A SPECTRIN-LIKE PROTEIN IN BOVINE RETINAL ROD PHOTORECEPTOR OUTER SEGMENTS AS DEFINED BY MONOCLONAL ANTIBODIES

By

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We accept this thesis as conforming to the required standard

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Biochemical and immunological studies indicate that rod outer segments (ROS) of bovine photoreceptor cells contain a Mr 240,000 polypeptide related to the α-subunit of red blood cell (RBC) spectrin. With the use of sodium dodecyl sulfate gel electrophoresis in conjunction with the immunoblotting technique, monoclonal antibody 4B2 was found to bind to a Mr 240,000 polypeptide in ROS that is distinct from the prominent Mr 220,000 concanavalin A binding glycoprotein. The Mr 240,000 polypeptide is highly susceptible to degradation by endogenous proteases. It does not appear to be an integral membrane protein but is tightly membrane associated since it can be partially extracted from ROS membranes with urea in the absence of detergent.

The 4B2 antibody cross-reacted with RBC ghost membranes and bovine brain microsomal membranes. Radioimmune assays and immunoblotting analysis of purified bovine RBC spectrin further revealed that the 4B2 antibody predominantly labelled the α-chain of RBC spectrin having an apparent Mr of 240,000. Monoclonal antibody 3A6 was found to bind to a polypeptide with a slightly lower Mr than the 4B2-specific polypeptide. It is also highly susceptible to degradation by endogenous proteases, but unlike the 4B2 antibody, it predominantly labelled the β-chain of RBC spectrin having an apparent Mr of 220,000. Polyclonal anti-spectrin
antibodies that bound to both the $\alpha$- and $\beta$-chain of RBC spectrin predominantly labelled a $M_r$ 240,000 polypeptide of ROS membranes. Two faintly labelled bands in the $M_r$ range of 210,000-220,000 were also observed. These components may represent variants of the $\beta$-chain of spectrin that are weakly cross-reacting or present in smaller quantities than the $\alpha$-chain.

Immunocytochemical labelling studies using the 4B2 antibody and immunogold-dextran markers indicated that the ROS spectrin-like protein is preferentially localized in the region where the discs come in close contact to the plasma membrane of ROS. Immunoblotting analysis indicated that rhodopsin and peripherin which constitute over 90% of total disc membrane proteins were selectively solubilized in Triton X-100, whereas a set of polypeptides including the 4B2-specific polypeptide and the $M_r$ 220,000 concanavalin A-binding glycoprotein was only partially soluble. Electron microscopy of a negatively stained Triton-extracted ROS pellet revealed a filamentous network.

These studies indicate that ROS contain a protein related to RBC spectrin, which may constitute a major component of a filamentous network lining the inner surface of the ROS plasma membrane as previously seen by electron microscopy. This membrane skeletal system may serve to stabilize the ordered ROS structure and maintain a constant distance between the rim region of the discs and the plasma membrane.
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<td>ATP</td>
<td>adenosine 5' triphosphate</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>Con A</td>
<td>concanavalin A</td>
</tr>
<tr>
<td>DEAE</td>
<td>diethylaminoethyl</td>
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<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
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<tr>
<td>EGTA</td>
<td>ethyleneglycol bis(β-amino ethylethyl)-N,N,N',N'-tetraacetic acid</td>
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<tr>
<td>FCS</td>
<td>fetal calf serum</td>
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<tr>
<td>GMP</td>
<td>guanosine monophosphate</td>
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<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
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<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
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<tr>
<td>HAT</td>
<td>hypoxanthine aminopterin thymidine</td>
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<tr>
<td>Ig</td>
<td>immunoglobulin</td>
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<tr>
<td>kDa</td>
<td>kilodalton</td>
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<tr>
<td>Mr</td>
<td>molecular weight</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PDE</td>
<td>phosphodiesterase</td>
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<tr>
<td>RBC</td>
<td>red blood cell</td>
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<td>RIA</td>
<td>radioimmune assay</td>
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<td>ROS</td>
<td>rod outer segment</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)amino-methane</td>
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<tr>
<td>WGA</td>
<td>wheat germ agglutinin</td>
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Many people have helped me throughout my graduate studies, but I will only name those people whose contributions I could not do without. Special thanks to Dr. Robert Molday, my supervisor of many years, for his expert guidance and constant encouragement. His supervision was excellent, and his choice of restaurant for a quiet dinner was "belly" good. Many thanks to my close colleagues, Dale Laird and Laurie Molday, for their superb technical assistance, much appreciated cooperations, and many valuable discussions. I would like to thank Dr. Dave Hicks who carried out the electron microscopic studies on the localization of the 4B2-specific protein in the ROS, and Dr. Delyth Reid for her assistance in preparing this thesis. I would like to thank Drs. James Richards and Pieter Cullis for being in my supervisory committee, and Dr. Everard Trip for giving me the opportunity to teach the undergraduate laboratory course. Also, I would like to thank my many fellow students for their good nature and companionships. Finally, I would like to dedicate this thesis to my family for their love and support.
INTRODUCTION

A. Vertebrate Retina.

The vertebrate retina is a thin layer of neural tissue lining the back of the eye chamber (Figure 1). It consists of neurons and glia. There are six types of neural cells: rod photoreceptors, cone photoreceptors, bipolar cells, horizontal cells, amacrine cells, and ganglion cells. The glial cells are also called Müller cells. These retinal elements are organized into distinct layers of cell bodies and cell processes (Figure 2) to perform the two functions of the retina. The first function, performed by the photoreceptors, is to transform light energy into nerve impulses. The second function, performed by the elaborate array of higher-order neurons, is to integrate the large number of photoreceptor signals and transmit the visual information about form, movement, and color to the brain via the optic nerve (reviewed in Shichi, 1983; Farber and Adler, 1986).

Given the key role of the photoreceptor cells in vision, it is not surprising that they have been the subject of considerable investigation in vision research.

B. Photoreceptor Cells and Visual Pigments.

The photoreceptor cells are closely packed in the retina
Figure 1. Gross Anatomy of the Vertebrate Eye. The major components of the vertebrate eye are shown in this diagram. The eye chamber is enclosed in three layers: the sclera, the choroid, and the retina. The fovea is the center of the visual field and the optic disc also known as the blindspot is where the retinal nerve fibers converge to form the optic nerve (modified after Walls, 1942).
Figure 2. Interrelationships of Retinal Neurons and Glia. The retinal layers and their synaptic relationships are illustrated in this diagram of the adult vertebrate retina. The retinal neurons are amacrine cells (A), bipolar cells (B), ganglion cells (G), horizontal cells (H), and rod (R) and cone (C) photoreceptor cells. Only the predominant glial cells, the Müller cells (M), are indicated. These retinal cells are organized into distinct layers of cell bodies and cell processes: outer segment layer (OSL), inner segment layer (ISL), outer limiting membrane (OLM), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (INL), ganglion cell layer (GCL), nerve fiber layer (NFL), and inner limiting membrane (ILM). The location of the retinal pigment epithelium (RPE) and the choroid are also indicated (modified after Dowling, 1970).
and axially oriented to the incident light. Light must pass through almost the entire thickness of the retina before impinging upon the photoreceptor cells. Light that passes through the photoreceptors is absorbed by the melanin granules of the posterior retinal pigment epithelium. In some animals, such as the cat, whose eyes are specialized for low light intensities, part of the pigment epithelium is transparent like the rest of the retina. This enables the light to reach the deeper-lying tapetum and to be reflected back through the photoreceptors giving them a second chance to absorb the light (reviewed in Rodieck, 1973).

There are two types of photoreceptor cells in the eyes of most vertebrates. They were originally called rods and cones because of their shapes. However, it is now evident that few, if any, of their morphological features are unique to either type of cell. Thus, functional properties such as photosensitivity and spatial and temporal resolution are also required to properly classify photoreceptors as either rods or cones (Fein and Szuts, 1982). The number of rods and cones and their distribution in a retina varies from species to species. In humans there are about 6.5 million cones and about 120 million rods distributed unequally throughout the retina (Pirenne, 1967). The highest concentration of cones is found in the center of the visual field called the fovea, whereas the frequency of rods increases towards the periphery of the retina. No rods are found in the fovea, the region of most distinct vision. Neither rods nor cones are found in
the optic disc, the region where the ganglion fibers converge to form the optic nerve.

Cones function in bright light and are responsible for color vision. Rods are much more sensitive to light than cones, so they become saturated and incapable of signalling in ordinary daylight. But their sensitivity enables them to function in dim light, so they are responsible for achromatic night vision. Both rod and cone vision are mediated by visual pigments. These light-absorbing molecules consist of an apoprotein, opsin, that is covalently linked to 11-cis-retinal in most cases (reviewed in Wald, 1953). Cone pigments are blue-, green-, and red-sensitive because of their absorption maxima at approximately 420 nm, 530 nm, and 560 nm, respectively (reviewed in Mollon and Sharpe, 1983). Rod pigment has an absorption maximum at 500 nm (Wald and Brown, 1958) and is called rhodopsin (Kühne, 1878).

C. Rod Photoreceptors.

The greater size and abundance of rods compared to cones dictate their use in many biochemical studies. Rods are slender and elongated and occur in a variety of sizes. For example, frog rods are typically 6-7 μ in diameter and 100-120 μ in length whereas bovine rods are typically 1-2 μ in diameter and 40-50 μ in length. However, rods have a basic structure which is similar in all vertebrate species. Each rod cell is composed of an outer segment, an inner segment, a
nucleus, and a synaptic body. The two segments are connected by a thin non-motile cilium (Figure 3).

The synaptic body is the site of information transfer to the higher-order neurons. It is characterized by the presence of many synaptic vesicles and the formation of chemical synapses with bipolar and horizontal cells. The inner segment contains all the metabolic and synthetic machinery of the cell and is metabolically very active. It consists of two distinct regions called ellipsoid and myoid. The ellipsoid is characterized by a very dense accumulation of mitochondria. The myoid is characterized by free ribosomes, rough endoplasmic reticulum, Golgi apparatus, and contractile proteins. In most vertebrates, with the exception of the rat, there are calycal processes (Cohen, 1963) that arise from the distal end of the inner segment and pass outward about the outer segment for about a third of its length. These fine processes are speculated to have a supportive role; perhaps to prevent the outer segment from rotating about the connecting cilium. A second cytoplasmic bridge may serve that function in many species, including the rat (reviewed in Rodieck, 1973).

The outer segment is a specialized organelle that serves as the site of phototransduction in vision. It is a highly organized structure consisting of hundreds of stacked photoreceptor disc membranes that are oriented perpendicular to its long axis and enclosed by a plasma membrane. The structure and function of frog and bovine rod outer segment
Figure 3. Diagram of a Vertebrate Rod Photoreceptor Cell. Common features among vertebrate rod photoreceptor cells are the outer segment, the inner segment, the nucleus, and the synaptic body. The two segments are connected by a thin non-motile cilium. The outer segment contains a stack of photoreceptor discs enclosed by a plasma membrane. The inner segment contains a mitochondria-rich region known as the ellipsoid and a region characterized by free ribosomes, rough endoplasmic reticulum, Golgi apparatus, and contractile proteins known as the myoid. The synaptic body contains synaptic vesicles.
(ROS) components have been investigated extensively since ROS can be obtained in high purity. Intact ROS membranes with enclosed cytoplasmic proteins and small molecules can be released from the retina by gentle homogenization and purified by flotation on a sucrose or Percoll gradient. The disc membranes are isolated by osmotically shocking the plasma membrane and sealed discs are purified by flotation on 5% Ficoll (Smith et al., 1975).

The disc membranes are composed of lipids and proteins in a bilayer configuration. Lipid to protein ratio, on a dry weight basis, is about one. Phospholipids represent more than 80% of the total lipid content; cholesterol accounts for only 3% (Anderson et al., 1975). The major phospholipids, on the basis of total lipid phosphorous, are phosphatidylethanolamine (44%), phosphatidylcholine (36%), and phosphatidylserine (15%). Polyunsaturation in more than 50% of the total fatty acid composition indicates the highly fluid nature of these disc membranes. The existence of a highly fluid lipid core in disc membranes is postulated to serve the purposes of phototransduction and ionic translocation (reviewed in Olive, 1980).

SDS-polyacrylamide gel electrophoresis of frog and bovine ROS disc membrane proteins demonstrates the presence of rhodopsin with apparent $M_r$ 34,000, a large protein with apparent $M_r$ 220,000, and several other minor components with apparent $M_r$ ranges from 36,000 to 300,000. Rhodopsin
accounts for 85-90% of the total disc membrane proteins whereas the 220 kDa-protein accounts for only 1-3% (Papermaster and Dreyer, 1974; Godchaux and Zimmerman, 1979a; Molday and Molday, 1979; Kühn, 1980; Kühn, 1981; Hamm and Bownds, 1986). In contrast, about 90% of the total ROS proteins is accounted for by three proteins: rhodopsin (70%), G-protein (17%), and cGMP-dependent phosphodiesterase (1.5%) [Hamm and Bownds, 1986]. The G-protein (Baehr et al., 1982) also called transducin (Fung et al., 1981) or guanine nucleotide binding protein (Godchaux and Zimmerman, 1979b) and the cGMP-dependent phosphodiesterase (Pannbacker et al., 1972; Chader et al., 1973; Miki et al., 1973) are two disc membrane associated proteins involved in cGMP hydrolysis. These two proteins and rhodopsin play central roles in the phototransduction process.

D. Phototransduction Process.

In the dark the Na$^+$ ion permeability of ROS plasma membrane is high, approximately $10^9$ Na$^+$ ions per second (Korenbrot and Cone, 1972; Hagins et al., 1976). Na$^+$ ions rapidly flow into the outer segment because of the existence of a large Na$^+$ concentration gradient across the plasma membrane. These Na$^+$ ions then diffuse through the connecting cilium and/or the cytoplasmic bridge to the inner segment and are extruded by Na$^+$-K$^+$-ATPase pumps in the inner segment plasma membrane to maintain the Na$^+$ concentration gradient.
Absorption of a photon causes the chromophore of rhodopsin, 11-cis-retinal, to undergo a series of transformations that result in the dissociation of all-trans-retinal from the protein to yield bleached rhodopsin or opsin (Wald, 1968; Ebrey and Honig, 1975; Honig, 1978; Birge, 1981). This conformational transition in a single rhodopsin molecule results in the closure of Na\(^+\) channels. Consequently, as many as a million Na\(^+\) ions are prevented from entering into the ROS (Stryer et al., 1981). The resulting hyperpolarization of the plasma membrane is transmitted to the synaptic terminal and communicated to other cells of the retina by suppressing neurotransmitter secretion into the rod synapse.

It is generally accepted that an internal messenger is essential to communicate between rhodopsin molecules in the disc membranes and the Na\(^+\) channels of the plasma membrane (Baylor and Fuortes, 1970). Both Ca\(^{2+}\) and cGMP have been proposed as intracellular messengers (Yoshikami and Hagins, 1971; Miller, 1981, 1986), but recent progress in the vision field has established cGMP as the likely messenger in rod (Caretta and Cavaggioni, 1983; Fesenko et al., 1985; Koch and Kaupp, 1985) and cone (Cobbs et al., 1985; Haynes and Yau, 1985) cells.

E. **Proposed Mechanism of Phototransduction.**

(1) **Role of cGMP**
The current hypothesis of the phototransduction mechanism is that the unusually high cGMP concentration (30-60 μM) in the ROS cytosol (Woodruff and Bownds, 1979) is required to maintain the opening of Na\(^+\) channels in the dark. This is supported by the fact that cGMP depolarizes the ROS plasma membrane within milliseconds after being experimentally injected into an isolated cell and increases the latency of the light-induced hyperpolarization (Miller and Nicol, 1979). Upon illumination the cGMP level decreases markedly. About a million cGMP molecules are hydrolyzed per second for each photolyzed rhodopsin molecule (Fung et al., 1981) resulting in the closure of Na\(^+\) channels.

The high rate of cGMP hydrolysis is due to the activation of an enzyme cascade (Liebman and Pugh, 1981). Photoactivated rhodopsin catalyzes the exchange of bound GDP for GTP on a regulatory protein called G-protein. The G-protein with bound GTP is able to activate a cGMP-dependent phosphodiesterase (PDE), probably by releasing an inhibitory factor (Hurley and Stryer, 1982). The activated PDE hydrolyzes cGMP to 5'-GMP. The hydrolysis of cGMP is highly amplified as one photolyzed rhodopsin can activate as many as 500 G-protein molecules (Fung et al., 1981), which in turn activates 500 PDE molecules. Each activated PDE molecule hydrolyzes about 2000 cGMP molecules per second (Figure 4).

