Articles

Expression of Cytochrome P-450 and Albumin Genes in Rat Liver: Effect of Xenobiotics†

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ABSTRACT: Thioacetamide, a hepatocarcinogen and an inhibitor of heme synthesis, blocks the phenobarbitone-mediated increase in the transcription of cytochrome P-450b+e messenger RNA in rat liver. This property is also shared by $CoCl_2$ and 3-amino-1,2,4-triazole, two other inhibitors of heme synthesis. Thus, it appears feasible that heme may serve as a positive regulator of cytochrome P-450b+e gene transcription. Thioacetamide enhances albumin messenger RNA concentration, whereas phenobarbitone decreases the same. However, these changes in albumin messenger RNA concentration are not accompanied by corresponding changes in the transcription rates. Therefore, drug-mediated changes in albumin messenger RNA concentration are due to posttranscriptional regulation. The property of thioacetamide to enhance the albumin messenger RNA concentration is not shared by $CoCl_2$ and 3-amino-1,2,4-triazole. Therefore, heme does not appear to be a regulatory molecule mediating the reciprocal changes brought about in the concentrations of cytochrome P-450b+e and albumin messenger RNAs.

Cytochrome P-450 and albumin are two major gene systems of the liver. There have been reports indicating that xenobiotics may precipitate a reciprocal response in the expression of these two gene systems. For example, it has been reported that phenobarbitone, which enhances cytochrome P-450b+e messenger RNAs in rat liver (Bhat & Padmanaban, 1979; Adesnik et al., 1981; Mizukami et al., 1983), decreases the levels of albumin messenger RNA (Hardwick et al., 1983). However, there have been earlier reports indicating that phenobarbitone administration has no effect on the levels of albumin messenger RNA (Gonzalez & Kasper, 1980; Philips et al., 1981). 3-Methylcholanthrene, which specifically increases cytochrome P-450c+d messenger RNAs (Bresnick et al., 1981; Morville et al., 1983) has been reported to decrease albumin messenger RNA (Pickett et al., 1982). On the other hand, thioacetamide, a slow-acting hepatocarcinogen, has been reported to increase the levels of albumin messenger RNA (Chakrabartty & Schneider, 1978), and we have shown that this chemical decreases the levels of translatable cytochrome P-450b+e messenger RNAs (Satyabhama & Padmanaban, 1984).

In this study, with the use of phenobarbitone and thioacetamide to modulate the cytochrome P-450 and albumin messenger RNA levels and cloned cDNA probes to quantify specific messenger RNA contents and run-off transcription rates, we have examined whether there is a reciprocal relationship in the expression of these two genes in the rat liver. Earlier, we have indicated that heme, the prosthetic group of cytochrome P-450, may also be a positive regulator of the transcription of this gene, in addition to the inducer (Ravishankar & Padmanaban, 1985). It is known that phenobarbitone enhances heme synthesis (Baron & Tephly, 1969), and we have shown that thioacetamide inhibits heme synthesis (Satyabhama & Padmanaban, 1984). Therefore, we have also addressed the question whether heme is a common denominator in influencing the reciprocal relationship, if any, between the expression of cytochrome P-450 and albumin genes.

Experimental Procedures

Treatment of Animals. Male albino rats of the Institute strain (90–100 g) received single or multiple injections of phenobarbitone (8 mg/100 g, ip), thioacetamide (5 mg/100 g, ip), cobalt chloride (6 mg/100 g, ip), and 3-amino-1,2,4-triazole (300 mg/100 g, ip), wherever indicated. The livers were isolated and processed for a variety of purposes.

Construction of cDNA Clones. A cDNA library for polyribosomal, poly(A)-containing RNA isolated from phenobarbitone-treated rat livers was constructed with *Pst*-cut pBR 322 and GC tailing procedures. The library was screened with single-stranded [^{32}P]cDNA prepared against enriched rat albumin messenger RNA. Albumin messenger RNA was purified by immunobinding polyribosomes isolated by the magnesium-precipitation procedure (Palmiter, 1974) to albumin antibodies and isolating enriched polyribosomes on protein A–Sepharose column (Kraus & Rosenberg, 1982). Poly(A)-containing RNA isolated from such enriched polyribosomes was found to be at least 50-fold enriched in albumin messenger RNA translatable activity compared to total polyribosomal poly(A)-containing RNA (Figure 1). The colonies that lighted up in the Grunstein–Hogness colony hybridization were further characterized by hybrid-selected messenger RNA translation, restriction mapping, and partial DNA sequencing by the chemical method. One of the clones had an insert of 1200 bp, and the restriction map and partial sequence data (Figure 2) indicated it to be an albumin clone covering nearly two-thirds of the albumin mRNA from the 3′-end. This clone, designated as pRSA-6, was used in this study. Standard methods were used for clone construction and characterization (Maniatis et al., 1982). A cytochrome P-450 cDNA clone designated as pP-450e-91 constructed in this

