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Articles

Lipofuscin and melanin of human retinal pigment epithelium

Fluorescence, enzyme cytochemical, and ultrastructural studies

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The life history of melanin and lipofuscin granules of human retinal pigment epithelium (RPE) was studied in 30 human eyes spanning nine decades of life. Autofluorescent granules in the cytoplasm of eyes over 30 years of age were shown, ultrastructurally and through lipid solvent extraction, to be lipofuscin granules. Sparse small fluorescent granules in infant eyes were secondary lysosomes containing small droplets of lipid. Fluorescent substances in RPE granules of eyes <50 years old were more readily extracted with lipid solvents than those in very old eyes (>70). Lipofuscin granules were positive for acid phosphatase and aryl sulfatase activity. Fusions between primary lysosomes and lipofuscin granules were common in older eyes, suggesting that the over-all degradative process involves repeated injection of lysosomal enzymes, i.e., the initial fusion of lysosomes with phagosomes (phagocytized outer segment disks) is only one of several attempts to hydrolyze the membranous material. Some melanin granules showed hydrolytic enzyme reactions. By use of enzyme cytochemistry, fluorescence microscopy, and lipid extraction two types of melanin-containing complex granules were identified: melanin with a cortex of lipofuscin (melanolipofuscin) and melanin with a cortex of nonlipid, enzyme-reactive material (melanolysosomes). These findings indicate that melanin commonly becomes incorporated into the lysosomal system of the RPE cell and suggests that it undergoes modification or degradation there. These studies indicate that a dynamic, complex interrelationship exists between the various components of the phagolysosomal system and the melanin granules in the RPE cytoplasm. Also, the observed variation from one human eye to another in the content and lipid extractability of RPE lipofuscin granules suggests that there may be differences in lipid composition of phagocytized photoreceptor disks and/or differences in the degradation of these lipids in the phagolysosomal system of the RPE cell.

Key words: aging, enzyme cytochemistry, fluorescence, human, lipofuscin, lysosomes, melanin, melanin degradation, retinal pigment epithelium

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Early histologists observed that human retinal pigment epithelium (RPE) contained two kinds of pigment granules, melanin and lipoidal granules. However, it was not until 1961 that a histochemical study by Streeten[1] established that the lipoidal granules were similar to lipofuscin, the "age pigment" or "wear-and-tear pigment" of brain and other organs. She also noted that certain staining features of these RPE lipoidal granules were common to rod outer segments (ROS). Lipofuscin granules of the brain were found by Essner and Novikoff[2] to be part of the lysosomal system, specifically, that they were the residual bodies of lysosomes. In 1965 we[3] described the ultrastructure of lipofuscin granules in the RPE as well as other bodies resembling portions of ROS in the apical cytoplasm of the RPE. Using the knowledge accrued to that time, we postulated that broken or detached portions of rod and cone outer segments were engulfed by the pigment epithelial cell and that during the life of the retina, which equals the life of the individual, fragments of outer segment were partially broken down by enzymatic digestion, leaving residues to accumulate as lipofuscin granules. Subsequently the elegant autoradiographic studies of Young[4] demonstrated that the photoreceptor outer segments are continually renewed; later, Young and Bok[5] showed that detached ROS terminal fragments were phagocytized by the RPE. Enzyme cytochemical studies by Ishikawa and Yamada[6] demonstrated that the phagosomes, composed of stacks of outer segment disks, contained acid phosphatase, indicating that they had been converted to phagolysosomes. Moreover, they showed that some lipofuscin granules in human RPE also had acid phosphatase activity. These findings established a biological paradigm which has guided research on the RPE during this decade: outer segment disks that are detached from the photoreceptor are phagocytized by the RPE and digested via the lysosomal system; for some unknown reason this process gives rise to lipofuscin granules in human RPE cells.

