PREPARATION AND CHARACTERIZATION OF BOVINE RETINAL PIGMENT EPITHELIAL CELL PLASMA MEMBRANE

by

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ABSTRACT

A 7-9 fold enriched preparation of bovine retinal pigment epithelial cell plasma membrane was prepared and characterized by enzymatic analysis. SDS-polyacrylamide gel electrophoresis and transmission electron microscopy revealed a large rod outer segment contamination in the preparation due to a tight retinal pigment epithelial cellular adhesion to the rod photoreceptor cells. The contaminating rhodopsin was partially removed by anti-rhodopsin immunoaffinity chromatography as determined by SDS-polyacrylamide gels stained with coomassie blue or silver.

Monoclonal antibodies raised against the retinal pigment epithelial plasma membrane preparation cross reacted with rod outer segment preparations. A monoclonal antibody, designated Rho-5A3, was classified as an IgG_3 kappa light chain immunoglobulin. It was shown to be specific for rhodopsin as determined by radioimmune labeling of bovine rod outer segment membrane proteins electrophoretically transferred to CNBr-activated paper. Limited proteolytic digestion of rhodopsin followed by electrophoretic transfer to CNBr- activated paper localized the binding site of this antibody to the N-terminal two-thirds of the rhodopsin molecule. Competition assays with rhodopsin polypeptides further defined the antigenic site to be within the 17-39 amino-acid segment of rhodopsin. The Rho-5A3 antibody did not bind to sealed ROS discs or frozen-thawed ROS discs but did bind to Triton X-100 solubilized discs indicating a detergent solubilization dependence for antigenic site accessibility.

Cultures of bovine retinal pigment epithelial cells were started by initial enzymatic isolation followed by recovery in RPMI-1640 culture medium. The retinal pigment epithelial cells established a doubling time of 52 hours until the cells reached culture confluency . The cells also maintained many of their in vivo characteristics such as a high degree of pigmentation and an abundance of microvilli.

Cell surface glycoproteins labeled with FITC-Con A, FITC-WGA, and FITC-RCA showed dense and random surface labeling patterns. Fluorescent labels were induced to redistribute to central spots and clear from the cell surface by incubating the labeled cells in buffer for 60 minutes at 37°C. Treatment by the appropriate saccharide inhibitors indicated that the labeled sites had undergone endocytosis by the cell. Continuous labeling experiments indicated that redistribution and internalization is constantly occuring so that previously unlabeled receptors become accessible for labeling. As a result a dense pattern of label on the cell surface was maintained.

The protein actin, with apparent M_r =46,000, was detected with rabbit anti-actin antisera labeling of the RPE plasma membrane preparation proteins electrophoretically transferred to nitrocellulose paper. Immunofluorescent labeling using the rabbit anti-actin antisera confirmed biochemical studies that actin was a major component of the bovine RPE cell. The activation of actin filaments may play an important role in the phagocytosis of bovine rod outer segments by retinal pigment epithelial cells in tissue culture.

Scanning electron microscopy and transmission electron microscopy have shown that 2 week old bovine retinal pigment epithelial cells *in vitro* can be induced to recognize, attach, and engulf dark adapted sealed rod outer segments.

In summary a monoclonal antibody was raised against a bovine retinal pigment epithelial cell plasma membrane preparation, however, the antibody raised proved to be specific for rhodopsin, a contaminating ROS protein found in the preparation. The monoclonal antibody, designated Rho-5A3, was fully characterized and its antigenic site determined. Finally, bovine retinal pigment epithelial cells grown in tissue culture acted as a model system for studying cell surface components and rod outer segment phagocytosis at the levels of fluorescence, scanning electron microscopy, and transmission electron microscopy.

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LIST OF ABBREVIATIONS

BSA -------- bovine serum albumin

CHAPS ------ 3-[(3-cholamidopropyl) dimethylammonio]
-1-propanesulfonate

Con A ------ concanavalin A

DMSO ------- dimethyl sulfoxide

EDTA ------- ethylenediamine tetraacetate

FCS -------- fetal calf serum

FITC ------- fluorescein isothiocyanate

HAT -------- hypoxanthine, aminopterin, thymidine

IMDM ------- Iscove's Modified Dulbecco's Medium

Ig --------- immunoglobulin

M_r --------- molecular weight

PBS -------- phosphate-buffered saline

PEG -------- polyethylene glycol

PMSF ------- phenylmethyl sulfonyl fluoride

RIA -------- radioimmune assay

RCA -------- Ricinius communis agglutinin

ROS -------- rod outer segment

RPE -------- retinal pigment epithelial

SDS -------- sodium dodecyl sulfate

SEM -------- scanning electron microscopy

TCA -------- trichloroacetic acid

TEM -------- transmission electron microscopy

Tris ------- tris (hydroxymethyl) aminomethane

WGA -------- wheat germ agglutinin

TABLE OF CONTENTS

EXPERIMENTAL PROCEDURES

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

RETINA

The retina has been extensively studied over the years as it represents a highly complex extension of the nervous system. Developmentally and functionally, the retina is an isolated part of the central nervous system to which it remains connected by the optic nerve.

The visual retina comprises a thin nervous layer lining the posterior part of the eyeball. In embryonic development, the optic vesicle becomes transformed into a two-layered optic cup, the outer layer forming the pigment epithelium, and the inner layer becoming the neural retina. Light passes through the neural retina layers consisting of the optic nerve fiber layer, ganglion cell layer, inner plexiform layer, inner nuclear layer, outer plexiform layer, outer nuclear layer and photoreceptor cells, respectively (Fig. 1).

PHOTORECEPTOR CELLS

Vertebrate photoreceptor cells consist of modified neurons called rod cells and cone cells. The cone cells are responsible for color vision while the more numerous rod cells are responsible for black and white vision. The bovine rod cells are slender cells approximately 60 um long with an average width of about 1.5 to 2 um. The rod cell is divided into a rod outer segment portion and a rod inner segment connected by a thin cilium (Fig. 2). The rod outer segment consists of stacks of disc

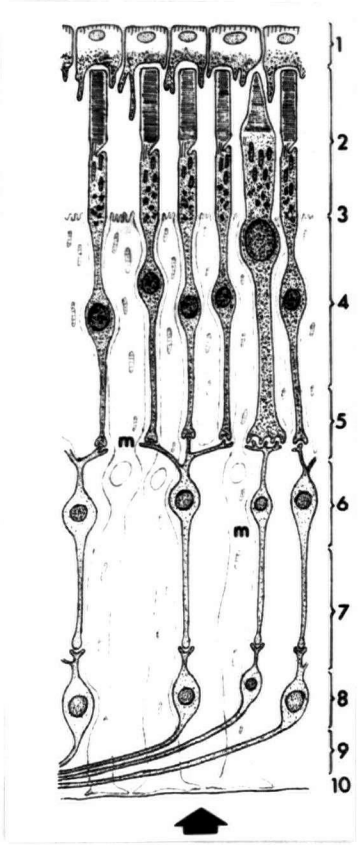


Figure 1. Diagram to illustrate the layers of the retina. (1.) Pigment epithelial. (2.) Layers of rods and cones. (3.) External limiting membrane. (4.) Outer nuclear layer. (5.) Outer plexiform layer. (6.) Inner nuclear layer. (7.) Inner plexiform layer. (8.) Ganglion cell layer. (9.) Optic fiber layer. (10.) Internal limiting membrane. The arrow indicates the direction of light. Diagram taken from reference 2.

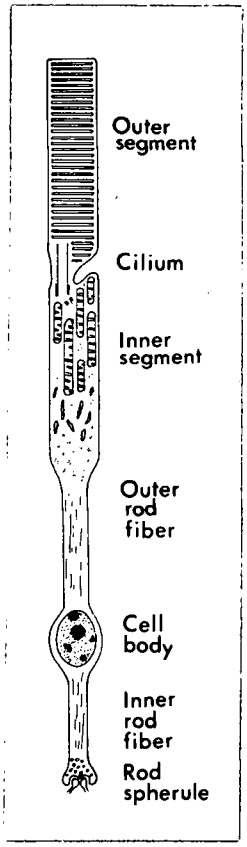


Figure 2. Diagram of a rod photoreceptor cell as seen by the electron microscope. Note the large portion of outer segment membrane. Diagram taken from reference 2.

membrane. The inner segment contains all the cytoplasmic organelles and metabolic machinery while 90% of the outer segment membrane is the photopigment rhodopsin. The tip of the outer segment is embedded in the pigment epithelium.

LOCATION OF THE RETINAL PIGMENT EPITHELIUM

The cuboidal monostratified retinal pigment epithelium (RPE) originating from the external layer of the optic vesicle, represents the outer most layer of the visual retina. The RPE covers the whole posterior part of the eyeball as far as the ora serrata and is predominantly dark-colored in most species owing to the melanin pigment in its cells. The basal face of the pigment epithelium rests on a basal lamina which represents the inner layer of Bruch's membrane. This structure functions in the adhesion of the pigment epithelium to the choroid. The apical surface of the retinal pigment epithelium has microvilli which interdigitate the outer segments of the photoreceptor cell (1).

THE FUNCTION OF THE RETINAL PIGMENT EPITHELIUM

RPE cells have been shown to be involved in the continuous renewal of membranes in the rod outer segments (1). The protein synthesized in the inner segment pass through the connecting cilium to form new discs at the base of the rod outer segment. At the same time, groups of discs are shed at the apex of the outer segment and engulfed in the cytoplasm of the pigment epithelium to form phagosomes. Finally, the phagosomes are degraded in these RPE cells.

It is now established that during light adaptation of the eye, the retinaldehyde liberated from visual pigment during bleaching in the outer segment is reduced to retinol and migrates into the pigment epithelium (1). The reverse process takes place during dark adaptation. Thus the cells of the pigment epithelium play a vital role in the visual process. Moreover the pigment epithelium prevents reflection by absorbing light and is essential for the formation of rhodopsin by storing and releasing vitamin A (2).

There exists in the RPE cells a very strong cellular polarity. The scleral face of the cell, with its basal infoldings giving rise to coated vesicles and the neighbouring mitochondria, represents a site of absorption and active transport in the vascular retina of mammals. The apical surface, with its pigmented processes in intimate contact with photoreceptor outer segments, indicates the complementarity of the pigment epithelium and the visual cells of the retina, since exchanges are important and continuous between these two layers.

PATHOLOGY OF RETINAL PIGMENT EPITHELIUM

The pathology of the RPE can be separated into two classes: dysfunction of polarization and pigmentation of the cell; and dysfunction of phagocytosis (1). In this paper attention is paid more to the latter. Several retinal pathologies are the result of a deficiency in the phagocytosis function, which can be expressed at different stages of the phagocytic process. The manifestation of pigment retinopathies of the retinitis pigmentosa type

involves derrangement of the first step of phagocytosis; that is, the recognition and adsorption of rod outer segment material by the apical processes. The rat Chimeras are particularly informative as the rdy^- gene is acting extrinsically to the photoreceptor cell, but also that the pigment epithelial cell is the actual site of the gene action (3).

Inherited retinal degeneration in the Royal College of Surgeons (RCS) rat has been shown to be a result of a structural or functional defect in the retinal pigment epithelial cells (3,4,5). This important diseased rat line has been used extensively in studying the mechanism of phagocytosis. Initially, in the RCS rat, normal photoreceptor shedding occurs during which there is a concomitant accumulation of lamellar bodies in the extracellular space between the photoreceptor outer segments and the RPE. This accumulation of lamellar material is accompanied by progressive deterioration of individual photoreceptor cells and a consequent decline in retinal sensitivity. The lysosomal system of the RPE cells has been reported to be deficient in the disease case resulting in the destruction of rod outer segments by the release of lytic enzymes into the intercellular space. Studies by Lavail as well as Essner and Gorrin have demonstated that macrophages invade the layer of debris that accumulates in the RCS interphotoreceptor space as the degeneration of the retina progresses (6,7).

There are several hypotheses as to the cause of the rod outer segment phagocytosis incapability in the RCS rat (8). First of all the pigment epithelial may lack some factor necessary for

phagocytosis or contain an abnormal factor that inhibits phagocytic activity. Alternately both the rod outer segments and the RPE may contain defects or complementary defects that are only expressed in connection with each other. Gery and O'Brien demonstrated that peritoneal macrophages from RCS rats exhibit phagocytic capability equal to that found in macrophages from normal strains of rat (8). Thus the genetic defect in RCS pigment epithelium is not expressed in the macrophages.

Both normal and dystropic retinal pigment epithelium are capable of phagocytizing carbon particles (9). However, when specfic photoreceptor packets are incubated with dystrophic RPE cells, no uptake occurs (4). Muller and Lavail have shown that ROS packets from normal or dystrophic animals are equally phagocytized by normal RPE indicating that the pigment epithelium is the primary site of the phagocytic defect (3). O'Brien proposed that RPE phagocytosis may be triggered by a change in terminal sugars such as fucose and galactose on plasma membrane glycoproteins of ROS (10). It is possible that terminal sugars are not recognized by defective RPE because membrane receptors for certain sugars are lacking or masked. Williams-Seyfried and Mclaughlin's results indicate that sugar coated beads when presented to retinal pigment epithelial explants show differences in phagocytosis (11). Fucose coated beads were not taken up by either normal or dystrophic explants while twice as many mannose beads are phagocytized by the normal as opposed to the dystrophic tissue.

PROCESS OF PHAGOCYTOSIS

The process of phagocytosis occurs in three fairly distinct steps; first, recognition and attachment of the rod outer segments to the RPE cell; second, engulfment; and finally, post engulfment phenomena which include sequential movement and fusion of lysosomes with the phagosome, a drop in phagolysosome pH, and digestion of the phagocytized material. The recognition process is most interesting to this study. It involves some ill defined electrical charge interactions or more specific interaction of cell surface molecules.

Mclaughlin et al. developed a method for replicating the membrane surfaces of rat retinal pigment epithelium explants during phagocytosis of latex beads (12). Surface replicas of initial stages of phagocytosis show the attachment and spreading of microvilli over the latex beads. This may be a morphological movement prior to ingestion that allows a sequential and circumferential interaction of receptors on the surface of the RPE membranes. The beads are engulfed by overlapping microvilli that resembles "Venus fly traps" in surface replicas. Following shortening of the microvilli the beads are engulfed leaving doughnut like impression in the surface membrane followed by flattened membrane domains (12). A similar study was done with human and bovine RPE with comparable results (13).

Many studies on phagocytosis have been done with embryonic RPE cells in tissue culture. Chaitin and Hall used a ROS antiserum and a double immunofluorescent labeling procedure for assaying the phagocytosis of ROS by cultured rat RPE cells (14).

Following the incubation of RPE cells with ROS, the rod outer segments attached to the surface of the cells were labeled with a ROS antiserum in conjunction with RH-GARG (tetramethylrhodamine conjugated goat anti-rabbit IgG). The same cells were disrupted under a graded acetone series and the internal ROS were labeled with the ROS antiserum followed by FITC-GARG (fluorescein isothiocyanate conjugated goat anti-rabbit IgG). ROS attached to the outer surface can be distinquished from the rods that have undergone phagocytosis by using different fluorescent filters. In the case where ^{14}C mannose and ^{3}H choline radiolabeled outer segments were used to study phagocytosis (15), no conclusive control was used to distinguish attachment from endocytosis. Chaitin and Hall‡s procedure showed that the attachment of ROS to dystrophic pigment epithelial cells occurred at a normal rate (14). However, only a small number of these ROS were ingested. Experimental evidence suggests that ingestion of most ROS occurs within 10 minutes to 1 hour incubation time once attachment has been made to the plasma membrane (14,15). Hall also suggests the possibility that two classes of receptors may exist for phagocytosis. One set of receptors that allows for rapid uptake of ROS and a second slower set of receptors that is also capable of phagocytosis of ROS. The dystrophic case would appear to lack the first of these two sets of receptors (14).

THE ROLE OF THE CYTOSKELETAL SYSTEM IN PHAGOCYTOSIS

With the conclusion that the ingestion phase of phagocytosis is defective in the dystrophic pigment epithelial cells, it

seemed possible that this defect might involve the mobilization or functioning of the contractile protein actin. Actin is directly involved in the ingestion mechanism in other phagocytic cells. Haley demonstrated with 2-dimensional gel electrophoresis coupled to fluoragraphy that actin is a major human RPE cell protein and that it is actively synthesized (16). Other distinct proteins of the RPE may represent the unique major cytoskeletal proteins. Studies with actin antibodies show that actin is distributed normally in dystrophic RPE cells (17). However, the ingestion mechanism involving actin becomes activated at only a few sites of ROS attachment. The array of actin filaments seen in RPE processes is not suited for ingestion of ROS since phagocytosis at the disc packets are surrounded by lateral protrusions of the RPE processes that extend inward from all sides. The ingestion of ROS disc packets would be expected to involve actin but actin other than that in filament arrays (17). It may be the changes in actin filaments to the cell periphery. In the case of neutrophilic polymorphonuclear leukocytes, an abnormal functioning actin, caused an impairment in locomotion and the ingestion of particles (18).

Indirect immunofluorescence and antibodies to tubulin were use to study the distribution of microtubules in rat RPE cell cultures (19). They found no apparent microtubule defect in both spreading and fully spread dystrophic RPE cells, rendering it unlikely that an altered distribution of microtubules is responsible for the phagocytic defect in these cells.

ENERGY SOURCE OF PHAGOCYTOSIS

A bovine RPE organ culture was used for the quantitative analysis of the phagocytosis of ^{125}I-labeled - globulin latex particles (20). Phagocytosis was significantly altered by 10^{-3} M colchicine and 10 ug/mL cytochalasin B indicating a possible cytoskeletal mechanism in phagocytosis. Also, apical microvilli appeared to be depleted in regions where many latex particles were ingested. Presumably the microvilli membrane was incorporated into the phagosomal membrane during endocytosis(20).

Masterson and Chader investigated the effect of phagocytosis on the metabolic machinery of cells of the pigment epithelium (21). Using embryonic chick RPE cells in tissue culture they were able to measure the phagocytosis of bovine ROS as measured by the uptake of ^{3}H-labeled outer segments. Phagocytosis was markedly inhibited by the lack of glucose and by two inhibitors of the tricarboxylic acid cycle-cytochrome system, dinitrophenol and malonate. The obvious conclusion is that tricarboxylic acid and its associated cytochrome systems play an important role as an energy source for phagocytosis in these cells.

LATENCY PERIOD IN PHAGOCYTOSIS

Several studies have been performed *in vitro* and *in vivo* designed to observe the latency period involved in phagocytosis (22,23,24). When polystyrene spheres and native *Sarcina subflava* were injected into the subretinal space of *Rana pipiens* tabpoles only the polystyrene spheres were actively phagocytized (22). Holleyfield‡s experiments concluded that the nature of the

material presented to the pigment epithelial cells can determine whether or not that material will be phagocytized (22). Quantitative studies done on the phagocytosis of latex beads showed a 12-17 h latency period in explant cultured calf and rabbit pigment epithelial cells while rat RPE cells showed no latent period as ingestion occured in the first hour (23,24). The abundance of apical processes does not appear to be directly related to the rate of phagocytosis.

SHEDDING OR "PINCHING OFF" OF ROS DISCS PACKETS

Considerable discussion has been made as to whether ROS fragments are shed prior to phagocytosis or whether the tips of the ROS are pinched off by the microvilli of the RPE. Recent evidence has shown microvilli evaginating into the ROS, seemingly pinching off a package of ROS discs. However, many researchers insist that ROS tips are not phagocytized by the pigment epithelium before being shed into the subretinal space (25). Since the phagocytic response of the RPE cells appears to be triggered by this shedding, some chemical change must occur to the tip of the ROS that allows the RPE to recognize the shed packet of discs as foreign and to initiate phagocytosis. Basinger showed that frogs undergo a peak of disc shedding 1 h after the onset of light indicating a light-triggered burst (25). Further experiments indicate that about 25% of the photoreceptors shed their apical tips each day (26). Studies done on the phagocytic patterns in fetal animals indicated that there was a 2-3 h phagocytic delay in the fetuses as opposed to 30 minutes in the

adult, upon the onset of light (27). This result indicates the first evidence of a cyclic pattern of phagocytosis in a population of fetuses during retinal development. An initial short latent period followed by a burst of phagocytic activity was observed in chick RPE cells. This would correspond with the initial increase in rate of phagocytosis of "primed" ROS followed by a subsequent decrease in the rate of phagocytosis of possibly "unprimed" ROS (15). This late non-specific phagocytosis may correspond to the basal levels of phagocytosis.

The phagocytic dependence on light was studied by exposing rats to a light-dark cycle. The RPE ability to phagocytize ROS increased prior to the onset of light indicating that light is not a definite prerequisite for phagocytosis (25).

RETINAL PIGMENT EPITHELIAL CELLS IN TISSUE CULTURE

One of the more recent developments in the study of structure and function of RPE cells is by the growth of these cells in tissue culture. RPE cells grown as explant cultures were maintained in culture for periods up to six months (28). Mosaic, tubular and spindle growth patterns were observed to evolve with a general decrease in pigment. Multinucleate cells were increasingly common as the duration of culture time increased with as many as six or eight nuclei observed in some cells. The morphological pattern of the colonies appeared to be influenced to some degree by the number of cells present. The mosaic pattern of cell growth developed adjacent to the original explants while spindle shaped cells were in the periphery of these colonies. In

areas at some distance from the original explants, tubular arrangements of epitheloid cells were observed (28). The cellular microenvironment appears to play a decisive role in the final expression of cell differentiation.

Whittaker found that in cultured chick embryonic RPE cells the doubling time was 36 h (29). There was a dilution of melanotic components by cell growth, decay in tyrosinase activity, and cessation of tyrosinase synthesis. Several prostaglandins and dibutyryl cyclic-AMP increase pigmentation and induced a more mature type of cell (30,31).

Berman, Schwell, and Feeney as well as Siakotos have undertaken the task of purifying pigment epithelial cells with moderate success (32,33). Heller and Jones have succeeded in purifying retinal pigment epithelial cells from bovine eyes by dissociation in calcium free buffers, centrifugation in a Ficoll density gradient followed by recovery in tissue culture with 90% viability (34). Primary goals were to detach the pigment epithelial cells from the basement membrane by as gentle a procedure as possible, to isolate the RPE cells from contaminating red blood cells, ROS, and pigment granules and finally to obtain intact and viable pigment epithelial cells at the end of the procedure. Emphasis must be placed on obtaining highly purified RPE cells for subcellular fractionation.