In order to restore PDE activity to the preactivation level, both bleached rhodopsin and G-protein must be
Figure 4. Enzyme Cascade of Vision. The current hypothetical model of signal transduction in the rod photoreceptor cell is illustrated in this schematic diagram. cGMP-dependent Na\textsuperscript{+} channels on the rod outer segment plasma membrane are kept opened in the dark by a high concentration of cGMP in the cytosol. Phosphodiesterase (PDE) and G-protein (G), two disc membrane associated enzymes involved in the hydrolysis of cGMP, are inactive in the dark. Absorption of light by rhodopsin (R), the photopigment in rod disc membranes, activates G-protein which in turn activates PDE*. The resulting decrease in cGMP concentration closes the Na\textsuperscript{+} channels (obtained from R.S. Molday).
inactivated (Liebman and Pugh, 1979). G-protein is inactivated by the hydrolysis of bound GTP to GDP due to its slow intrinsic GTPase activity (Kühn, 1980). Bleached rhodopsin is inactivated by phosphorylation (Sitaramayya and Liebman, 1983) due to a rhodopsin kinase activity (Wilden and Kühn, 1982). Only 1-2 PO$_4$/rhodopsin are required for the rapid, ATP-dependent inactivation of bleached rhodopsin as indicated by a recent report (Sitaramayya, 1986).

How the phosphorylation of rhodopsin results in the deactivation of PDE has been the subject of several recent studies. Kühn et al. (1984) have suggested that a soluble 48 kDa-protein competes with G-protein for binding sites at the rhodopsin surface following the phosphorylation of rhodopsin, thus preventing the activation of G-protein by bleached rhodopsin. This proposal is supported by the studies indicating that the 48 kDa-protein binds much more readily to the phosphorylated form of rhodopsin than to the unphosphorylated form (Kühn et al., 1984), and that rhodopsin phosphorylation is required for the 48 kDa-protein dependent quenching of PDE activation (Wilden et al., 1986). Zuckerman et al. (1984, 1985) also report the 48 kDa-protein participates in the rapid inactivation of bleached rhodopsin. However, they proposed that the 48 kDa-protein is first activated by phosphorylated rhodopsin before quenching PDE activation by direct interaction with the G-protein or PDE (Zuckerman et al., 1985; Zuckerman and Cheasty, 1986).

Contrary to these reports, Miller et al. (1986) have
indicated that rhodopsin phosphorylation alone rather than the 48 kDa-protein represents a major component of the PDE inactivation process. Using a preparation consisting of purified phosphorylated rhodopsin species reconstituted into phosphatidylcholine vesicles and an extract containing G-protein, PDE, and only a trace amount of the 48 kDa-protein, they found that 80-90% of the light-initiated PDE activation can be inhibited by rhodopsin phosphorylation. They have suggested that phosphorylation of rhodopsin results in the reduction of the binding affinity of G-protein for photoactivated rhodopsin. This proposal is supported by preliminary evidence indicating that the G-protein-dependent enhancement of metarhodopsin II formation (Emeis and Hoffman, 1981) is reduced by as much as 80% in high PO₄/rhodopsin vesicles relative to 0 PO₄/rhodopsin vesicles (Miller et al., unpublished observation). Obviously, more experiments are required to clarify the role of phosphorylation of rhodopsin and the importance of the 48 kDa-protein in rod cell transduction mechanism.

The preactivation level of cGMP is restored by guanyl cyclase which catalyzes the conversion of GTP to cGMP. Both light and Ca²⁺ may control guanyl cyclase activity (Cohen, 1981; Goldberg et al., 1983).

(2) 

The Ca²⁺ hypothesis states that Ca²⁺ ions are stored in
the intradiscal space in the dark. Light stimulates the release of Ca$^{2+}$ ions into the cytoplasmic space, and then Ca$^{2+}$ ions diffuse to the plasma membrane to block Na$^+$ channels. This hypothesis has been supported by electrophysiological experiments in which the addition of Ca$^{2+}$ to the retina mimics the effect of light (Hagins and Yoshikami, 1974; Brown et al., 1977; Lipton et al., 1977) and the addition of calcium chelators such as EGTA makes the rod less sensitive to light (Brown et al., 1977; Hagins and Yoshikami, 1977). Furthermore, many Ca$^{2+}$ ions are extruded from an illuminated ROS following a light pulse (Gold et al., 1980; Yoshikami et al., 1980), and sufficient Ca$^{2+}$ is present in ROS and in the discs for it to be available as a transmitter (Liebman, 1974; Szuts and Cone, 1977). However, recent experiments have shown that millimolar Ca$^{2+}$ has little effect on the opening of the light-regulated channel by cGMP in an isolated patch of ROS plasma membrane (Fesenko et al., 1985) or in isolated discs (Koch and Kaupp, 1985). Furthermore, Nicol et al. (1987) have shown that the normal flash responses of isolated rod photoreceptors persist after the depletion of intracellular calcium. Therefore, the Ca$^{2+}$ hypothesis needs to be modified, and a change in intracellular Ca$^{2+}$ concentration is not essential for excitation of photoreceptors by light.

The precise role of Ca$^{2+}$ in the phototransduction process remains to be determined, but its effect on the rod photoresponse could be explained by its action on the cGMP
metabolism in the photoreceptor (reviewed by Kaupp and Koch, 1986) because cGMP levels in living rods are $\mathrm{Ca}^{2+}$-dependent (Cohen, 1981; also reviewed by Korenbrot, 1985). Thus, further insights into the role of $\mathrm{Ca}^{2+}$ in the rods could be gained by studying the properties and regulation of ROS guanyl cyclase.

In contrast to guanyl cyclase, the key components of the enzyme cascade, rhodopsin, G-protein, and PDE are well characterized (reviewed in Applebury and Hargrave, 1986; Stryer, 1986).

(3) Rhodopsin

Rhodopsin, the vertebrate photopigment, has been a major focus in vision research. Several vertebrate opsins have been sequenced (Ovchinnikov et al., 1982; Hargrave et al., 1983; Pappin et al., 1984), and the genes for bovine rod and human rod and cone opsins (Nathans and Hogness, 1983, 1984; Nathans et al., 1986a & b) have also been characterized. Rhodopsin is a transmembrane glycoprotein with its N-terminus facing the intradiscal space and its C-terminus facing the cytoplasmic space (Clark and Molday, 1979), and is envisioned to traverse the disc membranes seven times in helical segments (Ovchinnikov et al., 1982; Hargrave et al., 1983). The N-terminus contains two carbohydrate chains which bind such lectins as Con A and WGA. The C-terminus contains phosphorylation sites for rhodopsin kinase (Wilden and Kühn, 1982). The cytoplasmic surface contains sites for light-
dependent binding of ROS proteins such as G-protein, rhodopsin kinase and the 48 kDa-protein (Kühn, 1981; Kühn et al., 1984; also reviewed by Hargrave, 1982; Applebury and Hargrave, 1986).

Rhodopsin appears to be related to some β-adrenergic receptors (Dixon et al., 1986; Yarden et al., 1986; Kobilka et al., 1987), a muscarinic acetylcholine receptor (Kubo et al., 1986), and a protein encoded by a cellular oncogene called mas (Young et al., 1986). Since rhodopsin acts through a G-protein belonging to a family of guanine nucleotide binding proteins (designated $G_o$, $G_i$, and $G_s$) whose functions are to regulate the activation or inhibition of adenylate cyclase by hormones and neurotransmitters (Gilman, 1984; 1986), it has been suggested that the mechanism of phototransduction in retinal rods may be a model for the early events of signal transduction in many cell types. This is further supported by the observation that the components of some of these systems are functionally interchangeable (Bitensky et al., 1982; Cerione et al., 1985). It is also interesting that cyclic-nucleotide-dependent channels are now being identified in other sensory systems (Johnson et al., 1986; Nakamura and Gold, 1987). These channels will no doubt be compared to the cGMP-dependent channel in retinal rods which has only recently been biochemically identified (Cook et al., 1987; Matesic and Liebman, 1987).
F. Rod Outer Segment.

In addition to the phototransduction process, ROS have also been investigated to further the understanding of the mechanism of biogenesis and renewal of ROS membranes, and how the highly ordered photoreceptor structure is maintained.

(1) Structure

The outer segment consists of a stack of hundreds of closed double-membrane discs equally spaced apart (Cohen, 1972). Both X-ray diffraction measurements on excised retinas (Blaurock and Wilkins, 1969) and freeze-fracture studies (Korenbrot et al., 1973) indicate that the center-to-center distance between adjacent discs has a constant value of about 300 Å. The internal membrane surfaces of a disc are separated by a 20 Å-wide aqueous space (Chabre and Cavaggioni, 1975), which is maintained over the entire extent of the disc (Figure 5). These specialized disc membranes occupy a relatively compact volume, but provide a large surface area for embedding a large number of photoreceptor molecules, which is required for the high sensitivity of rods (Fein and Szuts, 1982).

The discs are divided into lobes by deep invaginations of the disc membrane termed incisures. The number of incisures and discs in a ROS varies among the vertebrates, but invariably the assembly of discs is enclosed by a plasma membrane that is osmotically (Korenbrot et al., 1973) and electrically (Hagins and Ruppel, 1971) separated from the
discs over most of the length of the ROS (Figure 5). The discs are composed of two apparently distinct membrane domains, the flat lamellar domain and the highly curved rim region (Falk and Fatt, 1969).

(2) Biogenesis and Renewal

The disc are continuously renewed (Young, 1967; Young and Droz, 1968). Autoradiographic studies have shown that the new disc membrane components are synthesized in the inner segment at one or more sites and are then added to the plasma membrane near the ROS base where final membrane assembly takes place (Hall et al., 1969; Young, 1973). The discs are formed by invagination of the plasma membrane, but are pinched off to produce closed discs as they are displaced apically (Sjöstrand, 1959; Moody and Robertson, 1960; Cohen, 1961). However, it has also been proposed that the discs are formed by repeated evagination of the plasma membrane in the region of the connecting cilium (Eakin, 1973).

A recent model has been proposed for the differential biogenesis of rim and lamellae of discs, in addition to the incisures (Steinberg et al., 1980). In this model each of the two surfaces of an evagination of the ciliary membrane form one of the surfaces of adjacent discs. The disc rim is initially specified as a region of ciliary membrane between adjacent disc-surface evaginations. This region grows bilaterally around the circumferences of adjacent discs,
Figure 5. Fine Structure of Rod Outer Segment. (a) The outer segment consists of a stack of closed double-membrane discs equally spaced apart and enclosed by a plasma membrane that is separated from the discs over most of the length of the outer segment (modified after Young, 1970). (b) The center-to-center distance between adjacent discs is approximately 300 Å. The internal membrane surfaces of a disc are separated by a 20 Å-wide aqueous space. The discs are divided into lobes by the incisures. The discs are composed of two apparently distinct membrane domains, the flat lamellar domain and the highly curved rim region (modified after Steinberg et al., 1980).
zippering together the apposed surfaces to form the rim and completed disc. At the same time the plasma membrane edges of the evaginations which have become detached from the surfaces are sealed. The incisures form in rod discs by infolding of the rim and surfaces together. They begin to form before the rim is completed around the disc perimeter. The rate of disc synthesis varies from species to species. For frogs the rate is 1.2 discs per hour, and all 1700 discs in a frog ROS are renewed in two months (Young and Bok, 1969). For Rhesus monkeys all the discs in a ROS are renewed in about 10 days (Young, 1971).

To maintain the ROS at a constant length, the tips are periodically pinched off to be phagocytized and degraded by the retinal pigment epithelium. Each shedding event removes the distal 10-20% of the outer segment (Kaplan et al., 1987) and this shedding process has been found to follow a diurnal pattern in a large variety of species (Basinger and Gordon, 1982). The orderly process of disc synthesis and removal appears to be extremely crucial to photoreceptor function. If the retinal pigment epithelium is incapable of phagocytizing the shed discs, the rods eventually degenerate and die (Bok and Hall, 1971). The precise mechanisms of disc renewal however remain to be determined.

(3) Maintenance

The nature and morphology of the structural components responsible for maintaining a constant distance between
adjacent discs and discs to plasma membrane are largely unknown. However, Usukura and Yamada (1981) and Roof and Heuser (1982) have observed thin filaments extending from the rims of ROS discs by electron microscopy. In the latter study, one type of filamentous structure was observed to link adjacent stacked discs and another filamentous structure appeared to link the discs to the plasma membrane. Although the molecular composition of these filaments has not been determined, it has been suggested that these filamentous connections may serve as structural elements of a cytoskeletal network responsible for stabilizing the highly organized outer segment structure.

G. Cytoskeletal Network in Red Blood Cell.

To date the best understood membrane cytoskeleton in terms of the organization and associations of its protein components is that of the red blood cell plasma membrane (reviewed in Marchesi et al., 1976; Branton et al., 1981; Marchesi, 1983; Bennett, 1985). Mammalian red blood cells contain on the cytoplasmic surface of their plasma membrane, a two-dimensional meshwork of structural proteins that stabilizes the overlying lipid bilayer. This meshwork probably functions in the maintenance of cell shape and in the organization of the integral membrane proteins (reviewed in Cohen, 1983). The principal component of this meshwork is the protein spectrin (reviewed in Marchesi, 1980).
Spectrin is a flexible rod-shaped molecule composed of an $\alpha$ subunit of $M_r = 240,000$ and a $\beta$ subunit of $M_r = 225,000$. The $\alpha$ and $\beta$ subunits are aligned side-to-side to form heterodimers, and the dimers self-associate in a head-to-head orientation to form tetramers of 200 nm in length (Shotton et al., 1979; Branton et al., 1981). Spectrin tetramers form the meshwork by binding of their ends to short actin filaments and to a 78,000 $M_r$ protein named band 4.1 which modulates spectrin-actin interaction. This complex is attached to the membrane by association of spectrin with ankyrin, a membrane-associated protein which attaches to a membrane-spanning anion transporter protein known as band 3 (Figure 6) [reviewed by Speicher, 1986].

Ankyrin, originally designated as band 2.1 (Steck, 1974), is a globular protein with apparent $M_r = 200,000$. Ankyrin and its membrane-associated proteolytic fragments have been collectively referred to as syndeins (Yu and Goodman, 1979). Ankyrin is located at the cytoplasmic membrane surface and mediates the attachment of spectrin to the membrane by binding to the $\beta$ chain of spectrin and band 3. Therefore, ankyrin is largely responsible for maintaining the close association of the membrane skeletal proteins with the lipid bilayer.

Actin, originally designated as band 5 (Sheetz et al., 1976; Tilney and Detmers, 1975), has a monomer $M_r = 42,000$. It is a highly conserved protein that polymerizes into
Figure 6. The Red Cell Membrane Skeleton. The organization and associations of cytoskeletal protein components on the cytoplasmic surface of red blood cell plasma membrane are indicated in this schematic diagram. Spectrin-actin-band 4.1 and spectrin dimer-dimer interactions are thought to be important in forming a two-dimensional meshwork. This meshwork is attached to the plasma membrane by spectrin-ankyrin-band 3 interactions (modified after Palek and Lux, 1980).
filaments. Short filaments of actin (25 nm long) associate with spectrin to form the cytoskeletal meshwork in erythrocyte. Their association is promoted, strengthened, or stabilized by band 4.1 in an undetermined manner. The role of band 4.1 has been suggested to maintain membrane elasticity and shape (reviewed in Cohen, 1983).

Analogs of red blood cell membrane proteins including spectrin, ankyrin, band 3, and band 4.1 have been identified in other cell types using polyclonal antibodies as probes (reviewed in Baines, 1984; Bennett, 1985). The spectrin analogs have been most extensively characterized to date.

H. Spectrin-like Proteins.

Two distinct spectrin-like proteins have been isolated from brain and intestinal microvilli. The former is known as fodrin (Levine and Willard, 1981) or calspectin (Sobue et al., 1981) or brain spectrin (Bennett et al., 1982), and the latter is known as TW 260/240 (Glenny et al., 1982a). These proteins are considered spectrin-like because not only do they have \( \alpha \) and \( \beta \) subunits with \( M_r \) similar to that of spectrin and similar tetrameric rod structure, but they also crosslink actin filaments and bind to calmodulin and ankyrin (Bennett et al., 1982; Burridge et al., 1982; Burns et al., 1983).

The \( \alpha \) subunits in all avian spectrins bind calmodulin and appear to be a widespread unit of structure as
immunological equivalents are detected in a variety of mammalian cell types (Repasky et al., 1982). The \( \beta \) subunits of avian spectrins differ from each other in \( M_r \) (260,000 for TW 260/240, and 235,000 for fodrin compared to 225,000 for spectrin), and they have distinct antigenic sites and yield different peptide maps (Bennett et al., 1982; Burridge et al., 1982; Glenny et al., 1982a, 1982b). Since many of the functional properties of red blood cell spectrin such as phosphorylation (Cohen, 1983) and ankyrin binding (Bennett and Stenbuck, 1979) are characteristic features of the \( \beta \) subunit, it has been suggested that the diversity of the \( \beta \) subunit contributes to the functional differences between spectrin and spectrin-like proteins in this and other species. However, almost all spectrins of many avian and mammalian cell types have been localized by immunofluorescence to the plasma membrane (Goodman et al., 1981; Levine and Willard, 1981; Burridge et al., 1982; Repasky et al., 1982).

I. High Molecular Weight Proteins in Rod Outer Segment.

In view of the importance of spectrin in maintaining membrane integrity in the red blood cell and the widespread occurrence of spectrin-like proteins in nature, it is of interest to determine the existence of such a protein in the rod outer segment. Several high molecular weight proteins have been identified in frog and bovine ROS preparations,
however they have not been well characterized.