Lipofuscin pigment has been described as intracellular yellow-brown refractile granules exhibiting sudanophilic, osmiophilic, argyrophilic, and periodic acid–Schiff-positive and acid-fast staining characteristics.[7] Enzyme cytochemistry has revealed the presence of hydrolytic (i.e., lysosomal) enzymes, and the reduction of diaminobenzidine by lipofuscin granules has been interpreted as evidence of peroxidase activity.[8] Ultrastructurally, the granules are membrane bound, but their internal structure as well as their chemical composition vary with cell type and from one granule to another in a given cell. Such heterogeneity is not surprising, since the granules represent terminal stages of lysosomal digestion of various products the cell phagocytizes or autophagocytizes.[9, 10] Complex lipids, however, are the most characteristic components of lipofuscin.[11] Extracts of lipofuscin granules fluoresce at 430 to 470 nm when irradiated at 360 to 365 nm, reportedly due to the presence of Schiff-base fluorophore formed during peroxidation and polymerization of unsaturated lipids.[12, 13]

Melanin, an insoluble high-molecular-weight polymer derived from the enzymatic oxidation of tyrosine and dihydroxyphenylalanine, is contained in membrane-limited granules in the RPE. RPE melanin differs from that in melanocytes in its origin from neural ectoderm rather than neural crest and in its apparent static condition during adult life. Although the melanosomes of uveal and dermal melanocytes show ultrastructural and enzymatic properties suggestive of continual synthesis during life,[3, 14] RPE melanin granules are synthesized in utero, achieve maturity before the first decade of life[15, 17] and thereafter are thought to be unchanging structures in the cytoplasm of these essentially nondividing cells.

The RPE of old human eyes usually contains complex granules composed of both melanin and lipofuscin,[3] suggesting that these two components of RPE cytoplasm may have interrelated life histories. The present study was undertaken to examine the life history and composition of lipofuscin and melanin in human RPE cells. The characteristic fluorescence of lipofuscin was used to demonstrate these granules in wet preparations of

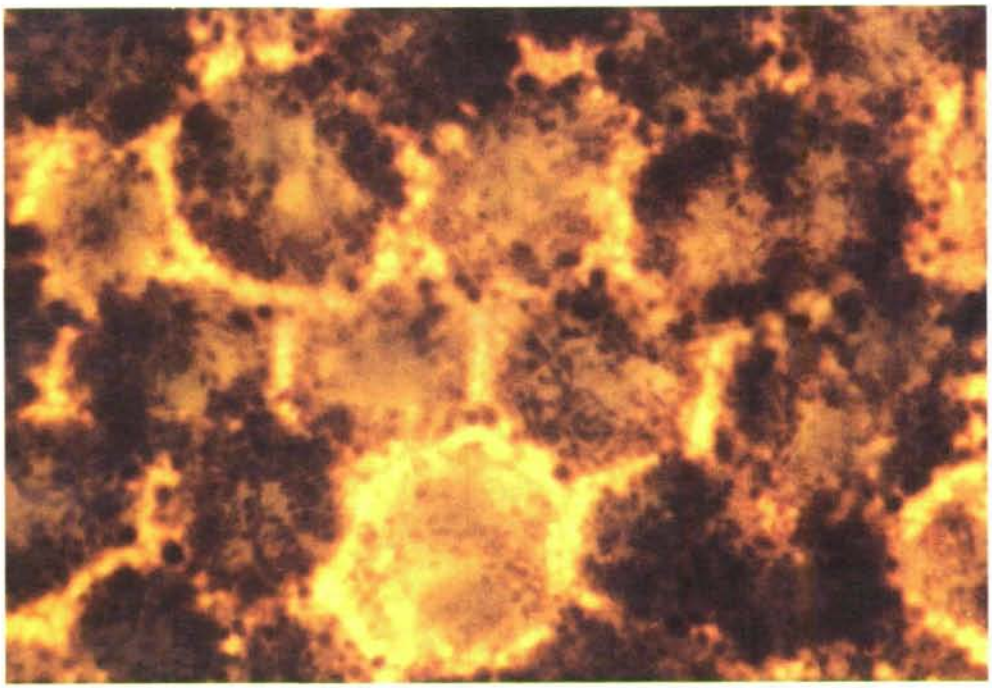

Fig. 1. Fluorescence photomicrograph of RPE of 1-year-old child. Fluorescent particles are seen mainly at the periphery of the cells. Dark elliptical melanin granules are abundant. (×1,200.)