EFFECT OF AGING ON RPE CELL MORPHOLOGY AND ENZYME ACTIVITY

Other studies done on RPE cells have looked at the morphological and enzymatic effects of aging (35). Lipofusion was

found to accumulate and increase in size during senescence in human and rat RPE. Apical microvilli are long and thin up to 11 months of age at which point they become more tubular in form. This corresponds with the reduction in phagosomes in rat RPE cells from 4 to 32 month old rats. The outer segment renewal rate may be slowed in older animals, perhaps in response to an impairment in phagocytic capability of the RPE. In terms of acid phosphatase activity there was no difference in the young and old rat RPE cells (35). The degradation of rhodopsin by a RPE lysosomal fraction was found to occur after the initial phase of degradation of other membrane proteins (36). Subsequently, rhodopsin is slowly but completely degraded as demonstrated by gel electrophoresis.

LECTIN CELL SURFACE LABELS

The lack of ROS phagocytosis found in dystrophic RPE cells may be the result of defective cell surface receptors or to the lack of such receptors. Carbohydrates on glycoproteins are thought to be involved in the recognition of molecules on cell surfaces. Recently, studies using lectins have been performed to find the distribution and arrangement of cell surface carbohydrates (37). Heath and Basinger looked at the hypothesis that sugar molecules might act as markers for ROS disc phagocytosis in the frog system. L-fucose, α-methyl-D-mannopyranoside, and D-mannose all significantly reduced the numbers of packets of ROS disc found in the RPE. D-fucose, L-mannose, D-fructose, D-galactose, D-glucose, and sucrose were

without significant effect at the same concentration. Ultrastructural examination indicated that the sugars were effective on the ROS disc shedding process rather than on phagocytosis of already shed disc packets (37).

Nir and Hall looked at the binding of ferritin-lectin complexes of Ricinus communis agglutinin (RCA), wheat germ agglutinin (WGA), and concanavalin A (Con A) to fixed frog RPE cells (38). They found that all three bound to the pigment epithelial cells quite clearly. Fer-WGA (ferritin-WGA) binding sites were somewhat more regularly distributed than the Fer-RCA (ferritin-RCA) and Fer-Con A (ferritin-Con A). Fer-RCA bound in a more clustered pattern to the microvilli separated by areas of plasma membrane which showed sparse binding of the lectin. Rat RPE cells also showed an irregular distribution of the Fer-Con A (39).

In both normal and dystrophic rats, WGA uniformly labeled both proximal and distal membrane surfaces of RPE microvilli whereas, RCA labeled primarily the distal regions (40). Con A labeled both normal and dystrophic RPE microvilli sparsely, and Lens culinaris agglutinin (LCA), specific for mannose and glucose, stained the RPE microvilli of normal rats more intensely than the diseased RCS rats. Differences in the accessibility or composition of certain cell surface sugars may be related to the diminished rate of phagocytosis in RCS retina.

IMMUNOLOGICAL APPROACHES TO STUDYING MEMBRANES

Immunological studies have been concerned with the site of

antibody-antigen action. Until recently all immunocytochemistry performed involved polyclonal antibodies. Hall used a polyclonal antisera directed against rod outer segments for studying phagocytosis in retinal pigment epithelial cells (14). A more significant advancement in immunological research was the production of monospecific (monoclonal) antibodies using cell fusion techniques developed by Kohler and Milstein (41,42). Antibodies of defined specificity produced by continuous cultures of monoclonal cell lines provide exquisite serological and biochemical probes. Labeled antibodies for light and electron microscopy provide insight into the location and distribution of specific components in cells.

Immunological studies over the past few years have resulted in several monoclonal antibodies being raised against retinal tissue. Anti-rhodopsin monoclonal antibodies designated Rho-4A2 and Rho-1D4 raised against rod outer segments have been localized and characterized fully as to their antigenic site of action (43). To date no monoclonal antibodies specific for the bovine retinal pigment epithelial cell plasma membrane have been reported.

THESIS INVESTIGATION

My thesis investigation was designed to characterize the bovine RPE cell plasma membrane and possible cell surface receptors involved in the phagocytosis of rod outer segments. The first approached used was to obtain a bovine RPE plasma membrane preparation and to characterize the polypeptides by SDS-

polyacrylamide gel electophoresis. Secondly, the monoclonal antibody technique was employed to raise antibodies to the RPE enriched plasma membrane preparation and RPE cells grown in tissue culture. Finally, bovine RPE cells were grown in tissue culture to assist in the identification of cell surface receptors as well as to establish a new assay for studying the phagocytosis of bovine rod outer segments.

EXPERIMENTAL PROCEDURES

1. MATERIALS

All general laboratory chemicals of reagent grade were obtained from Sigma Chemical, BDH Chemicals, or Fisher Scientific.

Ficoll 400 was purchased from Pharmacia Fine Chemicals. Dimethyl sulfoxide and cyanogen bromide were obtained from Fisher Scientific. BDH Chemicals supplied the sodium dodecyl sulphate (SDS) and the polyethylene glycol (PEG). B-mercaptoethanol used in SDS-gel electrophoresis was purchased from Eastman Organic Chemicals. Cytochrome c was supplied by Sigma. J.T. Baker Chemicals Co. supplied the silver nitrate.

Molecular weight standards purchased from Bio Rad Laboratories included: lysozyme M_r=14,400, soybean trypsin inhibitor M_r=21,500, carbonic anhydrase M_r=31,000, ovalbumin M_r=45,000, bovine serum albumin M_r=66,200, phosphorylase b M_r=92,500, B-galactosidase M_r=116,250, and myosin M_r=200,000.

Bovine rhodopsin N-terminus peptides were isolated from cyanogen bromide digest of rhodopsin according to the method of Hargrave *et al.*(44). The 2-39 and 2-16 N-terminal rhodopsin peptides which were purified by reverse-phase high preformance liquid chromatography (HPLC) and characterized by amino acid analysis were generously supplied by Piotr Czaykowski.

Goat anti-mouse Ig antisera and rabbit anti-actin antisera were purchased from Antibodies Incorporated and Miles-Yeda Ltd., respectively.

Iscove's Modified Dulbecco's Medium (IMDM) purchased from

GIBCO Laboratories was made up to one liter with distilled water with the addition of 3.7 g of sodium bicarbonate. The culture medium was filter sterilized and supplemented with penicillin (100 units/mL), streptomycin (100 ug/mL), and fungizone (1.25 ug/mL) as well as 10-20% fetal calf serum (FCS), all supplied by GIBCO Laboratories. RPMI 1640 culture medium was prepared in a similar manner. All cell cultures were maintained at 37°C in an atmosphere of 10% CO_2, 90% air in a humidified incubator. The trypsin and collagenase used were purchased from Difco Laboratories and Millipore Corporation, respectively.

Purified concanavalin A (Con A) was purchased from Vector Laboratories Inc; Ricinus communis agglutinin 1 M_r =120,000 (RCA) from Miles-Yeda Ltd.; and wheat germ agglutinin (WGA) from Boehringer Mannheim.

Buffer A contained 0.58 g NaCl, 1.22 g Tris, 1.02 g $MgCl_2.6H_2O$, 0.11 g $CaCl_2$, and 7 mg PMSF per liter of distilled water, pH 8.5. Buffer B contained one third of these concentrations with the exception of PMSF which was at equal concentration as Buffer A. Phosphate-buffer saline (PBS) included 8.0 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4 and 2.1 g Na_2HPO_4 per liter of distilled water at pH 7.4. RIA buffer consisted of 1% BSA, 0.1% NaN_3, and 2% FCS in PBS.

2. PROTEIN ASSAYS

All protein concentrations were determined by the method of Lowry et al.(45) using BSA as a standard.

3. ISOLATION OF BOVINE RETINAL PIGMENT EPITHELIAL CELLS

Usually 20 to 50 fresh bovine eyes (no more than 3 hours

post mortem) were transported on ice to the laboratory where they were cooled to a temperature of 10-12°C. Feeney-Burns and Berman demonstrated that the neural retina proper adheres more loosely to the epithelial layer when the bovine eyes are kept at this temperature (46).

The eye ball was initially punctured with the point of a pair of curved scissors and the anterior portion of the eye was cut at the level of the ora serrata. By tilting the eye and gently squeezing the optic cup, the lens and the aqueous and vitreous humor fell out leaving the sclera, choroid, and retina in the posterior portion of the optic cup. The translucent retina completely covering the posterior portion of the optic cup was collected around the optic nerve and removed in one piece. Care was taken not to severe the choroid or sclera layers.

After the retina had been removed the optic cup was rinsed once with 2 mL of Buffer A. This treatment removed any stray pieces of retina tissue and most of the erythrocyte contamination. In some cases the optic cup was rinsed again with an additional 2 mL of Buffer A containing 0.01% EDTA but without calcium and magnesium. This second wash helped remove some of the rod outer segments that were loosely attached to the RPE cells.

Several methods for removing the RPE cells from the optic cup were tried such as, aspirating and dissecting the cells from the tissue, but the most effective method was to brush the cells off the optic cup. Two mL of cold Buffer A were added to the optic cup and approximately 1 minute of gentle brushing was used to remove the melanotic and amelanotic RPE cells from the Bruch's

membrane. The cell suspension in the optic cup was transferred to a chilled 50 mL centrifuge tube. Another 1 mL of cold Buffer A was used to rinse the optic cup of any remaining RPE cells. The cell suspension was kept on ice for future cellular fractionation and plasma membrane isolation.

4. PREPARATION OF RETINAL PIGMENT EPITHELIAL CELL PLASMA MEMBRANE

The bovine retinal pigment epithelial cell suspension was washed twice in Buffer A by centrifugation at 1000g for 10 minutes at 4°C. The pellet of RPE cells from 50 bovine eyes was swelled overnight at 4°C in 30 mL of hypotonic buffer consisting of 0.007M Tris, pH 7.4, and 0.01% EDTA. The following morning the cells were homogenized with 5 strokes of a chilled Wheaton Potter-Elvehjem tissue grinder. The nuclei were subsequently pelleted by centifugation at 480g for 3 minutes. An equal volume of 10% Ficoll 400 was added to the homogenate and the total suspension was loaded on top of 42% (w/w) sucrose and spun in a Beckman SW 27.0 rotor at 25,000 rpm for 3 hours. The crude plasma membrane was collected at the Ficoll/sucrose interface with a 14 gauge needle. The plasma membrane suspension was diluted with one volume Buffer A and spun at 15,000 rpm for 30 minutes in a Sorvall SS 34 rotor. The plasma membrane pellet was resuspended in one volume Buffer A and one volume 20% sucrose, loaded onto a 20-50% (w/w) continuous sucrose gradient and spun overnight at 25,000 rpm in a Beckman SW 27.1 rotor. Two bands were collected between 34% and 40% (w/w) sucrose with a 14 gauge needle. Both bands were diluted separately with one volume Buffer A and pelleted in a Sorvall SS 34 rotor at 15,000 rpm for

30 minutes. Both bands were assayed for plasma membrane enrichment based on 5‡ nucleotidase and Na^+K^+ATPase specific activity. Contamination of the preparation by mitochondria and endoplasmic reticulum were quantitated by succinate cytochrome c reductase and NADH cytochrome c reductase, respectively as described below.

In order to remove the contaminating rhodopsin from the membrane preparation, detergent solubilized membrane was chromatographed on an anti-rhodopsin affinity column. Rho-1D4 monoclonal antibody]specific for rhodopsin as described by Molday and MacKenzie (43)[was covalently coupled to Sepharose 2BCl by the CNBr- activation method as described by Cuatrecasas (47). Generally 1 mL of membrane suspension (1 mg/mL) was solubilized in 1% CHAPS and incubated with 1 mL of Rho-1D4 antibody-Sepharose 2BCl beads for 30 minutes at room temperature in a Bioanalytical Systems Inc. microfiltration unit. The contaminating rhodopsin bound to the Sepharose beads while the remaining membrane components were eluted by centrifugation in a clinical bench top centrifuge at 2,500 rpm for 10 minutes. The eluted material was stored at -20°C until required.

5. ENZYME ASSAYS

A. 5'NUCLEOTIDASE

5' Nucleotidase was assayed , with modifications, according to Solyam et al.(48). Membrane protein (40-60 ug) was incubated in 0.5 M Tris buffer, 10 mM $MgCl_2$ and 10 mM adenosine 5' monophosphate, pH 7.5, in a total volume of 1 mL. After 20 minutes at 37°C the reaction was stopped by adding 1 mL of cold

10% TCA and the precipitate was pelleted by centrifugation at 3000 rpm for 5 minutes. A heat denatured membrane control was run under the same conditions. The inorganic phosphate generated was determined by the Fiske and Subbarow method (49). To a 0.5 mL volume of the assay sample 0.5 mL of the assay reagent, (0.1g ammonium molybdate dissolved in 10.0 mL of 0.5 M H_2SO_4 with 0.4 g of $FeSO_4.7H_2O$) was added and vortexed. The absorbance at 700 nm was measured for all samples between 15-30 minutes. Phosphate concentrations were calculated from a standard curve run simultaneously using inorganic phosphate.

B. Na^+ K^+ ATPASE

Na^+K^+ ATPase was assayed by a modification of the method of Costantino-Ceccarin et al.(50). Thirty uL samples (30-60 ug protein) were incubated in a total volume of 0.5 mL consisting of 50 mM Tris-Cl, pH 7.4, 100 mM NaCl, and 3 mM $MgCl_2$ with 3 mM Tris-ATP for 15 minutes at 37°C. The reaction was started by the addition of 20 uL of 0.5 M KCl in the presence or absence of 10^{-4} M ouabain. The reaction was run for 30 minutes prior to being terminated by the addition of 0.5 mL of ice cold 10% TCA. The samples were chilled on ice for five minutes and then centrifuged at 3000 rpm for 5 minutes to pellet the precipitate. Supernatant aliquots of 0.5 mL from each sample were assayed for inorganic phosphate by the method of Fiske and Subbarrow (49) as previously described.

C. NADPH CYTOCHROME C REDUCTASE

NADPH cytochrome c reductase activity was assayed by the method of Ragnotti et al. (51). A 50 uL sample (50-100 ug

protein) was added to 1.42 mL of a solution consisting of 66 umoles of potassium phosphate, 0.05 umoles KCN, 100 umoles KCl, and 0.075 umoles cytochrome c, at pH 7.6. The reaction was started by the addition of 30 uL of 3 mM NADPH. The increase in absorbance at 550 nm was recorded for 4 minutes. The specific activity was calculated from the molar extinction coefficient of 18.7 X $10^3 M^{-1} cm^{-1}$ for cytochrome c (reduced minus oxidized) at 550 nm.

D. SUCCINATE CYTOCHROME C REDUCTASE

Succinate cytochrome c reductase activity was measured by the method of King (52). A 50 uL sample (50-100 ug protein) was added to 0.92 mL of a solution consisting of 100 umoles potassium phosphate, 2.5 umoles KCN, 0.3 umoles EDTA, and 0.1 umoles cytochrome c, at pH 7.4. The reaction was started by the addition of 30 uL of 0.6 M succinate. The increase in absorbance at 550 nm was recorded for 3 minutes. The specific activity was calculated from the molar extinction coefficient of 18.7 X $10^3 M^{-1} cm^{-1}$ for cytochrome c (reduced minus oxidized) at 550 nm.

6. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS AND GEL TRANSFER

RPE cell plasma membrane or rod outer segment preparations (1.0-5.0 mg/mL) were solubilized in an equal volume of denaturing solution consisting of 5% SDS, 40% sucrose, 0.01 M Tris, pH 6.8, 10% B-mercaptoethanol and 4% bromophenol blue. Samples (15-25 uL) were applied to wells of a 6.5-15% polyacrylamide gradient slab gel or a 10% continuous polyacrylamide slab gel (0.75 mm X 5.0 cm or 0.75 mm X 12.0 cm) and electrophoresis was carried out according to the procedure of Laemmli (53). Gel slices were

either stained with coomassie blue according to Fairbanks et al.(54) or stained with silver according to Wray et al.(55).

In some cases gels were subjected to electrophoretic transfer as adapted from blotting transfer procedure of Towbin et al.(56). Briefly, SDS-gels were washed twice with 100 mL changes of 20 mM Tris-Acetate, 2 mM NaEDTA, and 0.01% SDS (transfer buffer). The washed gels were sandwiched against a wet sheet of pure nitrocellulose membrane and placed in a Bio Rad transblot apparatus. Electrophoretic transfer was carried out at 19 V and 0.5 A at 4°C in transfer buffer for 12-16 h. Following transfer the nitrocellulose paper was quenched of any remaining binding sites for 2-16 h at room temperature with immunoblot buffer consisting of 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, 1 mM NaN_3, and 2% Triton-X 100 with 4% BSA at pH 7.5. The nitrocellulose paper was rinsed in distilled water and incubated immediately with a monoclonal antibody.

In other cases, gel slices underwent electrophoretic transfer to CNBr-activated paper prepared by the method of Clark et al.(57). Prior to the transfer the gel was washed in three 100 mL changes of 0.1 M sodium phosphate, pH 7.4, containing 0.1% SDS for 5 minutes in each ,followed by two 100 mL changes in 0.02 M sodium phosphate, pH 7.4, for 5 minutes each. The transfer took place in a Bio Rad transblot apparatus at 35 V and 1.5 A at 4°C in 0.02 M sodium phosphate, pH 7.4, for 2-4 h. After the transfer the remaining CNBr- reactive groups were quenched in Tris buffer, pH 9.0, with 0.06 M glycine and 1% BSA overnight at room temperature. The following morning, the filter papers were rinsed

in distilled water and incubated with the appropriate monoclonal antibody.

7. PREPARATION OF GOAT ANTI-MOUSE Ig AND GOAT ANTI-RABBIT Ig ANTIBODY REAGENTS

Goat anti-mouse Ig and goat anti-rabbit Ig were purified by affinity chromatography on a mouse Ig or rabbit Ig Sepharose 4B column, respectively (58). Both goat anti-mouse Ig and goat anti-rabbit Ig antibodies were labeled with ^{125}I by the chloramine T method (59) to give specific activities of 1-2 x 10^6 dpm/ug. One mg of the antibody was dissolved in 0.1 M PBS followed by the addition of 500 uCi of $Na^{125}I$. The reaction was initiated by the addition of 5 uL of chloramine T (4 mg/mL) at room temperature. After 10 minutes the free $Na^{125}I$ was removed by centrifuging the reaction mixture at 1500-2000 rpm for 10 minutes through a Centrex Microfilter unit loaded with 0.5 g of AG 1-X10 anion exchange resin.

8. POLYPEPTIDE DETECTION BY ANTIBODY

Transfer papers were rinsed in RIA buffer and incubated with 10 mL of hybridoma culture fluid or rabbit anti-actin antisera (100 fold diluted) for 1-2 h at 23°C. The papers were then washed for 1-2 h with several changes of immunoblot buffer without BSA followed by two changes in wash buffer consisting of 2 M urea, 0.1 M glycine, and 1% Triton X-100. The papers were subsequently incubated with ^{125}I-labeled goat anti-mouse Ig or ^{125}I-labeled goat anti-rabbit Ig (1-2 x 10^6 dpm/mL) for 1-2 h at 23°C followed by extensive washing as before. The nitrocellulose papers were air dried and subjected to autoradiography on preflashed Kodak

Royal X-Omat film with an X-ray intensifying screen for 12-48 h at 23°C. In the case of CNBr- activated papers, all washing took place in several changes of PBS with 0.4% N-lauroyl sarcosine.

9. SILVER STAINING TECHNIQUE

SDS gels were washed for 2 h in 50% methanol/10% acetic acid and silver stained according to the protocol of Wray et al.(55). The fresh staining solution contained 0.8 g silver nitrate 21 mL of 0.36% sodium hydroxide in a total volume of 100 mL. The gel was stained for 15 minutes with constant gentle agitation followed by a 5 minute wash in deionized distilled water. The stain was developed in 2.5 mL of 1% citric acid and 0.25 ml of 38% formaldehyde in a total volume of 500 mL for less than 10 minutes. The development was stopped by placing the gel in 45% methanol/10% acetic acid. The gel was swelled to normal size in distilled water and photographed with Kodak high contrast film.

10. MONOCLONAL ANTIBODY TECHNIQUES

A. IMMUNIZATION OF BALB/C MICE

Technique for the production of monoclonal antibodies were developed by Milstein (41). Male or female BALB/C mice were immunized with a 100 ug of RPE plasma membrane preparation or RPE cells grown in tissue culture, emulsified with 0.1 mL of Freund's complete adjuvant. Three intraperitoneal injections were carried out three weeks apart with the spleen cell- myeloma cell fusion taking place four days after the last immunization.

B. PREPARATION OF TISSUE CULTURE MYELOMA CELLS

One vial of liquid nitrogen frozen NS-1 (myeloma) cells containing 1 x 10^7 cells was thawed in a 37°C water bath. The

cells were washed once in 10 mL of IMDM medium supplemented with 10% fetal calf serum (FCS) and pelleted at 1500 rpm for 5 minutes. The cells were resuspended in 5 mL of supplemented medium. A sample of the cells was diluted one to one with 0.4% trypan blue and the viable cells were counted on a haemocytometer. The cells were plated out at 1 x 10^5 viable cells per mL in 10 mL petri dishes. The cells were maintained between 5 x 10^4- 5 x 10^6cells per mL for 7-10 days before sufficient numbers were available for a cell fusion.

C PRODUCTION OF HYBRIDOMA CELLS

The myeloma cells grown in tissue culture were aspirated off the 10 mL petri dishes and collected in polystyrene sterile tubes. The cells were spun down at 1500 rpm for 5 minutes and collectively resuspended in 10 mL of serum free medium. Approximately 2 x 10^7 viable cells were used for a cell fusion.

A thymocyte cell suspension was prepared from one weaning rat which acted as a feeder layer for cell growth. The thymus was excised from above the rat heart and subsequently broken up in 10 mL of serum free IMDM medium by squeezing the thymus through a sterile piece of cheese cloth. The cell suspension was dispersed through a 20.5 gauge needle into a 10 mL polystyrene tube and centrifuged at 1500 rpm for 10 minutes. The thymocyte pellet was resuspended in 10 mL of IMDM medium supplemented with 100 uM hypoxanthine, 0.4 uM aminopterin, 16 uM thymidine (1 x HAT) and 20% FCS and stored at 37°C. A viable cell count was also performed on the haemocytometer as described above.

