

# The Fate of Immunoreactive Opsin Following Phagocytosis by Pigment Epithelium in Human and Monkey Retinas

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**Polyclonal and monoclonal antibodies to human rhodopsin were used to identify and localize this principal glycoprotein of the photoreceptor outer segment discs on thin sections of human and monkey retinal pigment epithelium (RPE) and on immunoblots of RPE subcellular fractions following gel electrophoresis. Antiopsin was visualized with protein A-gold labeling by electron microscopy or peroxidase-linked second antibody on immunoblots. In immunocytochemical studies using polyclonal antibodies, the rod outer segments (ROS) were heavily labeled whereas cone outer segments labeling was variable and more sparse. Phagosomes and other small bodies in the RPE, interpreted as secondary lysosomes, were labeled. In contrast, lipofuscin granules, osmiophilic residual bodies of the lysosomal system of the RPE, were negative. No reactive sites were found in Bruch's membrane or in drusen. A monoclonal antibody (MAB) specific for the amino terminus of human opsin and another MAB specific for the carboxy terminal region of bovine opsin produced labeling patterns similar to, but about one-half the density obtained with polyclonal antibodies. In the immunoblot analyses, the lipofuscin granule fraction from a sucrose density gradient of human RPE homogenates was positive for rhodopsin only in those specimens that were found, upon ultrastructural examination, to contain recognizable phagosomes. When phagosomes were lacking and therefore did not contaminate the lipofuscin granule fraction, the immunoblots were negative for opsin. Melanolipofuscin granule fractions were uniformly negative for opsin. We conclude that the superficial hydrophilic antigen binding sites on the opsin molecule for which the antibodies are specific have been altered or destroyed by lysosomal enzyme digestion within the phagolysosomal system of the RPE prior to formation of definitive lipofuscin granules. Thus, these antibodies are of limited value in revealing the ultimate fate of the whole rhodopsin molecule, eg, the hydrophobic sequences that are the most likely residues in lipofuscin granules. Invest Ophthalmol Vis Sci 29:708-719, 1988**

The daily phagocytosis of discarded photoreceptor outer segment disks by the retinal pigment epithelial (RPE) cells implies that these cells have an efficient system for degrading these membranous structures. After a lysosome fuses with a phagosome, creating a phagolysosome, hydrolytic digestion begins to alter the macromolecules of the engulfed membranous disks. Partial breakdown of proteins, lipids and polysaccharides and diffusion of resultant smaller molecules out of the phagolysosome reduces the size of

this organelle, theoretically to the vanishing point.<sup>1</sup> In young animals, most RPE phagolysosomes are reduced to less than half their initial size within 16 hr after a shedding event.<sup>2,3</sup> The rate of phagosome reduction or disappearance in eyes of old animals has not been determined.

RPE cells from older humans (>40 years) are filled with lipoidal granules (lipofuscin) thought to be the indigestible residues of phagolysosomes formed during earlier shedding events.<sup>4</sup> Lipofuscin granules stain similarly to photoreceptor outer segments with several histochemical stains<sup>5</sup> but the identity of their constituent molecules is largely unknown.

The principal protein component of the outer segment disk membranes is opsin, a glycoprotein that has been well characterized and therefore is a useful marker for the heterophagic pathway in RPE cells. We have used both polyclonal and monoclonal antibodies to human and bovine rhodopsin<sup>6</sup> to examine the fate of rhodopsin in the phagolysosomal pathway of human and monkey RPE employing biochemical and cytochemical methods.

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## Materials and Methods

### Rhodopsin Antibodies

Sensory retinas of 51 human eyes were dissected from posterior eye cups of eye bank eyes, rinsed by dipping in buffered saline, and stored frozen. The outer segments of the photoreceptors were isolated<sup>7</sup> and opsin was purified on a con-A sepharose column<sup>8</sup> in the laboratory of Dr. Paul Hargrave (see ref. 6).

Two rabbits were immunized with opsin as described.<sup>6</sup> Blood samples were taken before immunization and prior to each booster. ELISA tests were performed using bovine outer segment-coated plates. The polyclonal antisera were tested against cyanogen bromide peptide fragments from opsin and found to be reactive mainly to the amino terminus of the molecule.<sup>6</sup> The monoclonal antibodies (MAB) used in this study were MAB 1D4 to the carboxy terminus of bovine rhodopsin made in Dr. Robert Molday's laboratory,<sup>9</sup> and a MAB specific for the amino terminus of rhodopsin, R2-15, made in Dr. Hargrave's laboratory.<sup>6</sup>

Immunoglobulin G (IgG) of the preimmune and immune polyclonal sera was affinity purified on a protein A-sepharose column (Pierce, Rockford, IL) in our laboratory<sup>10</sup> and stored at  $-70^{\circ}\text{C}$  until used.

### Immunocytochemistry

Six human eyes were obtained from the Eye Bank and four monkey eyes from the Oregon Regional Primate Research Center. Ages and post mortem delay times are given in Table 1.

Eyes were bisected and one-half was placed in periodate-lysine-paraformaldehyde fixation (PLP)<sup>11</sup> for 1.5–3 hr at  $4^{\circ}\text{C}$ . The monkeys were treated in a manner that conforms with the ARVO Resolution on the Use of Animals in Research. The other half of each eye was immersed in one of the following fixatives to evaluate the effects of fixative composition, fixation times and temperatures on the immunocytochemical reactions: 1.5% phosphate buffered glutaraldehyde at  $20^{\circ}\text{C}$  for 1 hr; 4% paraformaldehyde in phosphate buffer at  $20^{\circ}\text{C}$  for 4 hr; and 2% glutaraldehyde-1% paraformaldehyde in cacodylate buffer at  $20^{\circ}\text{C}$  for 24 hr. Osmic acid was not used so that lipofuscin and melanin granules would be distinguishable in the electron beam and in order to preserve antigenicity. Tissue was dissected from macular, equatorial and peripheral zones of the retina, dehydrated in ethanol and embedded in L.R. White resin,<sup>12</sup> a hydroxyethyl methacrylate. The resin was polymerized at  $4^{\circ}\text{C}$ . Semithin sections were stained and examined by light microscopy. Thin sections were collected on formvar-coated gold grids. Nonspecific binding sites on the sections were blocked by

**Table 1.** Eyes used in this study

	Age (yrs)	PM time* (hr)
<i>1. Immunochemistry</i>		
Human	7	3
	26	4
	71	5
	74	5
	78	8
	82	2.5
Monkey	17	0.1
	21.5	0.1
<i>2. RPE Fractionation</i>		
Human	21	20
	38	24
	56	12
	64	16
	74	29
	91	6
Monkey	?	1

\* Death to fixation or to suspension of cells in protease inhibitor solution.

