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Protein Complement of Rod Outer Segments of Frog Retina†

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ABSTRACT: Rod outer segments (ROS) from frog retina have been purified by Percoll density gradient centrifugation, a procedure that preserves their form and intactness. One- and two-dimensional electrophoretic analysis reveals a smaller number of proteins than is observed in many cell organelles and permits quantitation of the 20 most abundant polypeptides. Rhodopsin accounts for 70% of the total protein (3×10^9 copies/outer segment), and approximately 70 other polypeptides are present at more than 6×10^4 copies/outer segment. Another 17% of the total protein is accounted for by the G-protein (3×10^8 copies/outer segment) that links rhodopsin bleaching and the activation of cyclic GMP phosphodiesterase (PDE). The phosphodiesterase accounts for 1.5% of the protein (1.5×10^7 copies/outer segment), and a 48 000-dalton component that binds to the membrane in the light accounts for a further 2.6%. The function of approximately 90% of the total protein in the outer segment is known, and two-thirds of the non-rhodopsin protein is accounted for by enzyme activities associated with cyclic GMP metabolism. The relative molar abundance of rhodopsin, G-protein, and PDE is 100:10:1. Apart from these major membrane-associated proteins, most of the other proteins are cytosolic. Thirteen other polypeptides are found at an abundance of one or more copies per 1000 rhodopsins, nine soluble and four membrane-bound, and their abundance relative to rhodopsin has been quantitated. ROS have been separated into subcellular fractions which resolve three classes of soluble, extrinsic membrane, and integral membrane proteins. A listing of the proteins that are phosphorylated and their subcellular localization is given. Approximately 25 phosphopeptides are detected, and most are in the soluble fraction. Fewer phosphorylated proteins are associated with the purified outer segments than with crude ROS. Distinct patterns of phosphorylation are associated with intact rods incubated with [^{32}P]P_i and broken rods incubated with [γ-^{32}P]ATP.

The outer segments of vertebrate retinal photoreceptors provide a unique preparation for studying the regulation of a hormonal signal because they can be obtained in quantities sufficient for biochemical analysis using gentle conditions that preserve their physiological viability (Biernbaum & Bownds, 1985a,b). More reactions potentially linking receptor activation to the output of the cell have been found than for many other cell types. Light activates an enzyme cascade that rapidly lowers cyclic GMP levels, and several lines of evidence suggest that this change plays a role in regulation of the conductance of the rod outer segment (ROS) plasma membrane (Miller, 1982; Capovilla et al., 1983; Cote et al., 1984; MacLeish et al., 1984; Fesenko et al., 1985). Other reactions include several light, calcium, or cyclic GMP dependent protein phosphorylations (Hermolin et al., 1982; Lee et al., 1981), reduction and oxidation of retinal (Zimmerman et al., 1975), retinol binding proteins (Heller, 1976), a light-induced calcium extrusion (Gold & Korenbrot, 1980; Yoshikami et al., 1980), the light-sensitive permeability mechanism, and sodium–calcium exchange (Hodgkin et al., 1984; Yau & Nakatani, 1984, 1985).

Given the amount of biochemical and electrophysiological data that are accumulating on this system, it seems appropriate to start a systematic list of ROS proteins, similar to those compiled for erythrocyte membranes (Fairbanks et al., 1971), ribosomes (Wittman, 1982), and cilia (Adoutte et al., 1980). It is possible using recent gel techniques to obtain more complete resolution and quantitation than has been possible in previous studies. This provides a context for studies for their individual functions and also is required as background for the studies that utilize monoclonal antibodies as probes of their function (Witt et al., 1984; Hamm & Bownds, 1984).

Several technical issues arise in efforts to purify ROS and establish their protein composition. Sucrose gradient separations have been used in several laboratories to show that at least 10 proteins copurify with rhodopsin (Papermaster & Dreyer, 1974; Godchaux & Zimmerman, 1979; Molday & Molday, 1979; Kuhn, 1980, 1981), but the sucrose gradient and washing procedures developed to minimize contamination can cause elution of minor proteins, including important enzyme activities. Robinson et al. (1980) have shown, for example, that the sucrose gradient procedure leads to loss of elements controlling the light-sensitive phosphodiesterase.

We have prepared ROS by procedures gentle enough to maintain their intactness and prevent elution of enzyme activities, and separated them from contaminating membranes and cells by density gradient centrifugation using Percoll, an isoosmotic low-viscosity suspension of small (<0.1 μm) silica particles coated with polyvinylpyrrolidine. This is the only study of intact rods besides that of Schnetkamp et al. (1979), who used Ficoll gradients to purify intact bovine ROS, and

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Shuster and Farber (1984), who purified rat rod outer segments on Percoll. Suspensions of ROS still attached to their inner segments that are prepared by this technique maintain their normal electrophysiology for several hours (Biernbaum & Bownds, 1985a). The intactness and purity of suspensions of isolated ROS prepared in this manner are discussed in this paper. We have used one- and two-dimensional gel electrophoresis to separate and characterize ROS proteins. Their phosphorylation state and localization to cytoplasm or membrane have been studied, and, for the more plentiful proteins, the number of copies per ROS has been determined.

Materials and Methods

Materials. Purified bovine photoreceptor G-protein was a gift of Dr. James Hurley; purified bovine photoreceptor cyclic GMP phosphodiesterase was a gift of Dr. Meredithe Applebury. Percoll was from Pharmacia. Acrylamide, *N,N'*-methylenebis(acrylamide), glycine, and Coomassie Brilliant blue R-250 were obtained from Bio-Rad. *N,N,N',N'*-Tetramethylethylenediamine (TEMED) and β-mercaptoethanol were from Sigma. Sodium dodecyl sulfate was from BDH. Tris(hydroxymethyl)aminomethane (Tris) base (ultrapure grade), Tris-HCl, and urea (ultrapure grade) were from Schwarz/Mann. Ampholytes were from LKB. NP-40 was from Bethesda Research Laboratories. [γ-^{32}P]ATP and [^{32}P]P_i were from New England Nuclear. All other chemicals were from standard commercial sources.

Isolation of Rod Outer Segments. Bullfrogs (*Rana catesbeiana*) were purchased from Acadian Scientific Supply, Baton Rouge, LA, and Central Valley Biologicals, Clovis, CA, and were kept in 18 °C holding tanks 2–4 weeks. They were fed 5 g of Purina dog chow (Ralston Purina Co., St. Louis, MO) with vitamin supplements 3 times a week and exposed to the photoperiod described by Woodruff and Bownds (1979). Animals were removed from the holding tanks 5–6 h before the end of the 12-h dark period and kept in the dark 1–2 h before decapitation. All experiments were done under infrared illumination using an image converter (FJW Industries, Mount Prospect, IL). Animals were killed, eyes removed, and retinas dissected as described by Woodruff et al. (1977). Retinas were gently rinsed in Ringer's solution [115 mM NaCl, 2.5 mM KCl, 2 mM $MgCl_2$, and 10 mM *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.5] containing 6% Percoll (Pharmacia, Uppsala, Sweden) to remove contamination from vitreous humor; then they were gently agitated for 2 min in three successive tubes containing 550 μL of Ringer's solution in 6% Percoll to release ROS from retina. Tubes were allowed to stand for 30 s to allow any cell clumps to settle. This shake procedure was gentle enough to maintain intactness of approximately 80% of the outer segments as judged by didansylcysteine (DDC) fluorescence (Yoshikami et al., 1974). The retinas remained intact during the shake, which, judged by microscopic examination of the suspension, did not cause major disruption of retinal cells.

Outer Segment Purification. During development of the Percoll purification method, two continuous gradients were evaluated to define the conditions for optimal ROS separation. (1) Isoosmotic Percoll gradient were performed by centrifugation of 50% isoosmotic Percoll at 17000*g* for 20 min in a Sorvall SS34 rotor, and ROS suspensions in 6% Percoll were layered onto the top of the gradients and centrifuged at 2500 rpm in an IEC rotor. (2) Suspensions of ROS in 6% Percoll were mixed with 60% Percoll to give a final concentration of 50% Percoll and centrifuged at 17000*g* for 20 min in a Sorvall SS34 rotor. The isopycnic density of the outer segments was determined by using colored density marker beads (Pharmacia). Red material banded at two densities, a lower band containing intact ROS at approximately 1.069 g/mL and an upper red band containing leaky ROS and ROS pieces at approximately 1.041 g/mL. A small white band was normally seen at the interface between the preformed gradient and the 6% Percoll suspension layered on the top. This contained retinal and pigment epithelium cells and debris, as well as occasional red blood cells (RBC) and bacteria. If the retina was shaken more vigorously, the banding pattern changed. Most of the ROS were found in the upper red band, and the white band containing cell debris was larger. The lower red band was smaller but still contained intact ROS with no noticeable increase in contaminants.