The spleen cell suspension was prepared from the immunized

BALB/C mouse. The spleen was removed from the mouse under sterile conditions and placed in a 10 mL petri dish containing serum free IMDM medium. The spleen was finely chopped in order to disperse the cells. The cell suspension was forced through a 20.5 gauge needle into a 10 mL polystyrene tube and spun at 1500 rpm for 5 minutes. The pellet was resuspended in 5 mL of serum free IMDM medium and incubated at 37°C. A sample of serum from the mouse was saved for a solid-phase radioimmune assay of the titre.

The cell fusion was carried out according to Galfre et al.(60). Immune spleen cells (2×10^8) and myeloma cells (2×10^7) were mixed in a centrifuge tube and pelleted at 1500 rpm for 5 minutes. The cells were washed in serum free IMDM and repelleted. The pellet of cells was placed in a 37°C water bath and 1 mL of 50% polyethylene glycol (PEG) 1500 was added over a period of 1 minute. The suspension was stirred for 1 minute followed by the addition of 2 mL of serum free IMDM over the following 2 minutes. An additional 7 mL of serum free medium was added to the cell suspension over the next 2-3 minutes. The cell suspension was spun at 1500 rpm for 5 minutes and the pellet was resuspended in the same IMDM medium and repelleted. Finally, the pellet was resuspended in 100 mL of IMDM 1 x HAT medium supplemented with 20% FCS and 2×10^6 thymocytes per mL. The cells were plated out in 1 mL volumes in multiwell dishes. After incubation at 37°C for one week, the old medium was aspirated off the wells and fresh IMDM 1 x HAT medium supplemented with 20% FCS was added. The wells were assayed for positive antibody producing cells by the solid-phase radioimmune assay method, 10-14 days

after the fusion.

D. STANDARD RIA

A standard solid-phase radioimmune assay was used to detect antibody secreting hybridomas according to the procedure developed by MacKenzie and Molday (58). The bovine RPE plasma membrane preparation or a rod outer segment preparation (1-2 mg/mL protein) was solubilized with 1% Triton X-100 and diluted to 0.25 mg/mL with distilled water. Aliquots of 25 uL were dried down on flex vinyl microtitre wells at 60^{o}C for 2 h. The plate was washed in distilled water followed by incubation with RIA buffer for 1 h to quench the non-specific binding sites. The vinyl plate was rinsed briefly in PBS buffer prior to incubation with 25 uL of culture fluid for 1 h at room temperature. The plate was washed extensively with PBS to remove unbound antibody and subsequently incubated with 25 uL of ^{125}I-labeled goat anti-mouse Ig (15-40 ug/mL; 1-2 x 10^{6} dpm/ug) for 1 h at room temperature. The plate was washed extensively as before and counted in a Beckman 8000 Gamma Counter.

In some cases rhodopsin, actin or other proteins were used as the solid phase antigen. Fixed bovine RPE cells, previously grown in vinyl flat-bottom microtitre wells, were also used as an antigen in radioimmune assays.

E. HYBRIDOMA CLONING

Hybridoma cells from positive microtitre wells were aspirated off the flat bottomed wells and transferred to a centrifuge tube and spun at 1500 rpm for 5 minutes. The pellet was resuspended in 1 mL of IMDM 1 x HT medium supplemented with 20% FCS and counted

on a haemocytometer. Approximately 750-1000 cells were plated out in 0.1 mL multiwell plates resulting in 5 cells/well or 1 cell/well with 2×10^6 thymocytes /mL. The remaining uncloned cells were pipeted into a 5 mL petri dish with 2×10^6 thymocytes/mL. One week later the wells were marked for single colony growth. The cloned cells were assayed 10-14 days later by RIA and the positive wells were recloned as before until virtually all wells were positive. The cells were then expanded for monoclonal antibody production either in culture fluid or in ascites fluid.

F. STORAGE OF VIABLE HYBRIDOMA CELLS

Hybridoma cells growing in log phase were aspirated off the petri dishes, transferred to a centrifuge tube and pelleted as before. The pellet was resuspended in 1.0 mL of 10% FCS supplemented growth medium plus 10% DMSO. The cells were frozen slowly to avoid ice crystal formation by placing vials in a foam insulated cell box located in a $-70^{\circ}C$ freezer. After 24 hours the vials were stored indefinitely in liquid nitrogen.

G. SOLID-PHASE RADIOIMMUNE COMPETITION ASSAYS

Competition assays were performed in order to more specifically define the antibody site of action. Briefly, 50 uL of varying concentrations of competing antigen was incubated at 23° C with 50 uL of hybridoma culture fluid. The culture fluid used was previously diluted to a concentration which gave 80-90% saturation of binding by the standard-solid phase RIA. After 1 h 50 uL of the mixture was removed and screened for remaining antibody activity by the standard solid-phase radioimmune assay

described above.

H. LOWICRYL THIN SECTION LABELING

Retina tissue was fixed in 1.25% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, plus 0.25 M sucrose for 30-60 minutes at 4°C. After washing in the same buffer for 1 h, the tissue was embedded in Lowicryl resin according to the method of Roth et al. (61). Briefly, the tissue was dehydrated in a graded series of aqueous dimethylformamide (DMF) at progressively lower temperatures: 50% at 4°C, 70% at -20°C, 90% at -35°C, and 2 changes of 100% at -35°C all for 30 minutes in each. The tissue was transferred to a mixture of 2 volumes of 100% DMF and 1 volume Lowicryl K4M resin for 1 h at -35°C followed by 1 volume 100% DMF and 1 volume Lowicryl K4M for 1 h at -35°C. After incubating the tissue in 100% Lowicryl K4M resin overnight at -35°C, the Lowicryl K4M resin was polymerized by ultraviolet irradiation overnight at -35°C.

Following polymerization of the Lowicryl resin embedded tissue, the blocks were trimmed and ultathin sections (60-70 nm) were cut and collected on clean copper grids. The grids were pre-incubated in 50 uL of PBS with 0.1% BSA for 10 minutes to block non-specific binding sites. The grids were then incubated in 50 uL of 10 fold diluted Rho-5A3 culture fluid for 30 minutes at room temperature followed by extensive washing in PBS and a 10 minute incubation in PBS with 0.1% BSA. Finally, the grids were incubated in 50 uL of 5 fold diluted goat anti-mouse Ig dextran-gold for 30 minutes at room temperature (62). After washing the grids extensively in PBS, they were stained with saturated uranyl

acetate for 5-7 minutes and saturated lead citrate for 1.5-2 minutes at room temperature. The sections were viewed on a Philips 200 electron microscope.

11. BOVINE RETINAL PIGMENT EPITHELIAL CELLS IN TISSUE CULTURE

A. CULTURING OF BOVINE RPE CELLS

Bovine RPE cells were cultured *in vitro* by modifications to the method of Basu *et al.* (63). Fresh bovine eyes were dissected as previously described under sterile conditions. The optic cup was rinsed once in sterile Buffer A. The optic cup was subsequently incubated with 2 mL of sterile PBS with 0.25% trypsin and 70 units/mL collagenase at 37°C with constant gentle agitation for 60-80 minutes. The RPE cells were subsequently aspirated off the optic cup and transferred to a centrifuge tube. The cell suspension was diluted with 3 volumes of culture medium consisting of RPMI-1640 medium supplemented with 10 mM Hepes, pH 7.2, buffer, antibiotics (penicillin 100 units/mL, streptomycin 100 ug/mL, and fungizone 50 ug/mL) and 10% FCS. The cell suspension was spun at 1500 rpm for 5 minutes. The pellet was washed in culture medium and repelleted as before. The RPE cells were plated out (approximately 5 x 10^5 cells/mL) in 10 mL Kimax glass petri dishes or 10 mL plastic petri dishes containing alcohol sterilized glass coverslips. The cells were grown at 37°C for 7-14 days or until confluent growth was reached at which point the cells were fixed in 0.2% glutaraldehyde, sub-cultured, or harvested and stored for future use.

B. FLUORESCENT DETECTION OF ACTIN IN RPE CELLS

Bovine RPE cells were grown on glass coverslips in tissue

culture for 2 weeks or until confluent growth was achieved. The coverslips were rinsed in PBS buffer with 0.81 mM $MgSO_4$ and 1.27 mM $CaCl_2$ prior to fixation in 1% paraformaldehyde in the same buffer for 30 minutes at 4°C. The coverslips were washed in PBS with Mg^{++} and Ca^{++} for 30 minutes. The cells were treated in 50% acetone for 3 minutes followed by 5 minutes in absolute acetone and replaced in 50% acetone for 3 minutes, all at 4°C. Again the cells were washed in PBS with Mg^{++} and Ca^{++} for 30 minutes. The RPE cells were incubated in 100 times diluted rabbit anti-actin antisera for 40 minutes at 4°C. The coverslips were washed extensively in three changes of PBS with Mg^{++} and Ca^{++}. The cells were incubated for 40 minutes at 4°C with 100 uL of FITC-labeled goat anti-rabbit Ig antibody. The cells were washed as before and mounted with 50% glycerol on a glass slide, prior to being viewed and photographed with a Leitz fluorescent microscope. Two controls were run with the test experiment. In one control the primary antibody was omitted in the first labeling step; in the second control, a solution of the primary antibody incubated with 100 ug of purified actin was used in the first step.

C. FLUORESCENT LECTIN LABELED BOVINE RPE CELLS IN VITRO

Fluorescent lectins are prepared according to Maher and Molday (64,65) by reacting the lectin with FITC (0.05 mg/mg lectin) in 0.02 M sodium carbonate, pH 9.0, for 2 h at 25°C. The fluorescent lectins were separated from free dyes on the appropriate affinity column: ovomucoid-Sepharose, for WGA: Sepharose 4B, for RCA: and Sephadex G-200, for Con A. FITC-lectin preparations typically had 495 nm/280 nm absorbance ratios of

0.5-1.0.

Coverslips of 1-2 week old bovine RPE cells were fixed with 1% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.2, with 0.18 M sucrose for 20 minutes at 4°C. The fixed or unfixed RPE cells were rinsed in PBS with Mg^{++} and Ca^{++} followed by incubation at 37°C for 5 minutes with 100 ug/mL of the appropriate fluorescent lectin. The excess or unbound lectin was washed off by successive washes in the same buffer. Controls were run by competing off the bound lectin for 15 minutes, at 37°C, with 0.2 M methyl mannoside, 0.02 M N-N-diacetyl chibitose, or 0.2 M D-galactose for Con A, WGA, or RCA, respectively. Fluorescent lectin treated RPE cells were observed and photographed under a Leitz fluorescent microscope.

D. DISCONTINUOUS FLUORESCENT LECTIN LABELING OF BOVINE RPE CELLS

Unfixed bovine RPE cells grown in tissue culture for 2 weeks were rinsed in PBS with Mg^{++} and Ca^{++} and incubated with 100 ug/mL of the appropriate fluorescent lectin for 5 minutes, at 37°C. The coverslips were rinsed in PBS with Mg^{++} and Ca^{++} followed by a 60 minute incubation period in the same buffer at 37°C. Subsequently the cells were either rinsed in the same buffer or incubated for an additional 15 minutes, at 37°C, with the appropriate lectin inhibitor and rinsed prior to observation.

E. CONTINUOUS FITC-LECTIN LABELING OF BOVINE RPE CELLS

Unfixed bovine RPE cells grown in tissue culture for two weeks were labeled with fluorescent lectins as before with the exception that the cells were continuously labeled for 60 minutes at 37°C. The coverslips were washed extensively in PBS with Mg^{++}

and Ca^{++} prior to observation under the fluorescent microscope.

11. PHAGOCYTOSIS OF BOVINE ROS BY BOVINE RPE CELLS IN VITRO

A. PREPARATION OF SEALED DARK ADAPTED BOVINE ROS

Twenty-five fresh bovine eyes collected from the local slaughter house were dark adapted at room temperature for 30 minutes. The retinas were carefully removed and placed in 10-15 mL of homogenizing solution consisting of 20% sucrose, 20 mM Tris, pH 7.2, 0.25 mM $MgCl_2$, 5 mM taurine, and 10 mM glucose. The ROS were broken loose from the retina by gently inverting the solution approximately thirty times, followed by filtration through cheese cloth. The procedure was repeated with an additional 1-2 mL of homogenizing solution. The solution was centrifuged for 2 minutes at 1000 rpm and the pellet discarded. The supernatant was filtered through cheese cloth once more, layered on a 27-60% (w/w) continuous sucrose gradient and spun in a Beckman SW 27.0 rotor at 25,000 rpm for 1.5 h. The band at the top of the gradient (approximately 30%) was removed and 1 volume was diluted with 2 volumes 0.01 M Tris buffer, pH 7.4. The solution was centrifuged at 5000 rpm at $4^{\circ}C$ for 5 minutes. The pellet was washed twice in Hank's Balance Salt Solution (HBSS) and centrifugation was repeated. The ROS were resuspended in 1 mL of RPMI 1640 medium supplemented with 10% FCS.

B. PHAGOCYTOSIS OF ROS BY ADULT BOVINE RPE CELLS IN VITRO

Two week old bovine RPE cells grown on glass coverslips were initially washed in PBS with Mg^{++} and Ca^{++} prior to incubation with dark adapted ROS. The cells were incubated in the dark for 5 h at $37^{\circ}C$ followed by extensive washing in the same buffer. In

order to enhance phagocytosis 10 mM glucose was also added to the ROS suspension in some cases. The cells were fixed in 0.2%-1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, and prepared for transmission electron microscopy (TEM).

C. PREPARATION OF SAMPLES FOR TRANSMISSION ELECTRON MICROSCOPY

Tissue samples were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, containing 0.25 M sucrose for 30-60 minutes at 4°C. The samples were washed in the same buffer prior to post fixing in 1% osmium tetroxide for 30-60 minutes at 4°C. After the samples were washed in the same buffer for 1 h, they were dehydrated in an ethanol series (50%,70%,90%, and two changes of absolute ethanol) for 15 minutes in each at room temperature. Dehydration was continued in a 50:50 mixture of absolute ethanol and propylene oxide for 15 minutes at room temperature followed by a 15 minute incubation in propylene oxide. Araldite/Epon resin penetration was facilitated by incubating samples in a 50:50 mix of propylene oxide and resin for 1-2 h followed by 24-48 h in 100% resin. The embedded samples were placed in a 60°C oven and allowed to harden for 24 h.

Seventy nanometer thick sections were cut on a Sorvall 5000 Ultra Microtome and supported on formvar coated S200 copper grids or uncoated S200 copper grids. Prior to viewing, the grids were stained with saturated uranyl acetate for 5-7 minutes at room temperature. The grids were also stained with saturated lead citrate for 1.5-2 minutes before being observed on a Philips 200 electron microscope.

D. PREPARATION OF SAMPLES FOR SCANNING ELECTRON MICROSCOPY

Unlabeled or labeled cells were washed in PBS and prepared for scanning electron microscopy as follows (66). The RPE cells were first fixed in 1% glutaraldehyde in 0.1 M cacodylate buffer for 1 h at 4°C followed by washing in the same buffer for 1 h. The RPE cells were stained with 1% osmium tetroxide (O) and 1% thiocarbohydrazide (T) in the sequence O:T:O:T:O for 30 minutes in each with extensive washing in cacodylate buffer between each step. The cells were dehydrated in a graded ethanol series (50%,70%,90%, and 2 changes of absolute ethanol) for 15 minutes each at room temperature. The cells were dried from CO_2 in a Polaron critical point drying apparatus (67) and coated with 200 nm of gold-palladium in a Technics Hummer V Sputter-Coater. Samples were viewed and photographed on a Cambridge 250 Scanning Electron Microscope (SEM).

SECTION 1

ANALYSIS OF BOVINE RETINAL PIGMENT EPITHELIAL PLASMA MEMBRANE PREPARATIONS

RESULTS

1. PREPARATION OF BOVINE RPE PLASMA MEMBRANE

The fractionation of bovine RPE cell membranes was monitored by following the distribution of markers for different subcellular components; Na^+K^+ ATPase (50,68,69) and 5‡ nucleotidase (48,70), for the plasma membrane; NADPH cytochrome c reductase (51,69) for endoplasmic reticulum; and succinate cytochrome c reductase (52,69) for mitochrondia. Table 1 follows the total activity and specific activity for each of these enzymes as well as their enrichment and recovery with respect to the cell homogenate.

After the initial hypotonic disruption of the RPE cells, the nuclei were pelleted yielding the 480g supernatant. Centrifugation of the supernatant on a Ficoll/ 42% sucrose discontinuous gradient yielded a 3.5 to 5.0 fold enriched plasma membrane preparation. Subsequent centrifugation on a 20-50% continuous sucrose gradient resulted in two plasma membrane enriched bands, the lower of which had a more turbid appearance.

The plasma membrane markers were enriched by 7-9 fold with respect to the cell homogenate with a recovery of about 18%. NADPH cytochrome c reductase specific activity decreased approximately 2-5 fold based on the homogenate. Succinate cytochrome c reductase specific activity was higher at the Ficoll/ sucrose interface but lower in the bands obtained from

TABLE 1

DISTRIBUTION OF MARKERS IN VARIOUS FRACTIONS RECOVERED DURING MEMBRANE ISOLATION

Fraction	5'Nucleotidase				Na^+K^+ ATPase			
	TA	%R	SA	PF	TA	%R	SA	PF
Homogenate	936	100	15.6	1	852	100	14.2	1
480 g supernatant	915	97.0	18.3	1.2	841	98.0	16.8	1.2
Interface	360	38.5	60.0	3.8	451	53.0	76.2	5.3
25,000 rpm Top Band	100	10.7	125	8.0	101	11.8	127	8.9
25,000 rpm Bottom Band	72.1	7.8	90.0	5.7	71.4	8.3	88.8	6.3

Fraction	NADPH Cyto C Reductase				Succinate Cyto C Reductase			
	TA	%R	SA	PF	TA	%R	SA	PF
Homogenate	1632	100	34.3	1	912	100	19.0	1
480g Supernatant	1497	91.7	39.0	1.13	916	100	23.5	1.2
Interface	197	12.0	47.0	1.14	182	20.0	43.3	2.27
25,000 rpm Top Band	27.2	1.6	16.0	0.46	15.7	1.7	9.1	0.47
25,000 rpm Bottom Band	17.6	1.1	8.0	0.23	54.3	5.9	24.7	1.30

Table 1. Distribution of markers in various fractions recovered during membrane isolation. The homogenate represents the cellular suspension after hypotonic disruption, while the 480g supernatant was the cell suspension after pelleting the nuclei. The interface is the membrane band collected from the Ficoll/42% sucrose interface. The 25,000 rpm top and bottom bands are the two bands collected from the 20-50% (w/w) continuous sucrose gradient. TA = total activity (nmoles/min). %R = represents the enzymatic recovery based on the cell homogenate. SA = specific activity (nmoles/min/mg). and PF = purification fold based on the cell homogenate.

the continuous sucrose gradient. However, the lower band had a higher succinate cytochrome c reductase specific activity than the homogenate indicating a mitochondria membrane contamination of this band.

Analysis of the membrane preparations by coomassie blue stained polyacrylamide gels indicated the wide diversity of polypeptide bands found in the RPE preparations ranging from an apparent M_r=20,000 to 200,000 (Fig.3 lanes b and c). In Fig.3 lanes b and c represent the top and bottom bands collected from the continuous sucrose gradient which differ considerably in staining pattern from a rod outer segment preparation (Fig.3 lane d). However, the RPE membrane preparations had a large rhodopsin contamination as seen by a major band at apparent M_r=34,000. A fractionation study of a RPE cell homogenate spun on a 30-50% (w/w) continuous sucrose gradient resulted in a high Rho-1D4 (anti-rhodopsin monoclonal antibody) binding level at the fraction where 5'nucleotidase activity was at a peak (Fig.4). This indicated that both the RPE and ROS membranes comigrate in the sucrose gradient. In an attempt to remove some of the rhodopsin contamination, the membrane preparation was solubilized in 1% CHAPS and passed through an immunoaffinity column consisting of Rho-1D4 antibody covalently linked to Sepharose 4B beads. The immunoaffinity column removed most of the rhodopsin as shown by a coomassie blue stained SDS-polyacrylamide gel (Fig. 3,lane a). Once the membrane preparation passed through the affinity column the polypeptides above an apparent M_r=100,000 and below M_r=45,000 were not seen by coomassie blue staining due to

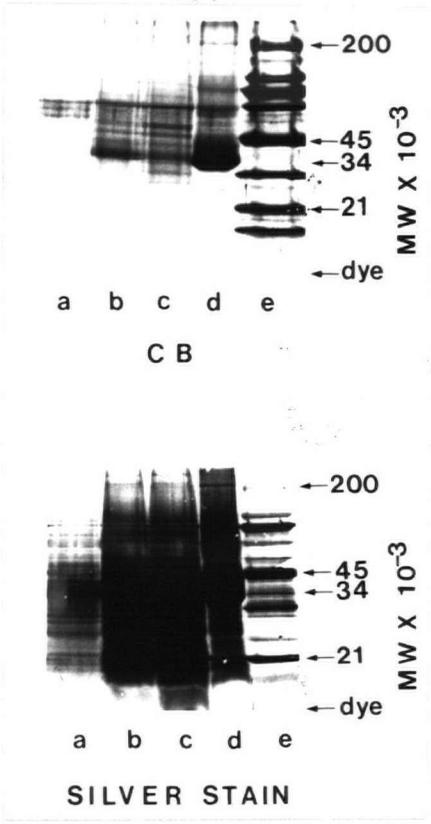


Figure 3. Coomassie blue and silver stained SDS-polyacrylamide gels of various membrane fractions. Membrane samples were solubilized in SDS in the presence of 2-mercaptoethanol and 25 uL were applied to each well. Electrophoresis was carried out on a 5-16% gradient SDS-polyacrylamide gel. Gels were stained with either coomassie blue (CB) or silver. a) 1% CHAPS solubilized bovine retinal pigment epithelial plasma membrane preparation passed through an anti-rhodopsin (Rho-1D4) Sepharose immunoaffinity column. The suspension was centrifuged and 25 uL of the eludent were loaded on the gel b) 25 uL of the top band collected from a 20-50% (w/w) continuous sucrose gradient centrifuged in an Beckman SW 27.1 rotor at 25,000 rpm for 14-16 h. c) 25 uL of the lower band collected from the same continuous gradient. d) 25 uL of a rod outer segment preparation. e) molecular weight standards. Lanes b-e were over developed when stained with silver to identify the polypeptide bands in lane a.

