Immunoelectrophoretic analysis of the labeled proteins of ROS demonstrated that only the newly synthesized protein band in the opsin region at 6 cm was reactive with antibodies prepared to opsin. Autoradiography of thin sectioned retina illustrated the assembly of labeled protein into ROS disks (Figure 3). This further supports our identification of this protein which has appropriate size, kinetics of labeling and transport, and antigenicity as newly synthesized opsin. Further immunochemical analysis of the labeled high molecular weight protein in ROS (approximately 300,000, Figure 5A, 1 cm) is in progress.

From the earliest period that we could detect newly synthesized opsin (60 min), it was membrane bound to either the "intermediate fragment" fraction 2.0 or the "microsomal" fraction 3. All membranous fractions were exposed to "soluble" proteins during early steps in the fractionation. No soluble proteins of larger or smaller size than opsin were transported at similar rates. Moreover, no proteins reactive with anti-ROS or anti-opsin were detected in the soluble fractions. During fractionation a "soluble" newly synthesized opsin could become artifactually bound or entrapped or might still be bound to ribosomes, but it is unlikely that only two of the many membranous fractions should become the sole fractions labeled by nonspecific binding. Transport on free ribosomes is unlikely since the buoyant density of fraction 2.0 is less than 1.19 g/ml. For these reasons the most apparent mode of transport seems to be on membrane surfaces readily sedimented by intermediate (50,000*g* max) and high (150,000*g* max) force fields. Since membranes may fuse or aggregate during homogenization and fractionation, it is not possible by these methods to determine the in vivo size of the membrane structures involved in transport.

The radioactivity of opsin and the 300,000 dalton protein was not detected in outer segments until 1.5 to 2 hr of incorporation had passed (Figure 6C). The kinetics of this transport closely resembles the ultrastructural reports of Young (1968) and Hall et al. (1969). Our radiolabeling studies were conducted in vivo in order to analyze synthesis while intercellular contact of ROS and pigment epithelium was maintained. These interactions may be important in 11-*cis*-retinal metabolism (Downing, 1960; Bridges, 1973; Hall and Bok, 1974) and may regulate localization of opsin in the plasma membrane of the outer segment. The intact eye also permits us to conduct the analysis under normal conditions of oxygenation and hydrostatic pressure.

What is the nature of the cellular surface involved in opsin transport? Our present fractionation techniques serve to simplify the analysis by localizing the transport to two fractions (2.0 and 3). These membrane fragments may arise from the same cell surface, but represent differing degrees of cellular disruption, or they may represent different structures. Golgi elements may contribute if opsin is glycosylated in a fashion comparable to glycoproteins excreted from other cells (Hall et al., 1969; Bok et al., 1974; O'Brien and Muellenberg, 1974). Hirano et al. (1972) have suggested that membrane glycoproteins may be transported as small vesicles released after completion of glycosylation in the Golgi zone. We have observed that the sedimentation behavior of the newly synthesized opsin suggests it is membrane bound to fairly sizable fragments easily sedimented at 15,000 rpm (cf. mitochondria) and at 50,000 rpm (cf. microsomes).

Embryological studies of developing photoreceptor cells have suggested that the disk membranes of the rod cell are created by an invagination of the plasma membrane near the connecting cilium to form free elements within the enveloping plasma membrane of the outer segment (Nilsson, 1964). This process may persist in the adult eye. One possible mechanism of transport of opsin may involve insertion of newly synthesized opsin into the plasma membrane of the inner segment. Opsin may travel in the inner segment plasma membrane toward the outer segment, where new disks are formed (Jan and Revel, 1974). However, the distribution of the radioactive isotope as it migrates through the inner segment in the autoradiographic studies of Young (1968) and Hall et al. (1969) makes this path seem unlikely. Palade (1959) has emphasized the continuity of biosynthetic membranes in exocrine cells and suggested that transport may involve direct connections from endoplasmic reticulum and Golgi membranes to the plasma membrane. Further autoradiographic and immunocytochemical analysis of opsin transport may distinguish among these pathways.

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Electrostatic Effects in Myoglobin. Application of the Modified Tanford–Kirkwood Theory to Myoglobins from Horse, California Grey Whale, Harbor Seal, and California Sea Lion†

S. J. Shire,‡ G. I. H. Hanania,§ and F. R. N. Gurd*

ABSTRACT: The modified Tanford-Kirkwood electrostatic theory (Shire et al., 1974a) was applied to ferrimyoglobins from the following animal species: sperm whale (*Physeter catodon*), horse, California grey whale (*Eschrichtius gibbosus*), harbor seal (*Phoca vitulina*), and California sea lion (*Zalophus californianus*). Computations were made of the overall hydrogen ion titration curves of the proteins, and of pH and ionic strength variations of ionization equilibria for individual groups in the protein, with particular reference to the hemic acid ionization of the iron bound water molecule. Coordinates and static solvent accessibility were estimated in terms of the sperm whale myoglobin structure. Where possible, theoretical results and experimental data are compared. Some comparative features of charge and ionization properties among the various myoglobins are presented.

We have previously shown (Shire et al., 1974a,b) that the Tanford-Kirkwood electrostatic theory (Tanford and Kirkwood, 1957) can be modified to take into account the specific accessibility to solvent of individual ionizing side chains in sperm whale ferrimyoglobin by introducing a set of solvent accessibility parameters (Lee and Richards, 1971). The modified theory was shown to account adequately for the hydrogen ion titration curve of the protein as well as the titrations of individual ionizing groups. Specifically, the theory predicts the pH and ionic strength variations of the ionization pK value for the iron-bound water molecule in reasonable agreement with the values determined experimentally. It also yields the ionic strength variation of the hydrogen ion titration curve of the protein, in agreement with experiment.

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‡ Present address: Biological Sciences Group, College of Liberal Arts and Sciences, The University of Connecticut, Storrs, Conn. 06268.

§ Present address: Department of Chemistry, American University of Beirut, Beirut, Lebanon.