Because retinal contaminants did not appear to enter the gradient after a gentle spin (2500 rpm, 4 min), a discontinuous density gradient composed of the following layers was designed to routinely separate intact ROS from broken ROS and contaminants: 0.25 mL of 6% Percoll (1.002 g/mL, refractive index n^{20}_D 1.3355) in Ringer's solution, 1 mL of 45% Percoll (1.055 g/mL, n^{20}_D 1.3420), and 0.5 mL of 68% Percoll (1.083 g/mL, n^{20}_D 1.3460). The ROS suspension of the retinal shake was layered on top of this gradient and centrifuged for 4 min at 2500 rpm in an IEC 816 rotor. Under the gentle shaking conditions, the major red band containing approximately 60–80% of the ROS was found at the 45–68% Percoll interface. The ROS in this band were osmotically intact as assayed by DDC exclusion and contained few contaminants visible in the light microscope (Figure 1B). The upper red band found at the 6–45% Percoll interface contained approximately 20–40% of the material, consisting of ROS and ROS fragments leaky to DDC. A small white band containing retinal contaminants, RBC, and pigment epithelial cells and fragments was found just above the red band and was not always well separated from this band. The lower band containing long, intact ROS was removed from the gradient by using a tuberculin syringe with a blunt 18-gauge needle. The material was diluted with 5 parts of Ringer's solution and centrifuged at 500*g* for 15 s. After this sedimentation, which was gentle enough to avoid disrupting the outer segments, the supernatant containing the diluted Percoll was removed. In a few experiments, suspensions were prepared in which 40–90% of the ROS remained attached to their mitochondria-rich inner segments. Procedures for making such suspensions are given in Biernbaum and Bownds (1985a). In other experiments, Percoll-purified intact ROS were mixed with 45% sucrose and layered underneath Ringer's solution. Rod outer segments were found at the sucrose–Ringer's interface after 20000*g* centrifugation for 30 min (Figure 1C).

Assessment of Outer Segment Purity. Several criteria were used in assessing the purity of ROS from the lower Percoll band. Light microscopic observation of several fields from each preparation was routinely used to assess the number of outer segments with inner segments still attached, and the number of other particles such as cones, erythrocytes, bacteria, and retinal cells. The presence of subcellular contaminants was assessed by gel electrophoretic techniques comparing polypeptide banding patterns from continuous and step Percoll gradients, and ROS with attached inner segments with normal preparations of ROS. The following considerations suggest that minimal contamination with subcellular contaminants occurs. (1) The methods used for shaking rod outer segments from the frog retina are gentle enough to disrupt mainly the fragile photoreceptor connecting cilium. Scanning electron micrographs of retinas that have been treated in this way show that only the outer segments are broken off at the ciliary stalk

and the rest of the retina is relatively unperturbed. Even most cone outer segments remain on the retina (M. D. Bownds and S. Carlson, unpublished results). The retinal shake is therefore gentle enough that it would not be expected to cause major cell disruption of other retinal cell types. This is in contrast to the chicken, rat, and bovine retinas where vortexing is needed to cause the much smaller outer segments to detach from the retina, and even more harsh treatment, homogenization, is a standard procedure for outer segment purification of bovine outer segments. According to Papermaster and Dreyer (1974), the more vigorous the treatment, the more contaminant organelles become trapped within the outer segment membranes. (2) If any subcellular particles are present, they would not be likely to enter the Percoll step gradient and sediment to the 45–68% Percoll interface during the 4-min, 2500 rpm centrifugation because their sedimentation coefficients are too low (Percoll manual, Pharmacia; Gratzl et al., 1981; Neat et al., 1981; Opperdoes, 1981; Khan et al., 1982). If subcellular particles did enter the step gradient, and sedimented to their isopycnic densities, mitochondria, nuclei, lysosomes, and erythrocytes would be expected to sediment to higher densities, while plasma membranes would sediment to lower densities; only ribosomes would be expected to sediment between 45% and 68% Percoll according to the Percoll manual. (3) Similar polypeptide patterns are obtained from ROS purified on either continuous Percoll gradients or various step gradients, suggesting that the ROS are relatively pure of contaminants. All early experiments were done on continuous Percoll gradients, either preformed gradients or self-forming gradients, and the one-dimensional Coomassie- or silver-stained protein patterns obtained from the intact ROS band were similar to that obtained with step gradients. We have also used the step gradients described by Biernbaum and Bownds (1985a) which contain an additional step in the gradient at 52% Percoll, 1.06 g/mL density. Rod outer segments tend to stay at this interface, while outer segments with inner segments attached go to the 52–68% Percoll interface. Again, the rod outer segments obtained at the 45–52% interface have a protein pattern similar to ROS from the 45–68% interface of the three-step Percoll gradient. (4) The main visible contaminant, the inner segment, contributes protein bands in regions of the gel that do not interfere with analysis of outer segment proteins. In preparations where inner segments remained attached to most ROS, extra protein bands were noted in regions of the gel marked by arrows in Figure 3. To test that these bands were of inner segment origin, experiments were carried out with preparations in which the proportion of ROS attached to their inner segments varied from 40% to 90%. The intensity of these bands on one-dimensional gels varied with inner segment content, whereas the proteins listed in Table I were present in constant ratio to the amount of rhodopsin present. Two-dimensional gel analysis was not done on such preparations; therefore, it is not known which of the more minor components of Figure 2 corresponding to arrows in Figure 3 might be contaminants of inner segment origin.

Subcellular Localization of ROS Proteins. Rod outer segments used in fractionation studies (Figure 4) were fractionated into soluble, peripheral membrane, and integral membrane fractions. The ROS were passed through a 27-gauge needle to break the ROS and release soluble proteins and then washed extensively with Ringer's solution containing 10^2 KIU (Kallikrein inhibitor units) of Trasylol/mL and 2 μM leupeptin. Samples were centrifuged in a Beckman Airfuge for 0.5 h at 100000g; the supernatant was removed, and the Ringer's wash was repeated 2 times, the pooled supernatants being the soluble proteins. The resulting pellet was then resuspended in water to remove proteins peripherally bound to the membrane and centrifuged as above. This procedure was repeated, once with water and once with 3 mM ethylenediaminetetraacetic acid (EDTA) (in water, pH 7.5). The pellet containing integral membrane proteins was resuspended in a small volume of Ringer's solution. Rhodopsin concentration was determined by difference spectroscopy (Bownds et al., 1971).

Phosphorylation Studies in Purified Rod Outer Segments. To monitor protein phosphorylation in suspensions of broken ROS, 50-μL samples of Percoll-purified ROS in Ringer's were permeabilized by passage through a 27-gauge needle and incubated for 8 min in Ringer's solution containing 10 μM [γ-^{32}P]ATP (20 Ci/mmol). For subcellular fractionation of phosphoproteins (Figure 4, lanes 10–12), the phosphorylation reaction was stopped by addition of ice-cold 5 mM KH_2PO_4 and 5 mM EDTA in Ringer's solution, and fractionation was performed as above. To measure kinase activities in the subcellular fractions, fractionation was performed as above, and fractions were incubated in Ringer's solution containing 10 μM [γ-^{32}P]ATP for 8 min. ROS and fractions were quenched with 200 μL of 20% trichloroacetic acid (TCA) and allowed to precipitate for 2 h at 0 °C. Control experiments that compared TCA-precipitated proteins with fractions directly quenched with sample solubilization buffer showed that all proteins were precipitated under these conditions. Endogenous protein phosphorylation levels in intact ROS were studied by incubating 50 μL of ROS in 0.5 mCi of carrier-free [^{32}P]P_i in Ringer's for 30 min. Samples were quenched after incubation by addition of 10% TCA, or for subcellular fractionation (Figure 4, lanes 7–9), samples were diluted with frog Ringer's solution containing 5 mM EDTA, 10 mM NaF, 10^{-8} M Ca^{2+}, 2 μM leupeptin, and 10^2 KIU of Trasylol. Fractionation was performed as above, except that centrifugation steps were performed for 5 min at 190000g. Fractions were quenched with 20% TCA.

Sample Preparation. Purified ROS were prepared for electrophoresis either by direct resuspension of the ROS pellet in solubilization solution (Polans et al., 1979) or by one of the following methods. (1) Precipitation of proteins by mixing ROS suspensions with 2 parts of cold 10% TCA and centrifugation at 5000g for 15 min, followed by resuspension of the pellet in 200 μL of Ringer's solution and centrifugation again at 5000g for 15 min. The final pellet was resuspended in solubilization solution. (2) For two-dimensional gels, the soluble and peripheral membrane protein fraction obtained by hypoosmotic shock of purified ROS was lyophilized and resuspended in a small volume of sample buffer. Control experiments showed that there were no significant differences in protein staining patterns or phosphorylation autoradiographic patterns between samples directly solubilized with sodium dodecyl sulfate (SDS) and those first precipitated with TCA, or lyophilized.