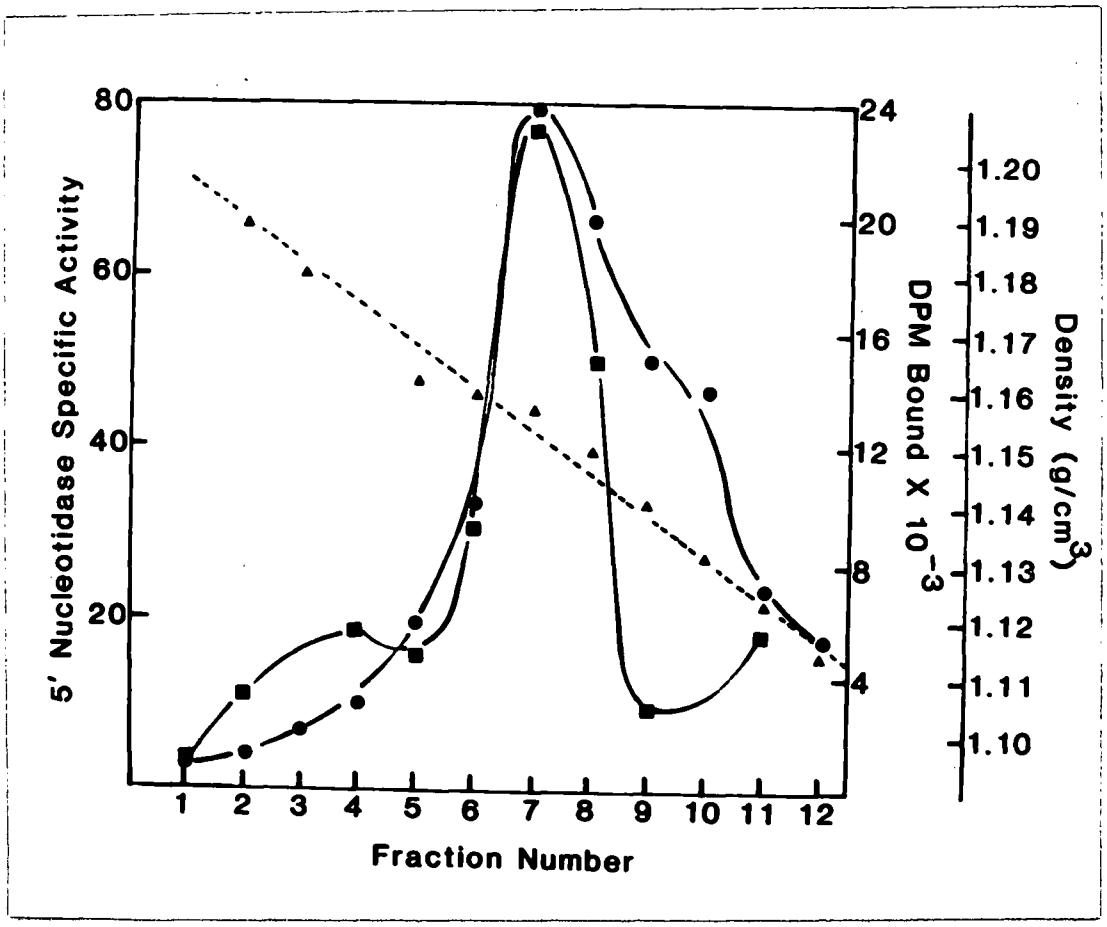


Figure 4. A RPE cell homogenate fractionation profile of a 30-50% (w/w) continuous sucrose gradient centrifuged for 14 h at 25,000 rpm. Fractions were collected with a fraction collector in 2 ml volumes. 5'nucleotidase specific activity (■—■) was in nmoles/min/mg. Mouse anti-rhodopsin Rho-1D4 hybridoma ascites fluid in conjunction with ^{125}I-labeled goat anti-mouse Ig was used to determine the amount of binding to each fraction by the standard RIA method (●—●) as described in Experimental Procedures. Sucrose densities were determined by measuring the refractive index of each fraction.

polypeptide dilution. Using a much more sensitive silver stain method most of the gel bands had been conserved after passing through the immunoaffinity column (Fig. 3 lane a). The gel that was silver stained had to be over developed in order to identify the polypeptide bands in lane a.

2. DETECTION OF ACTIN IN THE RPE PLASMA MEMBRANE PREPARATION

With the knowledge that actin is an important cytoskeletal protein, it was expected that actin would be a major RPE cell component due to the role of RPE cells in the phagocytosis of rod outer segments. The RPE plasma membrane preparation was run on a SDS-polyacrylamide gel and subsequently transferred to nitrocellulose where actin was detected by a rabbit anti-actin antisera at an apparent M_r=46,000 (Fig. 5). It was also noted that little actin was detected in the ROS preparation as opposed to the bovine RPE plasma membrane preparation. The possible significance of actin in these cells will be discussed later.

3. TEM OBSERVATION OF SUBCELLULAR FRACTIONATION

Rhodopsin was detected in the plasma membrane preparation by SDS-polyacrylamide gel electrophoresis which led to the possibility that the membrane preparation was contaminated with rod outer segment disc material. At the level of TEM, ROS disc-like material was found in the plasma membrane enriched bands collected from the sucrose gradient (Fig. 6). The plasma membrane fragments appeared as vesicles of varying size.

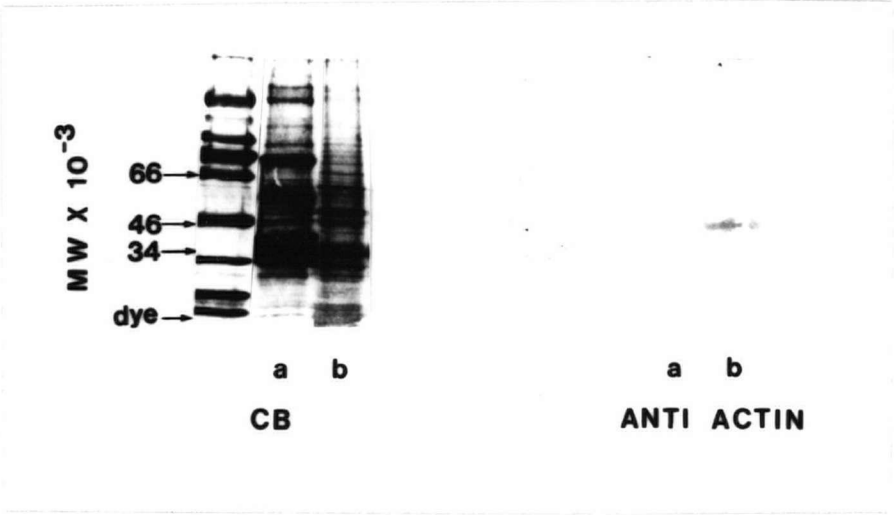


Figure 5. Detection of actin in ROS and bovine RPE plasma membrane preparation by SDS-polyacrylamide gel eletrophoresis in conjunction with immunoblot. Rod outer segments (a) and bovine RPE cell plasma membrane preparations (b) were solubilized in SDS in the presence of 2-mercaptoethanol and 25 uL of each was subjected to electrophoresis on a 5-16% gradient SDS-polyacrylamide gel. The gels were stained with coomassie blue (CB) or electrophoretically transferred to nitrocellulose paper. The nitrocellulose paper was incubated for two hours with rabbit anti-actin antisera, washed in immunoblot buffer and incubated for 1 hour in ^{125}I-labeled goat anti-rabbit Ig antibody (1-2 X 10^6 dpm/mL). The paper was washed as before and subjected to autoradiography.

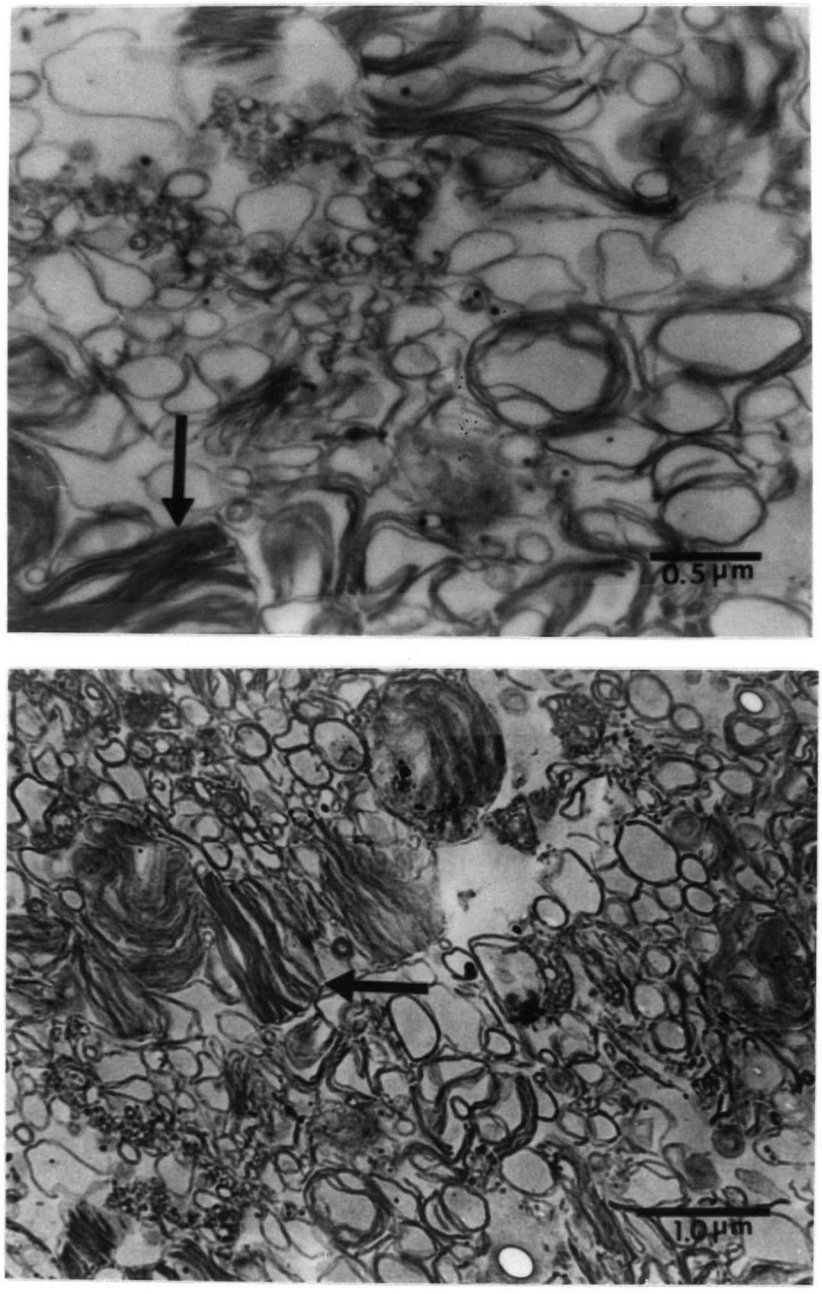


Figure 6. Transmission electron micrographs of bovine retinal pigment epithelial cell plasma membrane preparations collected from the top band of a 20-50% (w/w) continuous sucrose gradient. Arrows denote areas of possible contaminating ROS disc material isolated with the bovine retinal pigment epithelial cell plasma membrane.

DISCUSSION

The RPE plasma membrane preparation was 7-9 fold enriched based on the cellular homogenate. Both Na^+K^+ ATPase and 5'nucleotidase plasma membrane markers showed similar fold purification. Similar recoveries and increases in specific activities of plasma membrane markers have been found with several other cell lines with similar methods of purification (68,71). A minor contamination of the membrane preparation was mitochondrial membrane in the lower sucrose band. However the primary source of impurities in the RPE membrane preparation was rod outer segment membrane from rod photoreceptor cells.

In the adult bovine eye it appears that the indigitated junction between the RPE cells and the photoreceptor cells is extremely tight. The long and numerous microvilli of the RPE prevent the clean removal of all ROS with the retina. Subsequent washing with Buffer A containing EDTA failed to release the tightly bound ROS from the RPE. Analysis of the fractions from the continuous sucrose gradient designed to separate subcellular organelles demonstrated the comigration of ROS membrane and RPE plasma membrane. Disrupted RPE cells in 10% Ficoll were layered on top of 42% sucrose and centrifuged in an attempt to float the "sack like" sealed ROS discs on top of the Ficoll while collecting a crude membrane band at the interface. The material pelleted through this centrifugation was unbroken mitochondria, residual nuclei, and pigment. This method proved to be successful in removing approximately half the disc material consisting

primarily of rhodopsin based on coomassie blue stained gels. Considerable plasma membrane marker activity (50-60%) was lost when the membrane preparation was spun on top of the 42% sucrose as much of the plasma membrane got trapped with the pigment and pelleted through the sucrose.

Plasma membrane marker activity was found in both of the primary bands collected from the continuous sucrose gradient. The plasma membrane in the lower band probably migrated further in the sucrose due its aggregation with mitochondrial membrane fragments. Analyzing the crude plasma membrane and the upper and lower bands collected off of the continuous sucrose gradient by SDS gel electrophoresis revealed that many of the polypeptide bands were conserved through out the plasma membrane purification procedure. Most of the polypeptides seen in this SDS polyacrylamide gel electrophoretic system have apparent M_r ranging from 20,000 to 200,000. One primary band seen on coomassie blue stained SDS gels and identified by the immunoblotting technique was actin with apparent M_r= 46,000. Actin has been reported to be an important cytoskeletal protein in human RPE cells (16,17).

Ultrastructural examination of the RPE plasma membrane preparations indicated possible ROS disc material located amongst the enclosed membrane vesicles. In an attempt to remove the rhodopsin contamination in the plasma membrane preparation, the membrane was solubilized and passed through an anti-rhodopsin immunoaffinity column. The solubilized membrane was diluted 2-3 fold with PBS which resulted in a requirement for a more

sensitive silver stain for polypeptide band identification on SDS polyacrylamide gels. This procedure retained the majority of polypeptide bands with a considerable reduction in the rhodopsin contamination.

The primary goal for performing a RPE cell subcellular fractionation study was to obtain a reasonably enriched plasma membrane preparation for use as an immunogen in monoclonal antibody production. Consequently, the top band from the sucrose gradient and the anti-rhodopsin immunoaffinity column eludent were used to immunize BALB/C mice for the production of monoclonal antibodies against RPE plasma membrane components.

SECTION 2

THE PRODUCTION AND CHARACTERIZATION OF A MONOCLONAL ANTIBODY

RESULTS

1. PRODUCTION OF MONOCLONAL ANTIBODIES

Several attempts were made to raise monoclonal antibodies against bovine RPE plasma membrane preparations. Partially purified plasma membrane was injected into BALB/C mice and screened for antibody production by RIA. The mouse blood titre was found to have a half maximum antibody binding to the RPE plasma membrane preparation when diluted 350 times in buffer indicating that membrane preparation was antigenic to the mouse immune system (Fig. 7). When mouse myeloma cells were fused with spleen lymphocytes from an immunized mouse, hybridoma cell lines were obtained which secreted antibodies reactive towards Triton X-100 solubilized and SDS solubilized RPE plasma membrane preparations. Further testing indicated that these monoclonal antibodies cross-reacted with preparations of Triton X-100 and SDS solubilized ROS.

2. CHARACTERIZATION OF RHO-5A3

One particular monoclonal antibody designated as Rho-5A3 was found to be against rhodopsin. Rho-5A3 hybridoma culture fluid had a half maximum binding to solubilized rod outer segments at a 60 fold dilution (Fig. 8). Table 2 shows typical results for a RIA in which detergent solubilized ROS membranes immobilized in microtiter wells were sequentially treated with hybridoma cell supernatant and ^{125}I-labeled goat anti-mouse Ig antibody. Rho-5A3 antibody showed a 26% reduction in activity to ROS solubilized in

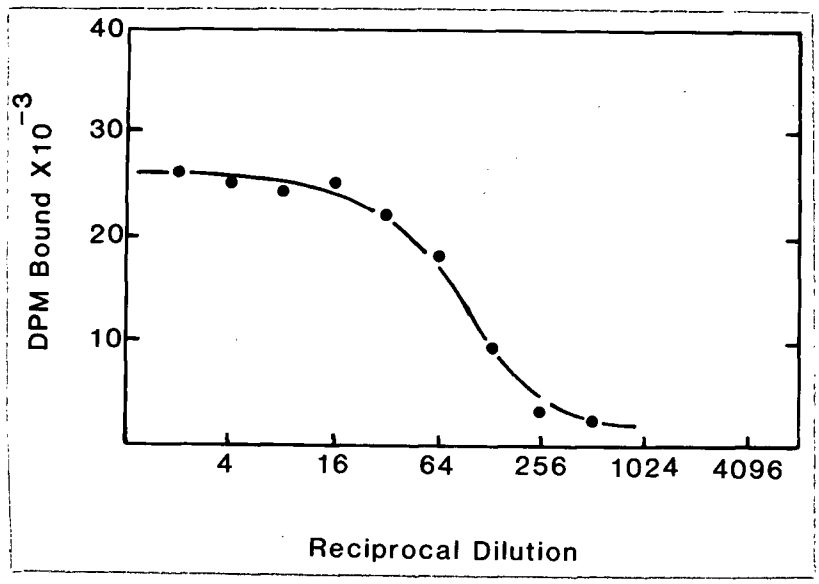


Figure 7. Antibody titration curve for blood from a BALB/C mouse immunized with bovine RPE plasma membranes. Bovine retinal pigment epithelial cell plasma membrane preparation solubilized in Triton X-100 was immobilized on microtiter wells and incubated with serial dilutions of the blood serum initially diluted 5 fold in RIA buffer. After washing in PBS the wells were incubated with ^{125}I-labeled goat anti-mouse Ig antibody.

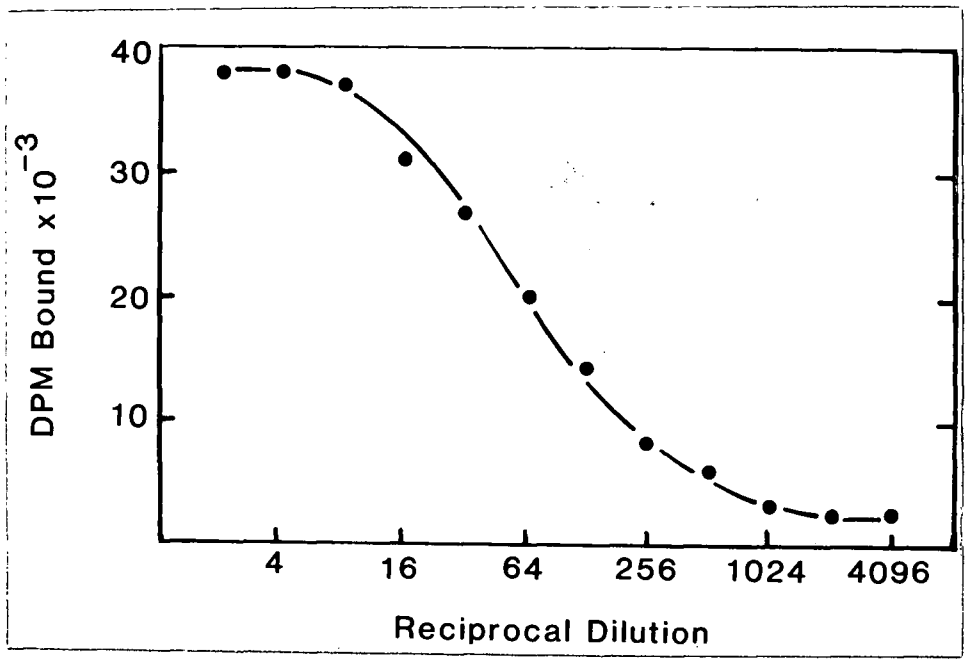


Figure 8. Titration curve of Rho-5A3 monoclonal antibody culture fluid against immobilized Triton X-100 solubilized rod outer segments. Solubilized rod outer segments were dried down on microtiter wells and incubated with serial dilutions of Rho-5A3 monoclonal antibody culture fluid. After washing with PBS, the microtiter wells were incubated with ^{125}I-labeled goat anti-mouse Ig antibody.

TABLE 2

SOLID-PHASE RADIOIMMUNE ASSAY FOR A ROS SPECIFIC ANTIBODY

HYBRIDOMA CELL SUPERNATANT	^{125}I-LABELED GOAT ANTI-MOUSE Ig BOUND (DPM)	
	TRITON X-100 SOLUBILIZED ROS	SDS SOLUBILIZED ROS
RHO-5A3	38,246	28,244
CONTROL	2,940	1,450

Control= non-specific antibody

Table 2. A comparison of Rho-5A3 monoclonal antibody binding to Triton X-100 solubilized and SDS solubilized rod outer segments. Rod outer segments (2.5 mg/mL) were solubilized in either 1% Triton X-100 or 1% SDS, diluted 10 fold in distilled water and 25 uL were dried down in microtiter wells. After blocking the non-specific sites in RIA buffer, the wells were incubated in Rho-5A3 hybridoma culture fluid, followed by washing and incubation with ^{125}I-labeled goat anti-mouse Ig antibody. The control was treated in a similar manner using a non-specific antibody.

SDS as opposed to ROS solubilized in Triton X-100. The control used was a primary non-specific antibody to ROS.

3. SUBCLASSIFICATION OF RHO-5A3 MONOCLONAL ANTIBODY

Solid-phase radioimmune assays were performed, as previously described, using Triton X-100 solubilized ROS immobilized on microtiter wells. The wells were incubated with Rho-5A3 culture fluid with an additional subclass Ig antibody incubation followed by detection with ^{125}I-labeled goat anti-rabbit Ig antibody. Results in Table 3 indicated that the Rho-5A3 monoclonal is an IgG_3 kappa light chain antibody.

4. IDENTIFICATION OF RHO-5A3 ANTIGEN OF BOVINE ROS MEMBRANES

In order to identify the antigenic site for Rho-5A3, ROS were subjected to trypsin and S.Aureus V-8 protease treatment with separation of the proteolytic fragments by SDS gel electrophoresis followed by transfer of the polypeptides to CNBr-activated paper. The paper strips were then directly treated with Rho-5A3 or Rho-1D4 antibodies and detection was made by using ^{125}I-labeled goat anti-mouse Ig as a second antibody. Results are shown in Fig.9, along with the coomassie blue staining pattern.