Papermaster et al. (1978) have used immunocytochemical techniques to localize a 290 kDa disc membrane protein, referred to as the rim protein. Szuts (1985) has reported that two proteins of 220 kDa and 240 kDa undergo a light-mediated phosphorylation reaction in frog ROS. The 220 kDa-protein appears to be equivalent to the rim protein. Molday and Molday (1979) have reported a 220 kDa transmembrane glycoprotein designated as ROS 1.2 in bovine ROS disc membrane preparations. Whether or not ROS 1.2 is equivalent to the rim protein remains to be determined as its localization to the rim regions of bovine ROS discs has not been reported. The functions of the rim protein and 240 kDa-protein in frog ROS and ROS 1.2 in bovine ROS are as yet unknown, although it has been suggested that the rim protein constitutes the filaments that link discs together in frog ROS (Roof and Heuser, 1982).

J. Monoclonal Antibodies to ROS Proteins.

In order to define in more detail the role of these and other minor proteins in the maintenance of the highly organized ROS structure, in the phototransduction process and in the other specialized processes such as disc renewal that occur in retinal rod cells, MacKenzie and Molday (1982) have used the technique of Köhler and Milstein (1975) to prepare monoclonal antibodies against bovine ROS proteins. The
majority of monoclonal antibodies generated were found to be specific for rhodopsin (MacKenzie and Molday, 1982). Monoclonal antibodies to rat rhodopsin (Fekete and Barnstable, 1983) and frog rhodopsin (Witt et al., 1984) have also been generated. These rhodopsin-specific monoclonal antibodies have been used as immunological probes to study the location, structure and function of rhodopsin. Polyclonal anti-rhodopsin antisera have been used for the immunocytochemical localization of the protein in the ROS disc and plasma membrane and in the inner segment subcellular organelles by several laboratories (Dewey et al., 1969; Jan and Revel, 1974; Papermaster et al., 1978). However, the major advantage of using monoclonal antibodies rather than polyclonal antisera in such studies is that monoclonal antibodies do not bind to multiple epitopes on the rhodopsin molecule. This is especially important in structural and functional studies, since monoclonal antibodies allow the immunological dissection of proteins.

Application of monoclonal antibodies as immunological probes for the structure and function of rhodopsin requires the localization of the epitopes for these antibodies. Recently, Molday et al. have reported the properties of several monoclonal antibodies against various segments of rhodopsin (MacKenzie and Molday, 1982; MacKenzie et al., 1984; Laird et al., 1987). The binding sites for these monoclonal antibodies in relation to the proposed transmembrane helical model of rhodopsin (Hargrave et al.,
1983) are shown in Figure 7. The general location of the epitopes for these rhodopsin-specific antibodies was determined by limited proteolysis of rhodopsin using trypsin and *S. aureus* proteases and subsequent immunoblotting analyses. More precise localization of antibody binding sites was determined by solid-phase radioimmune competitive inhibition studies using synthetic peptides and small peptides derived from chemical or proteolytic cleavage of rhodopsin.

Almost all of these antibodies were found to bind either the N-terminal or the C-terminal segments of rhodopsin, and only two antibodies were found to bind to other segments of rhodopsin. The reason for the high degree of antigenicity found for the N-terminal and C-terminal regions of rhodopsin (Hargrave et al., 1986; Laird et al., 1987) as well as many other proteins (Anderer and Schlumberger, 1965; Altschuh and Regenmortel, 1982; Quesnizux et al., 1983) is unknown. It has been hypothesized that the segmental mobility or flexibility of peptide regions is an essential component of antigenicity (Tainer et al., 1985). But in the case of scorpion neurotoxin, an exceptional surface exposure of relatively short loop segments rather than segmental mobility appears to make the best correlation (Novotny and Haber, 1986). One C-terminal specific anti-rhodopsin antibody referred to as rho-1D4 has been characterized extensively. This IgG₁ antibody binds to the 341-348 (1'–8') C-terminal segment and requires the terminal Ala amino acid for binding
Figure 7. Localization of Anti-Rhodopsin Monoclonal Antibodies. The binding sites of several monoclonal antibodies specific for rhodopsin are illustrated in this model for the organization of rhodopsin in rod outer segment disc membranes (modified after Hargrave et al., 1983). In this model, rhodopsin transverses the disc membranes seven times in helical segments with its N-terminus facing the intradiscal surface, while its C-terminus faces the cytoplasmic surface. The N-terminus contains two carbohydrate chains (solid squares and unmarked circles) that bind such lectins as Con A and WGA. The C-terminal segments and cytoplasmic loop regions of rhodopsin are accessible to proteases such as S. aureus and trypsin. Monoclonal antibodies rho-2B2, -4A2, -4A3, -4D2, and -5A3 bind to the N-terminal segment of rhodopsin. The C-terminal segment of rhodopsin contains binding sites for monoclonal antibodies rho-3D6, -1D4, -2C1, -3C2, -3A6, and -1C5. Only two monoclonal antibodies, rho-4B4 and rho-8A6, bind to the loop regions (modified after Laird et al., 1987).
to rhodopsin. Rho-lD4 has been successfully used in the purification of the 318-348 peptide from cyanogen bromide cleaved rhodopsin by immunoaffinity chromatography (MacKenzie et al., 1984; Laird et al., 1987). It has also been used with the immunogold-dextran labelling method to localize rhodopsin along the cytoplasmic surface of discs (Molday et al., 1987) and to study the properties of light-dependent phosphorylation of rhodopsin (Molday and MacKenzie, 1985). Because it has been well characterized, rho-1D4 has been routinely used as a specific probe for rhodopsin in many biochemical and immunocytochemical labelling studies.

Molday et al. (1987) have also reported monoclonal antibodies against a novel membrane protein, termed peripherin, with an apparent M_r of 33,000. Two monoclonal antibodies designated as 2B6 and 3B6 were found to bind to peripherin. Immunogold labelling of morphologically intact isolated discs indicated that peripherin is localized along the rim region of discs. Peripherin appears distinct from the M_r 290,000 rim protein in frog ROS previously described by Papermaster et al. (1978), although both proteins localized along the rim regions of discs.

In addition to monoclonal antibodies against rhodopsin and peripherin, MacKenzie and Molday (1982) have also reported a monoclonal antibody designated as 4B2 which bound to a high molecular weight polypeptide having a similar mobility to ROS 1.2, a 220 kDa Con A-binding glycoprotein, on SDS-polyacrylamide gels. This antibody is of interest
because very little is known about the structure and function of high molecular weight proteins found in vertebrate ROS. Although the 4B2-binding protein was shown to be associated with ROS disc membranes, its properties were not characterized in detail.

K. Thesis Investigation.

This thesis characterizes further the 4B2-binding protein in bovine ROS using the 4B2 monoclonal antibody as a molecular probe. In previous studies, the 4B2 antibody was shown to be an IgM molecule with \( K \) light chains. Although the 4B2 hybridoma cell line could be grown either in culture medium or as ascites tumors in mice, the concentration of 4B2 antibody in ascites fluid was generally 25- to 40-fold higher than in cell culture supernatant. In this thesis investigation, the 4B2 cell culture supernatant and ascites fluid rather than purified 4B2 antibody were used for identifying and characterizing the 4B2-specific protein in ROS membrane preparations because it was shown that the 4B2 antibody activity decreased at least 4-fold after the purification and lyophilization procedures. Moreover, this antibody could only be partially purified from ascites fluid by DEAE-Sephacel column chromatography (Wong, 1984).

There were four major objectives of this thesis. The first objective was to determine whether the 4B2-binding protein is the same as the 220 kDa-glycoprotein designated as
ROS 1.2. The second objective was to use both monoclonal and polyclonal antibodies as molecular probes to determine whether the 4B2-binding protein (or some other unidentified protein) is immunologically related to spectrin from red blood cells. The third objective was to use immunocytochemical techniques to determine the location of the 4B2-binding protein within the rod cell. The final objective was to use biochemical and immunoblotting techniques to devise a simple and efficient method for extracting the 4B2-binding protein from the ROS membranes.
MATERIALS AND METHODS

A. MATERIALS.

General laboratory chemicals of reagent grade were obtained from either Fisher Scientific Company (Ottawa, Ontario), Sigma Chemical Company (St. Louis, MO), or BDH Chemicals Canada Limited (Toronto, Ontario). Specialized materials and suppliers are listed below or indicated in the Methods Sections.

Cell culture media, fetal calf and heat-inactivated horse sera, penicillin and streptomycin, fungizone, and Freund's complete and incomplete adjuvants were obtained from Grand Island Biological Company (Grand Island, NY). Disposable and sterile centrifuge tubes (50 mL) and petridishes (100 x 20 mm) were obtained from Corning Laboratory Sciences Company (Oneonta, NY). Disposable and sterile needles and syringes, 96-well polystyrene and flexible vinyl plates for solid-phase RIA assays, 1- and 2-mL serological pipets, and petridishes (60 x 15 mm) were obtained from Falcon Labware, Becton and Dickenson Canada Limited (Mississauga, Ontario).

Bovine eyes and brain for antigen preparation were obtained from Olympic Intercontinental Packers Limited, a local slaughter house. Bovine blood samples were obtained locally either from J.L. Meats (Surrey, British Columbia), or
the Animal Science Farm of the University of British Columbia. BALB/c mice and New Zealand Whites rabbits for monoclonal and polyclonal antibody production were obtained from the Animal Care Unit of the University of British Columbia.

Acrylamide, ammonium persulfate, N-N’-methylene bisacrylamide, N, N, N’, N’- tetramethylethylenediamine, Coomassie blue R-250, and a Protean Dual Vertical Slab Gel Electrophoresis Cell apparatus for SDS-polyacrylamide gel electrophoresis were obtained from Bio-Rad (Richmond, CA). The power supply (Model ECPS 3000/150) used for gel electrophoresis was obtained from Pharmacia (Uppsala, Sweden). For electrophoretic transfers, a Bio-Rad power supply (Model 250/2.5) was used.

Lowicryl K4M embedding medium and basic supplies for electron microscopy were obtained from JB EM Services Incorporated (Dorval, Quebec). Uranyl acetate was obtained from Polysciences Incorporated (Warrington, PA). A Sorvall Ultra Microtome MT 5000 from DuPont Company (Wilmington, DE) was used to cut embedded specimens into thin sections for electron microscopy.

B. Hybridoma Cell Culture Conditions.

All cell culture procedures were performed in a sterile culture hood (NuAire Inc.). The window and working surface
area of the hood were washed with 70% ethanol before and after each usage and periodically irradiated with ultraviolet light for 24 h to keep the hood sterile. Glasswares, pipets and pipet tips, vials, and solutions were sterilized in an autoclave (Consolidated).

Hybridoma cells were grown in either RPMI 1640 or IMDM cell culture medium. The culture media were sterilized by passing them through a Sterivex-GS 0.22 μm filter unit (Millipore) under constant pressure of 15-20 psi, delivered by a peristaltic pump from Millipore (Bedford, MA), into sterile 500-mL bottles. The RPMI 1640 cell culture medium was supplemented with 2 mM L-glutamine, 24 mM sodium bicarbonate, 2 mM sodium pyruvate, 10% FCS, 5% heat-inactivated horse serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 1.25 μg/mL fungizone. The pH of this medium was adjusted to 7.2. The IMDM cell culture medium was supplemented with 36 mM sodium bicarbonate, 10% FCS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 1.25 μg/mL fungizone. The pH of this medium was 7.2.

C. Maintenance of Hybridoma Cell Lines.

Hybridoma cells in cell culture medium were grown at 37°C in an atmosphere of 5% CO₂ in a humidified incubator (National). Growing cultures were maintained in 10-mL culture plates at concentrations ranging from 4-10×10⁵ cells/mL. The concentration of viable cells was determined by staining cell
cultures with an equal volume of 0.4% Trypan blue and counting unstained cells with a hemacytometer (American Optical).

Hybridoma cells were stored by centrifuging 10 mL of a cell culture at 1,500 rpm for 5 min. Each cell pellet was resuspended in 5 mL of 10% dimethylsulfoxide in supplemented IMDM culture medium. Cells were then transferred to freezing vials (Nunc) and immediately placed in a box insulated with styrofoam. The box was left in a -70°C freezer (Kelvinator). After 24 h the vials were placed in a liquid nitrogen tank (Cryogenics).

Frozen cells were retrieved by quickly thawing and washing the cells with 10 mL of non-supplemented IMDM culture medium. The cell pellet was then resuspended in 10 mL of supplemented IMDM culture medium.

D. Cloning of Hybridoma Cell Lines.

Antibody-secreting 1D1, 3A6, and 4B2 hybridoma cell lines were cloned by limiting dilution. Approximately 230 live hybridoma cells were suspended in 4.6 mL of a cloning medium consisting of 10⁷ BALB/c mouse thymocytes per mL of supplemented IMDM culture medium. Aliquots (100 μL) of this suspension were used to plate 36 wells of a 96-well culture plate, such that an average of 5 cells were seeded in each well. To the remaining 1 mL of cell suspension, 4 mL of cloning medium were added. Aliquots (100 μL) of this
suspension were used to plate another 36 wells, giving an average of one cell per well.

Aliquots (100 μL) of the cloning medium were added to each well of the cloning plate at five and at twelve days later. The growth of cloned cells was monitored under a light microscope with phase-contrast optics (Zeiss). Wells with cell growth were then tested for antibody activity by standard RIA as described in Methods Section J. Positive clones were expanded and stored as described in Methods Section C.

E. Production of Monoclonal Antibody.

Culture supernatants and ascites fluids from antibody-secreting hybridoma cell lines were routinely used for antibody binding analyses. The 3A6 and 4B2 hybridoma cells were grown and expanded continuously for a 2-week period. Culture supernatants were collected by centrifuging the cells at 1,500 rpm for 5 min and stored in 0.1% sodium azide at 4°C. The 3A6 and 4B2 hybridoma cells were also grown as ascites tumors in BALB/c mice that had been injected intraperitoneally with 0.5 mL of pristane (Aldrich) 7 days prior to injection of 5-10X10⁶ cells. When ascites tumors became noticeable, ascites fluid was harvested. Ascites fluid was separated from the contaminating blood cells by centrifuging the mixture at 12000 rpm for 20 min and stored in 5 mL aliquots in a -70°C freezer.
F. Preparation of ROS and Disc Membranes.

Purified ROS membranes from frozen retinas (Hormel) were prepared by a modification of the procedure of Papermaster and Dreyer (1974) as follows: Fifty bovine retinas in 50 mL of a sucrose solution containing 0.15 M NaCl, 2 mM MgCl₂, and 10 mM Tris-acetate, pH 7.4 (Buffer B), and having a density of 1.16 g/mL, were swirled gently for 1 min and centrifuged at 4,000 rpm for 4 min in a SS-34 Sorvall rotor. Pellet was resuspended in 30 mL of the same sucrose solution, and then centrifuged at 8,000 rpm for 8 min. Supernatants were combined and passed through two layers of cheesecloth. Two volumes of cold Buffer B were added to the filtrate and the suspension was centrifuged at 10,000 rpm for 10 min. Pellet was resuspended in 15 mL of a sucrose solution containing Buffer B and having a density of 1.10 g/mL, and the suspension was centrifuged at 15,000 rpm for 30 min. Pellet was resuspended and homogenized twice in 20 mL of the same sucrose solution in a tight dual tissue homogenizer (Wheaton).

The homogenate (5 mL/gradient) was layered on a discontinuous gradient consisting of 4 mL of sucrose having a density of 1.15 g/mL, 11 mL of sucrose having a density of 1.13 g/mL, and 9 mL of sucrose having a density of 1.11 g/mL. After centrifugation at 25,000 rpm for 1 h in a SW-27 rotor, the band of purified ROS membranes was collected at the interface between the 1.11 g/mL and 1.13 g/mL sucrose
solutions, diluted with 30 mL of Buffer B, and washed twice by centrifugation at 15,000 rpm for 15 min in a SS-34 Sorvall rotor. The pellet was resuspended in Buffer B.

Purified ROS membranes from freshly dissected retinas were prepared as previously described (Wong and Molday, 1986). Fifty bovine retinas in 15 mL of homogenizing solution containing 20% sucrose, 0.25 mM MgCl₂, 10 mM taurine, 10 mM glucose, and 20 mM Tris-acetate, pH 7.4, were swirled gently for 1 min and filtered through one layer of cheesecloth. Retinas were then washed with an additional 5 mL of homogenizing solution, swirled, and filtered as above. Filtrates were combined, refiltered twice through two layers of cheesecloth, and placed on ice for 5 min. Filtrate (5 mL/gradient) was applied to four 22 mL 25-60% (w/w) linear sucrose gradients containing 10 mM taurine, 10 mM glucose, and 20 mM Tris-acetate, pH 7.4. The gradients were centrifuged at 25,000 rpm for 50 min in a SW-27 rotor. Purified ROS membranes were collected as a single band at the upper region of the gradient and washed in 20 mM Tris buffer, pH 7.4.