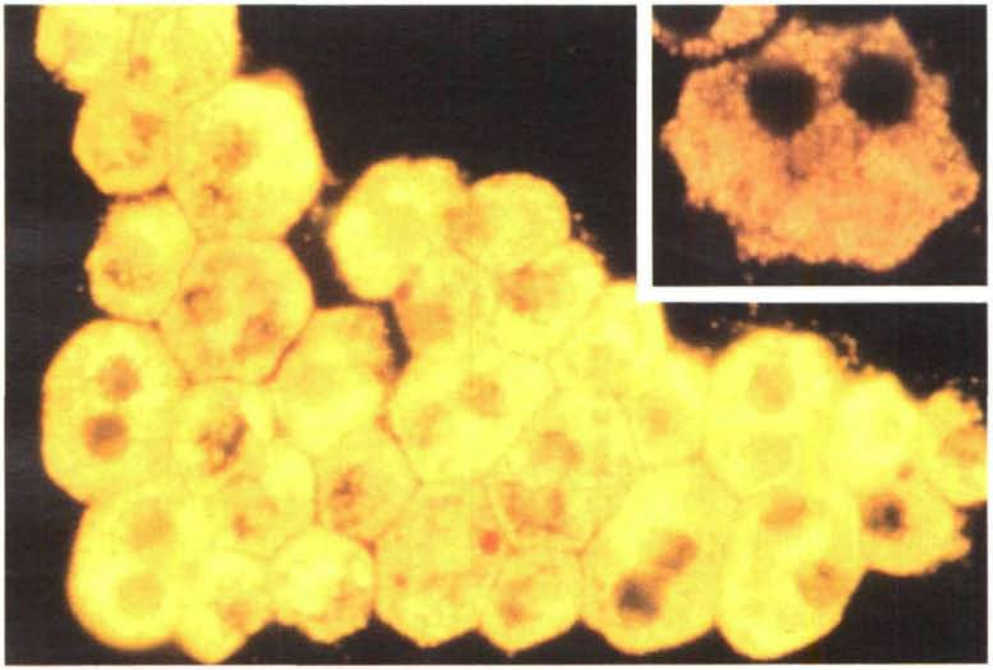

Fig. 2. Fluorescence photomicrograph of RPE of 49-year-old human being. Cells are brilliantly fluorescent owing to numerous fluorescent granules in the cytoplasm. Few melanin granules (dark spots) are seen. Many cells are binucleate. (×980.) Inset: Oil-immersion photomicrograph of cell from same individual. (×1,300.)