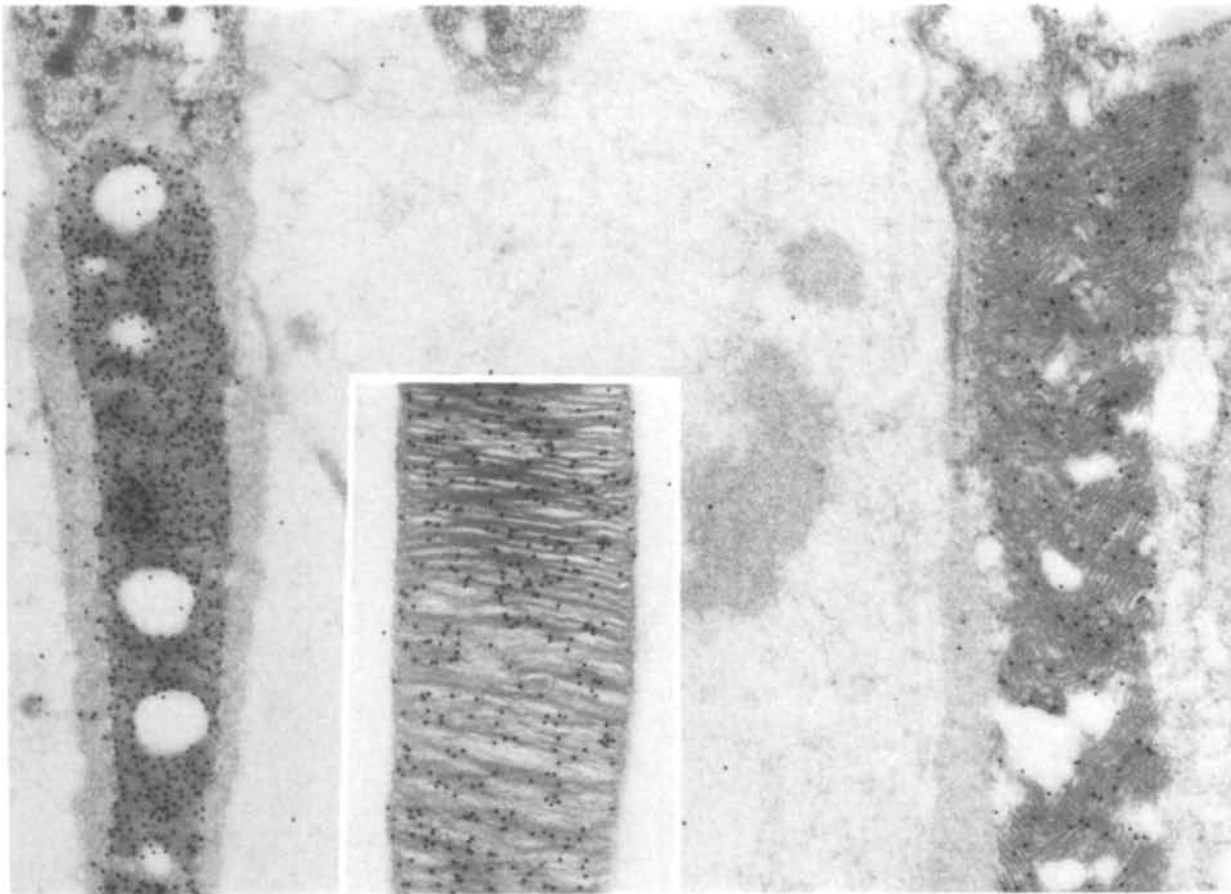
incubation with 4% bovine serum albumin prior to incubation for 4 hr with rabbit preimmune serum (control) or antirhodopsin IgG, (1 mg/ml) diluted 1:200 at  $4^{\circ}\text{C}$ . MABs (1 mg/ml) were diluted 1:25. The procedure used for reacting the sections with *Staphylococcus aureus* protein A-gold (15 nm) was that outlined by the manufacturer, Janssen Pharmaceutical (Beerse, Belgium). Grids were examined without staining for particle counting. Uranyl acetate and lead nitrate staining was done if more tissue contrast was desirable. Particle counts were done on three electron micrographs of each of two human specimens for photoreceptors. For RPE, Bruch's membrane and drusen at least five micrographs from each of the six specimens listed in Table 1 were counted to obtain the means and standard deviations shown.

### RPE Cell Fractionation

RPE cells were brushed from the human and monkey eyes described in Table 1. They were homogenized and fractionated individually (human) or in pairs (monkey) as described previously,<sup>4</sup> except that 1 mM protease inhibitor, phenylmethyl sulfonyl fluoride, was added to sucrose and saline solutions. Fractions aspirated from the sucrose density gradient were washed with phosphate buffered normal saline and centrifuged. The pelleted fractions were resuspended and a sample of each was prepared for electron microscopy as described previously.<sup>4</sup>

### Gel Electrophoresis

The remainder of the granule fractions were solubilized in a protein denaturing solution containing 4%



**Fig. 1.** Monkey rod (left) and cone (right), protein A-gold (15 nm) localization of antihuman opsin polyclonal antibody. Note greater density of rod labeling and low background in the interphotoreceptor space and matrix. Lucent defects in tissue are artefacts produced during polymerization of embedding medium and extraction of tissue components due to omission of osmium tetroxide from the fixation procedure. 17-yr-old rhesus, 4% paraformaldehyde fixative,  $\times 26,000$ . Inset: Amino terminus monoclonal antibody, same specimen. Label on rod is one-half the density of that with polyclonal antibody.  $\times 32,000$ .

SDS and 10% mercaptoethanol. Samples were boiled 10 min in a water bath or incubated at room temperature for 1 hr then applied to the wells of SDS running gel of 12% polyacrylamide. Proteins were electrophoresed using the buffer system of Laemmli<sup>13</sup> for 5 hr and stained with Coomassie blue.

#### Immunoblotting

Fractions from several pairs of eyes were pooled for electrophoresis and subsequent electroblotting. Three identical copies of each gel were made to analyze for the presence of opsin-derived polypeptides: one for amido black staining, one each for preimmune or immune serum incubation. Proteins were electroblotted (Trans Blot apparatus, BioRad, Richmond, CA) onto nitro-cellulose paper using Towbin's buffer system<sup>14</sup> for 4 hr. The blots were blocked with 5% bovine serum albumin. One set of lanes was incubated in rabbit anti-human opsin antiserum diluted

1:200 for 3 hr at room temperature or overnight at 4°C; the other set of lanes was incubated in an identical manner in preimmune serum. Both the experimental and control electroblots were then incubated at room temperature in secondary antibody: peroxidase-labeled goat anti-rabbit IgG diluted 1:1000. The enzyme-conjugated complex was visualized with 4-chloro-1-naphthal (Boehringer Mannheim, Indianapolis, IN) in 0.0015% hydrogen peroxide.