Both antibodies Rho-1D4 and Rho-5A3 bind to rhodopsin, the major coomassie blue staining glycoprotein of apparent M_r= 34,000. When ROS were treated with S.Aureus protease three major rhodopsin bands were located at apparent M_r= 33,000 ,25,000 and 12,000 (72,73). Rho-5A3 bound to the M_r=33,000 and 25,000 cleaved rhodopsin bands while Rho-1D4 only bound residual undigested rhodopsin (74).

When ROS were subjected to proteolysis by trypsin, one

TABLE 3

IDENTIFICATION OF Ig SUBTYPE OF RHO-5A3 MONOCLONAL ANTIBODY

Rabbit anti-mouse Ig subtype specific antibody binding to Rho-5A3

Anti-	(α)	-(γ1)	-(γ2a)	-(γ2b)	-(γ3)	-(μ)	-(κ)	-(λ)	control
DPM bound									
X 10^{-3}	2.2	2.7	3.0	2.5	6.0	2.2	9.6	2.6	2.9

Table 3. Immunoassay for the screening of Rho-5A3 monoclonal Ig antibody subtypes. Rho-5A3 hybridoma culture fluid was incubated with Triton X-100 solubilized rod outer segments dried down on microtiter wells. After washing in PBS buffer the wells were incubated with subclass specific rabbit anti-mouse immunoglobulin followed by washing as before and incubation with ^{125}I-labeled goat anti-rabbit Ig antibody.

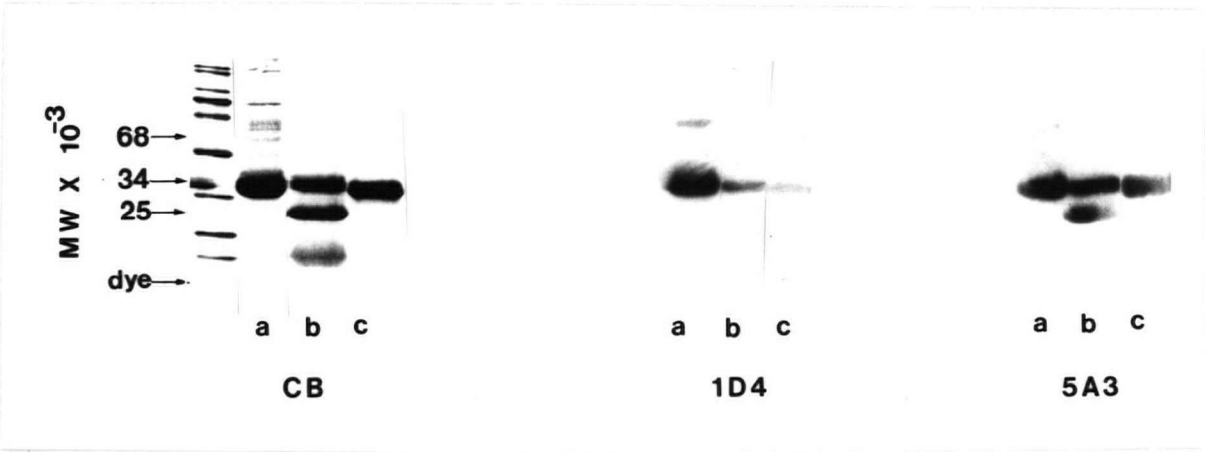


Figure 9. Analysis of polypeptides from untreated and protease digested rod outer segments which bind Rho-1D4 and Rho-5A3 anti-rhodopsin monoclonal antibodies. Rod outer segments were digested with S.aureus protease (lane b) and trypsin (lane c), washed by centrifugation, solubilized and electrophoresized on a 10% SDS-polyacrylamide gel. The polypeptides were either stained by coomassie blue (CB) or transferred to CNBr- activated paper. The latter was treated with Rho-1D4 or Rho-5A3 culture fluid. Identification was made using ^{125}I-labeled goat anti-mouse Ig antibody. Lane a was untreated rod outer segments.

fragment migrated just ahead of the leading edge of undigested rhodopsin. The Rho-5A3 monoclonal antibody binds strongly to this large (apparent M_r=32,000) fragment while Rho-1D4 binds only residual undigested rhodopsin. It has been shown that this fragment corresponds to rhodopsin with a 9 amino acid segment removed from the carboxyl terminus (74).

5. COMPETITIVE INHIBITION OF RHO-5A3 MONOCLONAL ANTIBODY

A competition assay was performed with Rho-1D4-Sepharose immunoaffinity purified rhodopsin (43) to determine the approximate concentration of rhodopsin required to give half maximum Rho-5A3 antibody binding. The inhibition profile (Fig. 10) indicated that half maximum binding was achieved at a concentration of 5 ug/mL.

The effectiveness of sealed ROS discs, frozen-thawed ROS discs, and Triton X-100 solubilized ROS discs to inhibit antibody binding to Triton X-100 treated, immobilized ROS discs was studied in order to determine the accessibility of the antigenic site (Fig. 11). Sealed discs and frozen-thawed discs showed no competition for the antibody even at high concentrations. Triton X-100 solubilized discs were found to inhibit Rho-5A3 antibody binding to immobilized and Triton X-100 solubilized discs at concentrations above 0.1 mg/mL.

6. ANALYSIS OF RHO-5A3 ANTIBODY BINDING PEPTIDE

Rhodopsin peptides were also used in an attempt to inhibit Rho-5A3 binding to Triton X-100 solubilized immobilized rod outer segments. When Rho-5A3 was competed with a 2-39 N-terminal polypeptide from rhodopsin, inhibition of Rho-5A3 binding to

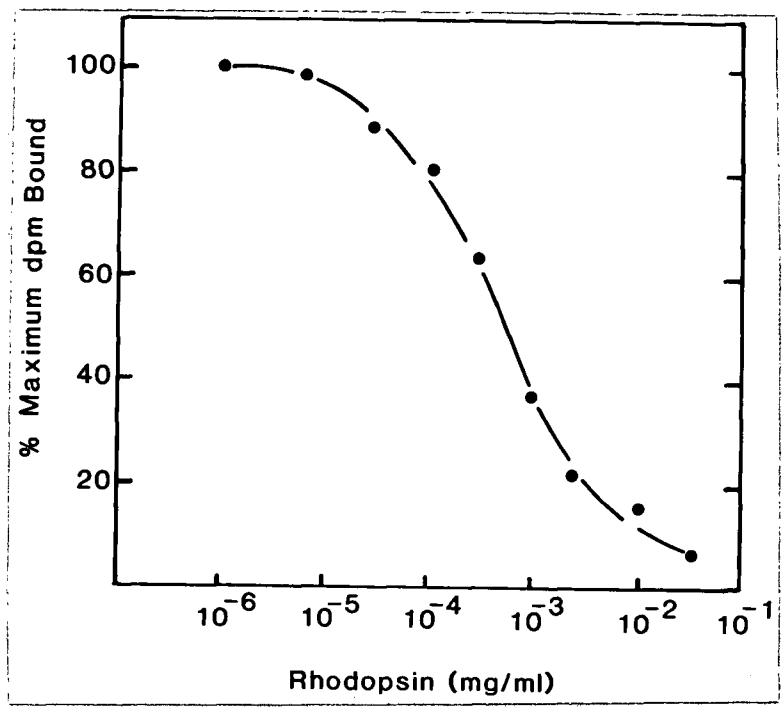


Figure 10. Inhibition of Rho-5A3 monoclonal antibody binding to Triton X-100 solubilized rod outer segments by incubation with rhodopsin. Serial dilutions of Rho-1D4 immunoaffinity purified rhodopsin were incubated with Rho-5A3 culture fluid in microtiter wells. Twenty-five uL from each well was incubated with dried down solubilized rod outer segments. Following washing in PBS, antibody detection was made using ^{125}I-labeled goat anti-mouse Ig antibody.

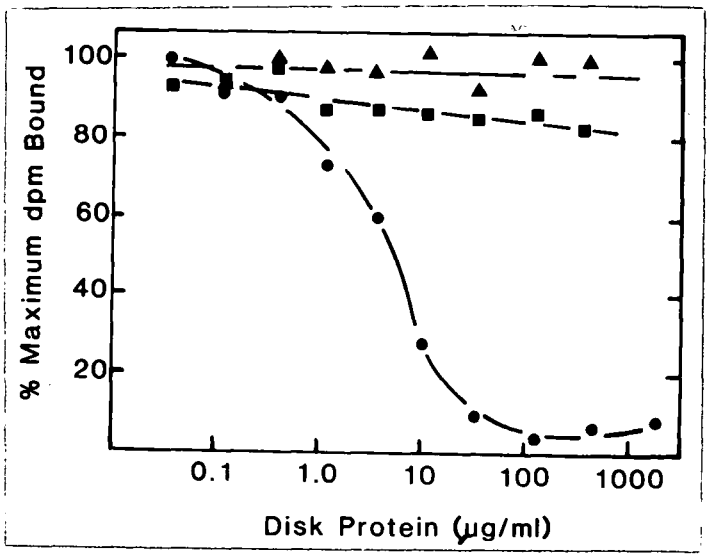


Figure 11. Inhibition of Rho-5A3 monoclonal antibody binding to Triton X-100 solubilized rod outer segment disc protein by frozen/thawed discs (▲—▲), sealed discs (■—■), and Triton X-100 solubilized discs (●—●). Serial dilutions of inhibitor were incubated with Rho-5A3 hybridoma culture fluid in microtiter wells. Twenty-five uL were removed from each well and incubated with Triton X-100 solubilized disc proteins dried down in microtiter wells previously incubated in RIA buffer. After washing in PBS the wells were incubated with ^{125}I-labeled goat anti-mouse Ig antibody.

immobilized Triton X-100 solubilized rod outer segments was observed at concentrations above 0.5 uM, but no inhibition was observed with the 2-16 N-terminal rhodopsin polypeptide (Fig.12).

7. LOWICRYL THIN SECTION LABELING

Thin sections of bovine photoreceptor cells, embedded in Lowicryl resin, were labeled with Rho-5A3 monoclonal antibody followed by goat anti-mouse Ig gold-dextran. Ultrastructural examinaton by TEM, revealed that the rod outer segments were heavily and randomly labeled while the rod inner segments had very little label (Fig.13).

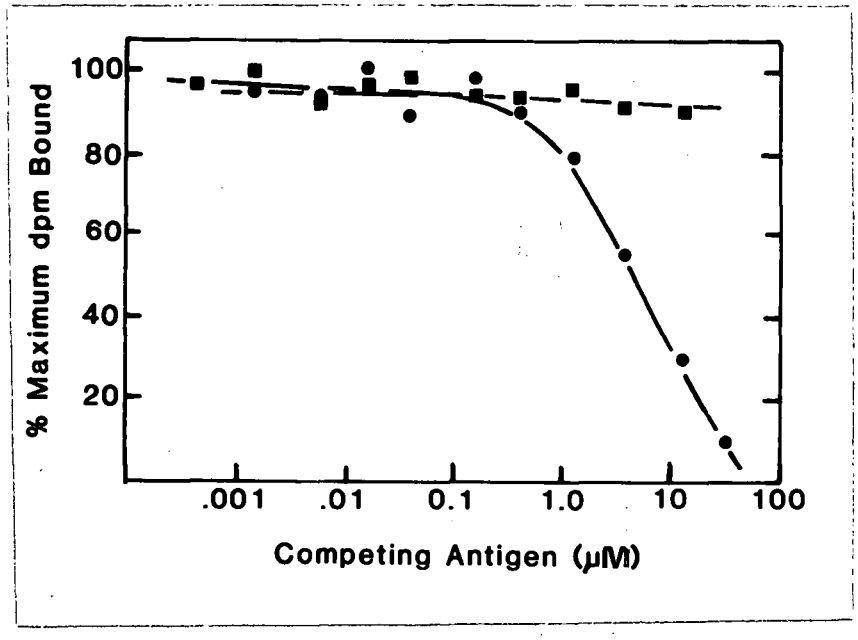


Figure 12. Inhibition of Rho-5A3 monoclonal antibody binding to Triton X-100 solubilized rod outer segments by rhodopsin 2-16 N-terminus polypeptide (■——■) and rhodopsin 2-39 N-terminus polypeptide (●——●). Serial dilutions of polypeptides were incubated with Rho-5A3 hybridoma culture fluid in microtiter wells. Twenty-five uL from each well was incubated with Triton X-100 solubilized rod outer segments dried down in microtiter wells previously incubated in RIA buffer. After washing in PBS the wells were incubated in ^{125}I-labeled goat anti-mouse Ig antibody.

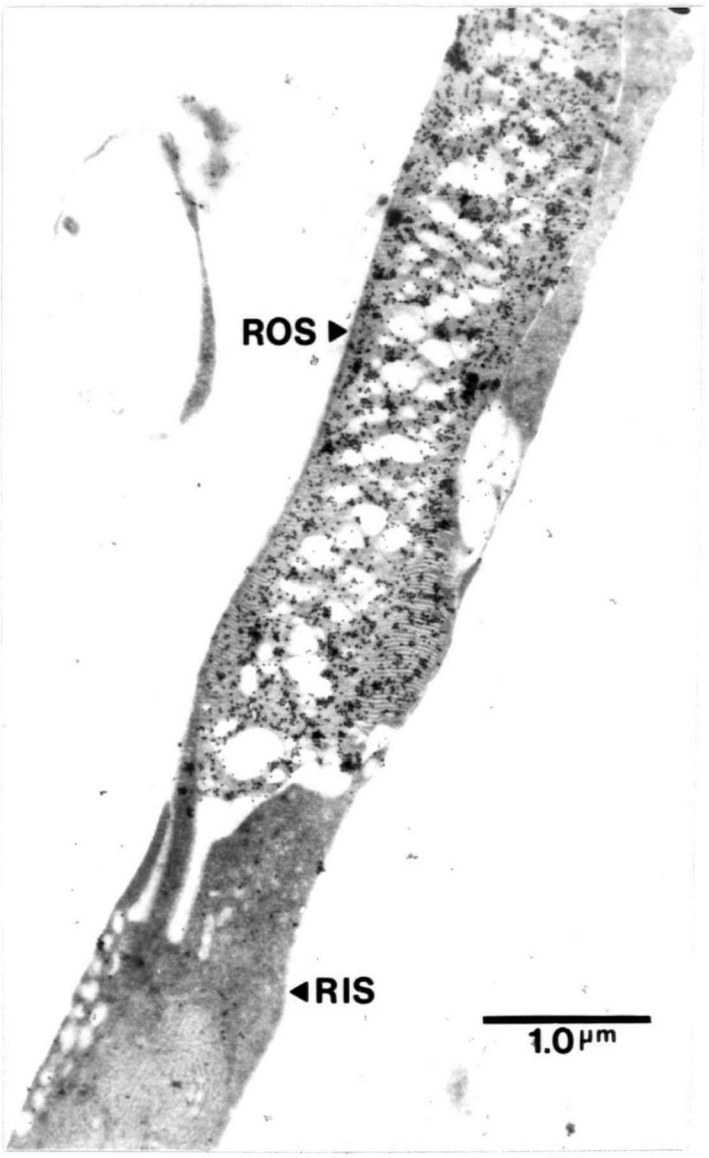


Figure 13. Transmission electron micrograph of a glutaraldehyde fixed bovine rod photoreceptor cell embedded in Lowicryl and labeled with Rho-5A3 monoclonal antibody hybridoma culture fluid followed by goat anti-mouse Ig-Au_{20} dextran. Notice the heavy labeling on the rod outer segment with little labeling on the rod inner segment.

DISCUSSION

Several cell fusions were performed with spleen cells from BALB/C mice previously immunized with RPE plasma membrane preparations for the production of monoclonal antibody secreting hybridoma cell lines. When the immunoaffinity purified RPE plasma membrane preparation was used to immunize BALB/C mice, no hybridoma cell lines were produced that secreted monoclonal antibodies specific for RPE plasma membrane preparations. This result probably reflects the lack of sufficient protein initially injected into the mouse. Alternatively the solubilized proteins injected were not antigenic to the immune system of the mouse. However, the fusion of spleen cells from a BALB/C mouse immunized with the RPE plasma membrane preparation top band collected from the continuous sucrose gradient and NS-1 (myeloma) cells resulted in a hybridoma cell line which secreted a monoclonal antibody specific for the membrane preparation. The monoclonal antibody, designated Rho-5A3, however was found to be specific for rhodopsin as determined by radioimmune labeling of ROS proteins electrophoretically transferred from SDS gels to CNBr- activated paper. Radioimmune competition studies localized the antigenic site to the N-terminus intradiskal region of the rhodopsin molecule (Fig. 14).

Analysis of immunoglobulin subtypes indicated that the Rho-5A3 monoclonal antibody was an IgG_3 kappa light chain antibody. IgG antibodies are the most useful type of antibody for serological labeling and immunological studies as their non-specific binding tends to be quite low.

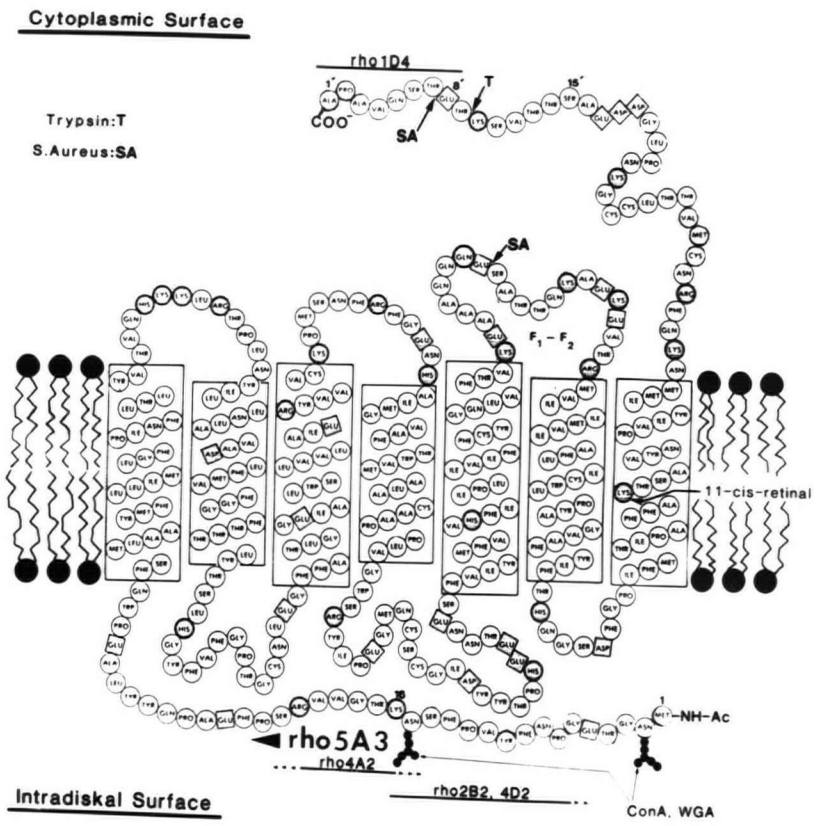


Figure 14. A diagrammatic model of rhodopsin, modified from Hargrave (75), and the location of the antigenic sites for a variety of monoclonal antibodies. Note the approximate location of the Rho-5A3 monoclonal antibody on the N-terminus of the rhodopsin molecule. The Rho-1D4 monoclonal antibody has been shown to be located on the carboxyl terminus (43).

The antigenic site for the monoclonal antibody, Rho-1D4, is along the first seven amino acids on the carboxyl terminus of rhodopsin.(43) With Rho-1D4 as a control, limited proteolytic digestion studies were performed to localize the site of Rho-5A3 action. Digestion of ROS material with S.aureus V-8 protease or trypsin which removed a seven or nine amino acid peptide from the C-terminus of rhodopsin resulted in the loss of Rho-1D4 binding while the antigenic site for Rho-5A3 binding was maintained. (Fig.9). In the case of S.aureus V-8 digestion, the Rho-5A3 monoclonal antibody also bound to the rhodopsin proteolytic fragment (M_r=25,000) designated the F_1 fragment which is known to contain the N-terminus (73). These results indicate that the Rho-5A3 antibody binds to an antigenic site along the amino-terminal two-thirds of rhodopsin (Fig.14).

With these results, it was interesting to explore the accessibility of the Rho-5A3 binding site. The Rho-5A3 antibody did not bind to sealed or frozen-thawed ROS disc rhodopsin material even at high concentrations. However, Rho-5A3 bound very well to rhodopsin when solubilized in Triton X-100 which is known to cause irreversible structural changes. Based on these results it appears that the Rho-5A3 antigenic determinant is inaccessible to the antibody when rhodopsin is in the disc membrane. The native rhodopsin protein conformation or the phospholipid bilayer may cause the inaccessibility of the Rho-5A3 determinant.

In an attempt to further identify the antigenic site of Rho-5A3 action, competition assays were performed with specifically prepared rhodopsin polypeptides. Previously, Rho-5A3 antibody had

been shown to compete with rhodopsin with half maximum antibody binding when the rhodopsin concentration was 5 ug/mL, quite similar to Rho-4A2 (another N-terminal rhodopsin binding monoclonal antibody) (43). Solid-phase competition assays demonstrated Rho-5A3 antibody's ability to compete with the 2-39 rhodopsin N-terminal peptide at a concentration above 0.5 uM. However, no competition was observed with the 2-16 rhodopsin N-terminal polypeptide up to a concentration of 10 uM. These preliminary studies indicate that the Rho-5A3 antigenic site is located in the 17-38 rhodopsin N-terminal peptide region.

Bovine ROS, previously fixed and embedded in Lowicryl, were labeled extensively and randomly by goat anti-mouse Ig gold dextran after treatment of the sections with the Rho-5A3 antibody (Fig.13). In these sections the antigenic sites of Rho-5A3 action are accessible to labeling techniques. Rhodopsin was found abundantly in the rod outer segments and not found at all in the rod inner segments.

The question remained as to the difficulty in obtaining antibodies specific for the bovine RPE plasma membrane. Another approach used to produce monoclonal antibodies specific for bovine RPE cells was to introduce the cells into tissue culture prior to immunization. Bovine RPE cells were grown in tissue culture and injected into BALB/C mice. Subsequent spleen cell and myeloma cell fusions resulted in two more hybridoma cell lines which secreted antibodies against ROS preparations but neither had rhodopsin as their antigenic determinant. It appears that ROS and residual ROS proteins associated with the RPE cells

are highly antigenic while RPE plasma membranes are not, possibly due to a blood-brain barrier.