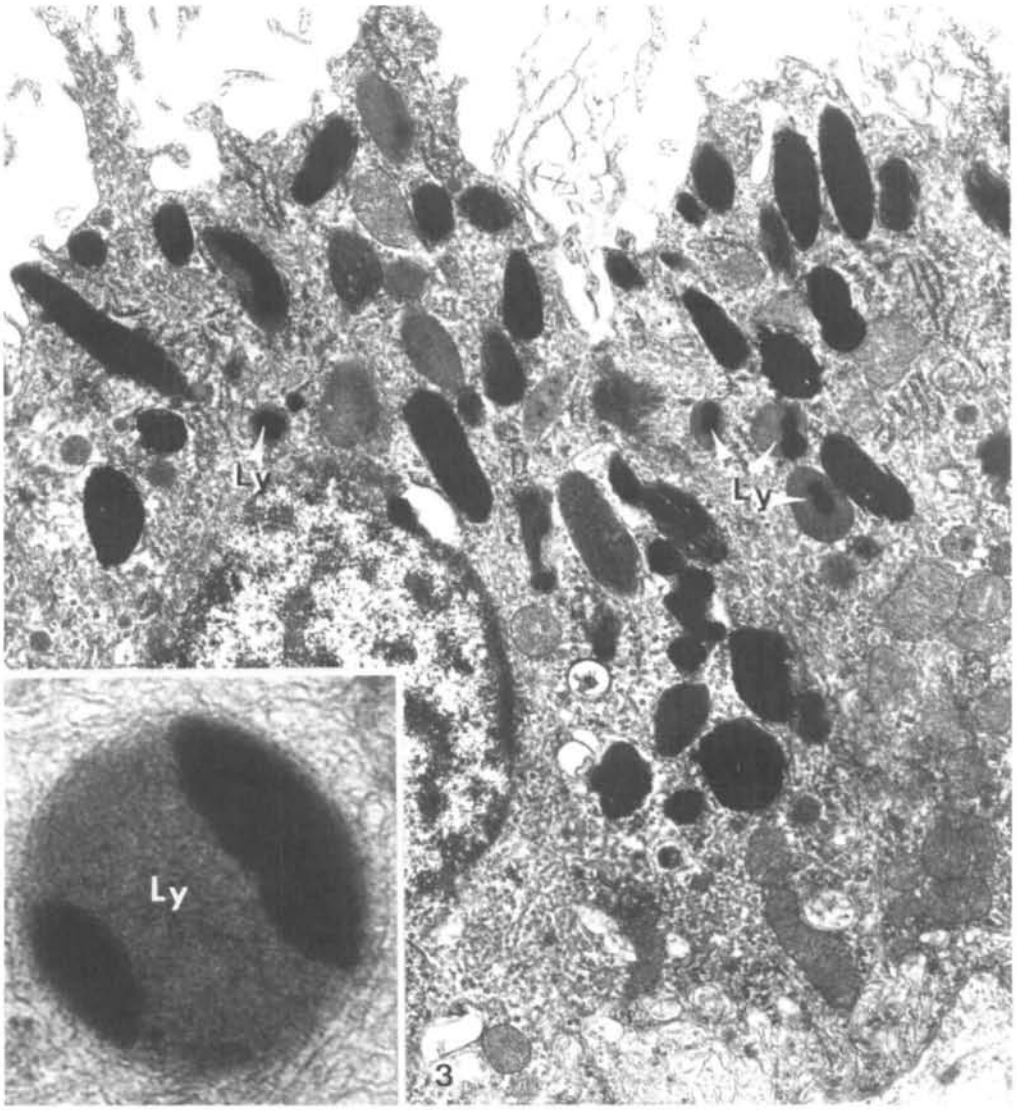

Fig. 3. Electron micrograph of RPE of 1-year-old child shown in Fig. 1. All but five or six of the osmiophilic granules in the cytoplasm are melanosomes in various stages of development. Small secondary lysosomes *(Ly)* containing osmiophilic lipid are seen (arrows); these are the apparent source of the fluorescence seen in cells in Fig. 1. (×11,500.) Inset: Typical secondary lysosome *(Ly)* of young RPE. (×90,000.)

unfixed RPE cells. The identical cells were subsequently fixed and examined ultrastructurally, before and after extraction of lipids, for the precise identification of the fluorescent components. These studies, carried out on eyes spanning nine decades of life, reveal differences in the structure and content of melanin and lipofuscin between childhood and old age. Enzyme cytochemical techniques for demonstration of acid hydrolases reveal a number of complex interrelationships among the lysosomes and lipofuscin and melanin granules of the RPE cytoplasm. These findings indicate that these subcellular