## Results

#### Immunochemistry

Gold-conjugated protein A has high affinity for the Fc portion of immunoglobulin G (IgG)<sup>15</sup>; therefore gold particles in the electron micrographs in this study are interpreted to mark the location of the antiopsin molecules. Outer segments of rods and cones of monkey and human retina were labeled with pro-

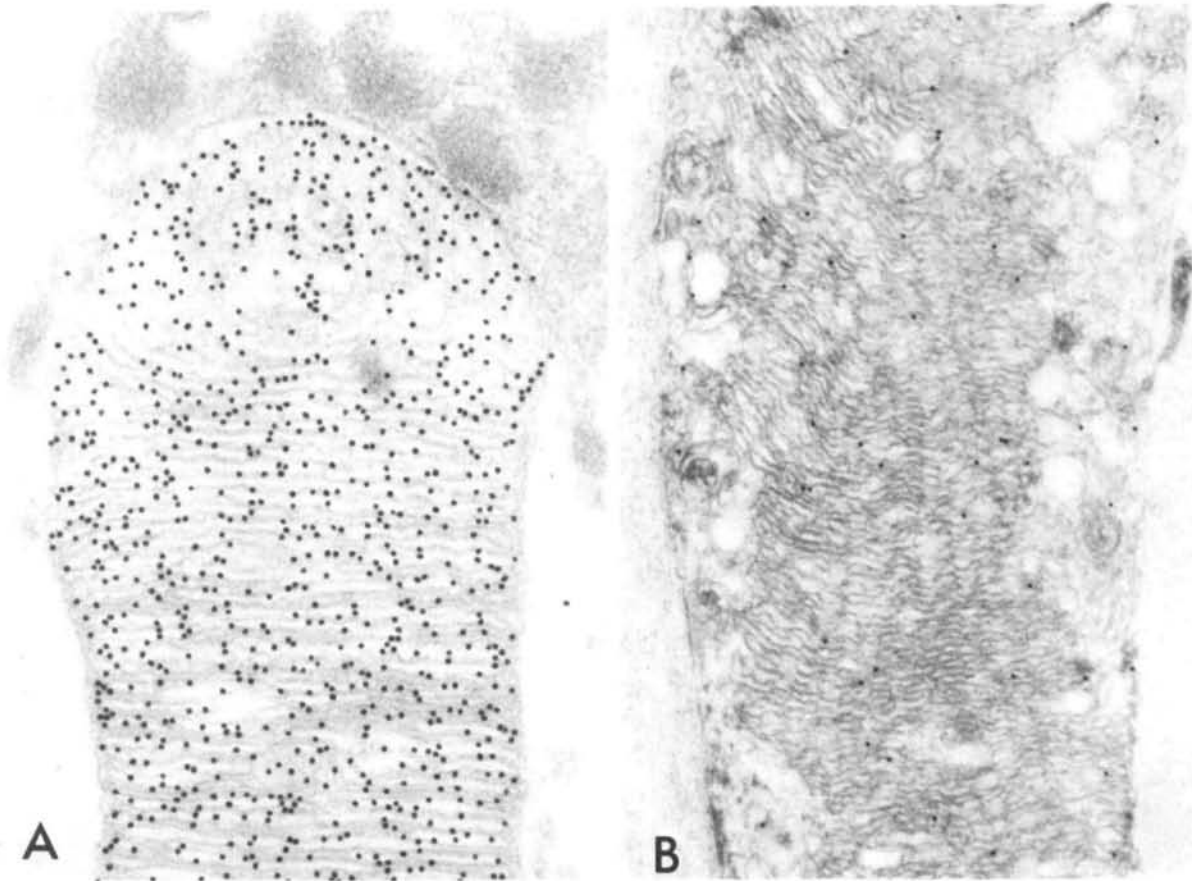


Fig. 2. Human rod and cone, polyclonal antibody. (A) Rod,  $\times 58,000$ . (B) Cone,  $\times 35,000$ , 82-yr-old, 2.5 hr post mortem PLP fixative.

tein A-gold (Figs. 1, 2). When polyclonal IgG was used as the primary antibody the density of gold particles was  $6\times$  (monkey) and  $9\times$  (human) greater on rods than on cones. The density of gold particles on sections of retina-choroid is shown in Figure 3. Outer segments had  $300 \pm 20$  particles per square micron whereas the ellipsoid of the inner segment had virtually none (Fig. 2A) and the myoid had only  $4 \pm 2$  particles.

The two monoclonal antibodies, one with specificity for the carboxy terminus of bovine opsin and another with specificity for the amino terminus of human opsin, gave similar labeling density on outer segment membranes. Each gave about one half the density of label of the polyclonal antiserum (Fig. 1, inset).

All four fixation procedures yielded specimens that gave positive immunocytochemical reactions. The PLP fixative specimens (1.5 hr at  $4^\circ\text{C}$ ) were deemed best for quantity of label and quality of morphology, and were therefore used for the quantitative data, shown in Figure 3. Subsequent studies have shown that even better morphology is achieved with fixation

in the 2% glutaraldehyde-1% paraformaldehyde at  $20^\circ\text{C}$  for 24 hr without appreciable loss of antibody labeling.

In RPE cells phagosomes were heavily labeled with antibodies to opsin (Figs. 3, 4). Some bodies smaller

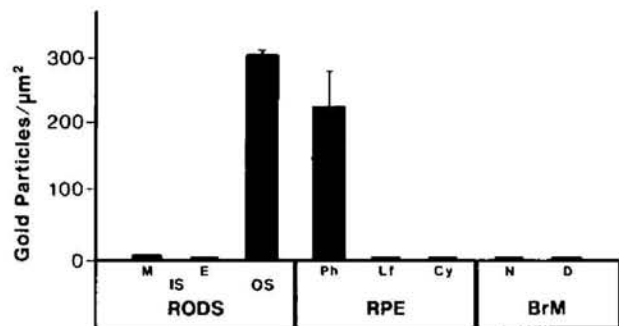


Fig. 3. Comparison of density of labeling sites (gold particles) in selected areas of rod photoreceptors and RPE cells, and in normal and drusenoid Bruch's membrane. In rods: M, E, myoid and ellipsoid respectively of inner segments IS; OS, outer segments. In RPE: Ph, phagosomes/phagolysosomes; Lf, lipofuscin granules; Cy, cytoplasm. Bruch's membrane: N, normal; D, drusen.

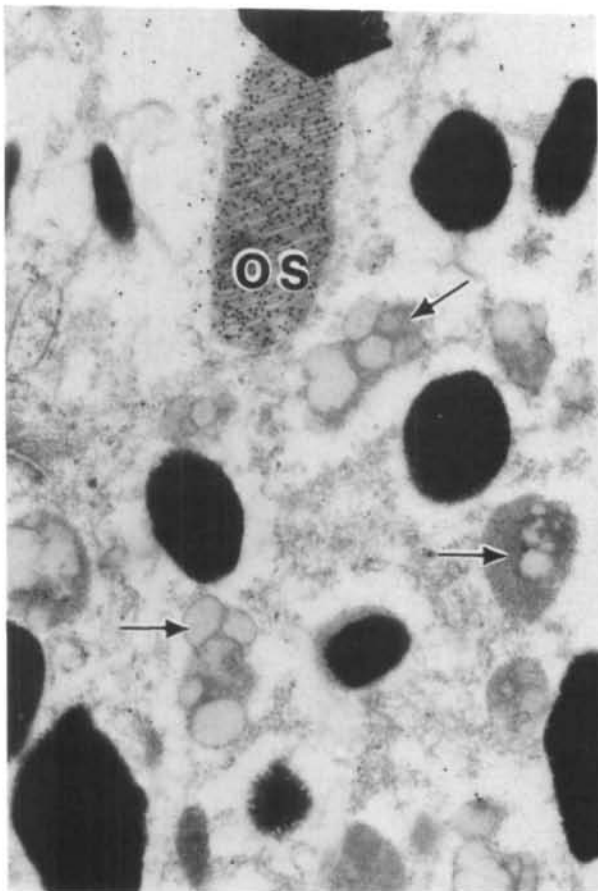


Fig. 4. Monkey pigment epithelial cell and distal end of an antiopsin labeled rod outer segment (OS). No label is seen in secondary lysosomes (arrows) (incipient lipofuscin granules). 21.5-yr-old rhesus, PLP fixative,  $\times 24,000$ .