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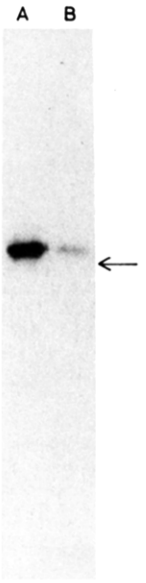

FIGURE 1: Cell-free translation of total poly(A)-containing RNA and enriched albumin messenger RNA. Total poly(A)-containing RNA (2 μg) and enriched messenger RNA (300 ng) were translated in the reticulocyte cell-free system in a total volume of 25 μL in the presence of 50 μCi of [^{35}S]methionine. The cell-free products were immunobound to albumin antibody, and the complex was isolated with *S. aureus* cells. The complex was dissociated with 2% (w/v) SDS and analyzed by SDS–PAGE (8% gels) and fluorography. (A) Product from enriched messenger RNA; (B) product from total poly(A)-containing RNA. Arrow indicates the position of migration of mature rat serum albumin.

laboratory (Ravishankar & Padmanaban, 1985) was used to quantify the phenobarbitone-inducible cytochrome P-450b+e messenger RNAs.

Quantification of Albumin and Cytochrome P-450b+e Messenger RNAs. Specific messenger RNAs were quantified by dot blot and northern blot hybridization procedures (Thomas, 1980; Gal et al., 1983) with nick-translated pRSA-6 and pP-450e-91 (5×10^7 cpm/μg of DNA). The translatable activities of these messenger RNAs were assessed in the reticulocyte cell-free system (Pelham & Jackson, 1976). Poly(A)-containing RNA isolated from guanidine hydrochloride extracted total RNA (Deeley et al., 1977) and total RNA isolated from nuclei (Guertin et al., 1983) were used in messenger RNA quantification experiments.

Table I: Runoff Transcription Rates for Albumin and Cytochrome P-450b+e Messenger RNAs with Nuclei Isolated from Drug-Treated Rats[a]

	messenger RNA hybridized (ppm)	
treatment	albumin mRNA	cytochrome P-450b+e mRNA
control	102 ± 10	7 ± 2
phenobarbitone	99 ● 8	125 ± 7
thioacetamide	110 ± 7	4 ± 2
phenobarbitone + thioacetamide	96 ± 4	21 ± 6

[a] The animals received one, two, or four injections of phenobarbitone and thioacetamide. Nuclei were isolated 6 h after a single injection of the drugs for measuring cytochrome P-450b+e messenger RNA transcription. Nuclei were isolated 24 h after two or four injections of the drugs for measuring albumin messenger RNA transcription. The results presented are those obtained after two injections for albumin mRNA transcription. The data obtained after four injections of the drugs are very similar except that the nuclei isolated under these conditions were very fragile. The RNA transcripts synthesized in vitro (10^7 cpm) were hybridized to pP-450e-91 or pRSA6 DNA loaded onto nitrocellulose filters. The results are expressed in terms of parts per million of RNA hybridized and represent the mean ± SD obtained from three experiments.

Cell-Free Nuclear Transcription. This was carried out by the method of Guertin et al. (1983) and has been described in a recent publication from this laboratory (Ravishankar & Padmanaban, 1985). Briefly, nuclei were isolated from drug-treated animals, and in vitro transcription was carried out in the presence of [α-^{32}P]UTP and other components. The labeled transcripts (1×10^7 cpm) were hybridized to pRSA-6 and pP-450e-91 DNA loaded on nitrocellulose filters. The radioactivity in the hybrid was quantified.