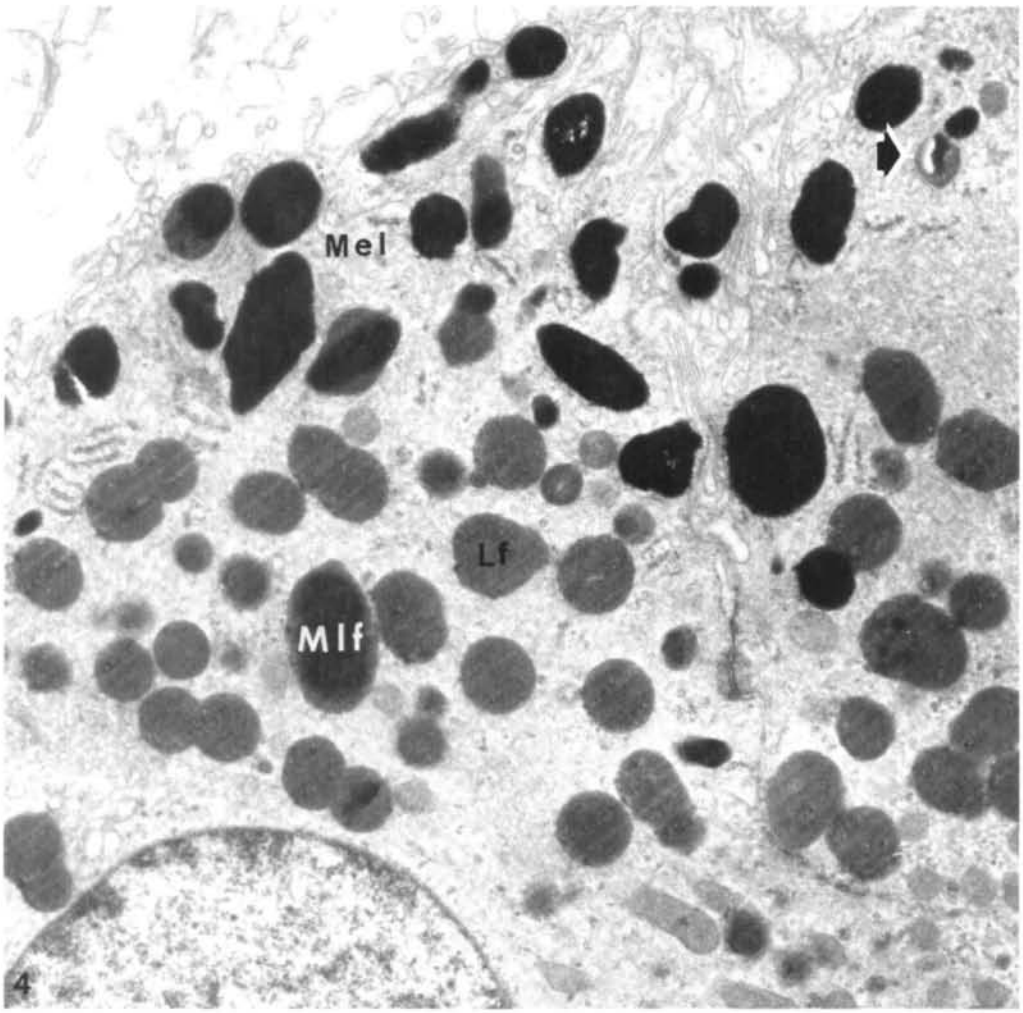

Fig. 4. Electron micrograph of RPE of 49-year-old person shown in Fig. 2. The majority of the osmiophilic granules in the cytoplasm are lipofuscin granules and secondary lysosomes (arrow). *MLF*, Melanolipofuscin; *Lf*, lipofuscin; *Mel*, melanin. (×11,500.)

organelles are in a dynamic state and capable of considerable rearrangement and alteration during the life of the RPE cell.

Methods

Thirty human eyes ranging in age from 7 weeks to 80 years* were obtained through the eye bank or surgical enucleations; no eye was more than 10 hr post-mortem. All specimens were examined by light and electron microscopy. Selected specimens from representative age groups were examined by fluorescence microscopy, and others were used in lipid solubility studies.

The anterior segment, lens, and vitreous were removed. For direct fixation of tissue the eye cup was dissected into posterior, equatorial, and peripheral pieces either before or after removal of the neural retina.

Light and electron microscopy. Tissues were placed in cacodylate-buffered paraformaldehyde-glutaraldehyde fixative for 1 to 2 hr, postfixed in 2% OsO_4 (except for solubility studies, see below), stained en bloc with uranyl acetate, dehydrated in acetone and propylene oxide, and embedded in Epon-Araldite. Thin sections were stained

*Ages: 7 weeks and 1, 8, 12, 21, 24, 30, 30, 31, 37, 37, 40, 47, 49, 51, 52, 55, 56, 57, 60, 62, 63, 66, 67, 68, 69, 71, 75, 75, 77, and 80 years.