than phagosomes and more electron-dense than the unsmicated lipofuscin granules, interpreted as partially digested phagosomes (phagolysosomes, some secondary lysosomes) were also labeled. However, lipofuscin granules, the lipoidal granules generally considered to be the residual bodies of the phagolysosomal system, were negative (Figs. 4-7). Also negative were melanolipofuscin granules, the product of fusions between melanin granules and lipofuscin granules.<sup>16</sup>

In monkey specimens the immunocytochemical results could be correlated with the time of light onset, subsequent outer segment shedding and phagocytosis of the opsin-containing membranes by the RPE. Specimens fixed 2 hr after light onset showed many labeled phagosomes (Fig. 5). They were located in the basal part of the cell, often under the nucleus. Some of these had recognizable outer segment disks whereas other appeared amorphous. Granules of the size and morphology of secondary

lysosomes were also labeled. Monkey lipofuscin granules, although morphologically different from those of human RPE, were for the most part negative for immunoreactive opsin (Figs. 4, 5).

Bruch's membrane was negative for immunoreactive opsin in all specimens examined so far by ultrastructural immunocytochemical methods (Fig. 6). Included in our examinations were eyes spanning the first to the ninth decades of life. Particular attention was given to zones of Bruch's membrane containing bodies that stained similar to outer segments of photoreceptors and/or boluses of RPE cytoplasm, in order to determine whether opsin was detectable in these structures. No immunoreactive opsin was found in any specimen.

Drusen from both macular and equatorial locations were examined for the presence of immunoreactive opsin. None was found (Fig. 7). The quantitative results of immunolabeling for opsin in rods, RPE cells, Bruch's membrane and drusen when polyclonal IgG was used is depicted in Figure 3.

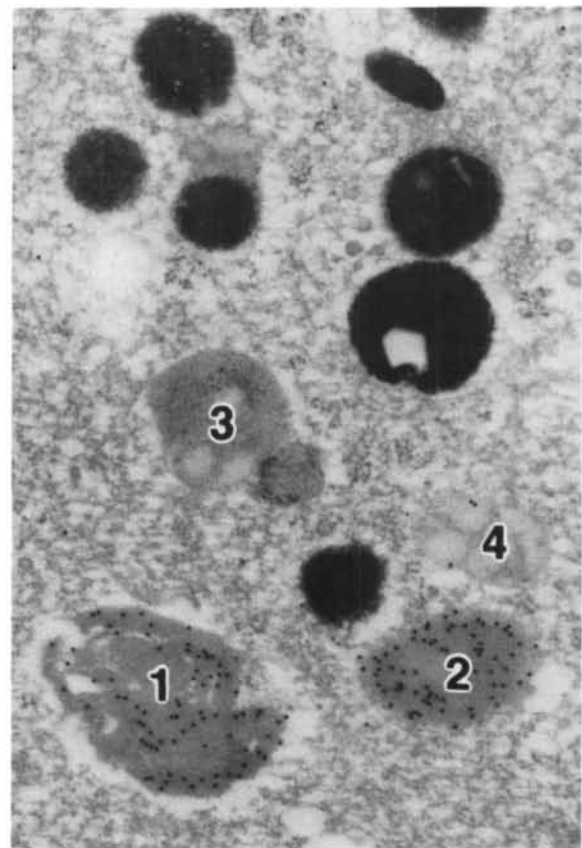


Fig. 5. Phagosomes in monkey RPE, 2 hr after light onset. The two basally located phagosomes (1, 2) are labeled whereas the more apical one (3) is not. The lipofuscin-like secondary lysosomes (4) has sparse label. 17-hr-old rhesus, glutaraldehyde-paraformaldehyde fixative,  $\times 30,000$ .