SECTION 3

ANALYSIS OF BOVINE RPE CELLS IN VITRO

RESULTS

1. ISOLATION OF BOVINE RPE CELLS

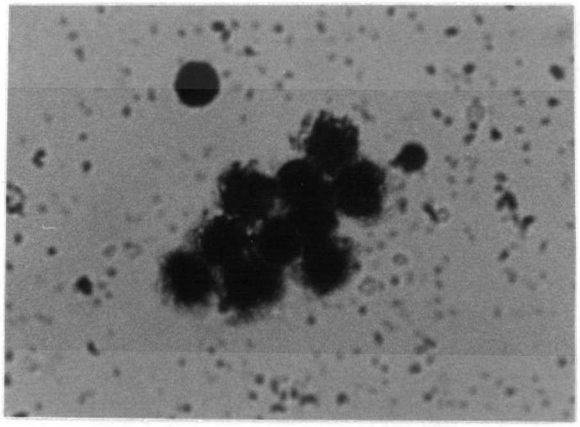
Several procedures for isolating RPE cells from the bovine eye optic cup were explored. Techniques such as scraping the RPE cells from the optic cup or dissecting out the RPE cells from the Bruch's membrane were performed with minimal results due to poor yields and cell breakage. The method which produced the best yield was enzymatic digestion with trypsin and collagenase. The RPE cells were plated out on a glass substrate which provided an excellent support for cell growth.

2. THE MORPHOLOGY OF BOVINE RPE CELLS IN VITRO

The bovine RPE cells after enzymatic treatment were cuboidal in shape and considerably reduced in size. After 4 days in tissue culture the cells still retained a cuboidal shape (Fig. 15a). The bovine RPE cells became established in tissue culture after approximately 1 week (Fig. 15b) and both the melanotic and amelanotic cells became more dispersed in appearance (Fig. 15c). As the cellular growth became confluent with a doubling time of 52 hours the pigmentation of the cells became less dense.

At the level of SEM, RPE cells appeared flattened after 10 days in tissue culture with several microvilli apparent on approximately one half of the cell surface (Fig. 16). The RPE cells also had scattered 1-2 um wide vesicles attached to the cell surface (Fig. 17). Underlying melanosomes bulged out against

a.



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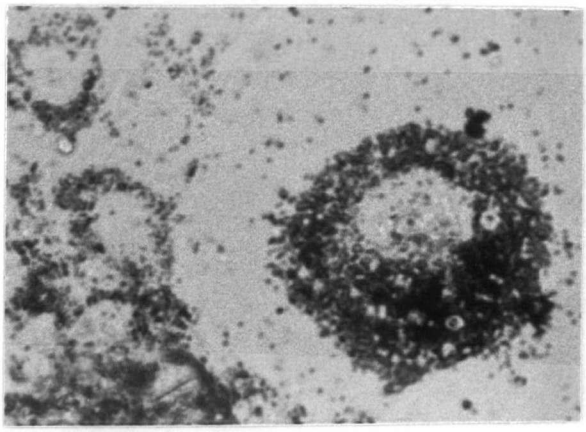


c.

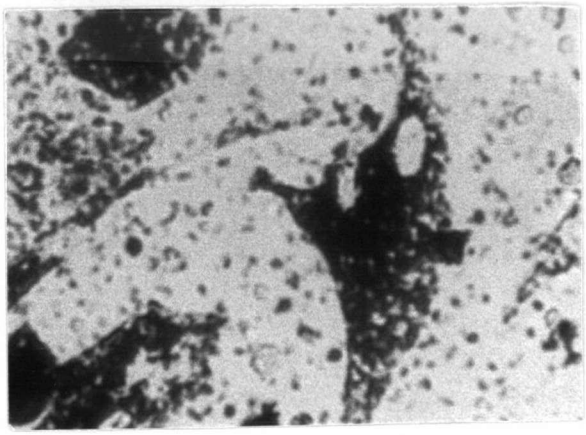


Figure 15. Bovine retinal pigment epithelial cells grown in RPMI-1640 medium on glass coverslips as seen by phase contrast microscopy. a) 4 days (1000x). b) 7 days (1460x). c) 18 days (1600x). Bovine RPE cells were isolated from the optic cup by enzymatic treatment with 0.25% trypsin and 70 units/mL collagenase dissolved in Buffer A. The cells were incubated at 37°C (approximately 5 x 10^5 cells/mL) in 10 mL petri dishes containing glass coverslips.

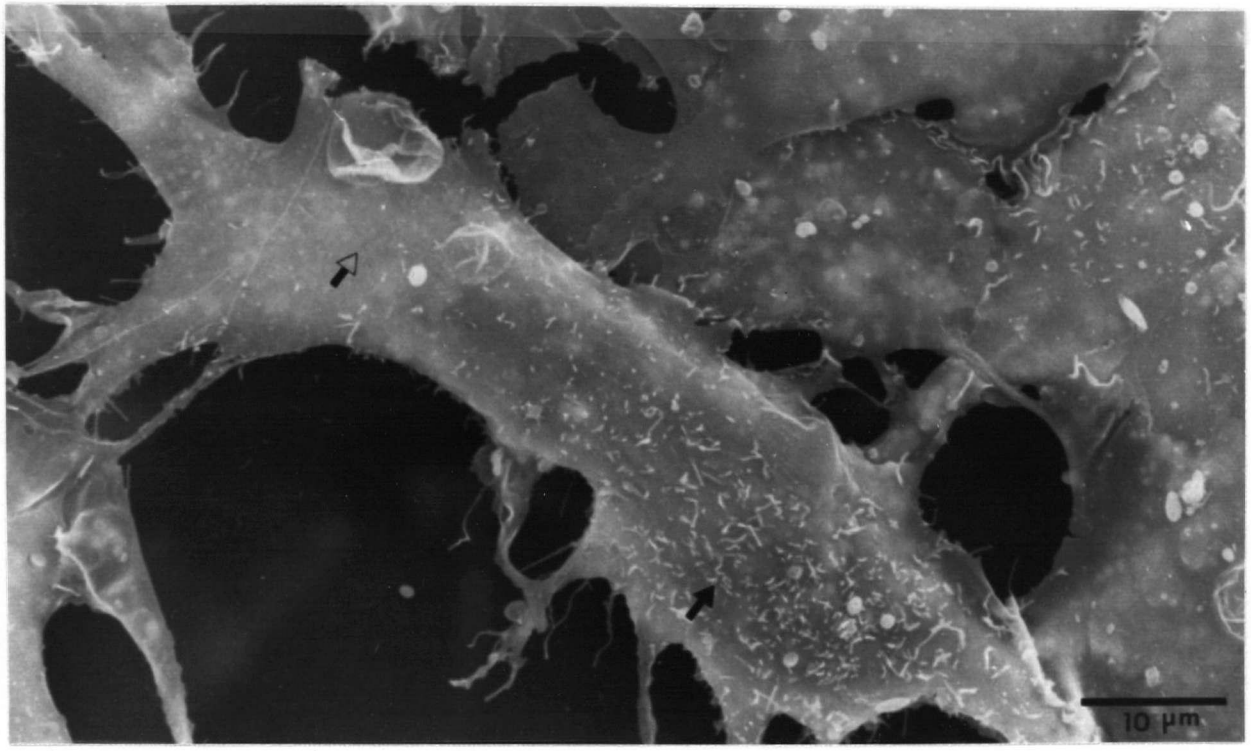


Figure 16. Scanning electron micrograph of a bovine retinal pigment epithelial cell grown on glass for 10 days in RPMI-1640 tissue culture medium. Clear arrow indicates area with few microvilli while dark arrow indicates area with dense microvilli.

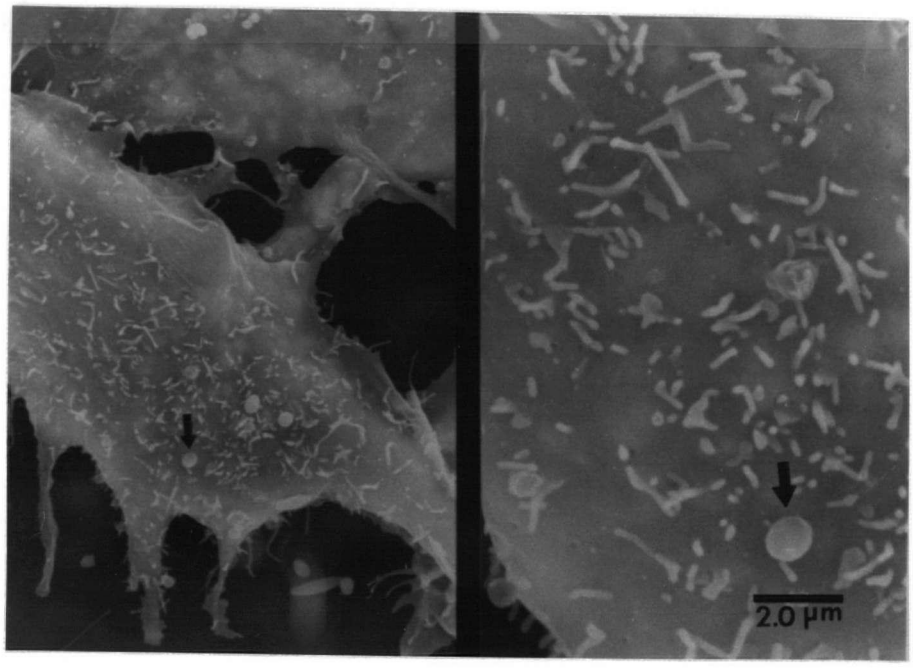


Figure 17. Scanning electron micrograph of a bovine retinal pigment epithelial cell grown on glass in RPMI-1640 culture medium for 10 days. Arrows denote vesicles, possibly rod outer segments attached to the cell surface.

the plasma membrane due to cell shrinkage during critical point drying of the samples for SEM (Fig.18). The general morphological features of the cell, however, were maintained after drying. Extreme caution had to be used in critical point drying of the RPE cells in the preparation for SEM as the plasma membrane tends to be quite fragile and easily disrupted.

3. FLUORESENT DETECTION OF ACTIN IN BOVINE CULTURED RPE CELLS

Paraformaldehyde fixed bovine RPE cells were treated with acetone and the cells were incubated with rabbit anti-actin antisera followed by FITC-labeled goat anti-rabbit Ig antibody. Results confirmed biochemical studies that actin was a major component of the bovine RPE cell. Fluorescence was concentrated throughout the cell with heavy fluorescence associated with the plasma membrane. When rabbit anti-actin antisera preincubated with purified actin was used as the primary antibody, only minimal fluorescence was detected in this control sample (Fig. 19).

4. DISCONTINUOUS FLUORESCENT LECTIN LABELING OF BOVINE RPE CELLS

Various fluorescent lectins were use to study the arrangement, distribution, and redistribution of sugar residues on the surface of bovine of RPE cells. Such lectins include FITC-Con A, specific for α-D-mannose and α-D-glucose; FITC-WGA, specific for N- acetylglucosamine oligomers; and FITC-RCA, specific for D- galactose.

Studies with 2 week old RPE cells incubated for 5 minutes at $37^{\circ}C$, with FITC-Con A showed extensive labeling on the cell surface (Fig. 20a). Similar results were found with cells fixed

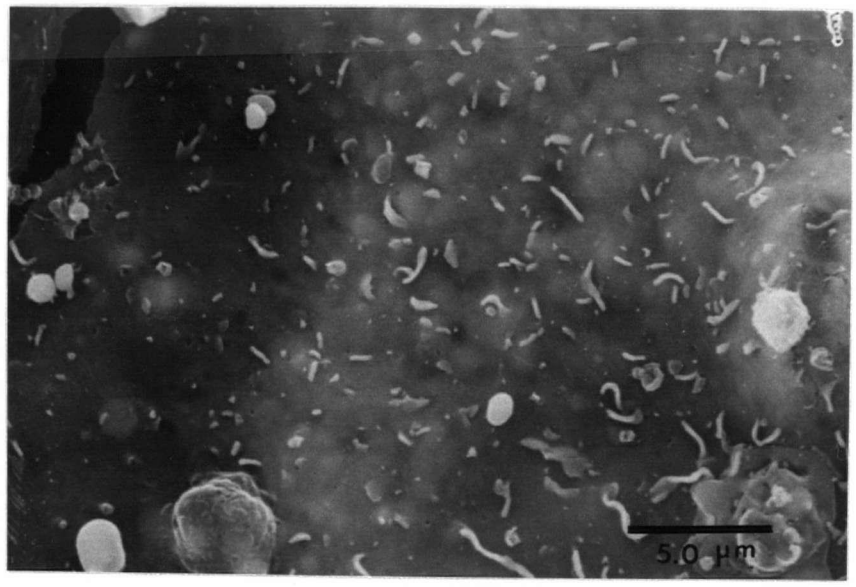
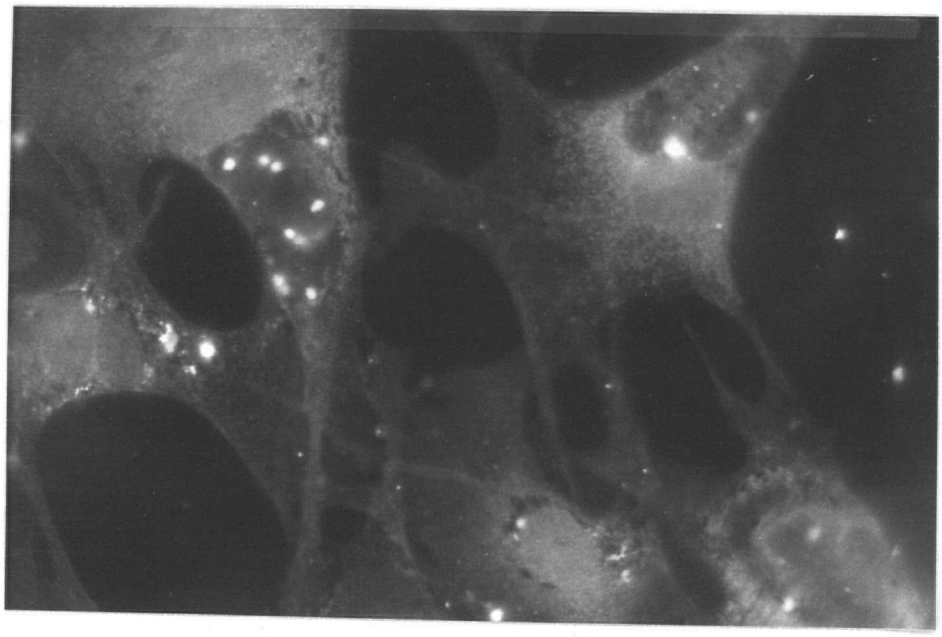


Figure 18. Scanning electron micrograph of a bovine retinal epithelial cell grown on glass for 10 days. Note the rough texture of the cell surface due to the abundance of melanosomes.

Figure 19.

a.



b.

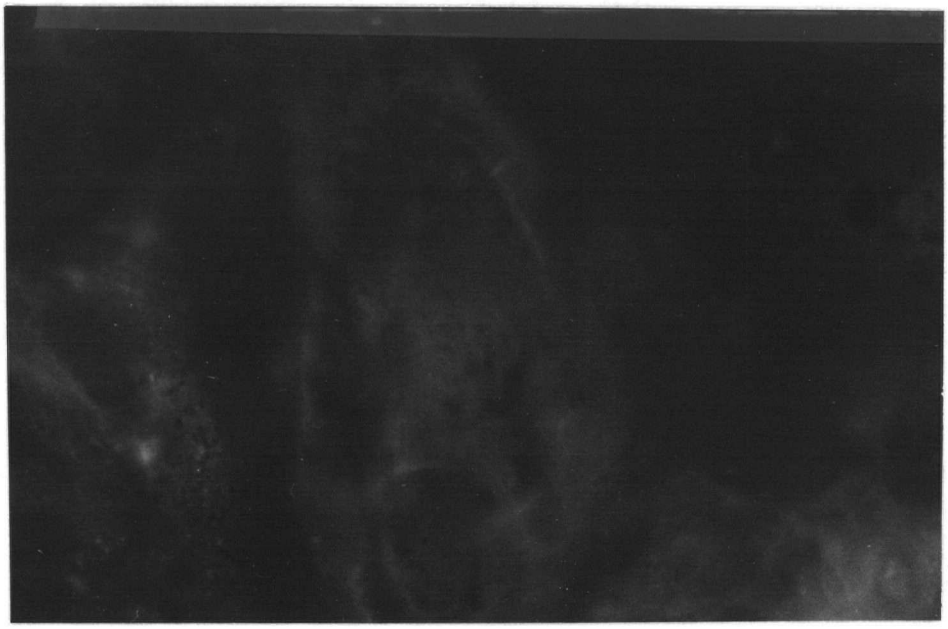


Figure 19. Fluorescent micrographs of paraformaldehyde fixed 2 week old bovine retinal pigment epithelial cells incubated with rabbit anti-actin antisera. a) RPE cells grown on glass coverslips were fixed in 1% paraformaldehyde followed by treatment in acetone. After washing in PBS buffer containing Mg^{++} and Ca^{++} the coverslips were incubated with rabbit anti-actin antisera followed by washing as before and incubation with FITC-labeled goat anti-rabbit Ig antibody. (2000x) b) As with a), with the exception that the primary antibody was previously incubated with 100 ug of purified actin. (2000x).

with 1% paraformaldehyde. As a control, the FITC-Con A lectin was competed off the cell surface with 0.25 M ∝ methyl mannoside (Fig. 20b). Labeling with FITC-Con A lectin for 5 minutes followed by a 60 minute incubation period in buffer resulted in patchy fluorescence indicating a possible internalization of the Con A receptors (Fig. 20c). Verification of receptor redistribution and internalization at the level of fluorescence, was achieved by an attempt to compete off the FITC-Con A lectin with methyl mannoside which proved to be unsuccessful (Fig. 20d).

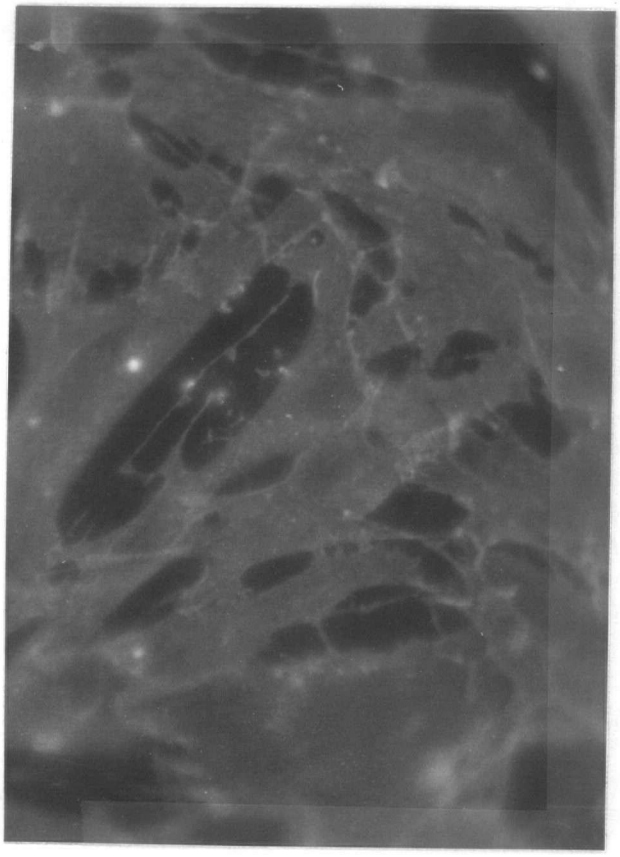
A similar study was performed using FITC-WGA and FITC-RCA. Labeling of unfixed RPE cells for 5 minutes gave a very uniform and intense fluorescence for FITC-WGA (Fig.21a), but the labeling with FITC-RCA was considerably less intense (Fig. 22a). In the control experiments labeling with FITC-WGA was competed off the cell surface by treating the cells with the saccharide inhibitor 0.02 M N-N diacetyl chitobiose at 37°C, for 15 minutes (Fig. 21b). FITC-RCA was competed off in a similar manner by 0.2 M D-galactose (Fig. 22b). Labeling RPE cells for 5 minutes with FITC-WGA followed by 60 minutes in buffer resulted in partial receptor redistribution with some apparent internalization due to the lack of competition with the appropriate saccharide inhibitor (Fig. 21c-d). Similarly, FITC-RCA when treated in the same way yielded patchy fluorescence due to receptor redistribution and internalization (Fig. 22c).

5. CONTINUOUS FLUORESCENT LECTIN LABELING OF RPE CELLS IN VITRO

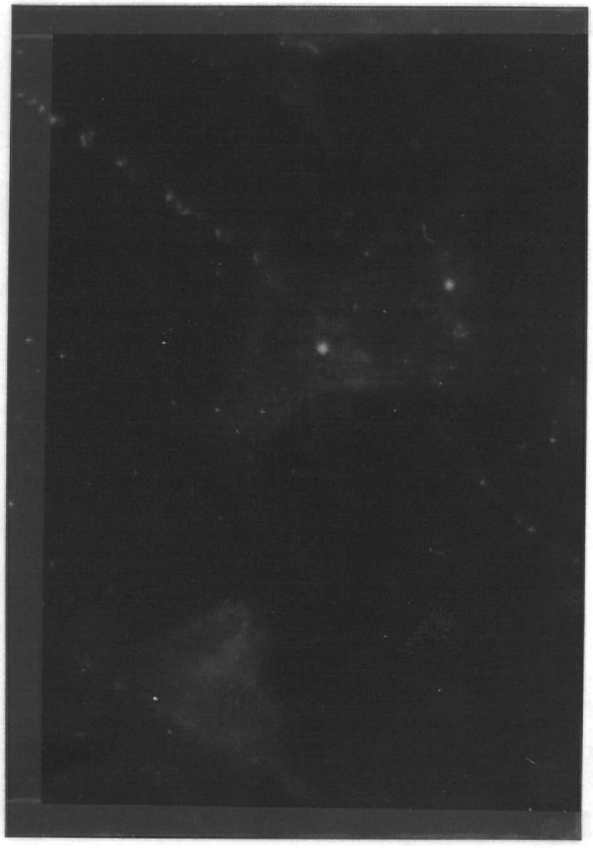
Bovine RPE cells were labeled continuously for 60 minutes

Figure 20.