Disc membranes were prepared from purified ROS by hypotonic lysis of ROS followed by flotation on 5% Ficoll according to the method of Smith et al. (1975). Briefly, ROS were slowly suspended in seven volumes of cold 7 mM Tris-acetate, pH 7.4, and centrifuged at 15,000 rpm for 15 min. Pellet was resuspended in 10 mL of 7 mM Tris-acetate, pH 7.4.
and kept in the dark inside a SW-27 bucket at 4°C for 30-60 min before adding 10 mL of 10% Ficoll 400 (Pharmacia). This mixture was shaken and overlayed with 2 mL deionized water, and left at 4°C for 15 min. After centrifugation at 25,000 rpm for 1 h in a SW-27 rotor, the disc membranes were collected at the interface between water and Ficoll and washed with 20 mM Tris-acetate (pH 7.4) by centrifugation at 15,000 rpm for 30 min in SS-34 Sorvall rotor. Membranes were either used immediately or stored in small aliquots in light-tight vials at -20°C. The 280- and 500-nm absorbance ratios ($A_{280}/A_{500}$) for membranes solubilized in 50 mM cetyltrimethyl ammonium bromide were typically 2.4-3.0.

G. Preparation of RBC Ghosts, Spectrin, and Brain Homogenate.

RBC ghosts were prepared from bovine blood by using the method of Dodge et al. (1963). Bovine brain microsomal membranes were prepared from bovine brain tissue by using the method of Burridge et al. (1982). Spectrin was purified from RBC ghosts by using the method of Marchesi and Steers (1968), or more recently the method of Speicher et al. (1980). Purified rabbit skeletal muscle actin was obtained from Sigma Chemical Company. Protein concentration was measured by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

H. Sources of Antibodies.
(1) **Monoclonal Antibodies**

The hybridoma cell technique of Köhler and Milstein (1975) was used to prepare monoclonal antibodies against ROS proteins. Monoclonal antibodies designated as 4B2 (MacKenzie and Molday, 1982), rho-1D4 (Molday and MacKenzie, 1983), and 3A6 (MacKenzie and Molday, unpublished result) were obtained from hybridoma cell lines generated by fusion of NS-1 mouse myeloma cells (a generous gift of Drs. Robert McMaster and Michael Weaver) with lymphocytes from mice immunized with ROS membranes.

A monoclonal antibody designated as 2C2 (MacKenzie and Molday, unpublished result) was obtained from a hybridoma cell line generated by fusion of NS-1 mouse myeloma cells with lymphocytes from a mouse immunized with a neuroblastoma cell membrane preparation. Monoclonal antibodies designated as 2A4 and 1H5 (Jarausch and Molday, unpublished results) were obtained from hybridoma cell lines generated by fusion of NS-1 mouse myeloma cells with lymphocytes from a mouse immunized with 1% Triton X-100-insoluble ROS proteins.

Monoclonal antibody designated as 1D1 was obtained from a hybridoma cell line generated by fusion of NS-1 mouse myeloma cells with lymphocytes from a mouse immunized with an urea extract of ROS membrane preparation. The immunization and cell fusion protocols are briefly described as follows: A female BALB/c mouse was immunized 3 weeks apart with two intraperitoneal injections of 10 μg of urea extractable ROS
proteins emulsified in 0.2 mL of Freund's complete adjuvant. The mouse was boosted 30 days after immunization with a similar intraperitoneal injection. The spleen of the immunized mouse was removed four days after the booster injection and used for cell fusion.

(2) **Cell Fusion**

Approximately $5 \times 10^7$ spleen cells were fused with $1 \times 10^7$ NS-1 mouse myeloma cells in 1.0 mL of 50% polyethylene glycol as described by Galfre et al. (1977). Briefly, the spleen and NS-1 cells were mixed in a 50-mL centrifuge tube and centrifuged at 1,500 rpm for 5 min to form a tight pellet. The cell pellet was gently stirred while 1-mL of warm polyethylene glycol was added over a 1-min period. After stirring the tube for an additional min, 1-mL of IMDM medium was slowly added to the mixture, followed by the addition of 7-mL of IMDM medium over a 2-3 min period. Following fusion, the cells were centrifuged at 1,500 rpm for 5 min and suspended in 50 mL of IMDM medium containing 100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine, and 20% FCS (HAT medium) and $2.5 \times 10^8$ BALB/c feeder thymocytes. Aliquots (100 μL) of cell suspension were seeded into 96-well culture plates and maintained at 37°C in an atmosphere of 5% CO$_2$ in a humidified incubator.

Approximately half of the HAT medium was carefully removed by aspiration and replaced by fresh HAT medium several days later. Two weeks after the fusion day, HAT
medium was replaced by IMDM medium supplemented with 100 μM hypoxanthine, 16 μM thymidine, and 20% FCS. Fifty μL of medium was removed from wells with almost confluent hybridoma cell growth and tested several times for antibody production using the standard RIA procedure as described in Methods Section J. Antibody-secreting hybridoma (1D1) was cloned by limiting dilution, expanded and stored as described in Methods Section C.

(3) Polyclonal Antibodies

Rabbit anti-human RBC spectrin polyclonal antiserum was a gift of Dr. Rinehart Reithmeier. Rabbit anti-bovine RBC spectrin and rabbit anti-bovine RBC ankyrin polyclonal antisera (Penz and Wong, unpublished results) were obtained from two New Zealand Whites rabbits immunized with 4 biweekly injections of purified bovine RBC spectrin (100 μg Protein/injection) or partially purified RBC ankyrin (10 μg Protein/injection) emulsified in Freund's adjuvant according to the procedure of Cooper (1978). Briefly, 20-200 μg of antigen in 1 mL of PBS was mixed with equal volume of Freund’s complete adjuvant and taken up in a 2-mL glass syringe. The mixture was repeatedly passed through a syringe having a 20 G-1 sterile needle and then through a 23 G-1 sterile needle. The emulsified antigens were injected into rabbits subcutaneously over 6-10 sites on their backs. For the second and subsequent immunizations, Freund’s incomplete adjuvant was used to emulsify the antigens. Rabbits were
bled before immunization, and one week after the second and each subsequent immunizations. Preimmune and immune sera were obtained by allowing blood clots to form at room temperature for 1-2 h and then centrifuging the blood at 15,000 rpm for 20 min. Sera were stored in 3-mL aliquots at -70°C.

Culture fluid obtained from hybridoma cells cloned at least twice and polyclonal antisera were passed through a 0.22 μm filter (Millipore) or centrifuged at 4,000 rpm for 5 min to remove any cellular debris and used in indirect immunochemical and immunocytochemical studies as described in Methods Sections J-N.

(4) Tracer Second Antibodies

Goat anti-mouse Ig was partially purified from goat anti-mouse Ig antisera (Antibodies Incorporated) by ion exchange chromatography on a DEAE-Sephacel column using the procedure of Garvey et al. (1977). Goat anti-rabbit Ig was purified by affinity chromatography on a rabbit Ig-Sepharose 4B column as follows: Goat anti-rabbit Ig antiserum (Miles) was applied to a rabbit Ig-Sepharose 4B column prepared by the method of Cuatrecass (1970). The column was washed with 2 column volumes of 0.01 M sodium phosphate buffer, pH 7.0, to remove unbound proteins. Goat anti-rabbit Ig was eluted by washing the column with 3 M sodium thiocyanate (Baker), dialyzed against several changes of 0.01 M sodium phosphate
buffer, pH 7.0, lyophilized and stored at -20°C.

I. **Radioiodination of Proteins.**

Goat anti-mouse Ig, goat anti-rabbit Ig, RBC spectrin, Con A (Sigma Chemical Company), and Protein A (Pharmacia) were labelled with carrier-free $^{125}\text{I}$ (New England Nuclear) by using the chloramine T (Eastman Organic Chemicals) method as described by Hunter and Greenwood (1962). The iodinated proteins (sp. act. 1-2 X $10^6$ dpm/μg) were separated from free $[^{125}\text{I}]$iodide and other reactants as previously described by Molday and MacKenzie (1985). Briefly, the reaction mixture was diluted with 0.5 mL of PBS and centrifuged through 0.5 g of AG1X10-C1 ion exchange resin (Bio-Rad) placed in the reservoir of a 0.2-μm microfiltration device (Schleicher and Schuell).

J. **Solid-Phase Radioimmune and Competitive Inhibition Assays.**

The binding of monoclonal antibodies to muscle actin, ROS membranes and RBC spectrin was measured by using indirect solid-phase RIA as previously described by MacKenzie et al. (1984). Briefly, bleached ROS membranes, RBC spectrin, or actin were solubilized with 1% Triton X-100, and 25 μL of a 0.25 mg/mL solution was dried onto flexible vinyl microtiter wells at 60°C. The wells were rinsed with water and incubated in RIA buffer (PBS containing 1% BSA, 1% FCS, and 0.1% sodium azide) for 60 min. The wells were then rinsed in
PBS and incubated with 25 μL of serially diluted hybridoma culture fluid containing antibody for 60 min at 23°C. Finally, the wells were rinsed in PBS and incubated with 25 μL of 125I-labelled goat anti-mouse Ig [10-30 μg/mL; (1-2)X10^6 dpm/μg] in RIA buffer for 30-60 min at 23°C. The plates were then rinsed in PBS and individual wells were counted in a Beckman 8000 gamma counter.

The binding of polyclonal rabbit anti-human RBC spectrin antibodies was measured by a similar procedure. Variable amounts of Triton X-100 treated ROS membrane proteins, bovine RBC spectrin, bovine RBC ghosts, or BSA were dried onto microtiter wells. In the first step the wells were treated with 25 μL of polyclonal rabbit anti-human RBC spectrin antibodies. After 30 min, the wells were rinsed and incubated with 25 μL of 125I-labelled Protein A [30 μg/mL; (1-2)X10^6 dpm/μg]. The wells were rinsed in PBS, and the radioactivity was measured as described above.

The effect of ROS membranes, RBC ghosts, and brain microsomal membranes on the binding of 4B2 monoclonal antibody to Triton X-100 solubilized ROS was studied by using RIA competitive inhibition assay previously described by Molday and MacKenzie (1983). Briefly, 25 μL of varying concentrations of 0.1% Triton X-100 treated bovine ROS membranes, bovine brain microsomal membranes, or bovine RBC ghosts in RIA buffer was incubated at 23°C with 25 μL of 4B2 hybridoma culture fluid diluted to a concentration that gave
80-90% saturation of binding by solid-phase RIA. After a 60-min incubation at 23°C, 25 μL of the mixture was removed and screened for remaining antibody activity by solid-phase RIA employing Triton X-100 solubilized ROS membranes as the immobilized antigen and ¹²⁵I-labelled goat anti-mouse Ig as a tracer second antibody.

K. SDS-Polyacrylamide Gel Electrophoresis and Gel Transfer.

Samples were solubilized in an equal volume of denaturing solution containing 5% SDS, 40% sucrose, 10 mM Tris, pH 6.8, 10% 2-mercaptoethanol (Eastman Organic Chemicals), and 4% bromophenol blue. Samples (10 μL) were applied to a 6% polyacrylamide (the ratio of acrylamide to N-N’methylene bisacrylamide is 30 to 0.8) minislab gel (0.75 mm thickness X 3.0 cm length) and electrophoresis was carried out by using the buffer system of Laemmli (1970). In one experiment, a 5-15% polyacrylamide gradient slab gel (0.75 mm thickness X 12.0 cm length) with 25% of regular N-N’methylene bisacrylamide concentration was used to increase the resolution of proteins in the 200,000 to 260,000 M₉ range.

Gel slices were either stained with Coomassie blue (Fairbanks et al., 1971) or silver (Wray et al., 1981) or subjected to electrophoretic transfer. In the latter procedure unstained SDS-polyacrylamide gels were washed over 20 min with two 50-mL changes of transfer buffer: 20 mM Tris-acetate, pH 7.4, containing 2 mM Na₂EDTA and 0.01% SDS.
Proteins were electrophoretically transferred from SDS-polyacrylamide gels to nitrocellulose paper (Towbin et al., 1979) in transfer buffer at 400 mA for 2-4 h in a Hoefer Transblot (Model TE 22) apparatus.

After gel transfer, nitrocellulose paper was stained with 0.025% Amido black (Merck) in 22.5% ethanol and 7.5% acetic acid in order to detect transferred proteins. The nitrocellulose paper was then incubated at 23°C for 1 h in immunoblot buffer: 0.15 M NaCl, 10 mM sodium phosphate, 1 mM Na₂EDTA, 1 mM sodium azide, 0.2% Triton X-100, and 2% BSA. The quenched paper was incubated with either 5-10 mL of hybridoma culture fluid or polyclonal rabbit anti-human or anti-bovine RBC spectrin antibodies for 30 min at 23°C and then rinsed 5 times with immunoblot buffer (without BSA), once with 2 M urea, 0.1 M glycine, and 1% Triton X-100, and once with PBS. After being washed, the paper was incubated with 5 mL of ¹²⁵I-labelled goat anti-mouse Ig (1.8 X 10⁶ dpm/µg; 2.3 µg/mL) or ¹²⁵I-labelled Protein A (0.8 X 10⁵ dpm/µg) in immunoblot buffer for 30 min at 23°C. Finally, the paper was washed as described and dried for autoradiography on Kodak Royal X-Omat film with an X-ray intensifying screen. For most experiments, exposure time was 1-3 days, but in the case of ¹²⁵I-labelled Protein A binding to ROS membranes, exposure time was increased to 5-7 days. In some experiments, quenched nitrocellulose papers were labelled with ¹²⁵I-labelled Con A (specific activity= 7.9 X 10⁵ dpm/µg).
In the case of 1D1 labelling of ROS membrane proteins, an immunostaining method according to the procedure of O'Connor and Ashman (1982) was used. Briefly, nitrocellulose paper containing electrophoretically ROS membrane proteins was quenched in 5% gelatin (Sigma) in PBS for 1 h. The paper was washed several times with 0.05% (v/v) Tween 20 in PBS for a period of 20 min and incubated with 1D1 cell culture supernatant for 2 h. The paper was again washed several times with 0.05% Tween 20 in PBS and then incubated with goat anti-mouse Ig-alkaline phosphatase conjugates (Sigma) in 0.2 M sodium barbital buffer, pH 9.5, for 2 h. The paper was washed several times with 0.05% Tween 20 in PBS for a period of 20 min and once with 0.2 M sodium barbital buffer, pH 9.5.

Before stain development, the paper was incubated with 5 mM of MgCl₂ in 0.2 M sodium barbital buffer for 15 min. The stain was developed by sequentially adding the substrate for alkaline phosphatase 5-bromo-4-chloro-3-indoxyl-phosphate (50 μg/mL) in dimethylformamide and nitro blue tetrazolium salt (100 μg/mL) in sodium barbital buffer, pH 9.5. Stain development was stopped by washing the paper with 0.05% Tween 20 in PBS. Finally, the paper was air dried and photographed.

L. Size Exclusion Chromatography of ROS Proteins on a Sepharose-CL 2B Column.

ROS membranes prepared from freshly-dissected bovine
retina as described in Methods Section F were centrifuged at 15,000 rpm for 30 min. ROS pellet (5 mg Protein) was solubilized in 1% SDS, 10 mM Tris-HCl, 0.5 mM 2-mercaptoethanol, 0.5 mM EDTA, 0.03 mM phenylmethylsulfonyl fluoride, and 0.02 % NaN₃, pH 8.5 and applied to a Sepharose-CL 2B column (1 cm in diameter and 42 cm in length), pre-equilibrated with the same buffer with 0.1% SDS. The column was eluted with the same buffer containing 0.1% SDS at a flow rate of 0.5 mL/min. Fractions (0.5 mL) were collected and assayed for absorbance at 280 nm. Fractions numbered 18, 20, 22, 24, 26 and 28 were analyzed by SDS-polyacrylamide gel electrophoresis and the immunoblotting technique before and after storage at 4°C for 7 days.

M. Lowicryl Thin Section Labeling.

Bovine retina was fixed in 1.25% glutaraldehyde (Merck) in 0.1 M cacodylate buffer, pH 7.2, and 0.2 M sucrose for 30-60 min at 23°C. After washing in the same buffer for 1 h, the tissue was cut into 1-mm² pieces. The samples were serially dehydrated in dimethylformamide (50%, 70%, 90%, and 100%) at -35°C and embedded in Lowicryl K4M medium at -35°C. Tissue was carefully oriented in gelatin capsules and polymerized under long wavelength ultraviolet irradiation overnight according to the method of Roth et al. (1981). Sections were cut, collected on clean copper grids, and preincubated in 50 μL of PBS with 0.1% BSA for 10 min to quench nonspecific binding sites. The grids were then
incubated in 50 μL of 4B2 hybridoma culture fluid for 30 min at 23°C, followed by extensive washing in PBS with 0.1% BSA. Finally, the grids were incubated in 50 μL of goat anti-mouse Ig gold-dextran (Hicks and Molday, 1986) for 30 min at 23°C. The grids were washed extensively in PBS, stained with saturated uranyl acetate and lead citrate and viewed under a Philips 200 or a JEOL 1200 EX electron microscope.