Other Procedures. RNA was isolated from the different fractions by the guanidine hydrochloride extraction procedure (Deeley et al., 1977). Commercial rat serum albumin (Sigma) was used as the antigen to elicit antibodies in rabbits. The cell-free translation products were immunobound to the antibodies, the antigen–antibody complex was adsorbed onto *Staphylococcus aureus* cells, and the complex was dissociated

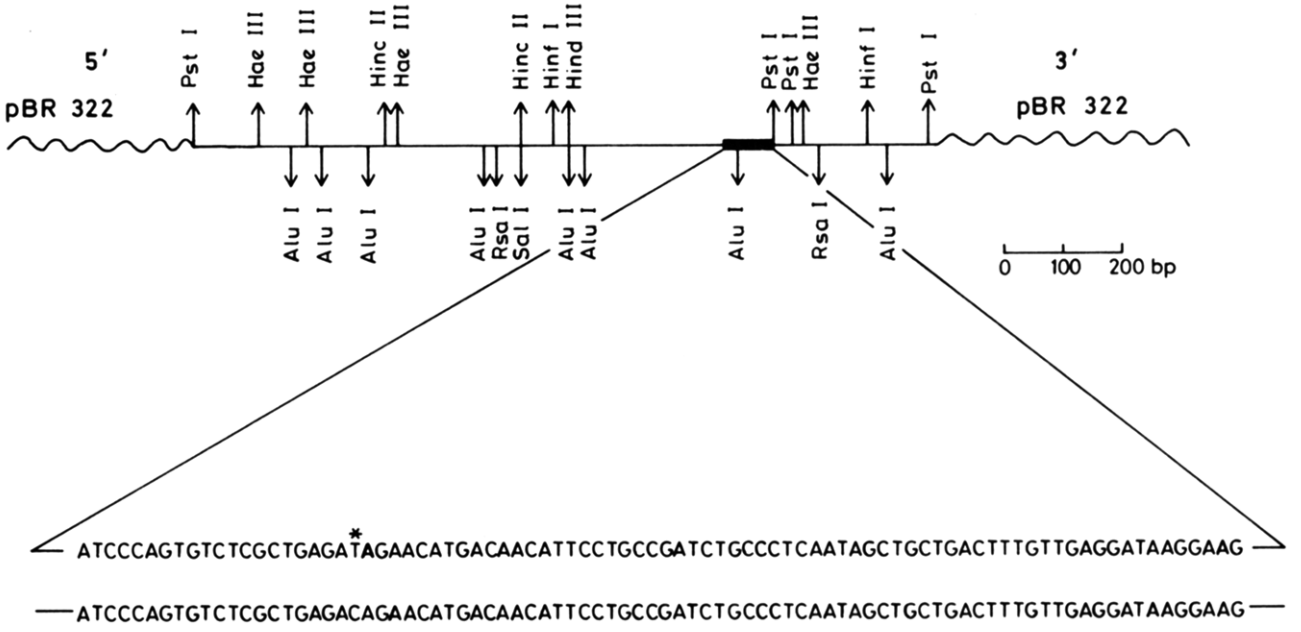

FIGURE 2: Restriction map and partial sequence of clone pRSA-6. The thick line represents the portion sequenced. The sequence obtained compares well with the reported sequence, given below the sequence for pRSA-6, except for one base change indicated by an asterisk.

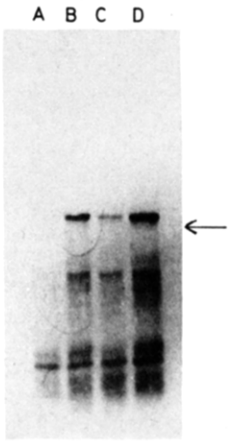

FIGURE 3: Effect of thioacetamide and phenobarbitone administration on the translatable activity of total cellular poly(A)-containing RNA for albumin. Total liver poly(A)-containing RNA was isolated from animals given four injections of the drugs. The RNAs were translated in the reticulocyte cell-free system and then analyzed by SDS–PAGE and fluorography. The other experimental details are as given in Figure 1. (Lane A) Phenobarbitone treated; (lane B) control; (lane C) phenobarbitone + thioacetamide treated; (lane D) thioacetamide treated.

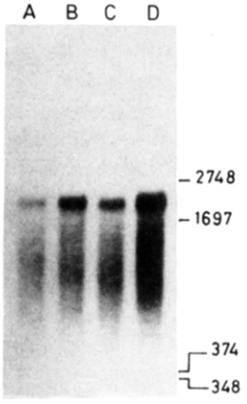

FIGURE 4: Northern blot analysis for total cellular poly(A)-containing RNA for albumin messenger RNA after thioacetamide and phenobarbitone treatments. Liver poly(A)-containing RNA (5 μg), isolated from the animals given the different treatments, was fractionated on 1.5% agarose–formaldehyde gels, blotted onto nitrocellulose, and then hybridized to nick-translated pRSA-6 insert. (Lane A) Phenobarbitone treated; (lane B) control; (lane C) phenobarbitone + thioacetamide treated; (lane D) thioacetamide treated. The size markers indicated were from a *Hpa*II digest of ϕ×174 DNA.

and analyzed by SDS–PAGE[1] (8% gels) and autoradiography (Maccecchini et al., 1979). Quantitation of radioactivity in gels was carried out by digesting the gel piece in H_2O_2 and then measuring radioactivity. Quantitation of radioactivity in the different blots was carried out by punching out the radioactive spot from nitrocellulose as revealed in the autoradiogram and then measuring its radioactivity.