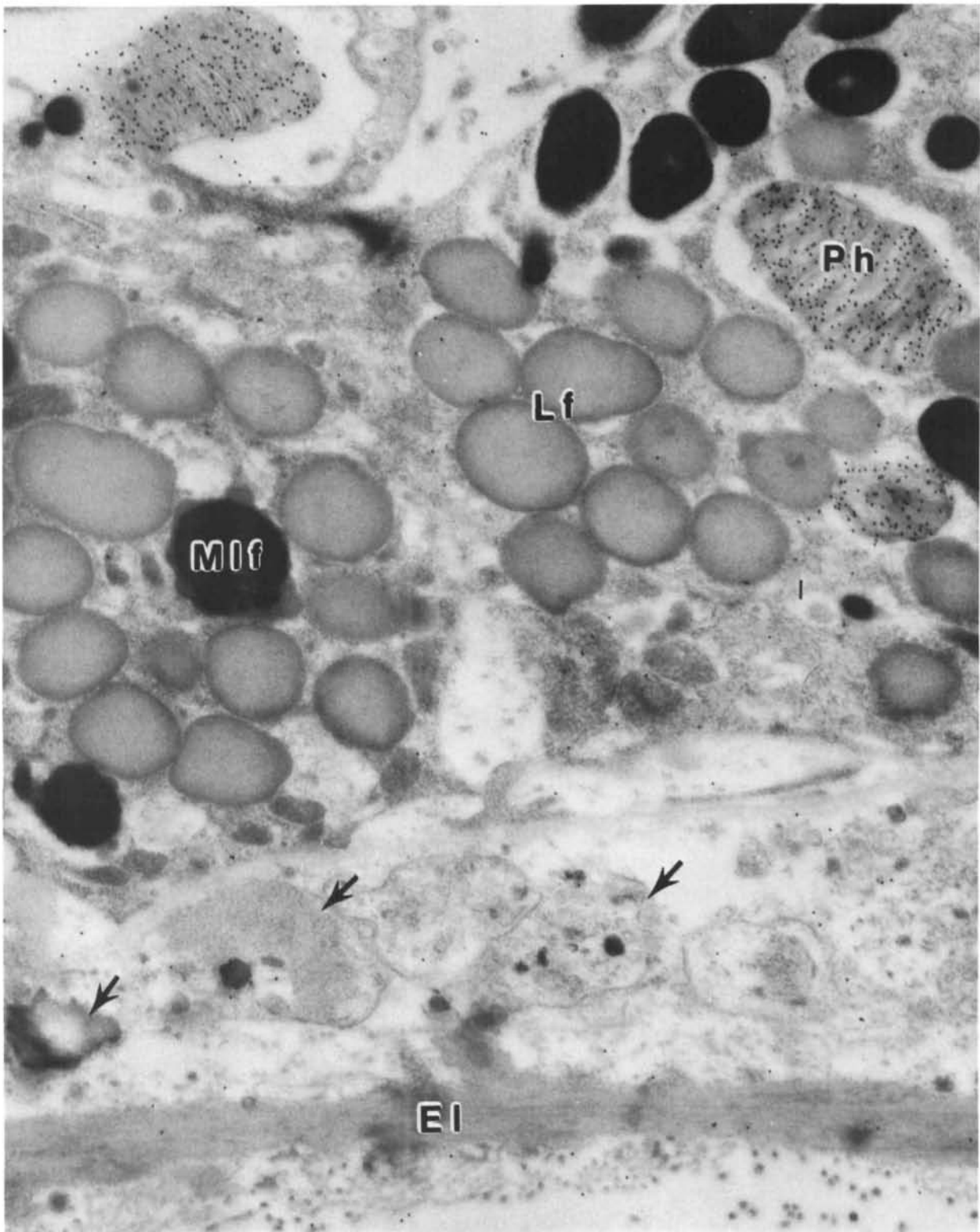


Fig. 6. Human RPE showing labeled phagosomes (Ph) and unlabeled lipofuscin (Lf) and melanolipofuscin granules (Mif). Debris (arrows) in Bruch's membrane shows no labeling. EI, elastic layer. 82-yr-old, 2.5 hr post mortem PLP fixative,  $\times 23,000$ .

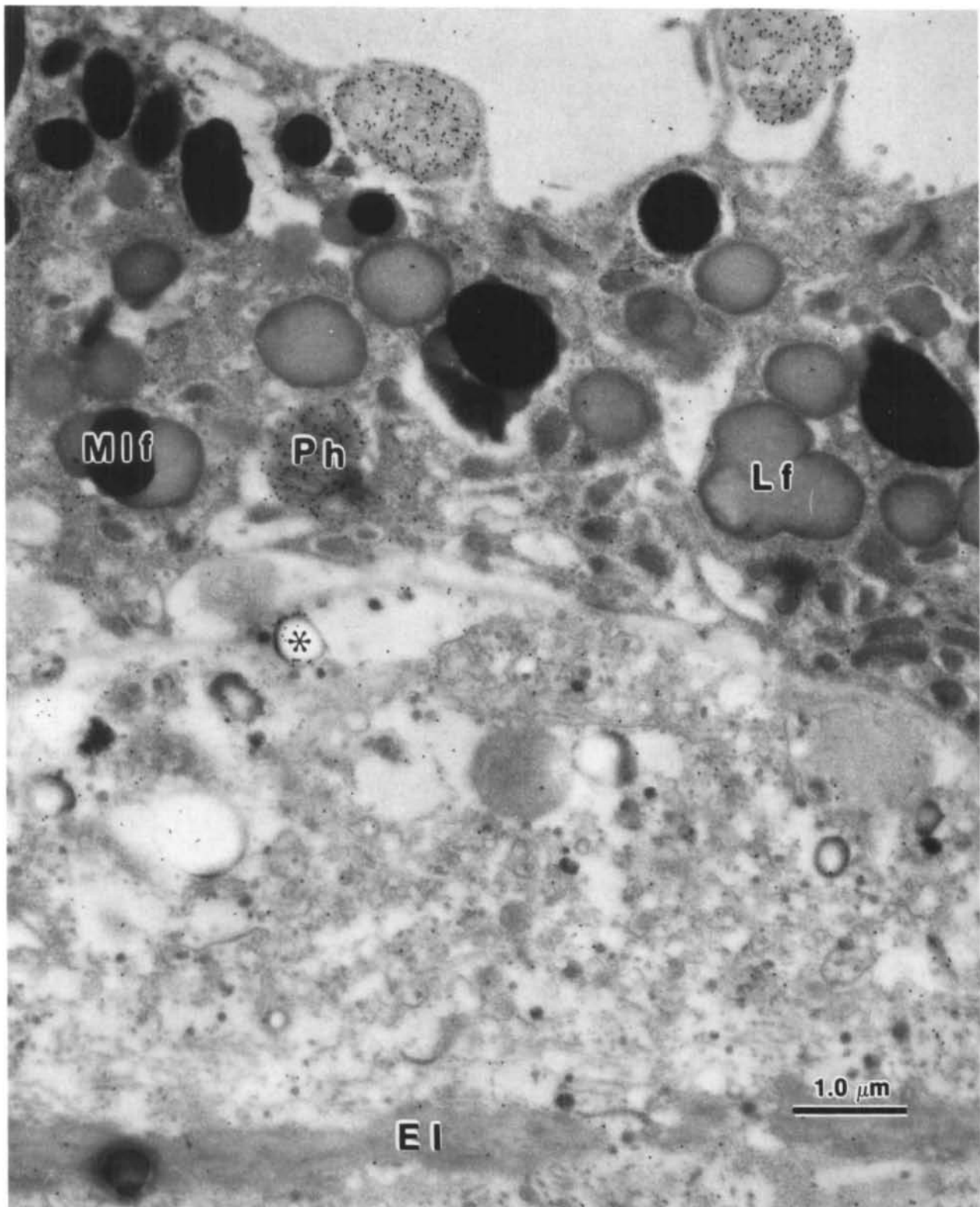


Fig. 7. Human RPE and granular drusen. No rhodopsin label above background is seen. Label at asterisk is nonspecific and occurs at a hole in the section, a common defect in sectioned drusen. EI, elastic layer of Bruch's membrane. MAB to carboxy terminus; background labeling is slightly higher than with polyclonal antiserum. 82-yr-old, 2.5 hr PM PLP fixative,  $\times 20,000$ .

### RPE Fractionation

Figure 8 shows the discontinuous sucrose density gradients of the post-nuclear (ie, nucleus-free) supernatant of RPE homogenates of a pair of monkey eyes and a single human eye, age 64. In white light the brown melanin of the monkey RPE masks some of the yellow color of the bands that is characteristic of the human specimen. Under UV light the meager fluorescence of the lipofuscin granule fraction of this adult (age unknown) monkey specimen is striking compared to the prominent, brilliantly fluorescent bands of the human specimen.

A typical lipofuscin granule fraction containing a phagosome/phagolysosome is shown in Figure 9. This degree of contamination was sufficient to produce an immunoblot positive for opsin.

### Gel Electrophoresis and Immunoblot Analysis

SDS-PAGE shows that the lipofuscin granule fraction contains numerous proteins; the pattern of bands is quite similar, even to the tenth decade. The melanolipofuscin granule fraction shows many fewer bands than the lipofuscin in Coomassie blue stained gels (Fig. 10). This difference is consistent throughout the age range 21–91 years. The entire fraction of melanolipofuscin granules from one eye was required to obtain the results shown in Figure 9, whereas the lipofuscin granule fraction from one eye provided sufficient material for three or four electrophoretic runs.