N. Extraction of ROS Membranes.

Four samples of dark adapted ROS membranes (6 mg protein/tube), prepared from fresh retinas as described in Methods Section F, were washed twice in 20 mM Tris buffer, pH 7.4, by centrifugation at 15,000 rpm in a SS-34 Sorvall rotor for 30 min. Each ROS pellet sample was resuspended with only one of the following solutions: 6 M ultrapure urea (Schwarz and Mann) in 20 mM Tris-acetate, pH 7.4; 20 mM Tris-acetate, pH 7.4; 0.3 mM ATP and 50 mM 2-mercaptoethanol in 0.3 mM sodium phosphate, pH 8.0; and 1 mM EDTA in 0.3 mM sodium phosphate, pH 8.0. The former sample was left for 24 h at 4°C while the latter three samples were dialyzed against their respective solutions for 24 h at 4°C. The mixtures were then centrifuged at 25,000 rpm in a SW-27 rotor for 1 h. Following centrifugation, the supernatant and the resuspended ROS pellet were dialyzed against 20 mM Tris buffer, pH 7.4. The dialyzates were subjected to SDS-polyacrylamide gel electrophoresis and immunoblot analysis as described in Methods Section K.
O. **Triton X-100 Extraction of ROS.**

Dark adapted ROS membranes prepared from 45 freshly dissected retinas as described in Methods Section F were washed twice with 20 mM Tris-acetate and 20 mM NaCl, pH 7.4, by centrifugation at 15,000 rpm in a SS-34 Sorvall rotor for 30 min. ROS pellet (5 mg protein) was resuspended in 5 mL of Tris buffer, pH 7.4, containing 75 mM KCl and 1% Triton X-100. Suspension was shaken continuously for 24 h at 23°C and then centrifuged at 15,000 rpm in a SS-34 Sorvall rotor for 30 min. The Triton X-100 pellet was resuspended in 0.5 mL of Tris buffer containing 75 mM KCl and 1% Triton X-100. Protein concentrations of the supernatant and pellet were determined by the method of Lowry et al. (1951). Equal volume of both fractions were subjected to SDS-polyacrylamide gel electrophoresis and immunoblot analysis as described in Methods Section K.

P. **Detergent Solubilization of ROS Membranes.**

Dark adapted ROS membranes, prepared from 100 frozen retinas as described in Methods Section F, were washed twice with 20 mM Tris-acetate and 20 mM NaCl, pH 7.4, by centrifugation at 15,000 rpm in a SS-34 Sorvall rotor for 30 min. ROS pellet (5 mg protein was resuspended in either 1 mL of 1% Triton X-100, or 1% SDS, or 1% octylglucoside, or 1% sodium cholate, or 1% (3-[(3-chloamidopropyl)dimethylammonio]
1-propanesulfonate) also known as CHAPS in Tris buffer, pH 7.4, containing 75 mM KCl. Suspensions were shaken continuously for 24 h at 23 °C and then centrifuged at 15,000 rpm in a SS-34 Sorvall rotor for 30 min. Supernatants were removed and pellets were subjected to SDS-polyacrylamide gel electrophoretic analysis as described in Methods Section K.

Q. Negative Staining of Triton X-100 Extracted ROS Pellet.

Triton X-100 extracted ROS prepared as described in Methods Section Q were resuspended in 0.1 ml of 75 mM KCl and 20 mM Tris-acetate, pH 7.4. Fifty μL of each samples was fixed with 100 μL of 1.25% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, and washed three times with the same buffer by centrifugation at 15,000 rpm in a SS-34 Sorvall rotor for 30 min. Pellet was resuspended in cacodylate buffer.

Formvar grids previously coated with 50 μL of 0.1% polylysine for 30 min and rinsed extensively in water were treated with 50 μL of the above sample solution for 1 h. After extensive washing in water, the grids were treated with 50 μL of 1% sodium phosphotungstate, pH 7.2, for 20-25 sec. Excess stain was removed by blotting the grids on their edges with filter paper. Grids were air dried before being viewed under a JEOL 1200 EX electron microscope.
A. High Molecular Weight Polypeptides of ROS Membranes.

When ROS membranes were subjected to SDS gel electrophoresis and stained with Coomassie blue, rhodopsin having an apparent $M_r$ 34,000 was observed as the major band and ROS 1.2 of apparent $M_r$ 220,000 was the second most intense band (Fig. 8). Bands faintly visible by Coomassie blue staining were intensified by using silver staining and several bands in the molecular weight range of 200,000-240,000 were seen.

The binding specificity of ConA and 4B2 for ROS polypeptides was determined by treating polypeptides transferred to nitrocellulose paper directly with $^{125}$I-labelled ConA or indirectly with 4B2 antibody followed by $^{125}$I-labelled goat anti-mouse Ig. With $^{125}$I-labelled spectrin as an external molecular weight reference, $^{125}$I-labelled ConA was found to label rhodopsin and ROS 1.2 at 220,000 as previously shown (MacKenzie and Molday, 1982). The 4B2 antibody, however, labelled a polypeptide chain of a slightly higher molecular weight (Fig. 8). This band had the same electrophoretic mobility as the $\alpha$ subunit of RBC spectrin and an apparent $M_r$ 240,000. A faintly labelled band at $M_r$ 220,000 was also seen upon prolonged autoradiographic exposure. In some preparations, bands in the molecular weight range of 95,000-150,000 were also labelled with the 4B2 antibody. These bands appear to represent proteolytic
Figure 8. SDS gel electrophoresis and immunoblots of ROS disc membranes. SDS-solubilized ROS disc membranes (20 μg/well) and 125-I-labelled bovine RBC spectrin (6 μg/well) were subjected to SDS gel electrophoresis on a 6% polyacrylamide slab gel. Gels were either stained with Coomassie blue (CB) or silver stain (SS) or transferred to nitrocellulose paper. Transfer papers were either directly labelled with 125I-labelled Con A or indirectly labelled with undiluted 4B2-antibody culture fluid and 125I-labelled goat anti-mouse Ig for autoradiography. ROS membrane proteins are shown in lane a, and 125I-labelled spectrin used as an external molecular weight marker for autoradiography is shown in lane b.
fragments of the $M_r$ 240,000 polypeptide (see below).

B. Degradation of 4B2-Specific Protein by an Endogenous Protease.

The sensitivity of the 4B2-specific protein and ConA-specific glycoproteins to degradation by an endogenous protease in ROS preparations was detected by immunoblotting analysis. As shown in Figure 9, the 4B2 antibody labelled only one major polypeptide of $M_r$ 240,000 in freshly prepared ROS membranes. When the ROS membranes were maintained at 4°C for 24 h, partial degradation of the $M_r$ 240,000 polypeptide to polypeptides of apparent $M_r$ 150,000, 120,000, and 95,000 was observed. In contrast, no degradation of rhodopsin or ROS 1.2 was detected by ConA labelling or by Coomassie blue staining. ROS disc membranes prepared by hypotonic lysis of freshly prepared ROS and flotation on 5% Ficoll were less prone to degradation under these conditions. Significant degradation of the 4B2-specific polypeptide was generally observed when ROS were prepared from frozen retina. Several protease inhibitors such as aprotinin, leupeptin, and phenylmethylsulphonyl fluoride, and a metal chelator EGTA have been found to be ineffective in preventing the proteolytic degradation of the 4B2-specific polypeptide since immunoblots of 4B2 labelling of ROS membrane proteins prepared in the presence of these inhibitors showed a band at $M_r$ 150,000 (not shown).

Size-exclusion chromatography of SDS-solubilized ROS
Figure 9. Proteolytic degradation of the 4B2-binding protein of ROS by endogenous proteases. ROS membranes prepared from freshly dissected retinas were either directly subjected to SDS gel electrophoresis (gel a) or stored at 4°C for 24 h prior to SDS gel electrophoresis (gel b). The gels were either stained with Coomassie blue (CB) or transferred to nitrocellulose paper and labelled with undiluted 4B2-antibody culture fluid and ¹²⁵I-labelled goat anti-mouse Ig or with ¹²⁵I-labelled Con A.
proteins on a Sepharose 2B column was attempted to separate the 4B2-specific protein from the endogenous protease. The protein components of the column fractions were analyzed by SDS gel electrophoresis and immunoblotting. As shown in Fig. 10, Coomassie blue staining of elutants after gel electrophoresis indicated the high molecular weight glycoprotein or ROS 1.2 with apparent $M_r$ 220,000 was well separated from rhodopsin with apparent $M_r$ 34,000. Two bands with apparent $M_r$ of 55-60,000 were most noticeable in fractions numbered 18, 20, and 28. This doublet appeared to be an artefact because it was present even in gel lanes that did not contain any column fraction sample. Immunoblots of the fractions before and after storage at 4°C for 7 days showed the intensity of bands was higher in immunoblot C than in immunoblot B due to differences in exposure time. However very similar 4B2 antibody labelling patterns were observed. This indicated that the 4B2-specific polypeptide with apparent $M_r$ 240,000 and its proteolytic fragments were not further degraded to smaller fragments to any significant extent during the storage period.

C. Urea Extraction of the 4B2-Specific Protein.

The extractability of the 4B2-specific protein and ConA-specific membrane glycoproteins with urea was studied to determine their interaction with the lipid bilayer. ROS disc membranes were treated with 6 M urea overnight and
Figure 10. Size exclusion Chromatography of SDS-solubilized ROS proteins on a Sepharose 2B column. ROS membranes prepared from freshly dissected bovine retina as described in Methods Section F were centrifuged at 15,000 rpm for 30 min. ROS pellet (5 mg Protein) was solubilized in 1% SDS, 10 mM Tris-HCl, 0.5 mM 2-mercaptoethanol, 0.03 mM phenylmethylsulfonylfluoride, 0.5 mM EDTA, and 0.02 % NaN₃, pH 8.5 and applied to a Sepharose-CL 2B column (1 cm in diameter and 42 cm in length), pre-equilibrated with the same buffer with 0.1% SDS. The column was eluted with the same buffer with 0.1% SDS at a flow rate of 0.5 mL/min. Fractions (0.5 mL) were collected and assayed for absorbance at 280 nm. Fractions numbered 18, 20, 22, 24, 26 and 28 were analyzed by SDS-polyacrylamide gel electrophoresis (Lanes 1-6 respectively). Gels were either stained with Coomassie blue (gel A) or transferred to nitrocellulose paper. Transfer papers were sequentially labelled with 4B2 cell culture supernatant and 125I-labelled goat anti-mouse Ig for autoradiography (gel B). The same fractions were again analyzed by gel electrophoresis and immunoblotting technique after they have been stored at 4°C for 7 days (gel C).
subsequently separated into a pellet and supernatant fraction by centrifugation at 25,000 rpm for 1 h.

Analysis of these fractions by SDS gel electrophoresis and immunoblotting indicated that a small quantity (5-10%) of the 4B2-specific $M_r$ 240,000 polypeptide and proteolytic fragments was extracted from the membrane (Figure 11). Extraction of the 4B2-binding protein by urea was reproducibly observed in three separate experiments. However, under these same conditions, no extraction of rhodopsin or ROS 1.2 into the supernatant was detected by $^{125}$I-labelled ConA blotting or Coomassie blue staining.

D. **Immunological Cross-Reactivity of Monoclonal Antibody 4B2 with Proteins from Other Cell Types.**

The presence of 4B2-binding proteins in cell types other than the rod photoreceptors was investigated by RIA competitive inhibition assays. As shown in Figure 12, both Triton X-100 solubilized bovine RBC ghosts and bovine brain microsomal membranes in addition to bovine ROS were able to inhibit the binding of the 4B2 antibody to Triton X-100 solubilized disc membranes immobilized on microtiter plates. Fifty percent inhibition of 4B2 antibody binding was attained at total protein concentrations of 300 and 400 $\mu$g/mL for brain membranes and RBC ghosts, respectively. ROS was a more effective inhibitor, requiring a 3-4 fold lower concentration to achieve 50% inhibition of 4B2 antibody binding. Accurate quantitative analysis of cross-reactivity, however, could not
Figure 11. Extraction of the 4B2-binding protein from ROS membranes by urea. ROS membranes were treated with 6 M urea at 4°C for 24 h. The membranes were then sedimented at 25,000 rpm for 1 h. The pellet was resuspended in 20 mM Tris-acetate, pH 7.4, and dialyzed against the same buffer for 24 h. The supernatant and pellet were solubilized in SDS and subjected to SDS gel electrophoresis on 6% polyacrylamide gels and to electrophoretic transfer. The supernatant (gel S) and the pellet (gel P) were labelled with either 4B2 antibody and 125I-labelled goat anti-mouse Ig or 125I-labelled Con A.
Figure 12. Competitive inhibition of 4B2 antibody binding to ROS membrane proteins by RBC membrane ghosts and brain microsomal membranes. Inhibition of 4B2 antibody binding to Triton X-100 solubilized, immobilized ROS proteins by Triton X-100 solubilized bovine ROS (solid circles), Triton X-100 solubilized bovine brain microsomal membranes (open circles), Triton X-100 solubilized bovine RBC membrane ghosts (solid triangles), and bovine serum albumin (open triangles).
be made since the quantity of cross-reacting antigen in these preparations was not known. Bovine serum albumin serving as a control did not inhibit 4B2 antibody binding. Competitive inhibition studies also indicated that human RBC ghosts bound the 4B2 antibody, but with a lower affinity than bovine RBC ghosts (not shown).

E. Identification of Spectrin as the RBC Antigen for the 4B2 Monoclonal Antibody.

Solid-phase RIA and immunoblot analysis were used to identify spectrin as the 4B2-binding protein of RBC. Figure 13 shows the effect of 4B2 antibody and rho-1D4 antibody dilution on the binding of these antibodies to purified bovine RBC spectrin immobilized on microtiter plates. The 4B2 antibody exhibited significant saturable binding. In contrast, rho-1D4 (an antibody that binds to the C-terminal region of rhodopsin) did not bind even at high antibody concentrations.

The subunit of bovine RBC spectrin that binds the 4B2 antibody was determined by sequentially labelling spectrin subunits separated by SDS gel electrophoresis with the 4B2 antibody and $^{125}\text{I}$-labelled goat anti-mouse Ig. As shown in Figure 14, the 4B2 antibody predominantly labelled the $\alpha$ subunit of bovine spectrin having an apparent $M_r$ 240,000 (Branton et al., 1981). In contrast, rho-1D4 did not label either subunit of bovine RBC spectrin. With prolonged exposure conditions, the 4B2 antibody was found to weakly
Figure 13. Binding of 4B2 and rho-1D4 antibodies to RBC spectrin. The effect of 4B2 and rho-1D4 antibody dilution on binding to bovine spectrin. Triton X-100 treated bovine RBC spectrin immobilized in microtiter wells was incubated with serial dilutions of culture fluid from either 4B2 or rho-1D4 hybridoma cells, rinsed and treated with $^{125}$I-labelled goat anti-mouse Ig.
Figure 14. SDS gel electrophoresis and immunoblots of bovine RBC spectrin. Bovine spectrin (2 µg/well) was subjected to electrophoresis on a 6% SDS-polyacrylamide slab gel and either stained with Coomassie blue (CB) or transferred to nitrocellulose paper. The transfer papers were treated with either 4B2 or rho-1D4 antibody and 125I-labelled goat anti-mouse Ig and were subjected to autoradiography. The α and β subunits of spectrin are indicated.
react with a band at \( M_r \) 220,000 (not shown).

F. Characterization of 3A6 Monoclonal Antibody.

A hybridoma cell line referred to as 3A6 was previously shown to secrete antibodies specific for disc membrane preparations by solid-phase RIA. Initial immunoblot analysis indicated that the 3A6 antibody labelled several ROS polypeptides in the \( M_r \) range of 100,000 to 220,000 (MacKenzie and Molday, unpublished results). Further characterization of this antibody was carried out to determine whether this antibody is similar to the 4B2 monoclonal antibody.

The 3A6 cell line was recloned and used to produce antibody-containing cell culture supernatants and ascites fluids. The effect of dilution of the 3A6 cell culture supernatant and ascites fluid on the binding of antibody to Triton X-100 solubilized ROS membrane proteins using an indirect solid-phase RIA is shown in Figure 15. Typically, half-maximum binding occurred at reciprocal dilutions of 8-16 for 3A6 cell culture supernatants and 500-1000 for 3A6 ascites fluids.

G. Binding of 3A6 Monoclonal Antibody to RBC Spectrin and ROS Membranes.

The polypeptides in bovine ROS and bovine RBC spectrin serving as antigens for the 3A6 monoclonal antibody were
Figure 15. Titration of 3A6 hybridoma cell ascites fluid (squares), 3A6 hybridoma cell culture supernatant (diamonds), and culture medium (triangles) against Triton X-100-solubilized ROS membranes. Solubilized ROS membranes immobilized in microtiter wells were treated with serial dilutions of culture supernatants or ascites fluid, rinsed and subsequently treated with $^{125}$I-labelled goat anti-mouse Ig.
identified by immunoblotting. As illustrated in Figure 16, the 3A6 antibody only labelled the $\beta$ band of RBC spectrin with apparent $M_r$ 220,000. In contrast the 4B2 antibody only labelled the $\alpha$ band of RBC spectrin with apparent $M_r$ 240,000 as previously indicated (Fig. 14). In ROS membranes, the 3A6 antibody labelled five ROS polypeptides with apparent $M_r$ 240,000, 220,000, 160,000, 150,000, and 80,000. The $M_r$ 240,000 polypeptide was the most intensely labelled band. The smaller polypeptides are believed to be the degraded fragments of the $M_r$ 240,000 polypeptide since the smaller polypeptides were more intensely labelled than the $M_r$ 240,000 polypeptide in some experiments. The 4B2 antibody also labelled several polypeptides with similar but not identical $M_r$. Interestingly the largest polypeptide in ROS labelled by the 4B2 antibody had an apparent $M_r$ 260,000 instead of 240,000 as previously determined. This difference in $M_r$ is most likely due to the higher resolving power of the more porous or less crosslinked polyacrylamide gradient gel used in this study. This result suggests that 3A6 and 4B2 antibodies are specific for two different ROS proteins.