RESULTS

First of all, we examined the effects of phenobarbitone and thioacetamide treatments on the levels of albumin messenger RNA in liver. Significant effects on albumin messenger RNA levels, especially of thioacetamide, were observed after multiple injections, and therefore, the animals received four injections of the drug, each injection being given at an interval of 24 h. The cell-free translation data presented in Figure 3 clearly reveal that thioacetamide administration resulted in a significant increase of translatable albumin messenger RNA levels, whereas phenobarbitone administration decreased it. A northern blot analysis using nick-translated pRSA-6 DNA indicates that the albumin messenger content, assessed in terms of hybridization, followed the same pattern as that of the translatable activity (Figure 4). The northern blot analysis also reveals that there was no gross change in the size of albumin messenger RNA (2100 ± 100 nucleotides) under the treatment conditions.

[1] Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

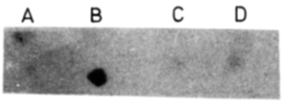

FIGURE 5: Dot blot analysis of cytochrome P-450b+e messenger RNA from phenobarbitone and thioacetamide treated rat livers. The animals were killed 6 h after receiving a single injection of the drugs, and poly(A)-containing RNA was isolated from the microsomes. Poly-(A)-containing RNA (0.4 μg) was loaded on nitrocellulose filters and analyzed by hybridization to nick-translated pP-450e-91 insert. (A) Control (40); (B) phenobarbitone treated (329); (C) thioacetamide treated (35); (D) phenobarbitone + thioacetamide treated (96). Values presented in parentheses are the actual radioactivity (cpm) measured from the blots.

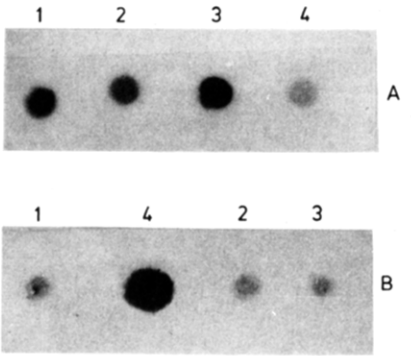

FIGURE 6: Dot blot analysis of nuclear RNA for albumin and cytochrome P450b+e premessenger RNA from phenobarbitone and thioacetamide treated rats. The animals were treated and nuclear RNAs isolated from the livers as described in the text. Nuclear RNA (10 μg) was loaded on nitrocellulose filters and hybridized to nick-translated pRSA-6 or pP-450e-91 DNA inserts. (A) albumin probe; (B) cytochrome P-450b+e probe. (1) Control (1038 and 57); (2) phenobarbitone + thioacetamide (873 and 87); (3) thioacetamide treated (1376 and 41); (4) phenobarbitone treated (221 and 2156). The values presented in parentheses are the actual radioactivity (cpm) measured for albumin and cytochrome P-450 messenger RNAs, respectively, from the blots.

The effect of drug treatments on cytochrome P-450 messenger RNA levels in liver was then examined. Since it is established that a single injection of phenobarbitone elicits a striking increase in the levels of cytochrome P-450b+e messenger RNA (Bhat & Padmanaban, 1979; Adesnik et al., 1981; Mizukami et al., 1983), the effect of thioacetamide administration on this induction process was examined. Dot blot hybridization data with nick-translated pP-450e-91 DNA clearly reveal (Figure 5) that phenobarbitone administration resulted in a striking increase in the levels of cytochrome P-450b+e messenger RNA and that the simultaneous administration of thioacetamide blocked this induction. These results are in agreement with the messenger RNA translation data provided earlier (Satyabhama & Padmanaban, 1984).