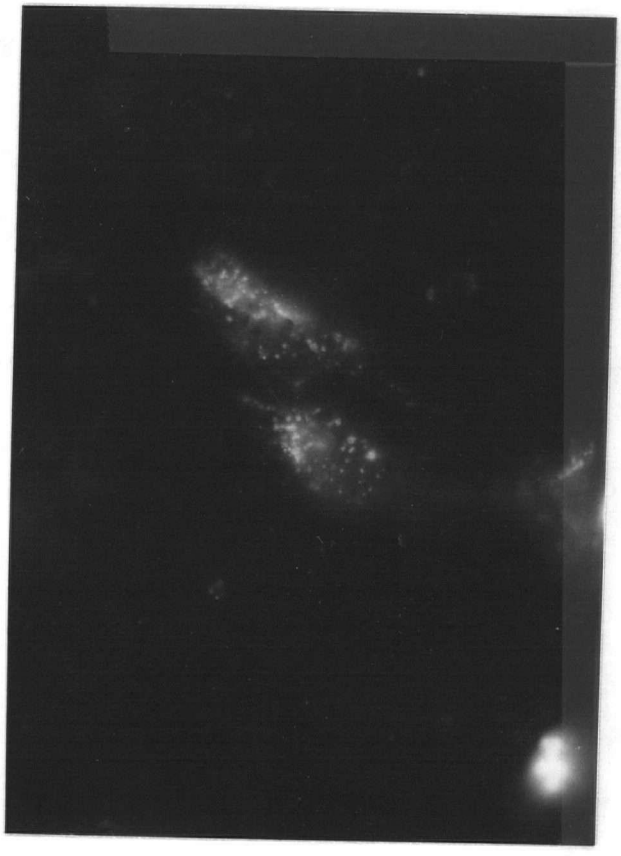
a.



b.



c.



d.

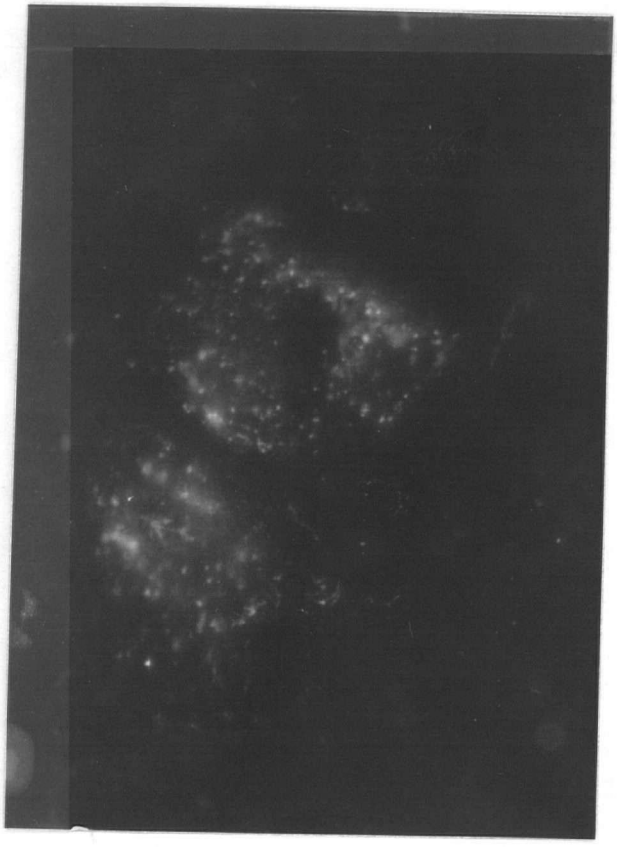
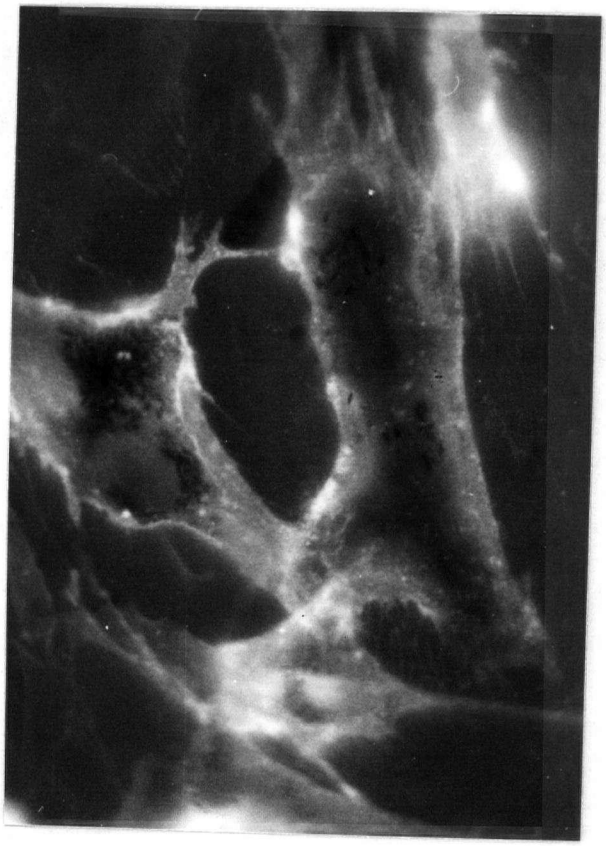


Figure 21.

a.



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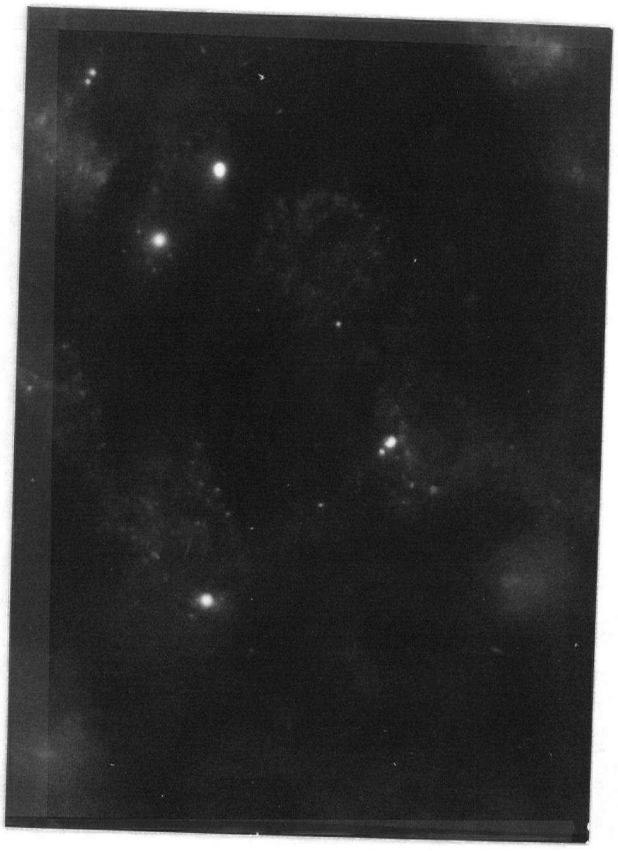

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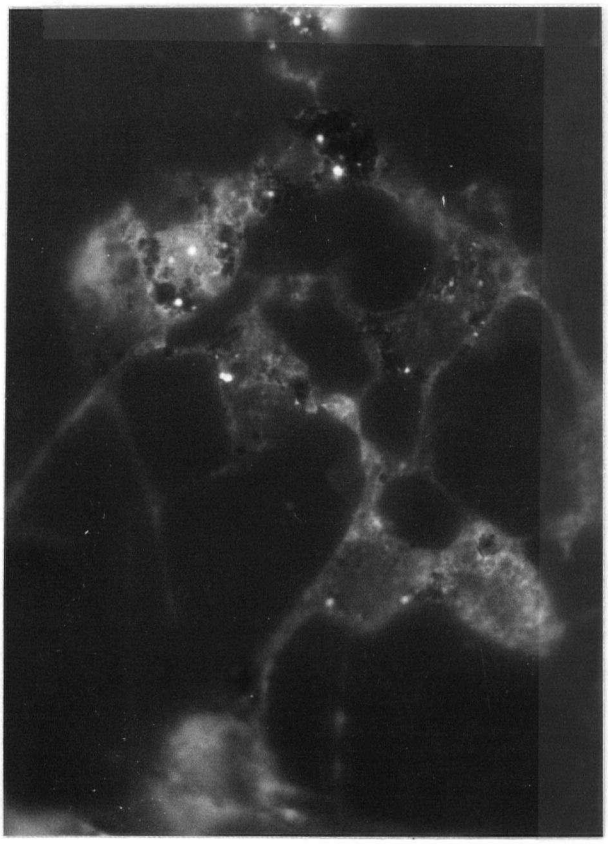

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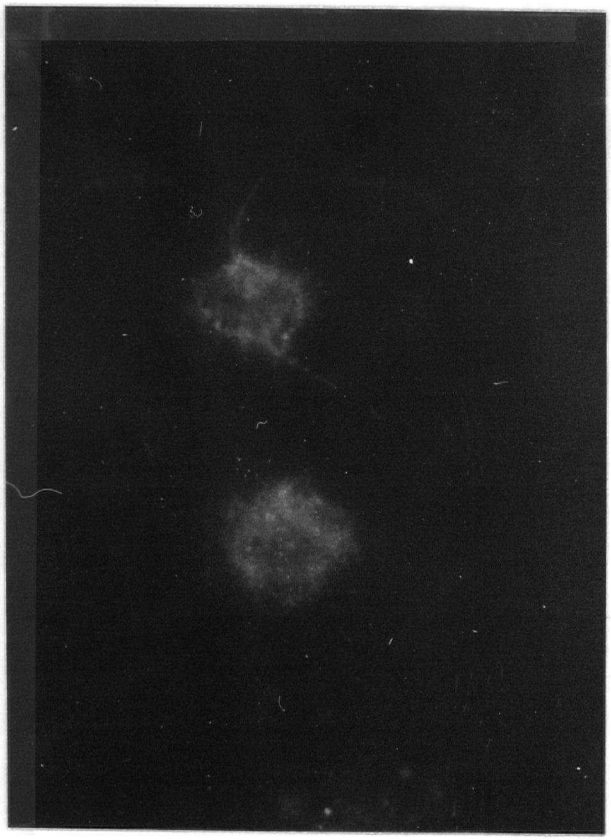
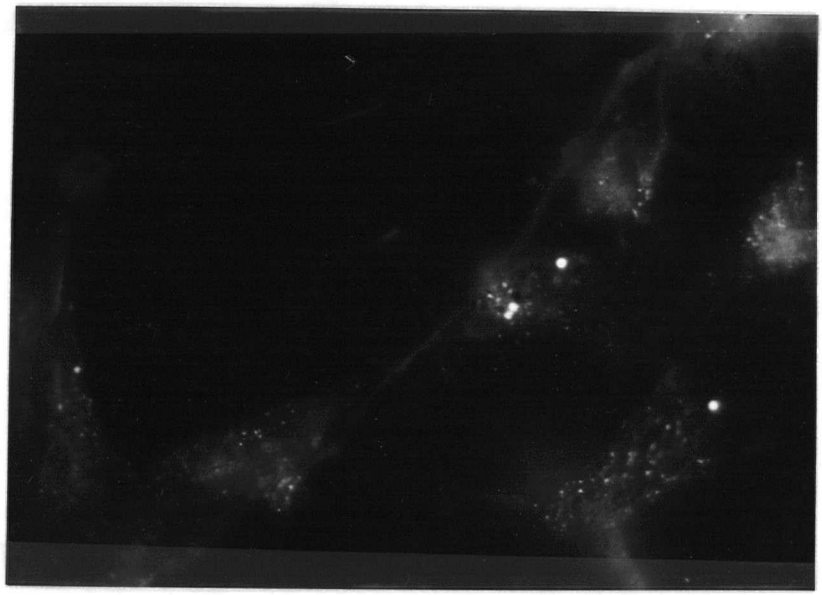


Figure 22.

a.



b.

c.

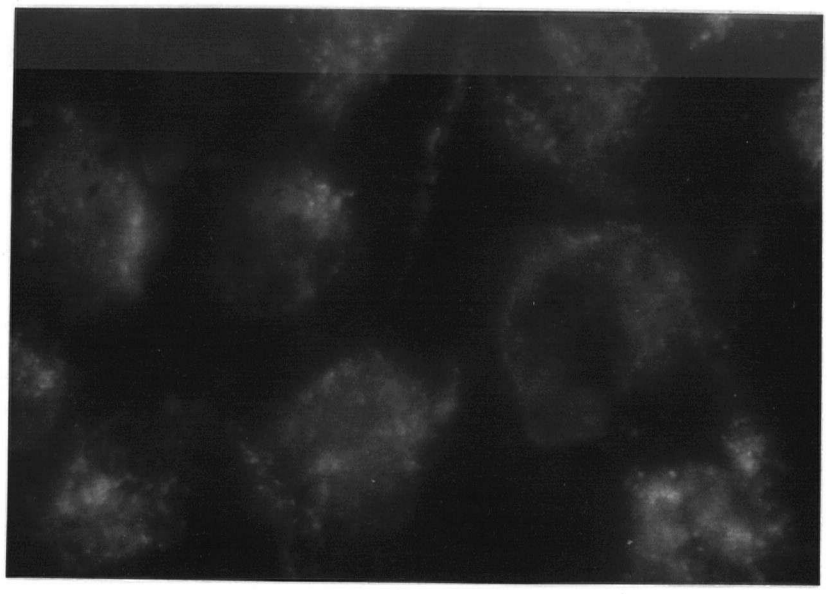
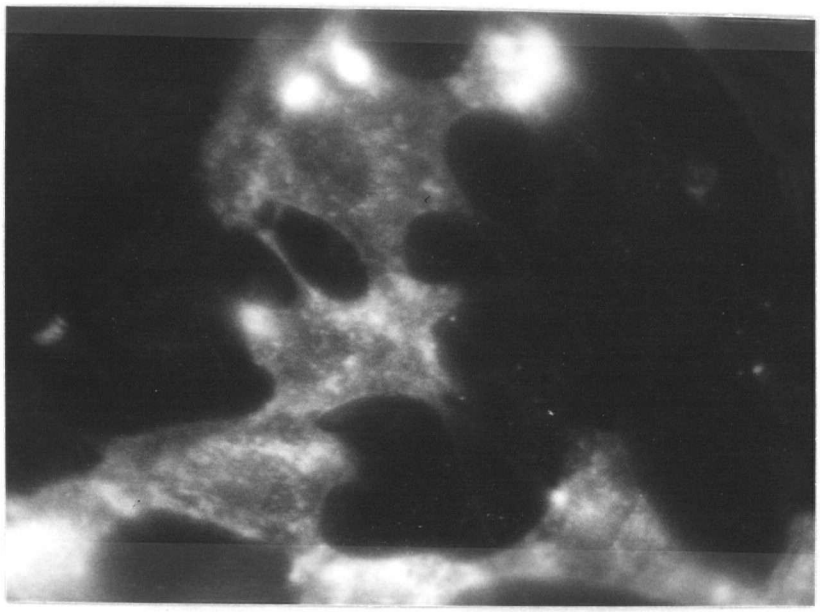
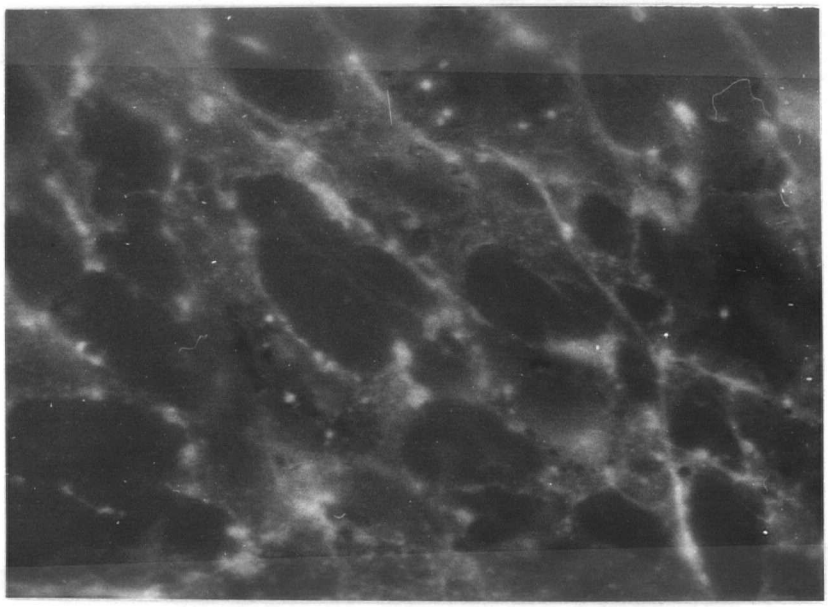


Figure 23.

a.



b.



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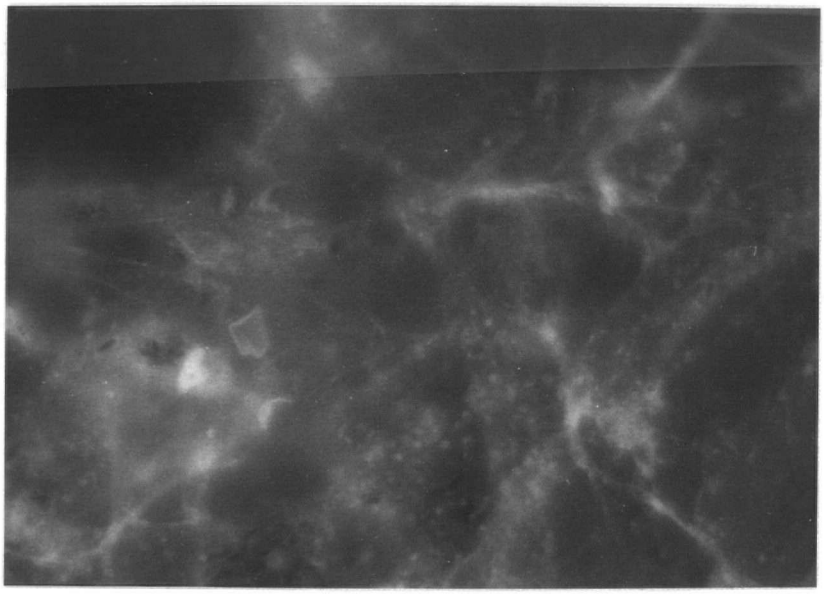


Figure 20. Fluorescent micrographs of unfixed RPE cells labeled with FITC-Con A for: a) 5 min.; b) 5 min. followed by a 15 min. incubation in 0.25 M ∝- methyl mannoside; c) 5- min. followed by wash in buffer and 60 min. incubation in buffer at 37°C; d) as in c) followed by a 15 min. incubation with 0.25 M ∝-methyl mannoside.

Figure 21. Fluorescent micrographs of unfixed RPE cells labeled with FITC-WGA for: a) 5 min.; b) 5 min. followed by a 15 min. incubation with 0.020 M N-N-diacetyl chitobiose; c) 5 min. followed by wash and 60 min. incubation in buffer at 37°C; d) as in c) followed by incubation in 0.020 M N-N-diacetyl chitobiose for 15 min.

Figure 22. Fluorescent micrographs of unfixed RPE cells labeled with FITC-RCA for: a) 5 min.; b) 5 min. followed by a 15 min. incubation in 0.2 M D-galactose; c) 5 min. followed by wash and 60 min. incubation in buffer at 37°C, plus 15 min. in 0.2 M D-galactose.

Figure 23. Fluorescent micrographs of RPE cells labeled for 60 minutes at 37°C with; a) FITC-Con A b) FITC-WGA c) FITC-RCA.

with FITC-lectins, at 37°C. In the case of Con A, there was extensive and intense fluorescence found through out the cell with some prominent large fluorescent patches (Fig. 23a). These results indicated that Con A receptors were induced to internalize but at the same time new receptors became available resulting in a heavy coat of label on the cell surface. Continuous labeling with FITC-WGA and FITC-RCA resulted in heavy fluorescence on the membrane with some apparent fluorescent patches (Figs. 23b-c)

6. PROBING BOVINE RPE CULTURED CELLS WITH RHO-1D4

Due to the presence of a high concentration of rhodopsin in the RPE plasma membrane preparation from fresh bovine eyes, it was interesting to explore whether rhodopsin could be detected in RPE cells grown in tissue culture. Using Rho-1D4 monoclonal antibody, specific for the C terminus of rhodopsin , bovine RPE cells grown in microtitre wells were solubilized in 0.1% Triton X-100 and assayed by the standard RIA method. Results indicated that Rho-1D4 bound to a residual rhodopsin component in the RPE cells at a high level for over 10 days *in vitro* (Fig. 24). Presumably, either rod outer segments are still associated with the RPE cells or rhodopsin has not been completely degraded by the cells in tissue culture.

7. PHAGOCYTOSIS OF ROS BY BOVINE RPE CELLS IN VITRO

When bovine ROS were incubated for 5 h with bovine RPE cells, SEM observation revealed that the ROS were attached to the cell surface (Fig. 25). Preliminary results indicated that the bovine RPE cells were recognizing and attaching to the rod outer

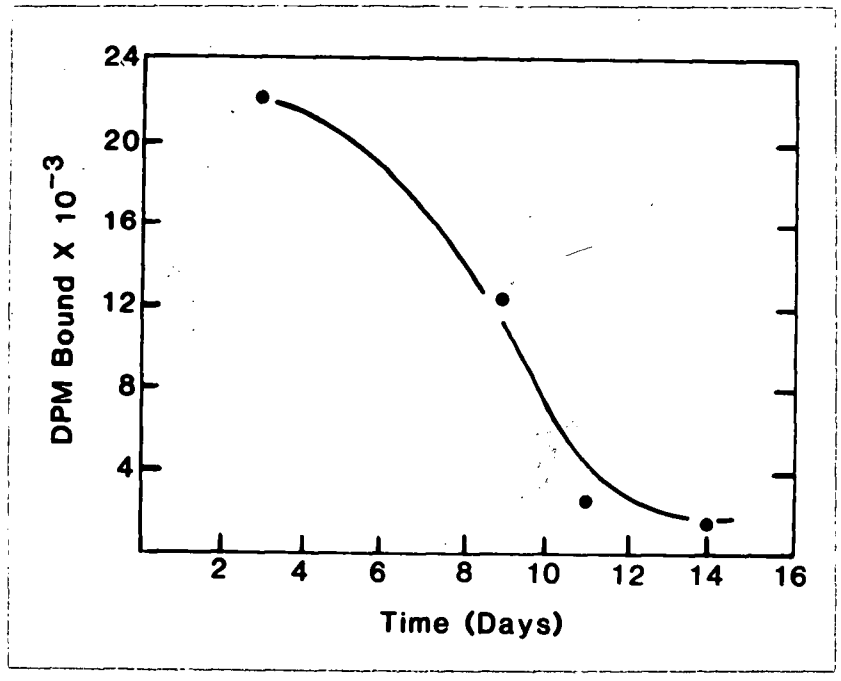


Figure 24. Rho-1D4 monoclonal antibody binding to Triton X-100 solubilized bovine RPE cells grown in microtiter wells. Bovine RPE cells grown in flat bottom microtiter wells were solubilized in 0.1% Triton X-100 and incubated with Rho-1D4 hybridoma culture fluid. Following washing in buffer, the wells were incubated with ^{125}I-labeled goat anti-mouse Ig antibody.