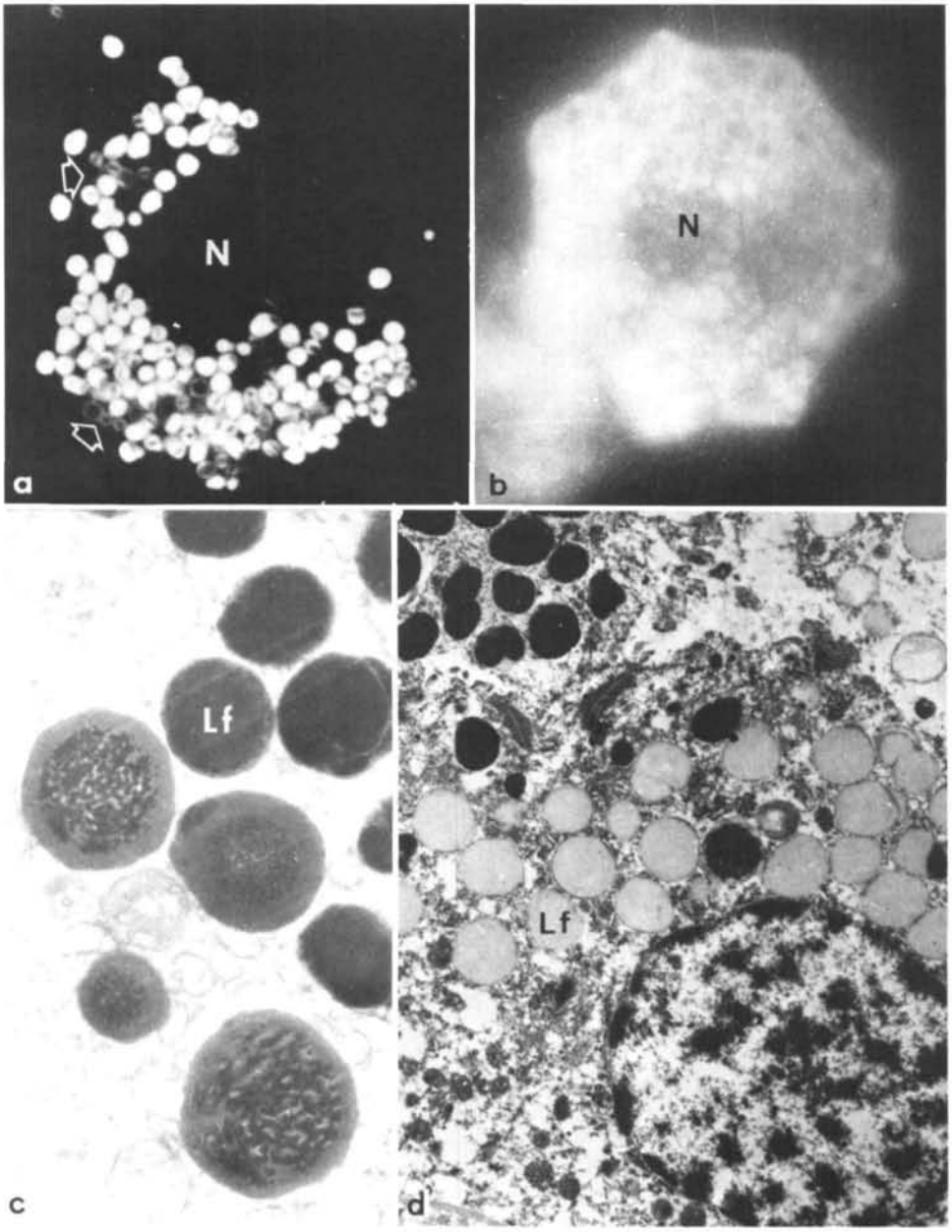

Fig. 5. a, Fluorescence photomicrograph of RPE cell of 46-year-old subject. Two types of fluorescent granules are seen: solid, uniformly sized granules, and granules with fluorescent rims and elliptical or round centers (arrows). *N* = nucleus. (×1,750.) **b,** Fluorescence photomicrograph of binucleate RPE cell after extraction with ethyl ether. The brilliant fluorescent granules seen in a are gone, but some fluorescence remains in the cell. (×1,750.) **c,** Electron micrograph of a portion of the same cell shown in **a.** The solid granules are typical lipofuscin granules *(Lf),* whereas those that had fluorescent rims and nonfluorescent centers are complex granules with a core of melanin. Note that the cortices of the complex granules are less osmiophilic than lipofuscin. (×24,000.) **d,** Electron micrograph of RPE cell extracted with chloroform : methanol prior to osmication and routine processing. Apical melanin granules are dense; basal lipofuscin granules have been extracted by the lipid solvents. (×8,000.)