The effects of drug treatment on nuclear premessenger RNA levels for albumin and cytochrome P-450b+e were assessed by dot blot hybridization, and the data are provided in Figure 6. Quantification of the radioactivity reveals that phenobarbitone decreased and thioacetamide increased the premessenger RNA levels for albumin. Phenobarbitone administration led to a striking increase in cytochrome P-450b+e

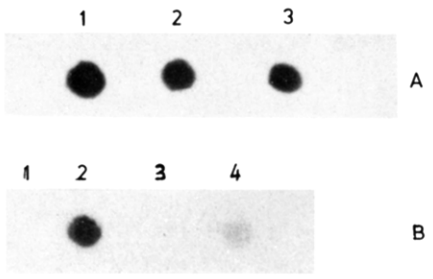

FIGURE 7: Effect of treatments with inhibitors of heme synthesis on albumin and cytochrome P-450 messenger RNA levels. The animals were treated as described in the text except that for albumin messenger RNA quantification the animals received only three injections of $CoCl_2$ or 3-amino-1,2,4-triazole, since the mortality rate was very high with the fourth injection. For cytochrome P-450b+e messenger RNA quantification, the animals received a single injection of the chemicals. Poly(A)-containing RNA was isolated from microsomes and 0.4 μg of RNA used for dot blot analysis with nick-translated pRSA-6 or pP-450e-91. (A) Albumin probe. (1) Control (1231); (2) $CoCl_2$ treated (723); (3) 3-amino-1,2,4-triazole treated (755). (B) Cytochrome P-450b+e probe. (1) Control (23); (2) phenobarbitone treated (497); (3) phenobarbitone + $CoCl_2$ treated (25); (4) phenobarbitone + 3-amino-1,2,4-triazole treated (76). The values presented in parentheses are the actual radioactivity (cpm) measured from the blots.

premessenger RNA levels, and the simultaneous administration of thioacetamide prevented this increase.

The effect of drug treatments on runoff transcription rates with nuclei isolated from the treated animals was then examined. The results presented in Table I reveal that none of the drug treatments resulted in any significant change of the albumin messenger RNA transcription rate. Phenobarbitone administration elicited a striking increase in the transcription rates for cytochrome P-450b+e messenger RNA, and the simultaneous administration of thioacetamide largely prevented this increase.

We have earlier shown that thioacetamide is an inhibitor of heme synthesis (Satyabhama & Padmanaban, 1984), and it was of interest to examine whether other inhibitors of heme synthesis such as $CoCl_2$ and 3-amino-1,2,4-triazole would elicit a response similar to that of thioacetamide. The dot blot hybridization experiments with total poly(A)-containing RNA reveal that multiple injections of $CoCl_2$ or 3-amino-1,2,4-triazole unlike thioacetamide failed to enhance the albumin messenger RNA levels. In fact, there was actually a decrease in albumin messenger RNA levels. However, all the inhibitors of heme synthesis blocked the phenobarbitone-mediated increase in cytochrome P-450b+e messenger RNA levels (Figure 7).

Discussion

In this study, it has been established that thioacetamide blocks the enhanced transcription of cytochrome P-450b+e messenger RNA brought about by phenobarbitone in rat liver. Thus, $CoCl_2$, 3-amino-1,2,4-triazole, and thioacetamide, which are inhibitors of heme biosynthesis, have been found to block cytochrome P-450b+e messenger RNA transcription. It can, therefore, be suggested that in addition to the drug an adequate supply of heme is required for optimal transcription of the cytochrome P-450b+e genes. The mechanism of this interaction needs further study.

Thioacetamide treatment is known to increase the translatable albumin messenger RNA levels (Chakrabartty & Schneider, 1978). This has been confirmed in this study with cell-free translation as well as northern blot analysis using a cloned probe. In addition, we have also demonstrated a clear-cut decrease in albumin messenger RNA levels after phenobarbitone treatment. The picture obtained with polyribosomal RNA is also reflected in the analysis of specific nuclear premessenger RNAs by dot blot analysis. However, the present studies show that the changes in albumin messenger RNA levels are not due to concomitant changes in the rates of transcription. Therefore, it is suggested that the albumin messenger RNA levels are modulated at a posttranscriptional level. This could involve processing, transport across the nuclear membrane, and stabilization of albumin messenger RNA. Thus, it is clear that although the drugs used bring about reciprocal changes in the concentrations of cytochrome P-450 and albumin messenger RNAs, they do so by acting at two different levels.

The present studies also reveal that while all the three inhibitors of heme biosynthesis tested block cytochrome P-450b+e messenger RNA induction, only thioacetamide enhances albumin messenger RNA levels. $CoCl_2$ and 3-amino-1,2,4-triazole actually decrease albumin messenger RNA levels. Thus, heme is, perhaps, not involved as a regulatory molecule in the reciprocal changes seen with the two messenger RNA populations under conditions of drug treatments.

Registry No. Co, 7440-48-4; thioacetamide, 62-55-5; cytochrome P-450b+e, 9035-51-2; 3-amino-1,2,4-triazole, 61-82-5; heme, 14875-96-8.

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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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