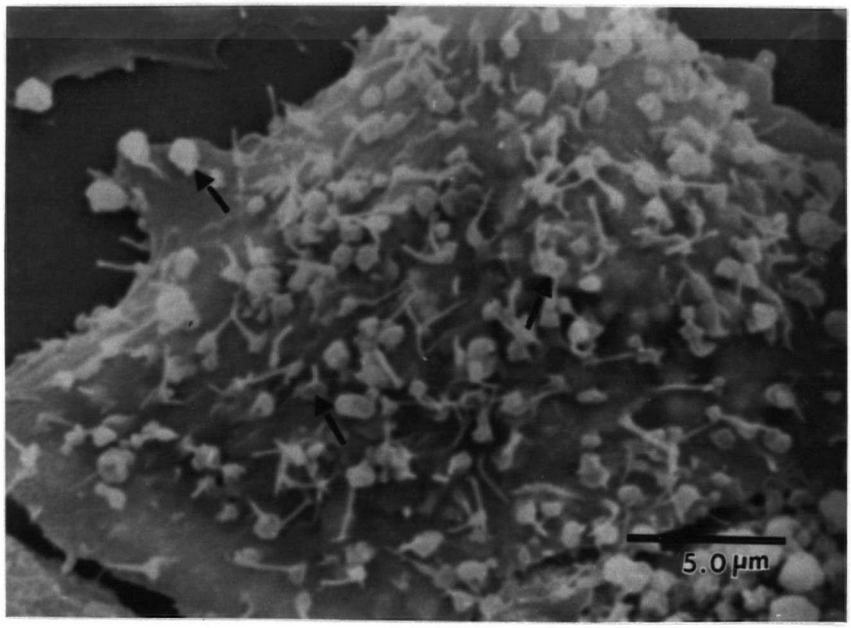
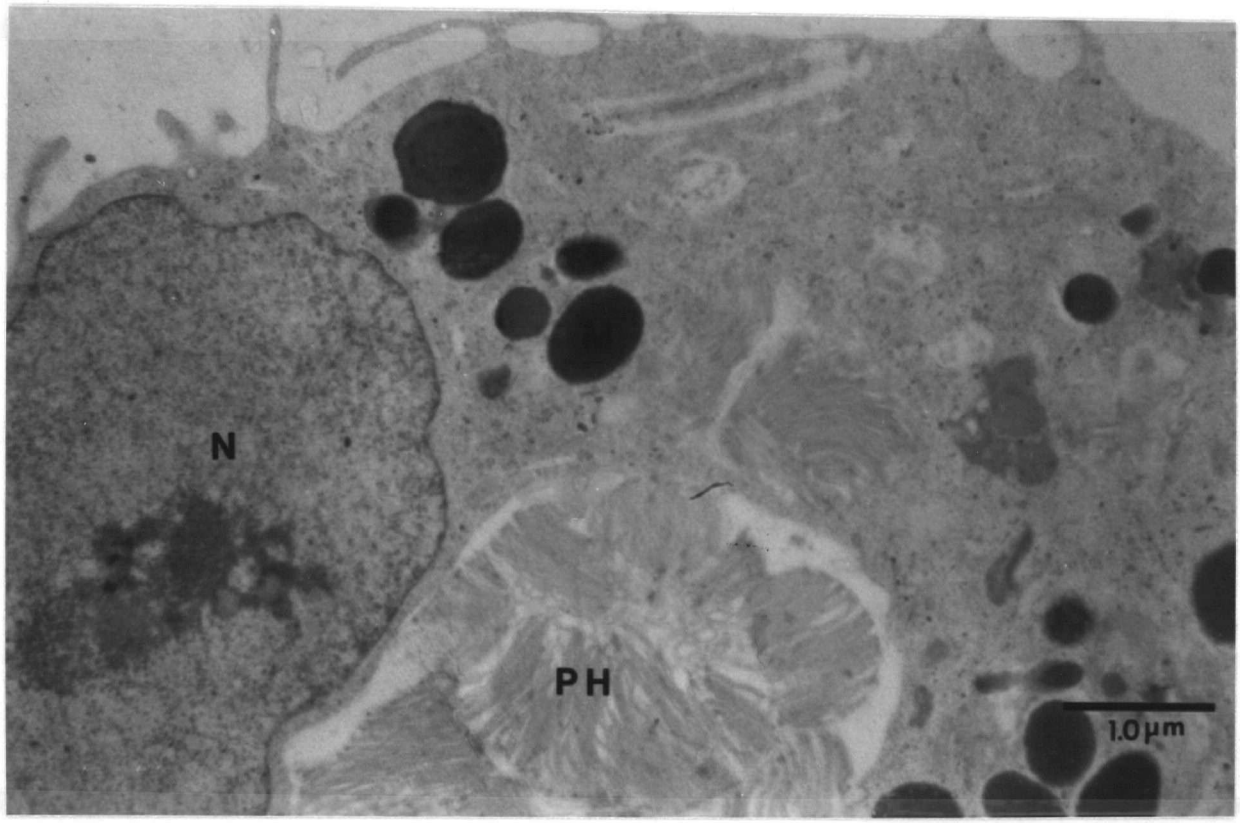


Figure 25. Scanning electron micrograph of a 2 week old bovine RPE cell incubated for 5 h with sealed bovine rod outer segments. Arrows indicate some of the numerous bound rod outer segments amongst the mat of microvilli.

segments. An assay condition was established for the phagocytosis of dark adapted ROS by 2 week old bovine RPE cells. Sealed ROS were collected from a sucrose gradient and incubated for 5 h, at 37^{o}C, with RPE cells grown on glass coverslips. Qualitative observations made by TEM indicated that some, but not all, adult bovine RPE cells <u>in vitro</u> were capable of phagocytizing ROS (Figs.26,27) These results established a new system for studying the phagocytosis of rod outer segments.

Figure 26.



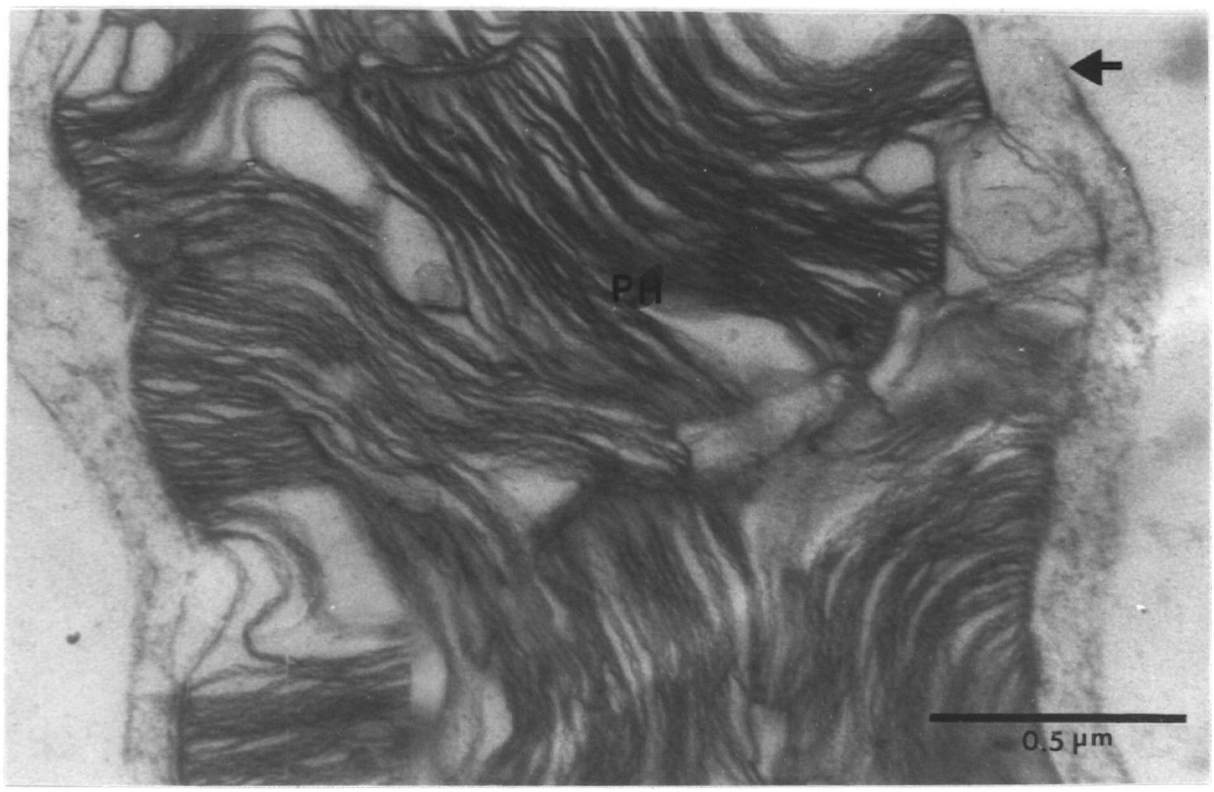
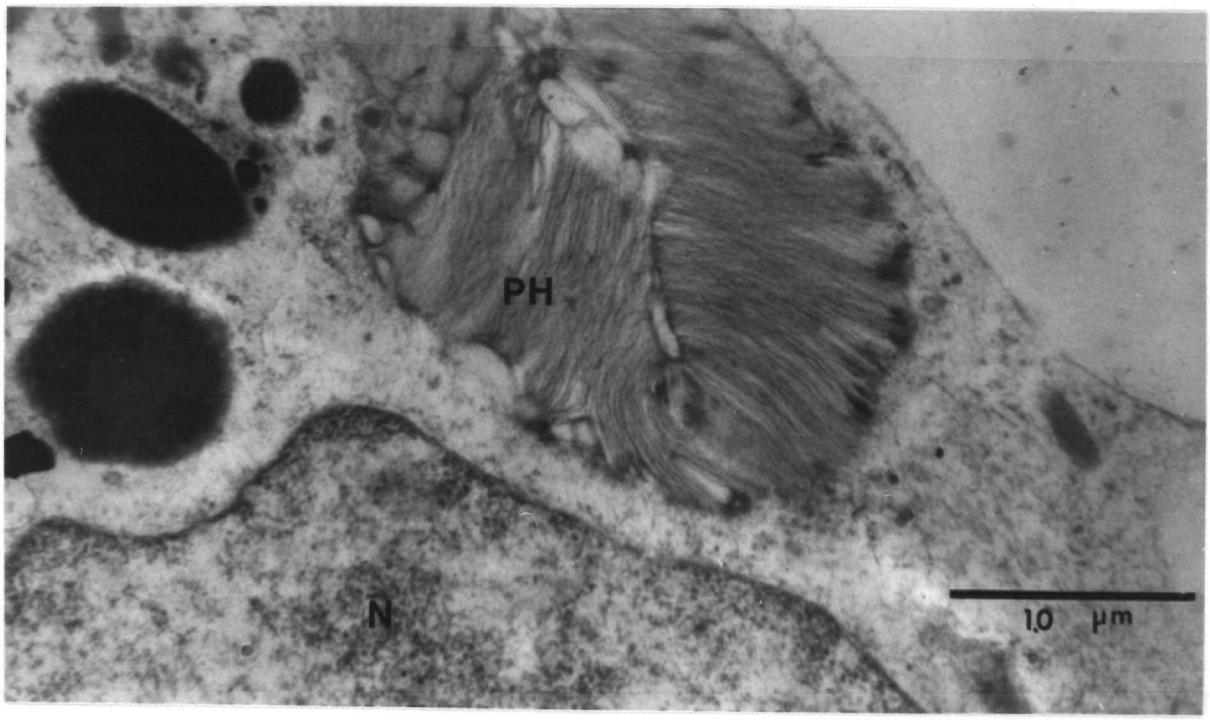


Figure 27.



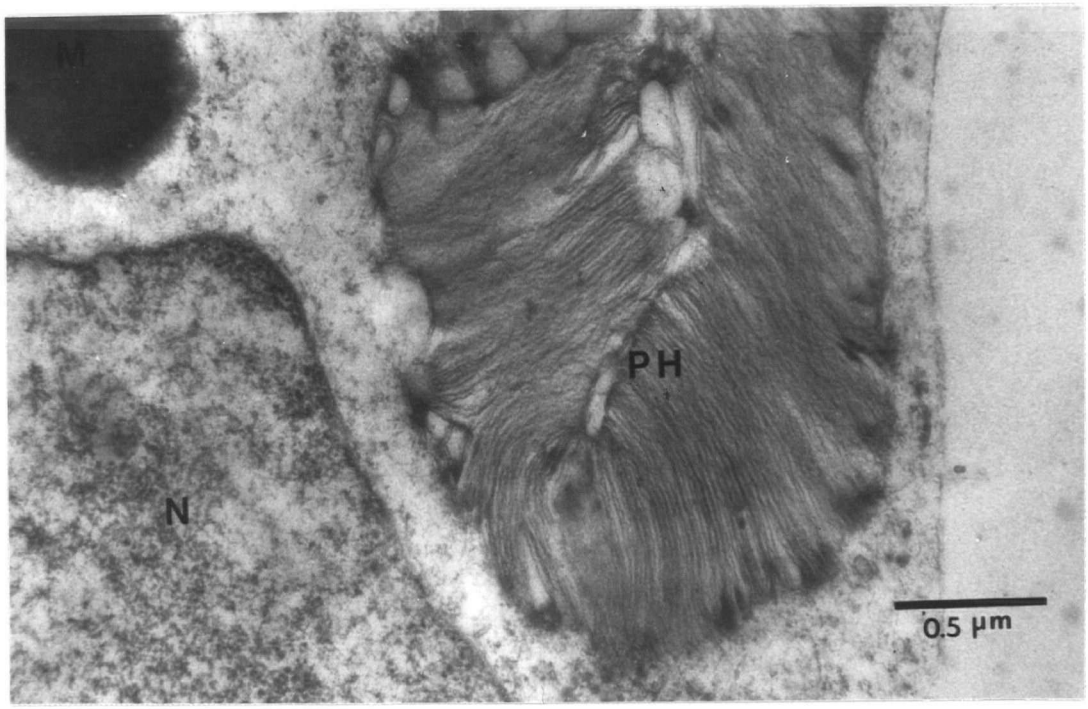


Figure 26. Transmission electron micrographs of 2 week old bovine RPE cells incubated with dark adapted sealed rod outer segments for 5 h. Arrow denotes the position of the plasma membrane. Note that the phagosome (PH) of ROS disc membrane is completely inside the cell. The nucleus (N) is quite evident along with several melanosomes (M).

Figure 27. Transmission electron micrographs of another 2 week old bovine RPE cell incubated with dark adapted sealed rod outer segments which further illustrates ROS phagocytosis. Notice the arrangement of stacks of ROS disc membrane forming the large phagosomes (PH). The nucleus (N) and melanosomes (M) are also evident.

DISCUSSION

The introducing of adult bovine RPE cells into tissue culture opened up a whole new approach in studying RPE cell surfaces and RPE-ROS interactions. Once the RPE cells recovered from the traumatic enzymatic treatment their cell morphology became less cuboidal as they established rapid growth *in vitro* as seen by other researchers (28,30,31). The normal and multinucleated cells, with primarily mosaic and spindle growth patterns developed a hardy growth rate (doubling time 52 h) until confluent cultures were obtained. At the level of light microscopy the cells demonstrated a flat highly pigmented nature as observed in intact tissue. Microvilli were found over a large section of the bovine RPE cell surface with interspersed attached vesicles. These vesicles on the cell surface have the appearance of ROS which still may be tightly associated with RPE cells even after 10 days in tissue culture.

From the fractionation and SDS gel electrophoresis of RPE cells grown in tissue culture there was still considerable rhodopsin associated with the RPE cells. Probing with the Rho-1D4 antibody (specific for carboxyl terminal of rhodopsin), rhodopsin was found to be associated with the bovine RPE cells in tissue culture for as long as 2 weeks. It appears that the membrane protein, rhodopsin, or at least the Rho-1D4 antigenic site, is slowly degraded in tissue culture with a possible initial latent period. This would support the proposed idea that the degradation of rhodopsin by RPE lysosomal fractions occurs after the initial phase of degradation of other membrane proteins (36).

Preliminary studies looked at the arrangement of certain cell surface glycoproteins that may be important in ROS-RPE interactions. Fluorescent lectins were used to look at the distribution of sugar residues on bovine RPE cells. Labeling both fixed and unfixed melanotic and amelanotic bovine RPE cells in vitro with FITC-Con A, WGA, and RCA at 37°C resulted in a dense uniform labeling at the fluorescent level of observation.

Labeling RPE cells with fluorescent lectins followed by 60 minutes in buffer resulted in the energy dependent redistribution of the label, as most of the fluorescence appeared as intense spots centralized in the extranuclear region of the cell. The staining patterns reflect an internalization of the label since a high concentration of inhibitor failed to remove the specific label. The cells treated with WGA still appeared to have labeled receptors on the membrane even after 60 minutes in buffer indicating that not all the labeled WGA receptors were induced to internalize or redistribute as seems to be the case with Con A and RCA. RCA peroxidase labeled sites on cultured embryonal neurons were shown by TEM to internalize (76). Cell surface binding sites continuously labeled with lectins at 37°C induced rearrangement for all three lectins used. At the level of fluorescence, all three lectin receptors appear to redistribute independently of their unlabeled counter parts when subsaturating concentrations of lectins were used. Redistribution and internalization is constantly occuring so that previously unlabeled receptors become accessible for labeling resulting in a dense pattern of label on the cell surface being maintained.

Further experiments using lectins are required to conclusively predict the mode of lectin receptor internalization. Differences in turnover rates of labeled receptors may play a role in the redistribution patterns. Double labeling experiments would further define the action of multiple receptor redistribution and internalization as Con A itself binds to many plasma membrane proteins (77).

Lectin studies are interesting in this paper as to their possible role in receptor-receptor interaction between rod outer segments and RPE cell surfaces. It is possible that sugar receptor arrangement may be involved in the recognition of ROS disc packets released from the rod outer segments. Carbohydrates as found on glycoproteins such as L-fucose and D-mannose significantly reduced the ROS disc packets phagocytized in the frog system (37). In the future, further studies at the level of TEM are required to quantitatively localize various lectins on the cell surfaces of normal and dystrophic rat RPE.

From the RPE plasma membrane preparation it was noted that actin was detected by immunoblot techniques. Acetone treated RPE cells also demonstrated specific anti-actin fluorescence concentrated primarily on the plasma membrane of the cells but also found through out the cell. The high degree of pigmentation tends to obscure much of the fluorescent preventing a good observation of the internal cytoskeletal system. Anti-actin fluorescence was non-uniform being more concentrated near the nuclear region interspersed with brighter fluorescent patches which may represent surface microvilli.

It appears from literature that the ingestion phase of phagocytosis is defective in the dystrophic pigment epithelial cells (3,9,14). Consequently, this defect may involve the mobilization or functioning of actin. Evidence from the arrangement of actin filaments in RPE cells points to needed actin activation and rearrangement to the cell periphery for the mechanistic phagocytosis of ROS (17). Possibly, in the dystrophic case, actin is only activated in a few sites when ROS become attached to the cell surface.

In order to further study the ROS-RPE receptor interaction a suitable assay system had to be developed that could be used repeatedly. Assay systems using chick and rat embryonic RPE explants have been used in the past to study the phagocytosis of non-specific particles as well as ROS (4,5,14,15), but bovine RPE cells in vitro have not been used previously in the study of phagocytosis of ROS. By using specially prepared dark adapted, sealed ROS, two week old RPE cells have been shown, not only to attach ROS, but also to phagocytize these same ROS. Rod outer segments have been seen attached to RPE cells by SEM (Fig. 25). At the level of TEM, large ROS packets were observed attached and also completely endocytosed by the bovine RPE cells (Figs. 26,27). Observations by TEM failed to provide any quantitative information in terms of the number of ROS phagocytized. Some cells in vitro failed to recognize or phagocytize ROS due possibly to a loss of receptor sites through cell divisions or a loss of needed microvilli. This new phagocytosis assay system opens up a whole new series of future experiments for studying

ROS receptor-RPE receptor interactions.

CONCLUSIONS

A 7-9 fold enriched RPE plasma membrane preparation was prepared and characterized by enzymatic analysis. The membrane preparation was found to have a large rhodopsin contamination which was partially removed by immunoaffinity chromatography. A monoclonal antibody, designated Rho-5A3, was raised against the RPE membrane preparation, but it was found to be specific for rhodopsin. The Rho-5A3 antibody bound to solubilized rod outer segments and was localized to the N-terminus of the rhodopsin molecule.

In order to further study bovine RPE cells, the cells were introduced into tissue culture where they maintained many of their *in vivo* characteristics. Fluorescent lectin labeled Con A, WGA, and RCA sites on the cell surface of bovine RPE were induced to constantly redistribute and internalize maintaining a dense pattern of label on the cell surface. Actin was identified as a major RPE plasma membrane and cytoskeletal protein. Finally a new assay system was established for studying the phagocytosis of ROS by bovine RPE cells *in vitro*.

Future investigation can take several approaches. First of all, a newly obtained RPE plasma membrane specific monoclonal antibody designated RPE-3A6 could be completely characterized along with its bovine RPE determinant. Secondly, an anti-N-terminus rhodopsin monoclonal antibody, designated Rho-4D2 which has the ability to bind to sealed ROS, could be used to

quantitatively study phagocytosis of ROS in conjunction with radioactive labels. Thirdly, the role of cell surface glycoproteins can be further studied by lectins at the fluorescent, SEM, and TEM levels. Finally , the problems of producing hybridoma cell lines that secrete monoclonal antibodies against RPE plasma membrane components should be solved and new monoclonal antibodies raised. All of these future investigations could eventually be applied to normal and dystrophic (RCS) rat photoreceptor-RPE systems.

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