One- and Two-Dimensional Polyacrylamide Gel Electrophoresis. Electrophoretic separation of ROS proteins was achieved by using the Laemmli method of one-dimensional gel electrophoresis described in detail in Polans et al. (1979) and the O'Farrell (1975) method of two-dimensional electrophoresis as modified by Ames and Nikaido (1976) for membrane proteins. In preparation for two-dimensional electrophoresis, ROS proteins were dissolved in 50 mM Tris-HCl, pH 6.8, and 0.5 mM $MgCl_2$, at 4 °C, and then diluted with 2 volumes of 9.5 M urea, 2% ampholines, 5%

β-mercaptoethanol, and 8% NP-40. After electrofocusing, proteins with an apparent isoelectric point (p*I*) higher than 8 were collected, precipitated with 95% EtOH at 20 °C overnight, and run separately on the second dimension (Figure 3, lane 3). Prior to electrophoresis in the second dimension, the tube gel was equilibrated in buffer solution containing SDS for 15 min. For the separation of proteins in the second dimension, discontinuous 10–20% gradient acrylamide gels were used (Polans et al., 1979). Molecular weight (in parentheses) determinations were made with reference to the mobility of standard proteins in the same gradient gels (Weber & Osborn, 1973): myosin (200 000); β-galactosidase (116 000); phosphorylase *b* (97 400); bovine serum albumin (66 000); ovalbumin (45 000); aldolase (40 000); glyceraldehyde-3-phosphate dehydrogenase (36 000); carbonic anhydrase (29 000); β-lactoglobulin (18 400); RNase A (15 800); lysozyme (14 300); cytochrome *c* (13 300). Protein phosphorylation was monitored by using the autoradiographic procedures described by Polans et al. (1979).

Protein Quantitation. Standard proteins were used to determine (1) the linearity of the protein staining intensity over a range of protein concentrations, (2) the variability of staining intensity between proteins, and (3) the limits of detectability of the protein stain. Coomassie brilliant blue and silver staining were compared as a test of reproducibility. Varying amounts (1 ng–1 μg) of five standard proteins [egg white lysozyme, M_r 14 300; β-lactoglobulin, M_r 18 400; trypsinogen, M_r 24 000; ovalbumin, M_r 43 000; albumin (bovine plasma), M_r 66 000] were analyzed by gel electrophoresis and stained with Coomassie brilliant blue (Fairbanks, 1971) or silver stain (Giulian et al., 1982), and staining intensity was determined by laser scanning densitometry (Bio-Med Instruments, Fullerton, CA, SL-504-XL). Under the conditions used in these experiments, the limit of detectability was 5–10 ng for silver and 10–20 ng for Coomassie brilliant blue. It was found that for each of the standard proteins, Coomassie brilliant blue was linear over a range of 20–250 ng, whereas silver was linear over a more limited range (25–100 ng): Coomassie brilliant blue and silver staining showed approximately the same variability over the linear range. A small error (10–20% standard deviation) was observed in scanning individual standard proteins at different loadings in different gels. A 1.5–2-fold variation in staining intensity between different proteins of similar molecular weights was observed by using both Coomassie brilliant blue and the silver stain. This variability could stem from variation in dye affinity for different proteins or error in the estimation of protein concentration of commercially obtained protein standards.

For quantitation studies of ROS proteins, intact ROS purified on Percoll gradients were lysed by hypoosmotic shock, and two fractions were obtained: (1) integral membrane proteins and (2) soluble and peripheral membrane proteins. A range of protein concentrations was analyzed by gel electrophoresis such that for all proteins present, a protein concentration in the linear range of staining intensity could be found. For the integral membrane fraction, 0.25–50 μg of total protein was loaded on gels and analyzed for Coomassie brilliant blue staining and 0.025–1.0 μg of protein for silver staining so that major proteins would be in their linear range at low protein loading, and minor proteins would be detectable at high loading. For soluble and peripheral membrane proteins, the range loaded onto the gel was 0.1–10 μg of total proteins for Coomassie brilliant blue and 10–400 ng for silver.

Silver-stained gels were scanned with a laser densitometer (633-nm light), and Coomassie brilliant blue stained gels were scanned by using a tungsten lamp with a 540-nm filter. It was calculated that proteins present at a concentration of 1 or more copies per 1000 rhodopsins would be present at levels high enough to be quantitated with respect to rhodopsin. The staining intensity for each protein at each loading was determined by integration of peak areas on scans where the peak was on scale. For each protein, four gel loadings where staining intensity was linear were used to compute a mean protein concentration ± SD with respect to rhodopsin whose concentration was determined independently by difference spectroscopy.

Results

Suspensions of Intact Outer Segments Can Be Purified by Percoll Gradient Centrifugation. In early experiments, continuous Percoll gradients were used to determine the isopycnic density of intact ROS. A Percoll step gradient was then designed that would separate intact ROS from leaky and broken ROS and other contaminants. The micrographs in Figure 1 compare ROS taken from the 46–68% Percoll interface (Figure 1B) with two other methods of preparing ROS: ROS freshly shaken from the retina (Figure 1A) and ROS further purified by flotation on 45% sucrose (Figure 1C).

Percoll-purified ROS (Figure 1B) are indistinguishable in morphology from ROS still attached to the living retina. The following observations suggest that they are intact: (1) More than 95% of the ROS present exclude both the vital stain trypan blue and the fluorescent dye didansylcysteine (Yoshikami et al., 1974); (2) they fail to incorporate the terminal phosphate of [γ-^{32}P]ATP into endogenous proteins in contrast to leaky ROS [see Schnetkamp et al. (1979), Miller et al. (1975), Paulsen & Schurhoff (1979), and Hermolin et al., 1982)]; (3) in response to hypoosmotic stress, they form rounded structures, as do ROS still attached to the living retina (Cohen, 1971); in contrast, ROS whose plasma membrane is disrupted unravel and elongate; (4) the 5–10% of the ROS that typically remain attached to the mitochondria-rich ellipsoid portion of the receptor cell maintain normal dark currents that can be suppressed by illumination (Biernbaum & Bownds, 1985); (5) the protein content of ROS is maintained even if intact ROS are left to stand in 45% Percoll for 6 h at 4 °C. The small amount of protein that appears in the supernatant can be accounted for by loss of soluble proteins from the 5–10% of the ROS that become permeable to didansylcysteine.

The appearance of the ROS prepared in Percoll can be easily contrasted either with ROS freshly shaken from the retina (Figure 1A) or with ROS further purified by flotation on 45% sucrose (Figure 1C). The freshly shaken material contains some broken ROS as well as clumps of cells, bacteria, cones, and occasional pigment epithelial cells and erythrocytes. This material, when dissolved and electrophoresed, yields a larger number of protein bands (Figure 1D, lane 1; arrows beside lane 1 denote contaminating bands) than Percoll-purified ROS (lane 2). The sucrose-purified ROS are distorted, beaded, and broken. They do not exclude the didansylcysteine dye. This is in agreement with other workers, who find that hyperosmotic sucrose treatment disrupts ROS which then appear broken (Kuhn, 1981), rounded (Godchaux & Zimmerman, 1979), or shrunken (Schnetkamp et al., 1979). A central point is that the sucrose-prepared ROS have lost a substantial amount of a number of the proteins present in the Percoll-prepared ROS (cf. Figure 1D, lanes 2 and 3, where missing or depleted protein bands are denoted by arrows to the right of lane 3), and thus it is not surprising that some enzyme controls are also eluted (Robinson et al., 1980).

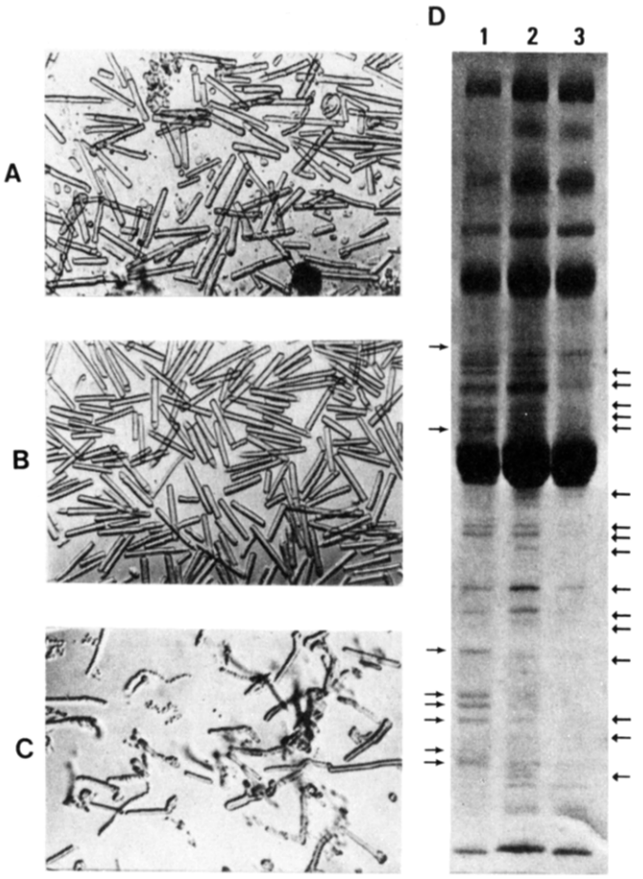

FIGURE 1: Percoll purification yields intact rod outer segments that have not lost minor components. (A) Rod outer segment preparation obtained by gentle shaking of the retina. Contaminants include varying numbers of retinal cells, bacteria, cones, and pigment epithelial cells. (B) Intact rod outer segments obtained by Percoll density gradient centrifugation. Average particulate contamination levels from light microscopic counts of 60 different preparations: cones and retinal cells present at 0.1–0.5%, and ellipsoids present at 5–10% of the number of outer segments. (C) Sucrose flotation of the Percoll-purified rods yields shrunken, broken rods which are leaky to vital dyes. (D) Gradient sodium dodecyl sulfate–acrylamide gel electrophoresis pattern of the three rod outer segment preparations shown on the left. Proteins were stained with Coomassie brilliant blue. (Lane 1) Crude rods contain contaminating protein bands (arrows at left of lane 1). (Lane 2) Percoll-purified rods contain fewer bands than crude, but more than sucrose-treated rods. (Lane 3) Sucrose-treated rods have lost rod-specific protein bands. Arrows to the right of lane 3 point to several of these.