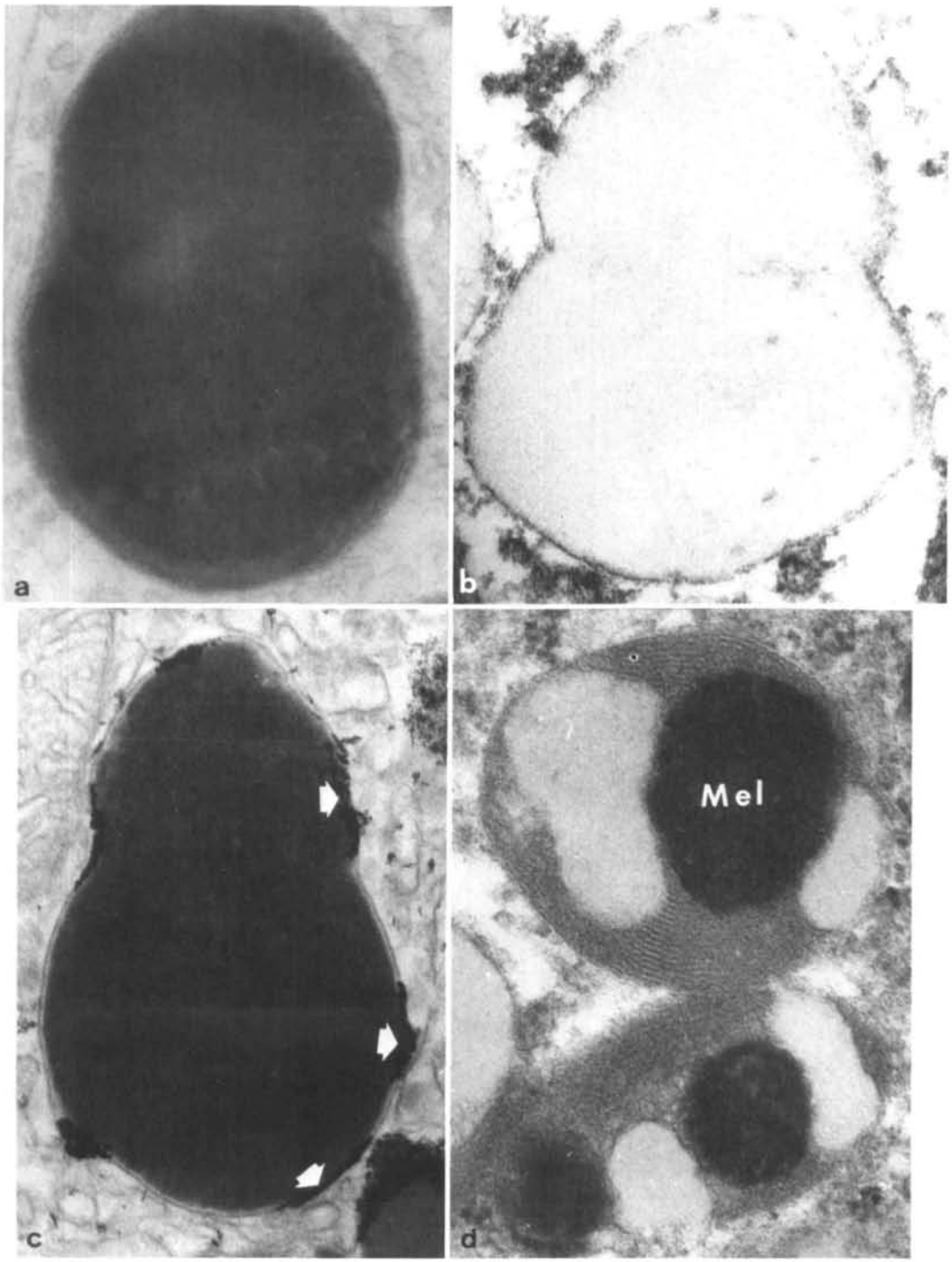

Fig. 6. a, Typical lipofuscin granules of human RPE as it appears with routine fixation (aldehydes and osmium tetroxide). (×70,000.) **b,** Typical lipofuscin granule fixed in aldehydes but without osmium tetroxide fixation. Most of the contents have been extracted by the dehydrating solvents, acetone and propylene. Note that some homogeneous background density remains. (×70,000.) **c,** Typical lipofuscin granule of human RPE (60-year-old), acid phosphatase reaction. Enzyme reaction product at arrows. (×65,000.) **d,** Granules from 12-year-old eye, lipid extracted. Note periodicity in proteinaceous component. *Mel,* Melanin. (×90,000.)