Immunoblot analysis was performed to determine whether or not opsin-related polypeptides were present among the proteins of the RPE subcellular fractions. In some specimens the RPE homogenate and the lipofuscin granule fraction showed anti-opsin binding (Fig. 11). In others these fractions were negative for opsin (data not shown).

When immunocytochemistry was performed on a sample from the same eye used for RPE fractionation and immunoblot analysis, we found that specimens with positive opsin immunoblots had antiopsin-labeled phagolysosomes in their RPE (Fig. 6). In contrast, donor eyes that gave negative results with antiopsin antibodies on immunoblots of lipofuscin fractions had no discernible RPE phagosomes when examined by ultrastructural immunocytochemical procedures (data not shown).

### Discussion

Opsin antibodies were effective in tracing early (ie, 2 hr after light onset) changes in, and translocations of phagosomes in the heterophagic (phagocytic) pathway of RPE cells. This study did not include

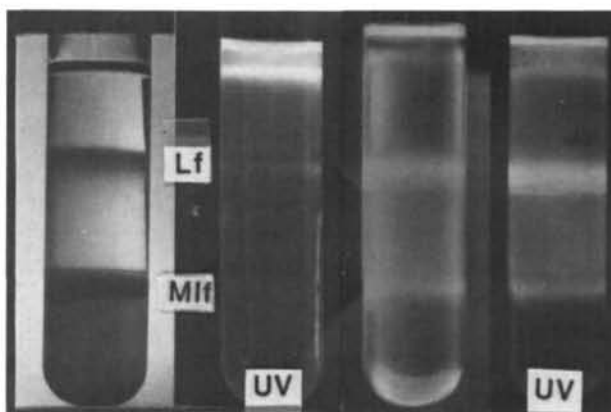


Fig. 8. Discontinuous sucrose density gradients (15 ml Cortex tubes) of the post nuclear supernatant of RPE homogenates of two monkey eyes (left pair) and one human eye (right pair) photographed under white and ultraviolet (UV) light. In white light the dark (brown) color of the monkey fractions differ from the light (yellow-white) color of the human fractions, reflecting their differing content of melanin and lipofuscin. In UV light, below the reflective meniscus is a greenish fluorescent zone containing small organelles (mitochondria, lysosomes, endoplasmic reticulum) and cytosol. The main upper band is lipofuscin, (LF) the lower band, melanolipofuscin (MLF). The pellet is melanin. Note the weak fluorescence of the monkey lipofuscin fraction compared to the human and the lack of fluorescence of monkey melanolipofuscin. Monkey, age unknown; human, age 64.

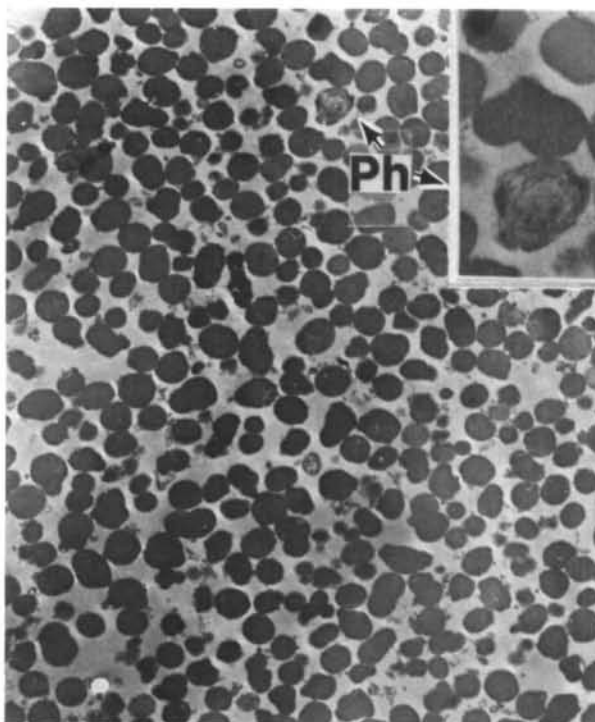
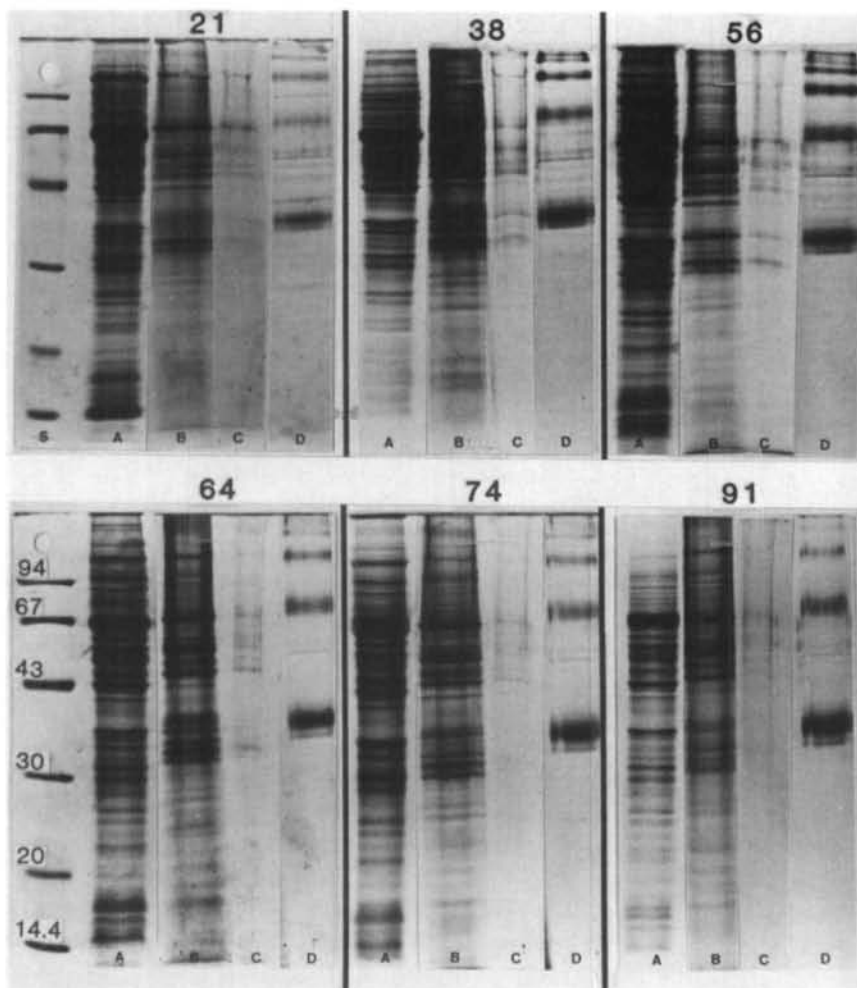


Fig. 9. Lipofuscin granule fraction of RPE of a specimen that gave a positive opsin reaction in the immunoblot owing to the minor contamination with phagosomes,  $\times 6000$ . Inset: enlargement of phagosome/phagolysosome,  $\times 16,000$ .