Schnetkamp et al. (1979) have shown that bovine ROS, unlike the frog material, can survive a sucrose and Ficoll preparation procedure osmotically intact, although some protein elution may have occurred.

The Percoll-prepared intact ROS (Figure 1D, lane 2) show a reproducible number of components from one preparation to the next; the pattern shown has been taken as a working reference. The protein pattern is similar to that noted by Kuhn (1980, 1981), Godchaux and Zimmerman (1979), Molday and Molday (1979), and Papermaster and Dreyer (1974) for purified cattle ROS (Pfister et al., 1983), except that more soluble proteins are present (arrows beside lane 3; also see below and Figure 4 where the proteins are further divided into soluble and membrane-bound components). The 280-nm:500-nm absorbance ratio for preparations solubilized in 40 mM hexadecyltrimethylammonium chloride was 3.3 (±0.82 SD, n = 23). This ratio is higher than in washed ROS membranes (Papermaster & Dreyer, 1974; Godchaux & Zimmerman, 1979; Schnetkamp et al., 1979) because of the presence of more soluble proteins, and perhaps other material, such as residual Percoll, absorbing at 280 nm. The major contaminant present is the inner segment portion of the rod receptor cell, which remains attached to 5–10% of the ROS present but does not make a significant contribution to the protein pattern observed upon electrophoresis. In preparations of ROS enriched in inner segments, several protein bands correlate with the presence of the inner segment (see below and arrows in Figure 3). Cones, bacteria, pigment, erythrocytes, or other cells or fragments visible in the light microscope are present at a frequency of about 1 per 200–1000 outer segments, depending upon the preparation. Simple calculations demonstrate that these would contribute protein well below the limit detectable by the staining techniques used. Subcellular contaminants not visible in the light microscope, such as membrane vesicles, mitochondria, lysosomes, ribosomes, etc., may be present. However, two considerations make this unlikely. First, the retinal shake is gentle enough to not cause major cell disruption and release of subcellular particles. Second, the sedimentation coefficients of subcellular particles are low, and thus it is unlikely that they would enter the Percoll gradient during the 2500 rpm, 4-min centrifugation used to purify outer segments. However, it is not excluded that such particles could stick to outer segment membranes and sediment with them. See Materials and Methods for a more complete discussion of possible contaminants.

Two-Dimensional Resolution of Outer Segment Proteins Reveals a Relatively Simple Pattern. Figures 2 and 3 show more detailed resolutions of ROS proteins. To improve visualization of the different proteins, the intact ROS are first disrupted and extracted (see Materials and Methods) to separate soluble and peripheral membrane proteins from rhodopsin and other intrinsic membrane proteins. This fractionation permits the loading on gels of greater quantities of the soluble proteins and the separate electrophoresis of membrane and soluble fractions. Proteins are then separated by two-dimensional gel electrophoresis by the method of Ames and Nikaido (1976).

The two-dimensional gel shown in Figure 2 separates soluble and peripheral membrane proteins with an apparent isoelectric point (pI) in the range of 4–7 (horizontal direction, first dimension) and an apparent molecular weight range of 5000–300 000 (vertical direction, second dimension). Lane A is a one-dimensional resolution of the remaining intrinsic membrane proteins. Lane B is a one-dimensional resolution of the soluble and peripheral membrane proteins having a pI higher than 7, and thus not included in the two-dimensional gel. (No proteins were detected with a pI of less than 5.)

Several major soluble and peripheral membrane proteins are easily identified by comparison with the one-dimensional gel shown in Figure 3. The subunits of the G-protein, the α and β subunits of cyclic GMP phosphodiesterase (PDE), and the 48 000-dalton protein that binds to rod membranes in the light (Kuhn et al., 1984; Pfister et al., 1984) are indicated in Figure 2. A number of proteins are clustered in narrow molecular weight ranges—at M_r 48 000, there are at least four—and it should be noted that in the quantitation of the one-dimensional pattern (see below) these are treated as one protein. Approximately 50 polypeptides can be counted in the two-dimensional resolution, an additional 12 are associated with the outer segment membranes (lane A, see also Figure 3, membrane proteins), and 8 soluble polypeptides have an apparent pI of more than 7 (lane B). Separate experiments using standard proteins have determined that the two-dimensional technique, together with staining by Coomassie brilliant blue, can reveal protein spots that contain as little as

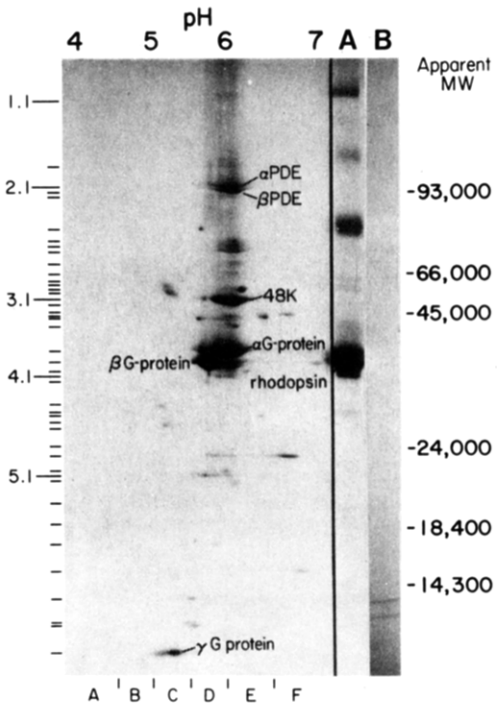

FIGURE 2: Two-dimensional separation of soluble rod outer segment proteins. Soluble and peripheral membrane proteins (200 μg) obtained by hypoosmotic washing of Percoll-purified rod outer segments are shown separated by two-dimensional gel electrophoresis. The membrane fraction (containing 50 μg of rhodopsin, lane A) and soluble proteins of apparent p*I* higher than 7 (lane B) were run in the second dimension only. Proteins were stained with Coomassie brilliant blue. The numbering and lettering system on the left and bottom is based on migration distance in the SDS–polyacrylamide gels and is similar to that employed for erythrocyte membranes (Fairbanks et al., 1971) and bovine ROS membranes (Molday & Molday, 1979). Identifiable marker proteins have been used to divide the gel into five numbered sections. Section 1 starts at the top of the gel, section 2 begins with the α subunit of the PDE, section 3 begins with a major soluble 48 000-dalton protein, section 4 begins with a 34 000-dalton marker protein just below rhodopsin, and section 5 starts with a 23 000-dalton protein. Decimal notation is used to divide these sections; the α and β subunits of PDE, for example, are designated as 2.1 and 2.2. For reference to the two-dimensional separations, a letter (A–F), corresponding to position in the pH gradient of the isoelectric focusing dimension, is placed before the number indicating migration distance in the SDS gels. Thus, the α subunit of PDE can be designated as 2.1 (in a one-dimensional separation) or E2.1 (in a two-dimensional separation).

10–20 ng of protein. This corresponds, for a 40 000-dalton protein, to a detection limit of (6–12) × 10^4 copies/outer segment (30–60 copies/disk membrane or 1:10 000 rhodopsins).

In Figure 3, soluble and peripheral membrane proteins have again been separated from integral membrane proteins by hypoosmotic extraction. The protein staining pattern in this silver-stained gel is similar to that seen with Coomassie stain, with one notable exception—the β subunit of the G-protein is not well stained. More minor integral membrane proteins can be seen than were resolved in Figure 2A; their molecular weights cluster between 30 000 and 50 000. The densitometric scans show relative staining densities in these two fractions, and the proteins that have been mentioned in the text are identified by name. Those present at more than 1 copy per 1000 rhodopsin molecules are identified by number, and others are identified only by lines. The arrows in Figure 3 indicate the position of several protein bands that are diagnostic for contamination by the mitochondria-rich inner segment that is attached to 5–10% of the outer segments in the purified and intact material that is used in obtaining the patterns shown.