H. Binding of Polyclonal Anti-Spectrin Antibodies to ROS Proteins.

In order to substantiate the findings that indicate an immunological equivalent of RBC spectrin exists in bovine ROS, polyclonal rabbit anti-human and anti-bovine RBC spectrin were tested for cross-reactivity to ROS membrane
Figure 16. SDS gel electrophoresis and immunoblots of ROS membranes. SDS-solubilized ROS membrane proteins (40 μg/well) and bovine RBC spectrin (6 μg/well) were subjected to SDS gel electrophoresis on a 5-15% polyacrylamide (acrylamide : bisacrylamide ratio was 150) gradient slab gel. Gels were either stained with Coomassie blue (CB) or transferred to nitrocellulose paper. Transfer papers were indirectly labelled either with undiluted 3A6 antibody or 4B2 antibody culture fluid and ¹²⁵I-labelled goat anti-mouse Ig for autoradiography. Lane a- ROS membrane proteins. Lane b- spectrin. Arrows indicate the bands of highest apparent Mr that were labelled with 3A6 and 4B2 antibodies.
proteins. The binding of polyclonal rabbit anti-human RBC spectrin to bovine RBC spectrin and bovine ROS was determined by the solid-phase RIA using $^{125}\text{I}$-labelled Protein A for detection. As shown in Figure 17, a linear increase in binding of the anti-spectrin antibody was observed when increasing concentrations of bovine spectrin or ROS membranes were dried onto the assay plates. This indicated that neither the primary antibody nor the $^{125}\text{I}$-labelled protein A was limiting under the conditions of this assay. For a given amount of protein, the anti-spectrin antibody was found to bind over 100 times greater to purified spectrin compared to total ROS membrane protein. This suggests that the ROS spectrin makes up less than 1% of the ROS membrane protein.

The immunoblotting technique was used to identify the polypeptides serving as antigens for the anti-spectrin antibody. As illustrated in Figure 18, the anti-spectrin antibody labelled with equal intensity the $\alpha$ and $\beta$ bands of bovine RBC spectrin. Faint labelling of a band near the top of the gel could also be seen. This may be undissociated $\alpha$-$\beta$ dimer. In ROS membranes, the anti-spectrin antibody predominantly labelled a $M_r$ 240,000 polypeptide having the same mobility as the $\alpha$ chain of spectrin. Two less intense bands were observed in the molecular weight range of 210,000-220,000. In addition, a band at $M_r$ 150,000 was also labelled with the anti-spectrin antibody. This same band is also labelled when ROS membranes are labelled with the 4B2 antibody and most likely represents the major proteolytic
Figure 17. The binding of rabbit anti-human RBC spectrin antibodies to purified bovine spectrin or ROS membrane proteins. (A) Variable amounts of RBC spectrin (squares) and (B) variable amounts of ROS membrane protein (solid circles) or BSA (open circles) were dried onto microtiter plates and sequentially labelled with rabbit anti-spectrin antibodies and $^{125}$I-labelled Protein A.
Figure 18. Immunoblots of bovine RBC spectrin and ROS membrane proteins labelled with polyclonal anti-human spectrin antibodies. Purified bovine RBC spectrin (6 μg) and ROS membrane proteins (30 μg) were subjected to SDS gel electrophoresis on 6% gels and transferred to nitrocellulose paper. The papers were sequentially labelled with rabbit anti-human RBC spectrin antibodies and 125I-labelled protein A. (Gel a) Autoradiograph of bovine RBC spectrin exposed for 5 h. (Gel b) Autoradiograph of ROS membrane proteins exposed for 7 days.
fragment of the $M_r$ 240,000 polypeptide.

An almost identical labelling pattern was obtained when polyclonal rabbit anti-bovine RBC spectrin was used instead of polyclonal rabbit anti-human RBC spectrin (Fig. 19). The only difference was that a band at $M_r$ 150,000 in the ROS membranes was not labelled with this anti-spectrin antibody. This further supports the suggestion that the $M_r$ 150,000 polypeptide may be the degraded fragment of the $M_r$ 240,000 polypeptide.

I. Extraction of 4B2-Specific Protein under Low Ionic Strength Conditions.

Spectrin along with actin and band 4.1 can be selectively eluted from erythrocyte membranes in low ionic strength buffer as described by Marchesi and Steers (1968) and Furthmayr and Timpl (1970). Approximately 70-90% of total spectrin could be extracted using low ionic strength buffers (Gratzer, 1982). Therefore the methods used for extracting spectrin from RBC membrane ghosts were tested for their efficiency in extracting the 4B2-specific protein from ROS membranes. ROS membranes were dialyzed against 0.3 mM sodium phosphate (pH 8) containing either 0.3 mM ATP and 50 mM 2-mercaptoethanol or 1 mM EDTA, for 24 h at 4°C and subsequently separated into a pellet and supernatant fraction by centrifugation at 25,000 rpm for 1 h. In control studies, ROS membranes were dialyzed against 20 mM Tris-acetate, pH 7.4.
Figure 19. Immunoblots of bovine RBC spectrin and ROS membrane proteins labelled with polyclonal anti-bovine spectrin antibodies. Purified bovine RBC spectrin (6 µg) and ROS membrane proteins (30 µg) were subjected to SDS gel electrophoresis on 6% gels and transferred to nitrocellulose paper. The papers were sequentially labelled with rabbit anti-bovine RBC spectrin antibodies and ¹²⁵I-labelled protein A. (Gel A) Autoradiograph of bovine RBC spectrin exposed for 1 day. (Gel B) Autoradiograph of ROS membrane proteins exposed for 5 days.
Analysis of these fractions by SDS gel electrophoresis and immunoblotting indicated that no significant quantity of the 4B2-specific protein was extracted from the ROS membranes under low ionic strength conditions as compared to the extraction by 6M urea. As previously indicated (Fig. 10) a small quantity (5-10%) of the 4B2-specific protein with apparent Mr 240,000 was extracted by urea. Similarly, under these same conditions, no extraction of ROS 1.2 (Mr 220,000) into the supernatant was detected by 125I-labelled Con A blotting (Fig. 20). In control studies, 4B2 antibody and Con A labelling of the pellet fraction of untreated ROS membranes showed the presence of 4B2-specific protein and its degraded fragments and ROS 1.2, respectively.

J. Association of 4B2-Specific Protein with ROS Membranes

The relative amount of 4B2-specific protein that remains with the disc membranes after hypotonic lysis of ROS was investigated to determine the interaction of the 4B2-specific protein with the disc and plasma membranes. The difference in the concentration of 4B2 antigen between ROS and ROS disc membranes was detected by immunoblotting analysis and quantified by competitive inhibition assays.

When equal amounts of ROS and ROS disc membrane protein were separated by SDS gel electrophoresis and labelled with 4B2 antibody followed by 125I-labelled goat anti-mouse Ig,
Figure 20. Extraction of ROS membranes under low ionic strength conditions. ROS membranes were treated with (2) water, (3) 0.3 mM sodium phosphate ,pH 8, containing 0.3 mM ATP and 50 mM 2-mercaptoethanol, (4) 0.3 mM sodium phosphate ,pH 8, containing 1 mM EDTA, and (5) 6M urea at 4°C for 24 h. The membranes were then sedimented at 25,000 rpm for 1 h. The supernatants (lanes 2-5) and the pellet from the water extraction (lane 1) were solubilized in SDS and subjected to SDS gel electrophoresis on a 6% polyacrylamide slab gel. The bromophenol blue dye marker was allowed to run off the gel in order to increase the separation of proteins with high apparent molecular weights. After electrophoresis, the samples were electrophoretically transferred to nitrocellulose paper. The transfer papers were labelled either directly with 125I-labelled Con A or indirectly with undiluted 4B2-antibody culture fluid and 125I-labelled goat anti-mouse Ig.
the intensity of labelling of the 240 kDa polypeptide in ROS was greater than that in ROS disc membranes (Fig. 21).

The degree of inhibition of rho-1D4 and 4B2 antibodies binding to Triton X-100 solubilized ROS membranes by Triton X-100 solubilized ROS and ROS disc membranes is shown in Figure 22. Half-maximum inhibition of an anti-rhodopsin antibody (rho-1D4) binding occurred at 0.5 μg/mL for both ROS and ROS disc membranes, but half-maximum inhibition of 4B2 antibody binding occurred at 60 μg/mL for ROS and at 110 μg/mL for ROS disc membranes. This suggests that the concentration of 4B2-specific protein in ROS is about 2-fold greater than in ROS disc membranes preparation.

K. Immunocytochemical Labelling of Rod Cells with the 4B2 Antibody and Immunogold-Dextran Markers.

Localization of the 4B2-specific protein in ROS was determined by sequentially labelling Lowicryl thin sections of bovine retina tissue with the 4B2 antibody and goat anti-mouse Ig-gold-dextran conjugates. As shown in Figure 23, the gold particles were preferentially distributed along the periphery of the outer segments where the rims of the discs are adjacent to the plasma membrane. Gold particles were often observed to extend inward from the plasma membrane up to 50 nm. In the basal portion of the rod outer segment, gold particles were also observed to line up in the region where the rims of the discs are adjacent to the ciliary spine extending into the outer segment from the connecting cilium.
Figure 21. Immunoblots of ROS and ROS disc membrane proteins labelled with 4B2 and Con A. ROS and ROS disc membrane proteins (20 µg/well) were subjected to SDS gel electrophoresis on a 6% polyacrylamide slab gel and transferred to nitrocellulose paper. The papers were sequentially labelled with undiluted 4B2 antibody culture fluid and ¹²⁵I-labelled goat anti-mouse Ig or directly labelled with ¹²⁵I-labelled Con A. (Lane R) ROS membrane proteins. (Lane D) disc membrane proteins.
Figure 22. Inhibition of 4B2 antibody (A) and rho-1D4 antibody (B) binding to Triton X-100-solubilized ROS membranes by Triton X-100-solubilized ROS membranes (solid circles) and ROS disc membranes (solid squares) using the solid-phase indirect radioimmune competition assay.
Figure 23. Transmission electron micrographs of ROS labelled with immunogold-dextran markers. Thin sections of Lowicryl-embedded ROS of bovine retina were sequentially labelled with either rho-1D4 or 4B2 antibody and goat anti-mouse Ig-gold dextran particles (diameter of gold, 10-15 nm). For 4B2 antibody labelling, the gold particles are primarily distributed along the periphery of the ROS where the rims of the discs come in close proximity to the ciliary spine (CS) and plasma membrane (magnification 25,000 X). For rho-1D4 antibody labelling, the gold particles are distributed uniformly throughout the ROS (magnification 22,000 X).
(Hicks and Molday, 1985). A few gold particles can be found in the central area of the ROS. It is not clear if this represents a low concentration of the 4B2-binding protein in this region or, alternatively, residual nonspecific binding of the 4B2 antibody, an IgM immunoglobulin. In contrast, outer segments labelled with anti-rhodopsin monoclonal antibodies were densely and uniformly distributed over the entire ROS (Fig. 23) as previously shown by Hicks and Molday (1986). No labelling was observed when a nonreactive monoclonal antibody was used in the first labelling step.

L. Detergent Solubilization of ROS Membrane Proteins.

The solubility of ROS membrane proteins in Triton X-100 was investigated because Triton X-100 has been used to obtain the red cell cytoskeleton from RBC ghosts membrane preparation (Yu et al., 1973). This mild nonionic detergent selectively solubilized all the membrane glycoproteins (principally bands 3 and PAS 1-3). The detergent-insoluble fraction contains nonglycosylated polypeptides and cytoskeletal proteins (principally spectrin, actin, and band 4.1). Dark adapted ROS membrane proteins were treated with 1% Triton X-100 and subsequently separated into a supernatant and pellet fraction by centrifugation at 15,000 rpm for 30 min. The supernatant changed from red to yellow in color upon exposure to light due to bleaching of photopigment rhodopsin, while the pellet remained white.
Protein assays of both fractions indicated that approximately 95% of the total ROS membrane protein was solubilized by Triton X-100 and the remaining 5% of the total ROS membrane protein constituted the pellet fraction. Protein components in both fractions were analyzed by SDS gel electrophoresis and immunoblotting. As shown in Figure 24, Coomassie blue staining and $^{125}$I-labelled Con A labelling of polypeptides from both fractions indicated rhodopsin with apparent $M_r$ 34,000 and its aggregated forms with apparent $M_r$ 68K, 102K, 136K, and 170K are the major polypeptides in the supernatant fraction. ROS 1.2 with apparent $M_r$ 220,000 represents a minor protein component in this fraction. In contrast Coomassie blue staining of polypeptides in the pellet fraction showed a series of equally intense bands with $M_r$ ranges from 34,000 to 220,000. Also the intensity of labelling of rhodopsin and ROS 1.2 by $^{125}$I-labelled Con A is about the same in the pellet fraction.

These fractions were further analyzed by immunolabelling with 4B2 antibody and a monoclonal antibody (2B6) against peripherin, a rim-specific protein previously described by Molday et al. (1987). The 4B2-specific protein and its major degraded fragment were found in the supernatant fraction as shown by the labelling of a $M_r$ 240,000 polypeptide and a $M_r$ 150,000 polypeptide with the 4B2 antibody. However, only the undegraded 4B2-specific polypeptide was present in the pellet fraction. The 2B6 antibody intensely labelled a band at about 34,000, the
Figure 24. Immunoblots of Triton X-100 extraction of ROS membranes. ROS membranes (1 mg protein/mL) prepared from freshly-dissected retinas were solubilized with 1% Triton X-100 for 24 h at 4°C. ROS membranes were then sedimented at 15,000 rpm for 30 min. Supernatant (45 μg protein/well) and pellet (5 μg protein/well) were subjected to SDS electrophoresis on a 8% polyacrylamide slab gel (lanes a and b, respectively). After electrophoresis, the proteins were either stained with Coomassie blue (CB) or electrophoretically transferred to nitrocellulose paper. Transfer papers were either directly labelled with 125I-labelled ConA or indirectly labelled with 4B2 or 2B6 antibody and 125I-labelled goat anti-mouse Ig.
approximate $M_r$ of peripherin (Molday et al., 1987), in the supernatant fraction. A very faint band was labelled in the pellet fraction (Fig. 24). These results suggested that peripherin and rhodopsin are almost completely solubilized by Triton X-100 while a set of polypeptides including the 4B2-specific protein and ROS 1.2 are only partially soluble. Thus they are present in both the supernatant and pellet fraction.

The solubility of ROS membrane proteins in zwitterionic and ionic detergents was compared to that of Triton X-100 in order to determine whether the selective solubilization of ROS membrane proteins (principally rhodopsin) could be further improved. The pellet fractions obtained from centrifuging the detergent solubilized ROS membranes at 15,000 rpm for 30 min were analyzed by SDS gel electrophoresis. As shown in Fig. 25, Coomassie blue staining of polypeptides in the pellet fraction obtained from either CHAPS, or octylglucoside, or cholate solubilization of ROS membranes showed a series of bands with $M_r$ ranges from 34,000 to 220,000. This polypeptide staining pattern is also observed in the pellet fraction obtained from Triton X-100 solubilization of ROS membranes and in the pellet fraction of non-solubilized ROS membranes. However, the intensity of the rhodopsin band at apparent $M_r$ 34,000 was relatively less in the Triton X-100 pellet than the other pellet fractions with the exception of the SDS pellet fraction. In the case of SDS solubilization of ROS membrane proteins, no band in the
Figure 25. SDS gel electrophoresis of detergent-treated ROS pellet. ROS membranes (5 mg protein) prepared from frozen retinas were solubilized with (1) 1% Triton X-100, (2) 1% CHAPS, (3) 1% octylglucoside, (4) 1% sodium cholate, and (5) 1% SDS for 24 h at 23°C. ROS membranes were then sedimented at 15,000 rpm for 30 min. The pellets (lanes 1-5 respectively) were subjected to SDS gel electrophoresis on a 8% polyacrylamide slab gel. Following gel electrophoresis, the gel was stained with Coomassie blue. Lane 6- pellet obtained from untreated ROS membranes.
pellet fraction was observed with Coomassie blue staining. These results indicate that SDS does not solubilize ROS membrane proteins selectively, and Triton X-100 is better than the other detergents tested for selective solubilization of ROS membrane proteins (principally rhodopsin).