**Fig. 10.** SDS-polyacrylamide gel electrophoresis of RPE homogenate, A; isolated lipofuscin, B; and melanolipofuscin granules, C; of RPE of six human donor eyes; ages are given above each set of lanes. S, low molecular weight standard, D, isolated human rod outer segments showing monomer, dimer and trimer forms of rhodopsin at approximately 37K, 68K, and 100 KMr. The melanolipofuscin fraction has five to eight bands whereas the lipofuscin fraction has 30 or more (Coomassie blue stain).

RPE specimens at known late intervals after light onset; therefore a detailed analysis of the time when all antigenic sites are lost during the degradative process was not possible.

The two monoclonal antibodies used in these studies are known to be specific for two different hydrophilic portions of the rhodopsin molecule, the 1-12 region of the amino terminus and residues 339-348 of the carboxy terminus, respectively. In our hands these MABs gave indistinguishable results in the amount and location of the 15 nm gold particles used to visualize the antigenic sites. The poorer morphology of unosmicated outer segment disks made it difficult to distinguish the internal from the external surface of disk membranes. Use of a smaller gold particle or a ferritin label would probably improve the localization of these reaction sites.

In this study large phagosomes in the RPE that retain morphologically recognizable outer segment discs were heavily labeled with antibodies to opsin. Other bodies that were smaller than either phago-

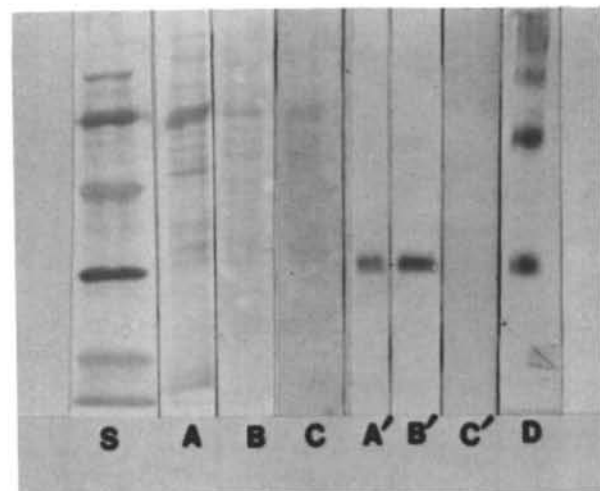
somes or lipofuscin granules were also labeled; these are interpreted as secondary lysosomes that are decreasing in size as cleavage products of enzymatic digestion are transported out of the lysosomes. Several of these small bodies presumably coalesce later to form the standard sized lipofuscin granule characteristic of older human RPE cells (Fig. 12). The exact criteria for distinguishing a secondary lysosome from a nascent lipofuscin granule have not yet been determined.

The degradative enzymes within the phagolysosomal system of the RPE that result in the disassembly of the photoreceptor membranes, the cleavage of constituent lipids, proteins and polysaccharides are just beginning to be investigated. Cathepsin D<sup>17</sup> and/or proteases with cathepsin D-like activity<sup>18</sup> have been identified in bovine RPE. This enzyme is slow-acting but nevertheless effective in degrading ROS protein. The proteases in lysosome-enriched fractions from RPE described by Zimmerman<sup>18</sup> are seven times more active than those in liver fractions in degrading

ROS, RPE lysosomes, and cathepsin D in particular, degrade photoreceptor membranes in a slow stepwise process that resembles closely the proteolytic action of other well studied proteases.<sup>19</sup> During the initial phase of degradation a specific 4000 dalton fragment appears to be removed from the opsin molecule, followed by a 6000 dalton fragment. Detailed investigations by Kean and coworkers,<sup>20,21</sup> carried out using highly purified labeled bovine rhodopsin and RPE homogenates, indicate that approximately 55% of the labeled rhodopsin is degraded after 2 hr incubation by what the authors term a "rhodopsin-cleaving enzyme," that has many properties of cathepsin D. The major product cleaved from rhodopsin was identified as an acidic glycopeptide of MW approximately 9000 (assumed to include the amino terminus of the molecule). This glycopeptide appears to be similar to that isolated by Regan and coworkers.<sup>19</sup> Moreover, it retains the full antigenicity of the intact molecule when tested with antibodies prepared against bovine rhodopsin. This appears relevant to the findings reported in this paper. Loss of these fragments with their antigenic binding sites may account for the lack of labeling in our study of lipofuscin granules, the presumed final indigestible residues of phagocytic activities of the RPE cell.

The absence of immunoreactive opsin in lipofuscin granules does not mean that other fragments of opsin molecule might not be present. The most likely opsin domain to escape proteolysis would be the hydrophobic sequences that are threaded through the membrane.<sup>22</sup> Indeed, when the time course of proteolysis of rhodopsin in disc membranes is examined by monitoring the chromophore that is buried in the hydrophobic domain, loss of spectral properties is not observed until more than 20 hr of incubation with cathepsin D.<sup>19</sup> In the RPE phagosome access to the hydrophobic interior of this transmembrane protein is probably facilitated by lipolytic lysosomal enzymes. Nevertheless, difficulties in the breakdown of both lipoidal and nonlipoidal components in phagolysosomes appears to contribute to the accumulation of lipofuscin residual bodies in aging RPE cells.