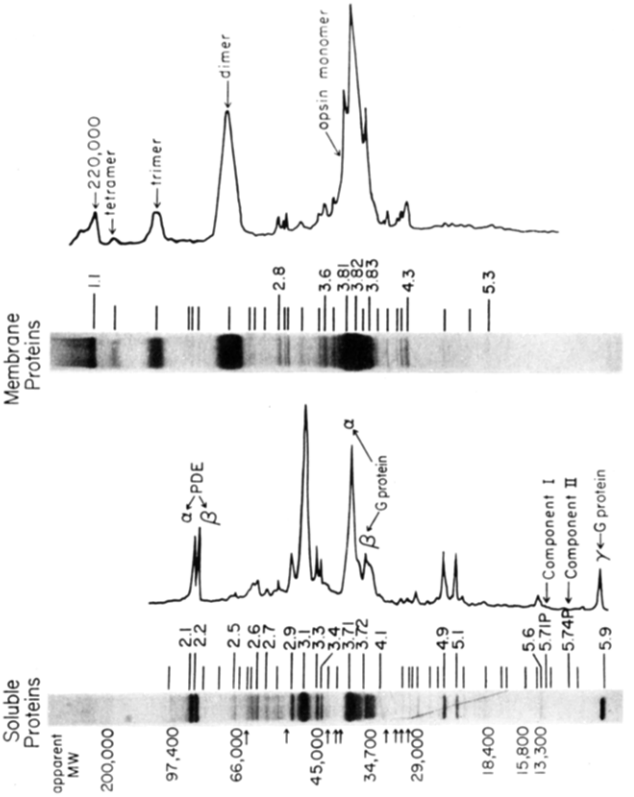

FIGURE 3: One-dimensional separation of outer segment proteins. Membrane proteins and combined soluble and peripheral membrane proteins are shown separated on gradient polyacrylamide gels. The gels were stained with silver (Giulian et al., 1983) and then scanned with a laser densitometer. From similar data, the numbers shown in Table I were calculated. Identified proteins are named; others are noted with the numbering system described in the legend of Figure 2. Proteins that are not detectable in Coomassie staining but which are phosphorylated (cf. Figure 4B) are designated by the letter P after the identifying number. Thus, components I and II, reported to undergo a light-induced dephosphorylation (Polans et al., 1979), are indicated as 5.71P and 5.74P. The arrows indicate the location of bands diagnostic for mitochondrial contamination.

In experiments in which the proportion of ROS still attached to their inner segments varied from 90% to 40%, the intensity of these bands varied with inner segment content, whereas the proteins listed in Table I were present in a constant ratio to the amount of rhodopsin present. Other potential sources of extraneous or misleading protein bands are minor by comparison (see above).

Known Activities Account for 90% of the Protein Mass. We quantitated proteins present at levels of 1 per 1000 rhodopsin molecules or more, and Table I lists the data obtained for these proteins. In a number of gels similar to the ones shown in Figure 3, two different protein stains were applied, Coomassie brilliant blue and silver. Then the staining intensity was determined by scanning densitometry, using the precautions described under Materials and Methods to ensure linear correspondence between protein amount and stain intensity. Only approximate values are obtained by the procedures used, because different known standard proteins showed a 1.5–2-fold variability in their staining intensity at nominally equal concentrations, and differing values are obtained depending upon protein stain used. Although in many cases the silver and Coomassie stain densities were in close agreement, there were some striking disagreements. Silver, for example, binds poorly to the β subunit of the G–protein (compare Figure 2 with Figure 3A). Apparent molecular weights, obtained by calibrating the gels with molecular weight markers, were used to estimate the molar quantities of each protein present. Even

Table I: Quantitation of the Major ROS Proteins[a]

protein	identification no.	app M_r	% of stain		molar ratio		copies/ROS	
			Coomassie	silver	Coomassie	silver	Coomassie	silver
rhodopsin	3.8	35000–39000	69 ± 6.7	68 ± 2.6	1000	1000	3×10^9	3×10^9
G-protein							$\sim 3 \times 10^8$	$\sim 3 \times 10^8$[c]
α	E3.71	40000	9.0 ± 1.18	11 ± 1.6	130	166		
β	E3.72	35000	7.3 ± 0.82	NS[d]	100	–		
γ	C5.9	5000	0.59 ± 0.04	1.4 ± 0.69	30	70		
48k	E3.1	48000	2.6 ± 0.08	2.6 ± 0.48	30	30	8.4×10^7	8.4×10^7
PDE							1.5×10^7	2.2×10^7[c]
α	E2.1	95000	0.87 ± 0.14	1.4 ± 0.54	5	9		
β	E2.2	94000	0.78 ± 0.14	1.4 ± 0.54	5	9		
220K	D1.1	220000	2.6 ± 0.43	11 ± 1.7	6	28	1.8×10^7	8.4×10^7
	F5.6	14000	0.43 ± 0.02	0.47 ± 0.09	15	19	4.5×10^7	5.7×10^7
	D,E5.1[b]	29000	0.57 ± 0.14	0.23 ± 0.05	11	5	3.2×10^7	1.3×10^7
	D,F4.9[b]	31000	0.71 ± 0.07	0.74 ± 0.05	12	13	3.6×10^7	3.9×10^7
	E3.4	45000	0.37 ± 0.08	–	4	–	1.2×10^7	–
	C,D,E,F3.3[b]	46000	0.77 ± 0.06	0.10	8	12	2.4×10^7	3.6×10^7
	E2.9	55000	0.77 ± 0.06	0.10	7	3	2.4×10^7	7.5×10^6
	E2.7	68000	0.14	–	1	–	3×10^6	–
	E2.6	70000	1.1 ± 0.06	0.23 ± 0.05	9	2	2.7×10^7	5.7×10^6
	E2.5	80000	0.47 ± 0.04	–	4	–	1.3×10^7	–
	5.3	20000	0.06 ± 0.007	0.15	2	4	5×10^6	1.2×10^7
	4.3	32000	0.18 ± 0.04	0.26	3	5	9×10^6	1.4×10^7
	3.6	43000	0.69	0.03	8	0.3	2.4×10^7	9.3×10^5
	2.8	56000	0.15 ± 0.01	0.08	1	1	3×10^6	1.7×10^6

[a] From left to right, the columns indicate the name of the protein (if known), the identification number assigned to the protein, the apparent molecular weight, the relative staining intensity with Coomassie and silver stains, the molar ratios with respect to rhodopsin taken as 1000, and an estimate of the number of copies of each protein per ROS. Each ROS contains approximately 3×10^9 rhodopsin molecules (Liebman & Entine, 1968). Protein masses were determined by integration of densitometer scans of stained acrylamide gels as described under Materials and Methods. Each result is the average (±SD) of several measurements obtained at four protein concentrations within the linear range of dye staining. Dashes indicate undetectable levels of stain. The relative abundance of each protein was calculated with respect to rhodopsin, whose concentration was known. Variability stems from different affinities of the proteins for each stain. [b] Represents one band in one-dimensional gels, multiple spots in two-dimensional gels. [c] See Results. [d] NS, nonstaining.

though the gel techniques used yielded a linear relationship between relative mobility and log molecular weight with protein standards spanning the 13000–92500-dalton range, some proteins (such as glycoproteins and acidic proteins) would be expected to behave anomalously (Weber & Osborne, 1973). The carbohydrates attached to rhodopsin are known to decrease its electrophoretic mobility (Baehr et al., 1982). The large protein of 220000 daltons is also known to contain carbohydrate (Papermaster et al., 1978; Molday & Molday, 1979).

Rhodopsin (G3.8) and its oligomers, identified by their light-induced phosphorylation, abundance, and similarity of apparent molecular weight with literature values, comprise approximately 70% of the total protein stain. This value is similar to that reported by Godchaux and Zimmerman (1979). Rhodopsin accounts for >95% of the Coomassie stain after hypoosmotic washing of the pellet, which removes soluble proteins along with peripheral membrane proteins such as the G-protein and PDE (see below). Rhodopsin monomer is seen at low gel loadings to consist of three bands of 35000, 36000, and 37000 daltons with an approximate stoichiometry of 1:2:1 in agreement with results by Molday and Molday (1979). Because rhodopsin is present at a known concentration on the gels, other rod outer segment proteins can be quantitated relative to it. [Porphyropsin accounts for less than 20% of the frog visual pigment (Bownds et al., 1971).]