M. Electron Microscopy of Triton X-100 Extracted ROS Pellet.

Electron microscopy of the RBC cytoskeletal structure that remains after the ghost membrane is extracted with Triton X-100 showed a filamentous meshwork (Yu et al., 1973; Hainfeld and Steck, 1977). Recently, this same approach was attempted using ROS membranes to determine whether the Triton-extracted pellet consisted of a filamentous meshwork and could be detected by electron microscopy. Triton-extracted ROS pellet was fixed in glutaraldehyde and adsorbed onto polylysine-coated formvar grids. The pellet was then negatively stained and viewed under an electron microscope.

At low magnification, the electron micrographs from two separate experiments showed a network of fine filamentous material. At high magnification, the network appeared to consist of interconnecting filaments of variable length and a sheet of material surrounding the filaments (Fig. 26). The filamentous material was not observed when no sample was applied to grids. This result suggests that Triton-extracted ROS pellet may contain fibrous proteins.
Figure 26. Negative staining of Triton X-100 ROS pellet. ROS membranes prepared from freshly-dissected retinas were solubilized with 1% Triton X-100 for 24 h at 4°C. ROS membranes were then sedimented at 15,000 rpm for 30 min. The pellet was fixed with 0.8% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. Pellet was washed three times by centrifugation at 15,000 rpm for 30 min and resuspended in 0.1 M cacodylate buffer, pH 7.2. Sample solution was placed on polylysine-coated formvar grids. The grids were washed extensively in water and stained with 1% sodium phosphotungstate, pH 7.2. Electron micrographs of negatively stained Triton X-100 ROS pellet from two separate experiments (A) and (C). (B) and (D) are higher magnification of (A) and (C) respectively. Bars represent 2, 0.5, 2, 0.2 μm for (A)-(D) respectively.
N. Antibody Probes for Identifying Protein Components of ROS Cytoskeleton.

In order to identify the protein components which comprise the ROS cytoskeleton as observed by electron microscopy, several monoclonal and polyclonal antibodies were generated against actin and ankyrin (two components of the RBC cytoskeleton), urea-extractable and Triton X-100 insoluble ROS membrane proteins.

(1) **Anti-Actin Monoclonal Antibody**

The presence of actin in ROS membrane preparations was detected by solid-phase RIA. The effect of dilution of an anti-actin monoclonal antibody, referred to as 2C2 (MacKenzie and Molday, unpublished result), on the binding of the antibody to Triton X-100 solubilized ROS membrane preparation and purified actin is shown in Figure 27. The 2C2 antibody was active against actin and solubilized ROS membrane preparation. There was a higher degree of binding of 2C2 antibody to actin than to ROS membrane preparation. In control studies, rho-1D4 and 4B2 which are specific for rhodopsin and a \( M_r 240,000 \) polypeptide respectively were inactive against actin.

(2) **Polyclonal Anti-Ankyrin Antibodies**

The binding specificity of polyclonal rabbit anti-bovine RBC ankyrin antibodies to bovine RBC membrane ghosts
Figure 27. Binding of an anti-actin monoclonal antibody to ROS membranes and actin. Titration of 2C2, 4B2, and rho-1D4 hybridoma cell culture supernatants against Triton X-100 treated rabbit muscle actin (solid circles) and 2C2 hybridoma cell culture supernatant against Triton X-100 solubilized ROS membranes (solid squares). Solubilized ROS membranes and actin immobilized in microtiter wells were treated with serial dilutions of cell culture supernatant, rinsed, and subsequently treated with $^{125}$I-labelled goat anti-mouse Ig.
preparation was determined by immunoblotting. As shown in Figure 28 (Gel C), the anti-ankyrin antibodies intensely labelled two polypeptides with apparent $M_r$ 240,000 and 210,000. Two faintly labelled polypeptides with apparent $M_r$ 220,000 and 180,000 could also be seen. In contrast polyclonal rabbit anti-bovine RBC spectrin antibodies labelled two polypeptides with apparent $M_r$ 240,000 and 220,000 (Gel B). These results indicate polyclonal anti-ankyrin and anti-spectrin antibodies have different binding specificity. The polyclonal anti-spectrin antibodies have been shown to be specific for the $\alpha$ and $\beta$ chains of purified spectrin (Fig. 18). However, the polyclonal anti-ankyrin antibodies have not been tested against purified ankyrin. When the polyclonal anti-ankyrin antibodies were tested against ROS membrane proteins by immunoblotting, no bands were labelled even after prolonged exposure.

(3) Characterization of 1D1 Monoclonal Antibody

The 1D1 hybridoma cell line was generated from a fusion of NS-1 mouse myeloma cells with spleen cells from a mouse immunized with urea extractable ROS membrane proteins. This cell line secreted antibodies specific for ROS membrane preparations as determined by solid-phase RIA (not shown).

The binding specificity of 1D1 antibody for ROS polypeptides was determined by treating polypeptides transferred to nitrocellulose paper indirectly with 1D1 antibody followed by goat anti-mouse Ig-alkaline phosphatase
Figure 28. Immunoblots of bovine RBC membrane ghosts labelled with polyclonal anti-bovine ankyrin antibodies. RBC ghosts (20 μg protein/well) were subjected to SDS gel electrophoresis on a 8% polyacrylamide slab gel. After gel electrophoresis, the proteins were either stained with Coomassie blue (gel A) or electrophoretically transferred to nitrocellulose paper. Transfer papers were sequentially labelled with either polyclonal anti-bovine spectrin (gel B) or polyclonal anti-bovine ankyrin (gel C) antibodies and $^{125}$I-labelled Protein A.
conjugates. Immunostaining of the paper showed three bands at 100,000, 60,000, and 40,000 (Fig. 29). These three polypeptides have not been characterized previously. The $M_r$ 60,000 and 40,000 polypeptides could be either degraded fragments of the $M_r$ 100,000 polypeptide or immunologically related to it. The 1D1 antibody labelled these polypeptides weakly by both immunostaining and immunoblotting techniques. Due to its low affinity for ROS membrane proteins, the 1D1 antibody was not used to detect cross-reacting protein in the RBC system.

(4) Characterization of 1H5 and 2A4 Monoclonal Antibodies

The 1H5 and 2A4 hybridoma cell line were generated from a fusion of NS-1 mouse myeloma cells with spleen cells from a mouse immunized with Triton X-100 insoluble ROS membrane proteins (Jarausch and Molday, unpublished results). The binding specificity of these antibodies for ROS polypeptides was determined by immunoblotting. As shown in Figure 30, 1H5 antibody intensely labelled a $M_r$ 240,000 polypeptide and a $M_r$ 150,000 polypeptide in the supernatant fraction obtained from the solubilization of ROS membrane proteins by Triton X-100 and a $M_r$ 240,000 polypeptide in the pellet fraction. This labelling pattern is almost identical to that observed for the 4B2 antibody labelling of these fractions (Fig. 24). The 2A4 antibody labelled several polypeptides with $M_r$ ranges from 68,000 to 270,000 in the supernatant fraction, but only
Figure 29. Immunoblot of bovine ROS membrane proteins labelled with 1D1 monoclonal antibody. SDS solubilized ROS membrane proteins (20 μg/well) were subjected to SDS gel electrophoresis on a 8% polyacrylamide slab gel. Gels were either stained with Coomassie blue (gel a) or transferred to nitrocellulose paper. Transfer paper was sequentially labelled with once cloned 1D1 cell culture supernatant and goat anti-mouse Ig conjugated to alkaline phosphatase. Transfer paper was then incubated with substrate 5-bromo-4-chloro-3-indoxyl-phosphate and stained by nitro blue tetrazolium salt (gel b).
two polypeptides with apparent $M_r$ 270,000 and 240,000 were labelled in the pellet fraction (Fig. 30).

Since the 1H5 antibody appears to be similar to the 4B2 antibody in binding specificity, it was tested for immunological cross-reactivity with RBC proteins. Immunoblots shown in Fig. 31 indicated that 1H5 cross-reacts somewhat better with the $\alpha$ than the $\beta$ band of RBC spectrin, whereas 4B2 only cross-reacts with the $\alpha$ band under the same conditions. This suggests that 1H5 antibody recognizes a different epitope on the $\alpha$ subunit of RBC spectrin than the 4B2 antibody and this epitope shares homology with an epitope on the $\beta$ subunit. As was previously shown in Figures 24 and 30, both antibodies labelled a $M_r$ 240,000 polypeptide in the pellet fraction.
Figure 30. Immunoblots of Triton X-100-treated ROS membranes labelled with 1H5 and 2A4 monoclonal antibodies. ROS membranes were solubilized with 1% Triton X-100 for 24 h at 4°C. ROS membranes were then sedimented at 15,000 rpm for 30 min. Supernatant (45 μg protein/well) and pellet (5 μg protein/well) were subjected to SDS electrophoresis on a 8% polyacrylamide slab gel. After gel electrophoresis, the proteins were either stained with Coomassie blue (CB) or electrophoretically transferred to nitrocellulose paper. Transfer papers were indirectly labelled with either 1H5 or 2A4 antibody and 125I-labelled goat anti-mouse Ig for autoradiography. (Lane a) Supernatant. (Lane b) Pellet.
Figure 31. Immunoblots of bovine RBC membrane ghosts and Triton X-100 ROS pellet labelled with 1H5 and 4B2. RBC membrane ghosts (20 μg protein/well) and Triton X-100 ROS pellet (5 μg protein/well) were subjected to SDS gel electrophoresis on a 8% polyacrylamide slab gel. After gel electrophoresis, the proteins were either stained with Coomassie blue (CB) or electrophoretically transferred to nitrocellulose paper. Transfer papers were indirectly labelled with either 1H5 or 4B2 antibody and 125I-labelled goat anti-mouse Ig for autoradiography. (Lane a) RBC membrane ghosts. (Lane b) Triton X-100 ROS pellet.
A. Differences between 4B2-Specific Protein and ROS 1.2.

Results of this study indicate that 4B2-specific protein of apparent \( M_r \) 240,000 is distinct from ROS 1.2 based on their electrophoretic mobilities on SDS-polyacrylamide gel, sensitivity to endogenous proteases, and extractability by urea. The 240K polypeptide specifically binds the 4B2 monoclonal antibody, but not Con A, comigrates with the \( \alpha \) chain of bovine RBC spectrin on 6% polyacrylamide gels, is highly susceptible to degradation by endogenous proteases, and can be extracted from the membrane, although to a limited degree, with urea in the absence of detergent. In contrast, ROS 1.2 binds Con A but not the 4B2 antibody, migrates with a slightly greater mobility indicative of an apparent \( M_r \) 210-220K, is not readily degraded by endogenous proteases, and like rhodopsin, is not extracted by urea, but requires detergent for solubilization. The 240K polypeptide is not simply a contaminant of the ROS preparation since immunocytochemical labelling studies using immunogold-dextran markers clearly show that this protein is localized within the ROS organelle.

It is important to show the differences between the 4B2-specific protein and ROS 1.2, because ROS 1.2 has been proposed to be involved in disc-disc interaction (Roof and Heuser, 1982; Corless et al., 1987) whereas the 4B2-specific protein has not been characterized previously.

The 4B2-binding protein of bovine ROS appears to be related to spectrin of RBC. The apparent molecular weight of the 4B2-binding polypeptide is similar to that of the \( \alpha \) subunit of bovine RBC spectrin. More importantly, the 4B2 monoclonal antibody cross-reacts with purified bovine RBC spectrin and specifically with its \( \alpha \) subunit as shown by RIA and immunoblotting studies. It also cross-reacts with brain microsomal membranes which are known to contain a spectrin-related protein called fodrin (Levine and Willard, 1981). Like the 4B2-binding protein, fodrin is known to be highly susceptible to degradation by proteases during its purification. The major proteolytic fragments of fodrin have apparent \( M_r \) of 150,000 and 160,000 (Burridge et al., 1982) which are comparable in size to the major proteolytic fragment of 4B2-binding protein of apparent \( M_r \) 150,000.

The identity of the endogenous protease responsible for the degradation of fodrin is not known, but it has been suggested that a \( Ca^{2+} \)-activated protease is involved (Burridge et al., 1982). A family of \( Ca^{2+} \)-dependent thiol-proteases known collectively as calpain I and II (reviewed by Murachi, 1983) have been found to be specific for fodrin as well as spectrin (Siman et al., 1984). In neurons calpain-mediated degradation of fodrin has been suggested to play a
role in controlling cell shape and the disposition of cell surface proteins on the basis of the results indicating both fodrin (Levine and Willard, 1981) and calpain I (Siman et al., 1983) are part of the submembrane cytoskeleton and the fact that spectrin serves such a function in erythrocytes.

Whether or not calpain exists in ROS remains to be determined. Like calpain with subunits of apparent $M_r$ 72-82,000 and 25-30,000, the endogenous protease in ROS appears to be of small molecular weight since size-exclusion chromatography of SDS-solubilized ROS membrane proteins on a Sepharose CL-2B column was shown to be effective in separating the 4B2-specific protein and its degraded fragments from the protease. However, it is also possible that the protease was denatured by SDS treatment. Future studies are needed to clarify this point. Preliminary studies indicate that EGTA, a Ca$^{2+}$ chelating agent, and leupeptin, an inhibitor of calpain (Siman et al., 1984), are ineffective in preventing the proteolytic degradation of 4B2-binding protein since immunoblots of 4B2 labelling of ROS membrane proteins prepared in the absence or presence of these inhibitors showed a band at $M_r$=150,000, the major proteolytic fragment of the 4B2-binding protein (not shown). However, the effectiveness of these two inhibitors could be better assessed if the purified 4B2-binding protein and its specific protease in ROS were used, because the 4B2-binding protein (240 kDa) could have been degraded before ROS membrane
preparation.

The 3A6-binding protein has a similar electrophoretic mobility on 6% SDS-polyacrylamide gels as the 4B2-binding protein. In order to show their difference in electrophoretic mobility, a 5-15% gradient polyacrylamide gel with 25% of normal crosslinker concentration was prepared for SDS gel electrophoresis. In this gel system, the 4B2-binding protein has an apparent $M_r=260,000$ and the 3A6-binding protein has an apparent $M_r=240,000$. Like the 4B2-binding protein, the 3A6-binding protein is also highly susceptible to degradation by endogenous proteases. More importantly, the 3A6 monoclonal antibody cross-reacts specifically with the $\beta$ chain of RBC spectrin as determined by immunoblotting.

Although monoclonal antibodies are more suitable than polyclonal antibodies as specific probes of protein fine structure and function, they are less useful in determining the extent of homology between two proteins, because they bind to single antigenic determinants which comprise only a few amino acid residues. In this study, it was shown that polyclonal anti-RBC spectrin antibodies cross-reacted with polypeptides of ROS having molecular weights equivalent to the $\alpha$ and $\beta$ chains of RBC spectrin. The $\alpha$ subunit of $M_r 240,000$ was the major unit of ROS spectrin detected by these polyclonal antibodies in this study. Several bands in the molecular weight range of 215,000-220,000 that labelled
with polyclonal anti-spectrin antibody may represent variants of the $\beta$ subunit of spectrin (Nelson and Lazarides, 1983) that are weakly cross-reactive, more susceptible to proteolytic degradation, or present in lower quantities than the $\alpha$ subunit in ROS.

A number of spectrin-like proteins has been identified on the basis of antigenic cross-reactivity. For example, a pair of polypeptides of $M_r$ 240,000 and 230,000 found in embryonic chick cardiac myocytes, 3T3 fibroblasts, and rat hepatoma cell have been shown to cross-react with anti-spectrin antibodies (Goodman et al., 1981). Similarly, antibodies specific for the chain of avian erythrocyte spectrin have been shown to cross-react with proteins of nearly identical $M_r$ (240,000) in cells from such diverse tissues as small intestine, liver, lens, brain, cilia, and striated muscle (Repasky et al., 1982).

Based on antigenic cross-reactivity, it is apparent that ROS contain a spectrin-like protein and that ROS spectrin may also consist of an $\alpha$ and a $\beta$ chain. However it is difficult to assess how the ROS spectrin is similar to RBC spectrin in structure or function based solely on antigenic cross-reactivity. The degree of cross-reactivity of both monoclonal and polyclonal antibodies does not necessarily correlate with either structural or functional homology among the spectrin family of proteins. For example, brain and RBC spectrins are similar in their amino acid composition, their tetrameric structure, and their ability to cross-link actin
filaments and bind calmodulin and ankyrin, yet only about 1% of epitopes in the two proteins were strongly cross-reactive as measured in a study by Harris et al. (1985). Similarly, immunological and biological data indicate that chicken nonerythroid and human erythroid α-spectrin are two of the more widely diverged members of the group of spectrin proteins. However, comparison of the amino acid sequence of human erythroid α-spectrin with the derived amino acid sequence of the chicken nonerythroid α-spectrin cDNA clone revealed a striking similarity between these two molecules (Birkenmeier et al., 1985). Therefore, it is not surprising to find that polyclonal anti-bovine RBC spectrin antibodies reacted more strongly with bovine RBC spectrin than with ROS spectrin from the same species. Furthermore, the 4B2 monoclonal antibody raised against the ROS antigen, reacted more intensely with ROS spectrin than with the RBC protein.