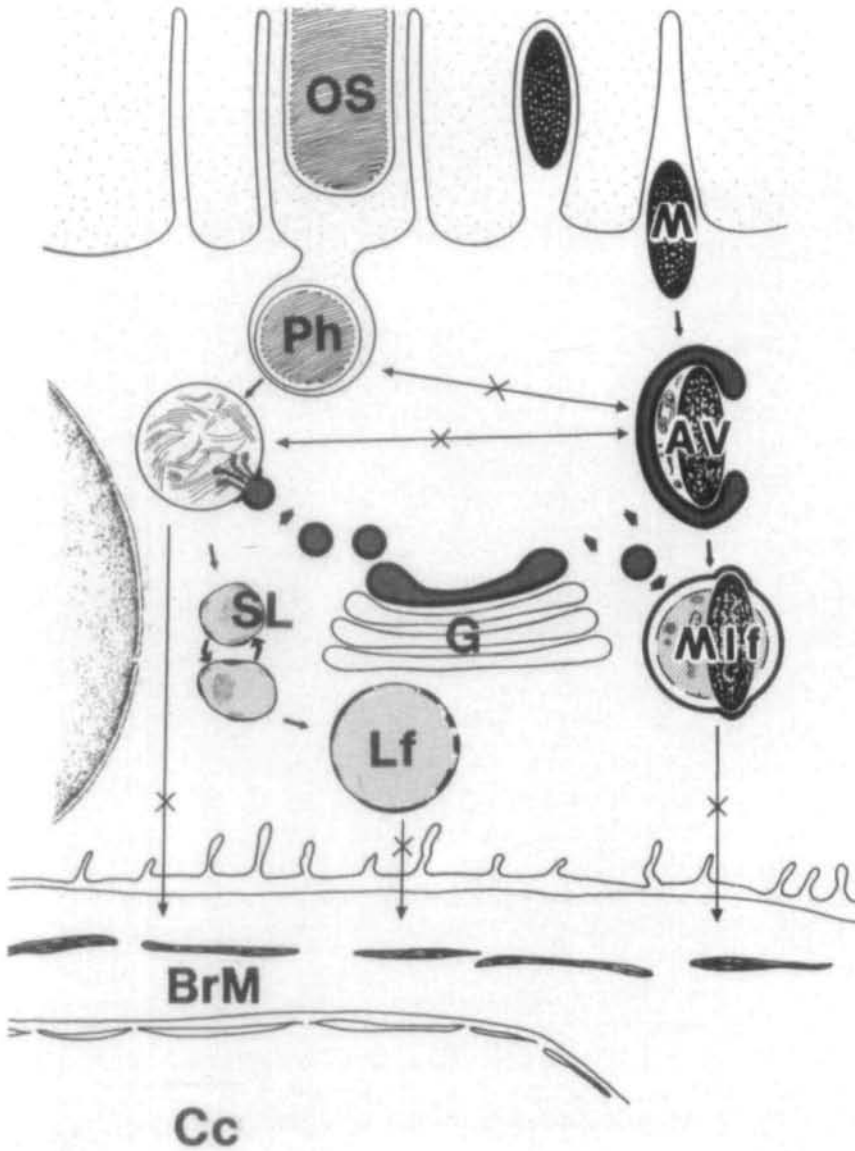
Lipofuscin granules have some staining characteristics similar to outer segments, eg, they are periodic acid-Schiff (PAS)-positive.<sup>5</sup> The PAS stain identifies the 1,2-glycols in the carbohydrate moieties of glycoproteins, and the supposition has been that some of the PAS staining of lipofuscin granules was due to opsin's oligosaccharide chains. The early loss of these opsin carbohydrate fragments in the phagolysosome and their total absence in lipofuscin granules suggests that lysosomal enzymes, which are themselves glycoproteins rather than opsin may be the chemical basis



**Fig. 11.** Immunoblot analysis of RPE fraction using rhodopsin antiserum. Left lanes, amido black stain of nitrocellulose strips showing electrotransferred low molecular weight standards, S; RPE post-nuclear-spin supernatant, A; lipofuscin granule fraction, B; melanolipofuscin granule fraction, C; (compare with Fig. 10). Right lanes, duplicate nitrocellulose strips were incubated with antiserum to human rhodopsin. D, isolated rod outer segments. Rhodopsin was detectable in the crude supernate and in a phagosome-containing lipofuscin granule fraction but not in melanolipofuscin granules.

for the PAS-positive staining. Alternatively, triglycerides may be converted to a 1,2-glycol form by the action of lipases in the phagolysosome to produce PAS-positive staining material in lipofuscin granules. This possibility is supported by preliminary observations (unpublished data) showing that chloroform-methanol extracts of lipofuscin give PAS-positive reactions in fractions running at the front in SDS-PAGE electrophoresis; it is known from studies on erythrocyte membranes that these are glycolipids. Other investigations with Bazan and coworkers have indicated a complex lipid pattern in lipofuscin<sup>23</sup>; these studies are still in progress.

Our preliminary studies had shown that antiopsin binding polypeptides were detectable in RPE of some donor eyes and not in others when the lipofuscin granule fraction was analyzed by the immunoblot technique.<sup>24</sup> These results have persisted despite refinements in the procedure designed to eliminate outer segment contamination, such as analysis of eyes obtained less than 2 hr post mortem and institution of a thorough washing procedure to remove all contaminating outer segments from the apical surface of the RPE prior to harvesting the RPE cells. In the present study using both morphologic and biochemical methods on the same donor eye, we were able to show that those specimens that had rhodopsin in their lipofuscin granule fraction also had opsin-positive phagolysosomes in immunocytochemical



**Fig. 12.** Diagram showing postulated mechanism of formation of lipofuscin and melanolipofuscin granules of RPE cells. In the RPE cytoplasm the stack of Golgi cisternae (G) separates the heterophagic (left) and autophagic (right) pathways of lysosomal degradation of exogenous and endogenous materials respectively. Left: The phagosome containing detached distal disks of the outer segments (OS) of photoreceptors receives lysosomal enzymes (Ly) from Golgi-derived (G) primary lysosomes. Phagolysosomes (PI) are reduced to smaller secondary lysosomes (SL) by hydrolysis of macromolecules; the contents became autofluorescent by processes still poorly understood. Small granules coalesce to form definitive lipofuscin granules (Lf). Right: A Golgi cisterna, the GERL of Novikoff<sup>29</sup> envelopes endogenous, presumably damaged or old RPE cytoplasmic organelles including a melanin granule (M) and cytosol, creating an autophagic vacuole (AV). Melanin is resistant to digestion and remains as a marker of the autophagic pathway. Immunolabeling of rhodopsin did not reveal fusions between phagosomes or phagolysosomes and autophagic vacuoles; also exocytosis of phagosomes, phagolysosomes, lipofuscin or melanolipofuscin granules was not observed (X on various potential pathways). IPM, interphotoreceptor matrix; N, nucleus; BM, Bruch's membrane; cc, choriocapillaris.

preparations. Conversely, those specimens that gave negative immunoblot reactions with antiopsin contained no recognizable phagosomes in thin sections of their RPE. Thus, the biochemical approach (ie, tissue homogenization fractionation, electrophoresis) to the question of whether or not isolated lipofuscin granules contain opsin gave ambiguous information until morphologic methods were used to visualize the status of the RPE phagolysosomal system.

The very sensitive immunoblot analyses of individual melanolipofuscin fractions of RPE from more than a dozen donor eyes failed to reveal the presence of opsin in this isolable class of autophagic vacuoles. Additionally, no opsin label was seen in these granules using immunocytochemical methods. Therefore,

we conclude that the heterophagic (phagocytic) vacuoles of the RPE normally do not interact with autophagic vacuoles in early stages of phagosome processing (Fig. 12). However, as noted above, since the antibodies used in the present study do not reveal the intramembrane sequences of rhodopsin it is possible that these two pathways interact at later stages of intracellular digestion and reorganization of residual bodies.

It has been proposed that aged Bruch's membrane and drusen contain phagosomes and lipofuscin granules that have been passed through or exocytosed by the adjacent RPE cell.<sup>25-28</sup> No evidence for such activity was found in this study wherein opsin antigenic sites served as tracers or in a previous study where

lysosomal enzyme labeling was employed.<sup>29</sup> However, the process of apoptosis, ie, shedding of RPE cytoplasm into Bruch's membrane, may result in accumulation of debris having marked heterogeneity, particularly in aged human<sup>30,31</sup> and monkey<sup>32</sup> eyes and in retinas damaged by intraocular drugs.<sup>33</sup>

**Key words:** Bruch's membrane, drusen, immunoblot analysis, lipofuscin, retinal pigment epithelium

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