Two proteins are present at more than 1 copy per 100 rhodopsins. A 48000-dalton protein (E3.1) present at approximately 3 copies per 100 rhodopsins was identified as the "48K" protein described by Pfister et al. (1984) by its molecular size, abundance, and light- and ATP-dependent binding to the membrane as reported by Kuhn (1980) and Kuhn et al. (1984). This protein appears to be an ATP binding protein (Zuckerman et al., 1982, 1985) and has recently been shown to be functionally, immunologically, and chromatographically identical with the S antigen responsible for autoimmune uveoretinitis (Pfister et al., 1984). G-protein (α subunit, D3.71; β subunit, D3.72; γ subunit, C5.9), identified by comigration with purified bovine G-protein on SDS gels, and purification by the method of Kuhn (1981), has approximately 10 copies of the α and β subunits per 100 rhodopsin molecules, and less of the γ subunit (3–7 copies, see estimate of error above). The G-protein stoichiometry is in agreement with several other studies on bovine G-protein [Kuhn, 1980, 1981; Kuhn et al., 1981 (6–8 G-proteins per 100 rhodopsins); Godchaux & Zimmerman, 1979 (7% of total rod outer segment protein)] and higher than the level reported for frog ROS G-protein by Shinozawa and Bitensky (1981) (1 per 400 rhodopsins). Recent studies suggest the subunit composition of the G-protein is $\alpha(\beta\gamma)$ under physiological conditions (Baehr et al., 1982; Fung et al., 1981; Fung, 1983); this would give approximately 10 G-protein complexes per 100 rhodopsins. There is diversity in the nomenclature for the guanine nucleotide binding protein of photoreceptor membranes, just as is the case for the homologous protein found in adenylate cyclase systems. The photoreceptor protein has variously been called GTPase, GTP binding protein, transducin, and G-protein. Until some name is generally agreed on for this generic class of protein [cf. Manning & Gilman (1983)], we will follow Applebury (Baehr et al., 1982) in referring to the photoreceptor protein as G-protein.

A total of 16 proteins are present at 1:100 to 1:1000 copies of rhodopsin. The function of only one of these is known. The PDE α and β subunits (E2.1, E2.2), identified by comigration with purified bovine PDE on SDS–polyacrylamide gels and by Ca^{2+}-dependent reconstitution studies (Robinson, 1981), are present at a 1:1 stoichiometry with approximately 5–10 copies of each per 1000 rhodopsins. The γ subunit (Hurley & Stryer, 1982) has not been identified. A 14000-dalton peptide (F5.6) that comigrates with purified bovine PDE γ

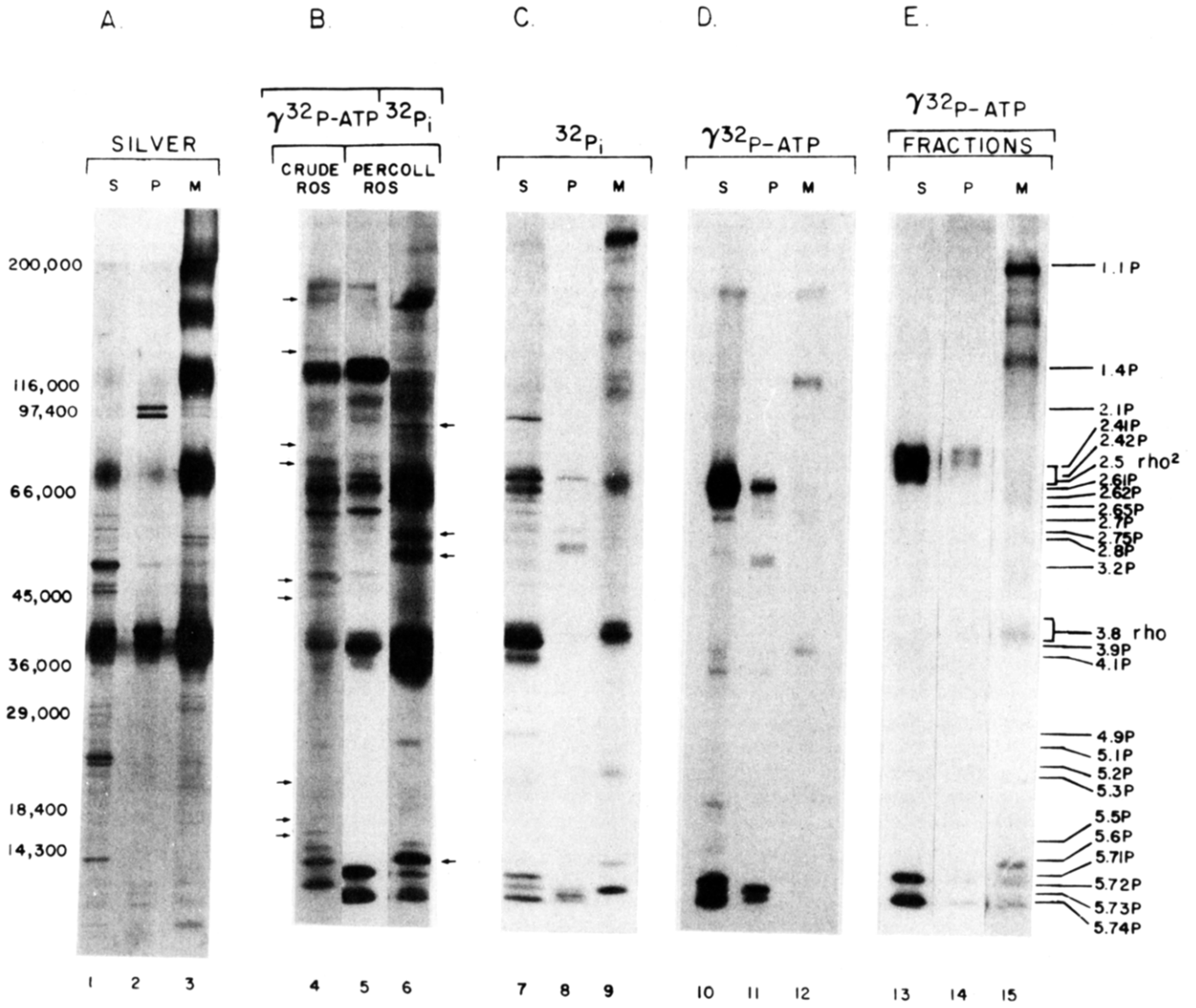

FIGURE 4: (A) Silver-stained proteins in three subcellular fractions. Soluble proteins (lane 1) are defined as those remaining in the supernatant after ROS membranes are washed several times in Ringer's solution and centrifuged. Peripheral membrane proteins (lane 2) are defined as those that are removed by several extractions with water containing 3 mM EDTA. Finally, land 3 shows the residual integral membrane proteins. Protein identification is as in Figure 2. (B) Protein phosphorylations in crude, intact, and disrupted ROS. Crude ROS freshly shaken from the retina (lane 4) or Percoll-purified ROS (lane 5) were disrupted and incubated in the dark with [γ-^{32}P]ATP. Percoll-purified intact ROS were incubated with [^{32}P]P_i (lane 6). (C) Subcellular distribution of proteins phosphorylated with [^{32}P]P_i in intact rods. Percoll-purified ROS were incubated with [^{32}P]P_i as in lane 6, and phosphorylated proteins were fractionated into soluble (lane 7), peripheral membrane (lane 8), and integral membrane fractions (lane 9) as described under Materials and Methods; then fractions were quenched and subjected to electrophoresis and autoradiography. (D) Subcellular distribution of proteins phosphorylated with [γ-^{32}P]ATP in broken rods. Percoll-purified ROS were disrupted and incubated with [γ-^{32}P]ATP as in lane 5, and phosphorylated proteins were fractionated into soluble (lane 10), peripheral membrane (lane 11), and integral membrane fractions (lane 12). (E) Partial separation of kinases and their protein substrates can be made by fractionating ROS and then adding isotope to each fraction. Proteins were first fractionated, and then reaction mixture containing [γ-^{32}P]ATP was added to each fraction. Fractions were quenched and subjected to electrophoresis and autoradiography.

subunit is present at 15–20 copies per 1000 rhodopsins, but fractionation studies show that it is a cytoplasmic component (Figure 4, lane 1). Baehr et al. (1979) have shown the subunit composition of bovine PDE to be $\alpha\beta\gamma$. If frog subunit composition and stoichiometry are similar, then PDE is present at roughly 5–10 copies per 1000 rhodopsins. Other studies give similar results: Baehr et al. (1979) estimate bovine PDE to be 1:40 to 1:140 rhodopsins, and Kuhn (1981) reports 1:120 rhodopsins. The 220 000-dalton glycoprotein (Papermaster et al., 1978; Molday & Molday, 1979) stains more intensely with silver (yielding an estimate of 28 copies per 1000 rhodopsins) than with Coomassie (6 copies per 1000 rhodopsins). The function of the other 14 polypeptides is not known. They are reproducibly present in gels at levels too high to be accounted for by contaminating tissues.