A low level of cross-reactivity between ROS spectrin and RBC spectrin does not necessarily imply limited structural or functional homology. For example, detailed studies on the antigenic variation of the influenza virus hemagglutinin protein indicate that marked reductions in monoclonal antibody binding are caused by even a single conservative amino acid substitution (Wiley et al., 1981). It is also noteworthy to find that the erythrocyte spectrin is not related to any other proteins whose sequence was known (Speicher et al., 1983a; 1983b; Speicher and Marchesi, 1984).
The primary structure of erythrocyte spectrin has been extensively investigated. The sequence of 1,435 amino acid residues or 35% of the total molecule has been determined (Speicher et al., 1983a; 1983b; and Speicher and Marchesi, 1984). Analyses of the amino acid sequences obtained to date indicate the presence of 22 homologous repeat units throughout both subunits of spectrin. It was suggested that each repeat unit, which contains 106 amino acid residues (Mr 12,000) may be folded into a triple helical structure with a short non-helical region connecting adjacent repeat units. Another 16 repeat units are expected to be present if most of the spectrin molecule is composed of this single type of repetitive structure. This has to be confirmed by further sequencing studies.

The location of the 3A6 and 4B2 antibody binding sites on the erythrocyte spectrin molecule has not been identified. But the fact the 3A6 antibody cross-reacts only with the \( \beta \) chain and the 4B2 antibody cross-reacts only with the \( \alpha \) subunit of RBC spectrin, suggests that these antibodies may perhaps bind to regions of functional specialization. It has been known that the \( \alpha \) subunit of avian spectrin binds calmodulin (Glenney et al., 1982) while the \( \beta \) subunit binds ankyrin (Bennett and Stenbuck, 1979) and probably band 4.1 (Coleman et al., submitted). However, it is also possible that these two monoclonal antibodies simply recognize two non-identical sequences located in non-functional regions of the spectrin chains.
C. Extraction of ROS Spectrin.

Spectrin from RBC (Marchesi et al., 1976) and spectrin-related proteins from other cell types (Glenney and Glenney, 1983) are nonintegral, membrane-associated proteins consisting of an elongated $\alpha-\beta$ heterodimer having a length of about 100 nm (Branton et al., 1981; Shotton et al., 1979). Two heterodimers further associate end to end to form a flexible 200-nm-long chain as part of a fibrous skeletal network lining the cytoplasmic surface of the plasma membrane. In the case of RBC this fibrous network interacts with the anion transport protein "band 3" primarily through interaction with ankyrin (Bennett and Davis, 1982). This membrane skeletal system appears to stabilize the plasma membrane and maintain the shape of RBC.

ROS spectrin also appears to be a nonintegral, membrane-associated protein that can be partially extracted from ROS membranes with urea under conditions in which the integral ROS membrane proteins are not extracted. A strong association of ROS spectrin with disc and plasma membranes exists, however, since relatively small amounts of this protein are extracted under the conditions used in this study. Also ROS spectrin was not extracted in low ionic strength buffer in the presence of chelating agents, i.e., conditions used to extract RBC spectrin. In this respect ROS spectrin is different from RBC spectrin.
D. Association of 4B2-Specific Protein with ROS Membranes.

The strong association of ROS spectrin with ROS membranes was also suggested by the fact that disc membranes prepared by hypotonic lysis of ROS membranes contained ROS spectrin as determined by immunoblotting. Competitive inhibition assays indicated a 2-fold difference in 4B2 antigen concentration between ROS and disc membrane preparations. This is unexpected because it suggests that 50% of total ROS spectrin was lost when disc membranes were prepared. Since ROS spectrin was not extracted in low ionic strength buffer, it would appear that the lost ROS spectrin could be found in the pelleted membrane fraction consisting of unsealed plasma and disc membranes and possibly other contaminating retinal debris. However, this membrane fraction only represents about 15% of the total ROS membranes (Molday and Molday, unpublished results). Accurate quantitative determination of 4B2 antigen concentration using $^{125}$I-labelled 4B2 antibody in a direct radioimmune assay has not been attempted because 4B2 antibody was never purified to homogeneity.

The association of ROS spectrin with ROS disc membrane fragments has been previously shown by immunocytochemical labelling of disc membrane preparation with the 4B2 antibody (MacKenzie and Molday, 1982). But it is still uncertain whether ROS spectrin associates with the disc and/or plasma membrane since Molday and Molday (1987) have shown that disc membranes prepared from hypotonic lysis of ROS contained
inside out or inverted plasma membrane vesicles. In order to show the association of ROS spectrin with plasma membrane, a reliable procedure for purifying ROS plasma membrane must be available. Although a method for isolating the plasma membrane has been described (Kamps et al., 1982), the purity of the plasma membrane preparation could not be assessed due to the lack of a plasma membrane specific marker. Plasma membrane specific markers (Molday and Molday, 1987) and a new method for isolating the plasma membrane (Molday and Molday, in press) have recently been described. Thus, it is possible in the future to investigate the association of 4B2-specific protein with the ROS plasma membrane.

E. Localization and Possible Function of ROS Spectrin.

The molecular structure and function of ROS spectrin is not yet known. Immunocytochemical labelling studies, however, indicate that this protein is localized along the periphery of the ROS organelle in the region where the discs come in close contact to the plasma membrane and ciliary spine. In the same region, filamentous structures linking discs to the plasma membrane have been seen in frog ROS under the electron microscope by Usukura and Yamada (1981), using freeze-deep etched replica techniques. Roof and Heuser (1982) have also observed specialized filaments connecting the disc rims to each other and different filaments linking the discs to the plasma membranes.
Filaments connecting the discs to each other are more numerous and have been suggested to be composed of the rim protein first characterized by Papermaster et al. (1978) in frog ROS. This protein may be related to ROS 1.2 glycoprotein of bovine ROS (Molday and Molday, 1979) although direct evidence for this is lacking. The molecular composition of the less numerous filaments connecting the discs to the plasma membrane that may be related to the filamentous network observed by Usukura and Yamada (1981), is not known. Results of this study indicate that the $M_r$ 240,000 spectrin-like protein is present in significantly lower amounts than the $M_r$ 220,000 glycoprotein ROS 1.2 as observed by Coomassie blue and silver staining of ROS proteins separated by SDS gel electrophoresis (Figure 8). RIA studies comparing the binding of anti-spectrin antibodies to RBC spectrin and ROS further suggest that ROS contains a protein that is immunochemically related to spectrin, and this spectrin-like protein constitutes less than 1% of the ROS protein (Figure 17). It would appear that the 240K polypeptides and fragments recognized by polyclonal anti-RBC antibodies are identical with those recognized by the 4B2 monoclonal antibody. This can be tested by immunoprecipitating ROS proteins with the polyclonal antibodies and measuring binding of monoclonal antibodies to the complex.

On the basis of these studies and analogy with RBC spectrin, the ROS spectrin protein is suggested to be a major
constituent of the filamentous network lining the cytoplasmic surface of the ROS plasma membrane as observed by Usukura and Yamada (1981), and more specifically, may comprise the filaments extending from the discs to the plasma membrane observed by Roof and Heuser (1982). This filamentous network, which probably interacts with integral membrane proteins, would serve to maintain a constant distance between the plasma membrane and discs as seen by electron microscopy and would generally stabilize the ROS structure. The observation that gold labelling with the 4B2 antibody extends inward from the plasma membrane up to 50 nm may indicate that the spectrin network interdigitates between the rims of the discs. A speculative model on the organization of ROS spectrin and other ROS proteins (R.S. Molday, personal communication) is illustrated in Figure 32. In this model, rhodopsin is mainly distributed in the lamellar region of disc membranes whereas peripherin (33 kDa rim-specific protein) and ROS 1.2 (220 kDa glycoprotein) reside in the rim regions. A spectrin-like protein (240 kDa) in the cytoplasmic space either directly or indirectly associate with both the disc and plasma membrane. The plasma membrane contains two glycoproteins of $M_r$ 230,000 and 110,000 as well as rhodopsin.

F. Detergent Solubilization of ROS Membrane Proteins.

Triton X-100 has been found to be better than some ionic
Figure 32. A speculative model for organization of proteins in ROS membranes is illustrated in this schematic diagram. Rhodopsin molecules are mainly distributed in the lamellar regions whereas peripherin and a 220 kDa glycoprotein are distributed in the rim regions of disc membranes. A spectrin-like protein in the cytoplasmic space either directly or indirectly associate with both the disc and plasma membrane. Two glycoproteins of M, 230,000 and 110,000 as well as rhodopsin are found in the plasma membrane (obtained from R.S. Molday).
and zwitterionic detergents in selectively solubilizing ROS membrane proteins. Results of the study on Triton X-100 extraction of ROS membrane proteins indicate that rhodopsin and peripherin which constitute over 90% of total disc membrane proteins (Molday et al., 1987) are almost completely solubilized by this detergent. In contrast, ROS 1.2 glycoprotein which constitutes 1-3% of total disc membrane protein (Molday and Molday, 1979) is relatively insoluble as determined by SDS gel electrophoresis and immunoblotting analyses. These results suggest that rhodopsin and peripherin may be more hydrophobic than ROS 1.2 and hence have stronger association with the lipid core in disc membranes. When the hydrophobic lipid core is disrupted by Triton X-100, rhodopsin and peripherin become soluble. ROS 1.2 glycoprotein, however, may have strong polar associations with other ROS proteins. Since polar protein-protein associations are not disrupted by Triton X-100, ROS 1.2 remains insoluble. The identity of the remaining ROS polypeptides in the Triton-insoluble fraction as observed by Coomassie blue staining of these polypeptides separated by SDS gel electrophoresis has not been investigated mainly due to the lack of previously characterized antibody probes. Only the 4B2-binding protein (ROS spectrin) has been identified.

Electron microscopy of a negatively stained Triton-extracted ROS pellet revealed a filamentous network. The molecular composition of this filamentous network is as yet
unknown, but the 4B2-binding protein is likely a component of this network since it is present in the Triton-extracted ROS pellet. Moreover, spectrin and spectrin-like proteins are known to be components of cytoskeletal system in other cell types.

G. Identification of ROS Cytoskeletal Components.

Studies have been initiated to identify the components that constitute the ROS cytoskeleton. Actin and ankyrin are two proteins that interact with spectrin in the RBC cytoskeletal system. Since a spectrin-like protein has been identified in ROS, it is of interest to determine whether actin- and/or ankyrin-like proteins also exist in this organelle. A monoclonal antibody specific for actin and polyclonal antiserum raised against a partially purified erythrocyte ankyrin preparation were tested for immunological cross-reactivity with ROS proteins.

Actin was detected in some ROS membrane preparations as determined by solid-phase RIA. However, on the basis of immunocytochemical labelling experiments which localized actin only to the distal connecting cilium in photoreceptors of many species including the cow (Chaitin et al., 1984; Chaitin and Bok, 1986), it is unlikely that actin is a component of the filamentous network lining the cytoplasmic surface of the outer segment plasma membrane. Nevertheless, there remains the possibility that the concentration of actin
in the rod outer segments is too low to be detected by the immunocytochemical labelling technique.

Ankyrin is a likely candidate for an attachment protein that links ROS spectrin to ROS plasma and/or disc membranes, since it serves such a function in erythrocytes. However, initial attempts to detect an ankyrin-like molecule in ROS membrane preparations using polyclonal anti-RBC ankyrin antibodies were not successful. This suggests that ROS may not contain an ankyrin-like molecule or there is limited cross-reactivity between erythrocyte and ROS ankyrins. Since immunologically related forms of ankyrin have been detected in a wide variety of non-erythroid cells, including platelets (Bennett, 1979; Bennett and Davis, 1981), ankyrin would also be expected to be found in ROS. Thus, it is necessary to use several polyclonal anti-ankyrin antibodies to further test for cross-reactivity with ROS proteins.

Further characterization of the Triton-extracted ROS pellet by both monoclonal and polyclonal antibodies specific for cytoskeletal elements will determine the similarities and differences between the erythrocyte cytoskeletal network and that found in ROS. Several antibodies including 1H5 and 2A4 presented in this thesis, were generated against Triton-extracted ROS pellet (J. Jarausch, personal communication). One antibody (1D1) was raised to urea-extractable ROS membrane proteins, and polyclonal anti-RBC ankyrin antibodies were generated against partially purified RBC ankyrin. However, further characterization of these antibodies is
required prior to their use as molecular probes.

H. Rod Photoreceptor Cytoskeleton.

Several cytoskeletal elements have been identified in the rod inner segment and in the non-motile cilium that connects the inner segment to the outer segment. The cilium consists of nine microtubule doublets primarily composed of a protein known as tubulin. Actin has been localized in the periciliary ridge complex (Peters et al., 1983), a specialized cellular structure that surrounds the cilium at the apex of the inner segment. This protein has also been localized in the basal discs, and the distal end of the ciliary process (Chaitin et al., 1984). Myosin, another contractile protein, has been localized throughout the ciliary extension (Chaitin and Bok, 1984). Furthermore, a bundle of microtubule-like elements has been found to be enclosed by the disc incisures (Fig. 5).

On the basis of its localization at the site of disc formation, actin has been suggested to play a role in opsin transport and incorporation into the outer segment membrane during disc formation (Chaitin et al., 1984). It has also been suggested that in lower vertebrate rods, actin may play a similar role in the assembly of discs (Burnside and Deary, 1986). Interestingly, the cytoskeletons obtained from Triton extraction of whole teleost retina were found to contain actin filaments, microtubules, the connecting cilium, and a
240 kDa calmodulin-binding protein (Nagle and Burnside, 1984). This protein appears to be similar to the ROS spectrin described in this thesis. It comigrates with the chain α of brain spectrin and cross-reacts with antibodies specific for rat brain spectrin (Nagle and Burnside, 1984). This spectrin-like protein and actin are thought to provide scaffolding for the large membrane surface of the long delicate teleost rods (Burnside and Dearry, 1986). In contrast, the bovine ROS spectrin-like protein identified in this investigation is proposed to be a constituent of a cytoskeletal network that stabilizes the highly-ordered ROS structure. Elucidation of the precise functions of ROS spectrin and other cytoskeletal elements requires further investigation.

CONCLUSIONS

This thesis investigation indicates bovine rod photoreceptor outer segments contain a 240 kDa spectrin-like protein that is distinct from the previously described ROS 1.2 glycoprotein. This spectrin-like protein is highly sensitive to proteolytic degradation by an endogenous protease and localizes along the preiphery of the ROS organelle in the region where the discs come in close contact to the plasma membrane. It associates strongly with the plasma membrane and/or disc membrane and due to these strong interactions with the membranes, it is not easily extracted.
However, this spectrin-like protein is enriched in Triton-extracted ROS pellet because it is a constituent of a fibrous matrix that is insoluble in Triton X-100 whereas the major component rhodopsin is soluble.

**FUTURE DIRECTIONS**

Future studies should be directed toward the understanding of the molecular structure and properties of ROS spectrin. This will require the purification of the 4B2-specific protein from ROS membrane preparations. Several difficulties in this regard are suggested by this thesis investigation. Firstly, the 4B2-specific protein constitutes less than 1% of the total ROS protein. Secondly, it is highly sensitive to proteolytic degradation by an endogenous protease whose identity and specific inhibitor are yet unknown. Finally, it associates strongly with ROS membranes, presumably due to strong interactions with other ROS proteins.

Nevertheless, a purification method can be devised to take advantage of the fact that the undegraded 4B2-specific protein is enriched in Triton X-100-extracted ROS pellet. In this method, ROS membranes are extracted with Triton X-100 and centrifuged at high speed. The resulting pellet fraction is then solubilized and applied to an anti-spectrin antibody column. The 4B2 monoclonal antibody is not suitable for this purpose because it has been found to be unstable and
difficult to purify from ascites fluid. Purified polyclonal anti-spectrin antibodies and IgG monoclonal antibodies specific for the 4B2-binding protein are more suitable for this purpose.

Another approach to study ROS spectrin would be to determine its gene sequence. An oligonucleotide probe corresponding to a partial amino acid sequence of RBC spectrin could be used to screen a bovine retinal genomic or cDNA library; alternatively, the 4B2 monoclonal antibody or polyclonal anti-spectrin antisera could be used to screen a bovine retinal cDNA expression library.

The interaction of the 4B2-specific protein with other ROS proteins should also be investigated in future studies in order to understand its possible function in the maintenance of ROS structure, phototransduction, and other specialized processes such as disc renewal that occur in the ROS organelle. Several major questions to ask are: (1) How does the ROS spectrin attach to the plasma membrane and/or disc membrane? (2) If ROS spectrin is part of a cytoskeletal network that maintains the shape of the rod outer segment, what happens to this network when discs are shed during the disc renewal process? (3) Does ROS spectrin or other ROS cytoskeletal components serve any role in the opening and closing of sodium channels during the phototransduction process or in the organization of the integral membrane proteins of the plasma and disc membranes? (4) How do the cytoskeletal elements assemble and migrate from the rod
inner segment to the outer segment? Future investigation will help to provide answers to these and other questions concerning the various specialized processes that occur in the retinal rod photoreceptor cell.


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