The functionally identified proteins make up almost 90% of the total ROS proteins (percent of Coomassie stain): rhodopsin, 70%; G-protein, 17%; PDE, 1.7%. Their relative molar abundance is approximately 100:10:1. G-protein and PDE, the first two enzymes of the cyclic GMP pathway, account for two-thirds of the non-rhodopsin protein. The function of the other proteins (comprising approximately 10% of the total protein) is not known. In cattle ROS, a 68 000-dalton protein has been identified as the kinase that mediates the light-induced phosphorylation of rhodopsin (Kuhn, 1978; Lee et al., 1981). Among the enzyme activities that remain to be assigned in frog ROS are the kinases mediating the several protein phosphorylations, the enzymes that oxidize and reduce retinaldehyde, and the Na/Ca exchanger and channel proteins known to be important in outer segment physiology.

Resolution of Soluble Proteins and Peripheral Membrane Proteins. In Figure 4A, conventional criteria have been used to divide ROS proteins into three classes. Most of the minor proteins are found in the silver-stained soluble fraction under these conditions, including the bands shown in Figure 1 to be preferentially depleted from ROS by sucrose purification (arrows, Figure 1D, lane 3). A major soluble protein, at about 48 000 daltons, has been shown by Kuhn (1980) to bind to the membrane in the light. A soluble 14 000-dalton peptide (labeled 5.6 in Figure 3) comigrates with the γ subunit of bovine PDE. The most prominent of the peripheral membrane proteins are the G-protein and PDE. In this gel, the γ subunit of the G-protein is not well stained by silver. The reason for this is not known, since it is well stained by silver in the gel of Figure 3. After extensive washes, the membrane fraction contains more than 95% rhodopsin, the other major membrane protein being the 220 000-dalton glycoprotein (Papermaster et al., 1978). Twelve minor bands are visible on silver-stained gels of the integral membrane fraction (see also Figure 3). Three of these are present at more than 1 copy per 1000 rhodopsins (5.3, 4.3, and 3.6; see Table I).

Protein Phosphorylation in Intact and Disrupted Outer Segments. The ROS protein phosphorylation pattern was investigated as a further characterization of Percoll-treated ROS. The right-hand panels of Figure 4 contain a listing of the ROS proteins phosphorylated under several conditions.

To further characterize the extent of purification of Percoll-purified rods, their phosphorylation pattern was compared with the phosphorylation pattern obtained from rods freshly shaken from the retina [lanes 4 and 5; see also Hermolin et al. (1982)]. Crude (lane 4) and Percoll-purified ROS (lane 5) incubated with [γ-^{32}P]ATP contain a similar set of phosphorylated proteins, with crude rods consistently containing a few contaminant phosphorylations (arrows next to lane 4 point to some of these). Several Ca^{2+}-sensitive phosphorylations noted by Hermolin et al. (1982) in crude preparations are not present in Percoll-purified ROS (data not shown). The protein phosphorylation pattern seen in lane 5 is very reproducible in over 60 such experiments and is taken as a standard set of ROS phosphorylations. It should be noted, however, that phosphorylated bands cannot unambiguously be associated with ROS, since a very minor but highly phosphorylated protein could be associated with the inner segment present on about 10% of the ROS. Thus, the same quantitative arguments used earlier about Coomassie-stained bands being of outer segment origin do not apply here. At the right of the figure, a numbering system is given similar to those in previous figures, with the letter P at the end denoting a phosphorylated band. The major bands are rhodopsin (3.8P), components I and II [5.71P and 5.74P; see Polans et al. (1979) and Hermolin et al. (1982)], and an unidentified component (1.4P) at 120 000 daltons.

In lane 6, intact ROS were incubated for 30 min with [^{32}P]P_i and allowed to incorporate this precursor into endogenous [γ-^{32}P]ATP. The ROS remain intact when this protocol is used, allowing an examination of which proteins are phosphorylated in situ, in their normal compartments and concentrations. Several proteins are phosphorylated which are never seen in broken preparations. Arrows point to the most prominent examples; 2.1P, 2.7P, 2.8P, and 5.6P. Much more phosphorylation of the rhodopsin monomer region of the gel (38 000 daltons) is seen here compared to lane 5, partly because in intact rods a cytoplasmic component (3.9P) is highly phosphorylated (see lane 7) and partly because the 5 mCi [^{32}P]P_i used in the incubation gives off a substantial amount of visible Cerenkov radiation causing some light-induced phosphorylation (compare lanes 9 and 12). Recent experiments (Brewer & Bownds, 1986) suggest that only the approximately 10% of the outer segments containing the mitochondria-rich inner segment have the ability to take up the tracer and incorporate it into nucleoside triphosphate. Thus, the phosphorylated bands marked with arrows at the right of lane 6 that were seen in intact ROS but not in disrupted rods phosphorylated by incubation with [γ-^{32}P]ATP may be of inner segment origin or may depend on the intactness or compartmentalization of the ROS.

Lanes 7–9 show the subcellular localization of the phosphorylated proteins from lane 6. Most of the phosphorylated proteins are found in the soluble fraction (lane 7), including a major phosphorylated band of approximately 38 000 daltons (3.9P) which may be similar to a protein described by Lolley (Lee et al., 1984) and components I and II (5.71P and 5.74P) which in their phosphorylated form are mainly soluble. Several minor bands are clustered around 65 000–70 000 daltons, including 2.41P, 2.42P, and 2.61P. This is the molecular weight range for rhodopsin kinase which is known to be autophosphorylated (68 000 daltons; Kuhn, 1978; Lee et al., 1982). Only a few phosphorylated bands are found in the peripheral membrane fraction (lane 8). One of these, a 12 300-dalton peptide (5.73P) is one of a group of four peptides (5.71–5.74P) whose phosphorylations are regulated by light and cGMP. This one is only phosphorylated in intact ROS (compare lane 8 with lane 5). In the integral membrane fraction (lane 9), the major phosphorylated species are rhodopsin and its dimer and the 220 000-dalton glycoprotein which can be seen as the top band in lanes 3, 6, 9, 12, and 15 (1.1P). A minor band at 5.6P is only phosphorylated in intact ROS (see also lane 6).

In lanes 10–12 are shown the soluble, peripheral, and membrane fractions of disrupted ROS that had been incubated in the presence of [γ-^{32}P]ATP (as in lane 5). The soluble fraction (lane 10) again contains most phosphorylated bands, although the relative intensities of phosphorylations are different, with components I and II (5.71P and 5.74P), 5.72P, and the cluster of proteins around 68 000 daltons more heavily phosphorylated here than in lane 7. Component I is present in the soluble fraction only, while component II and 5.72P are distributed between the soluble and peripheral membrane fractions (see also lanes 7 and 8). One membrane phosphoprotein, 1.4P, is seen only in broken ROS (see also lanes 4 and 5).

A further point made by Figure 4 is that a partial separation of kinases, cofactors, and protein substrates can be made by fractionating ROS and then adding [γ-^{32}P]ATP to each fraction. More bands are phosphorylated in lanes 10–12 than in lanes 13–15 (soluble, peripheral membrane, and integral membrane fractions). The mechanism of this regulation such as separation of cofactors, inhibitors, or kinases and their substrates into different fractions was not investigated. Components I and II (5.71P and 5.74P) are most phosphorylated in the soluble fraction; thus, it is possible that the kinase responsible for this phosphorylation is present in the soluble fraction. Another major phosphorylated band is at approximately 68 000–70 000 daltons—this can be shown to be a complex of several bands and is similar to this region of lane 10. Only two peripheral membrane proteins are phosphorylated—a lesser amount of the 68 000–70 000-dalton complex and a small amount of components I and II. In the integral membrane fraction, the major phosphorylated species is the 220 000-dalton glycoprotein. Rhodopsin (3.8P) is

phosphorylated, and a 14000-dalton protein (5.6P) that was phosphorylated only in intact rods (see arrow, lane 6) is phosphorylated under these conditions, suggesting that there may be an inhibitor in the other fractions. Lanes 5 and 10–15 show proteins phosphorylated in disrupted preparations, and thus normal compartmentation of kinases and their substrates, as well as normal concentrations, is lost. Under such conditions, it might be possible to observe new kinase substrates and specificities. In fact, the number of phosphorylated bands is decreased after ROS disruption. Comparing lanes 7–9 with lanes 10–12 and lanes 13–15 shows that in ROS few "new" substrates are uncovered after disruption and fractionation.

Discussion

The preparation of intact and purified ROS by the Percoll gradient centrifugation procedure has allowed determination of both the approximate number of proteins present in an outer segment and a more definitive specification of those that are substrates for endogenous protein phosphorylation reactions. The preparation procedure appears to be a reasonable compromise between the need for purity and the need for physiological viability. The preparation conditions used in this work have been shown to allow ROS attached to their inner segments to maintain normal dark currents and light sensitivity for several hours after their isolation from the retina (Biernbaum & Bownds, 1985a). Another paper (Biernbaum & Bownds, 1985b) deals in more detail with the light-sensitive chemistry and physiology observed in Percoll suspensions of ROS and rod outer segments still attached to inner segments.

This study suggests that a larger number of polypeptides are associated with ROS than reported previously (Kuhn, 1980, 1981; Godchaux & Zimmerman, 1979; Molday & Molday, 1979; Papermaster & Dreyer, 1974). A comparison of the protein patterns shown in Figure 1 with those studies suggests that the difference lies in the retention of the protein fraction designated as "soluble" in Figure 4. The preparation procedures used in the earlier studies would be expected to have eluted most of these proteins, leaving only peripheral and intrinsic membrane proteins. [It seems likely that the three proteins of molecular weight 37000, 41000, and 95000 reported as soluble by Godchaux and Zimmerman (1979) are peripheral membrane proteins remaining bound during sucrose purification.] Even taking into account the added soluble proteins, the protein pattern obtained from outer segments in two-dimensional separations appears relatively simple compared to those obtained from whole cells or other organelles. As an example, the ROS (which is a modified cilium) with its 70 major polypeptides would appear to have a simpler composition than highly purified somatic cilia from *Paramecium* which contain at least 250 polypeptides (Adoutte et al., 1980).

It should be emphasized that the estimate of approximately 70 polypeptides might overlook components important to the physiology of the cell but present in amounts not detectable by the staining methods used [fewer than (6–12) $\times 10^4$ copies/outer segment]. It has been suggested that the outer segment may contain as few as 100000 light-sensitive cation channels and the number of sodium/calcium exchanging sites may be small as well. The use of the more sensitive radioactive labeling procedures would have made possible the detection of more than the 70 major polypeptides observed, but distinguishing polypeptides clearly associated with ROS from those associated with contaminating retinal material would be difficult.

It proved possible to demonstrate that the 20 proteins quantitated in Table I were present in approximately constant proportion to the amount of rhodopsin (i.e., outer segment membrane) present. However, several possible sources of error are not excluded: reproducible nonspecific sticking to ROS of contaminant material not visible in the light microscope; contamination of ROS with subcellular organelles with the same sedimentation behavior; or missing small amounts of proteins actually associated with the ROS but variably eluted for unknown reasons. Thus, some of the 70 bands we associate with outer segments could be contaminant proteins (see Materials and Methods for a discussion of purity criteria). Also, some of the observed bands could be proteolytic breakdown products of other bands or oligomers as is the case with rhodopsin.

A next step in this work will be the association of the various polypeptide and phosphorylated bands with enzyme activities known or suspected to be important in ROS function using biochemical techniques or immunological cross-reactivity. At present, rhodopsin, G-protein, and PDE are the only functionally identified proteins. Several other proteins and enzyme activities have been reported to be present in ROS (although the purity of some of the preparations used might be questioned). These include cyclic nucleotide related enzymes [guanylate cyclase (Fleishman & Denisovich, 1979; Berger et al., 1981), 5′-nucleotidases, 75000 and 67000 daltons (Fukui & Schichi, 1981, 1982), cyclic nucleotide dependent protein kinase, 165000 daltons (Lee et al., 1981), rhodopsin kinase, 67000–68000 daltons (Kuhn, 1978; Lee et al., 1981) or 50000–53000 daltons (Shichi & Somers, 1978)], metabolic enzymes [transphosphorylases (Berger et al., 1980; Schnetkamp & Daemen, 1981)], proteins involved in the visual cycle [retinol binding proteins (Heller, 1976), retinol dehydrogenase (Zimmerman et al., 1975)], and other enzymes [superoxide dismutase (Hall & Hall, 1975), Ca^{2+}-ATPase (Puckett et al., 1985), Mg^{2+}-ATPase (Uhl et al., 1978)]. There are several phosphorylated species, an acylphosphorylated polypeptide of 160000 daltons (Thatcher, 1981) and components I (13000 daltons) and II (12000 daltons), which are light, Ca^{2+}, and cGMP sensitive (Polans et al., 1979).

Other proteins are thought to be present on more functional grounds: the light-regulated conductance mechanism of the plasma membrane (Hodgkin et al., 1984), a Na^+–Ca^{2+} exchange mechanism (Hodgkin et al., 1984), and a Ca^{2+} extrusion mechanism (Yoshikami et al., 1981; Gold & Korenbrot, 1980). Components mediating Ca^{2+} sensitivity of biochemical reactions are suspected because of Ca^{2+} effects on several light-stimulated reactions including PDE activity (Robinson et al., 1980), rhodopsin phosphorylation (Hermolin et al., 1982), and guanine nucleotide binding to the G-protein (Robinson, 1981). Various structural proteins are probably present including tubulin and microtubule-associated proteins (Fleishman & Denisovich, 1979), myosin (Hesketh et al., 1978), and other as yet unidentified structural proteins (Roof & Heuser, 1982).

In summary, the identity of ROS proteins accounting for 90% of the total protein is known, and information on the relative abundance of these proteins offers the opportunity of constructing more detailed models of their interactions. The ability to clearly separate and identify the proteins has aided studies in this laboratory characterizing monoclonal antibodies that appear to block light-activated pathways (Hamm & Bownds, 1984; Witt et al., 1984).

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Registry No. cGMP phosphodiesterase, 9068-52-4.

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Monoclonal Antibodies to the Porcine Intestinal Receptor for 1,25-Dihydroxyvitamin D_3: Interaction with Distinct Receptor Domains†

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ABSTRACT: Monoclonal antibodies to different domains of the porcine intestinal 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] receptor have been produced. A nuclear extract enriched in the 1,25-$(OH)_2D_3$ receptor was prepared from small intenstinal mucosa of young pigs. The receptor was purified an additional 6600-fold by chromatography on DNA–cellulose, ammonium sulfate precipitation, gel filtration high-performance liquid chromatography, and DEAE-Sepharose chromatography, with an overall yield of 23% and an average purity of 24%. A BALB/c mouse immunized with this material developed serum polyclonal antibodies to the 1,25-$(OH)_2D_3$ receptor, as demonstrated by a change in sedimentation of the porcine receptor on sucrose gradients. Spleen cells from this animal were fused with mouse myeloma cells (P3-NSI/1-Ag4-1, SP2/0-Ag14), and 24 hybridomas secreting antibodies to the 1,25-$(OH)_2D_3$ receptor were identified by both a radiometric immunosorbent assay and an immunoprecipitation assay. Twenty-one hybridoma lines were cloned by limiting dilution and further characterized as subclass IgG_1 antibodies with the exception of one which is an IgA. All but two of the antibodies cross-react with the 1,25-$(OH)_2D_3$ receptor from both mammalian (human, monkey, and rat) and avian (chicken) intestine; two antibodies recognize only porcine intestinal receptor. All antibodies are unreactive to the vitamin D serum transport protein. Eight of the antibodies bind denatured receptor on an immunoblot. A solid-phase competition assay was used to identify four groups of antibodies that bind to distinct epitopes on the 1,25-$(OH)_2D_3$ receptor. One antibody from each of the four groups was used to examine the effect of antibody binding on the DNA-binding activity of the receptor–hormone complex. One antibody completely inhibited the binding of the 1,25-$(OH)_2D_3$ receptor complex to DNA–cellulose, suggesting that the epitope for this antibody may be located in the polynucleotide binding domain of the protein. Antibodies from two additional groups only slightly perturbed DNA binding, while one had no effect, suggesting that these antibodies bind to receptor epitopes distant from the region of the polypeptide directly involved in polynucleotide binding. These antibodies that are directed to several different binding sites on the 1,25-$(OH)_2D_3$ receptor provide important new tools to probe the biochemistry and topology of the 1,25-$(OH)_2D_3$ receptor and to investigate its role in mediating target tissue response to hormone.

The 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] receptor plays a central role in target tissue responsiveness to hormone (Franceschi et al., 1981; Link & DeLuca, 1985). In mammals, 1,25-$(OH)_2D_3$ stimulates a complex biphasic response of intestinal calcium transport in the small intestine (Halloran & DeLuca, 1981); in concert with parathyroid hormone, it functions in the mobilization of calcium from bone and is believed to act on the distal renal tubule to facilitate reabsorption of calcium (DeLuca, 1983; DeLuca & Schnoes, 1983). Tissue responsiveness is mediated by 1,25-$(OH)_2D_3$ binding to a intracellular receptor protein (Brumbaugh & Haussler, 1973; Kream et al., 1976). The hormone–receptor complex is believed to stimulate the transcription of proteins involved in calcium and phosphorus transport and homeostasis. The ultimate function of 1,25-$(OH)_2D_3$ is the maintenance of blood calcium and phosphate levels to support normal bone growth and mineralization. The receptor may also mediate less well-elucidated functions of the hormone in other tissues and cells in which the protein has been described (Stumpf et al., 1979, 1980, 1981; Narbaitz et al., 1983; Haussler et al., 1984; Eisman, 1984; Suda et al., 1984).

The 1,25-$(OH)_2D_3$ receptor has been studied extensively in crude extracts from a variety of tissues and species by using radiolabeled hormone–receptor binding techniques (Link & DeLuca, 1985). The mammalian receptor sediments at 3.1–3.2 S on high-salt sucrose gradients (Kream et al., 1977)

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