Table I. Effects of lipid solvents on fluorescence and ultrastructure of human retinal pigment epithelium granules

	*Fluorescence**	*Ultrastructure†*		
I. Fresh, unfixed	++++			
acetone/propylene oxide	+			
chloroform:methanol	+			
ether	+			
		Lipofuscin	Melanolipofuscin	Melanolysosome
II. Aldehyde-fixed	++++			
acetone/propylene oxide	++			
chloroform:methanol	++			
ether	++			
ethanol	++			
III. Osmic acid	−			
Aldehyde + osmic acid	−			

*Zeiss UGl excitation filter, 440 nM barrier filter; dark field condenser, 40X objective.
†After dehydration and embedment in epoxy resin; see text for ultrastructural details.

with uranyl and lead salts and examined in a Siemens Elmiskop 101 (Siemens Corp, Iselin, N. J.).

Fluorescence microscopy. RPE cells were scraped from Bruch's membrane with a spatula, dispersed on a microscope slide in a drop of saline or aldehyde fixative, and either air-dried or cover-slipped and sealed with silicone grease. They were examined in a Zeiss Photoscope II with ultraviolet light of an HBO 200 high-pressure mercury bulb, excitation filter UG1 (which has a sharp wavelength peak at 365 nm), a darkfield condenser, and a 440 nm barrier filter. Micrographs were taken with daylight Kodak High-Speed Ektachrome ASA 160 or Tri-X black and white film. Some aldehyde-fixed cells were dried onto collodion-coated slides and photographed first by fluorescence microscopy and then postfixed in osmic acid, dehydrated and embedded in plastic. The cells were subsequently stripped from the slide, thin-sectioned, and photographed by electron microscopy.

Lipid solubility experiments. Slides of RPE cells which had been photographed by fluorescence microscopy were then placed in a Coplin jar with the lipid solvents (acetone/propylene oxide, chloroform:methanol 2:1, ethyl ether, ethanol). The slides were air-dried, and the identical cells were re-examined for changes in fluorescence characteristics. Alternatively, cells were extracted with the lipid solvents in a test tube, and an aliquot was cover-slipped and compared with unextracted cells of the same eye. For ultrastructural evaluation of the effects of lipid solvents, pieces of fresh RPE-choroid were fixed (1) directly in buffered 2% OsO_4 or (2) in paraformaldehyde-glutaraldehyde without postfixation in OsO_4. Tissues were then dehydrated in acetone–propylene oxide and embedded. Other specimens were fixed in the aldehyde fixative, then extracted with either chloroform:methanol, ethyl ether, or ethanol prior to rehydration and postfixation in OsO_4.

Enzyme cytochemistry. Pieces of RPE-choroid from four eyes (ages 52, 60, 67, and 69), were fixed in 1.5% glutaraldehyde for 1 hr, washed, and incubated in substrates for acid phosphatase[18]; or aryl sulfatase[19] for 1 to 2 hr. Controls for acid phosphatase were deletion of substrate or incorporation of NaF or ouabain[18]; for aryl sulfatase, deletion of substrate or incorporation of taurodeoxycholate.[19] Tissues were osmicated and processed for electron microscopy.

Results

Whole adult human RPE cells, whether unfixed, aldehyde-fixed, wet, or dry, showed bright golden-yellow fluorescent granules in their cytoplasm when irradiated with ultraviolet light. Aldehyde-fixed specimens showed sharper detail than unfixed cells, but there was no apparent change in fluorescence. Whole mounts of RPE from young individuals contained few autofluorescent granules compared to older (>50) individuals (Figs. 1 and 2). In younger RPE the fluorescence was seen mainly at the cell periphery, perhaps owing to the thinner layer of melanin-containing cytoplasm here (Fig. 1). By fine focusing of the oil-immersion lens, the granular character of the major fluorescent component was apparent. The nucleus was obscured by dark nonfluorescent cytoplasm. Adult RPE fluoresced brilliantly from all parts of the cell except the nucleus and small dark spots

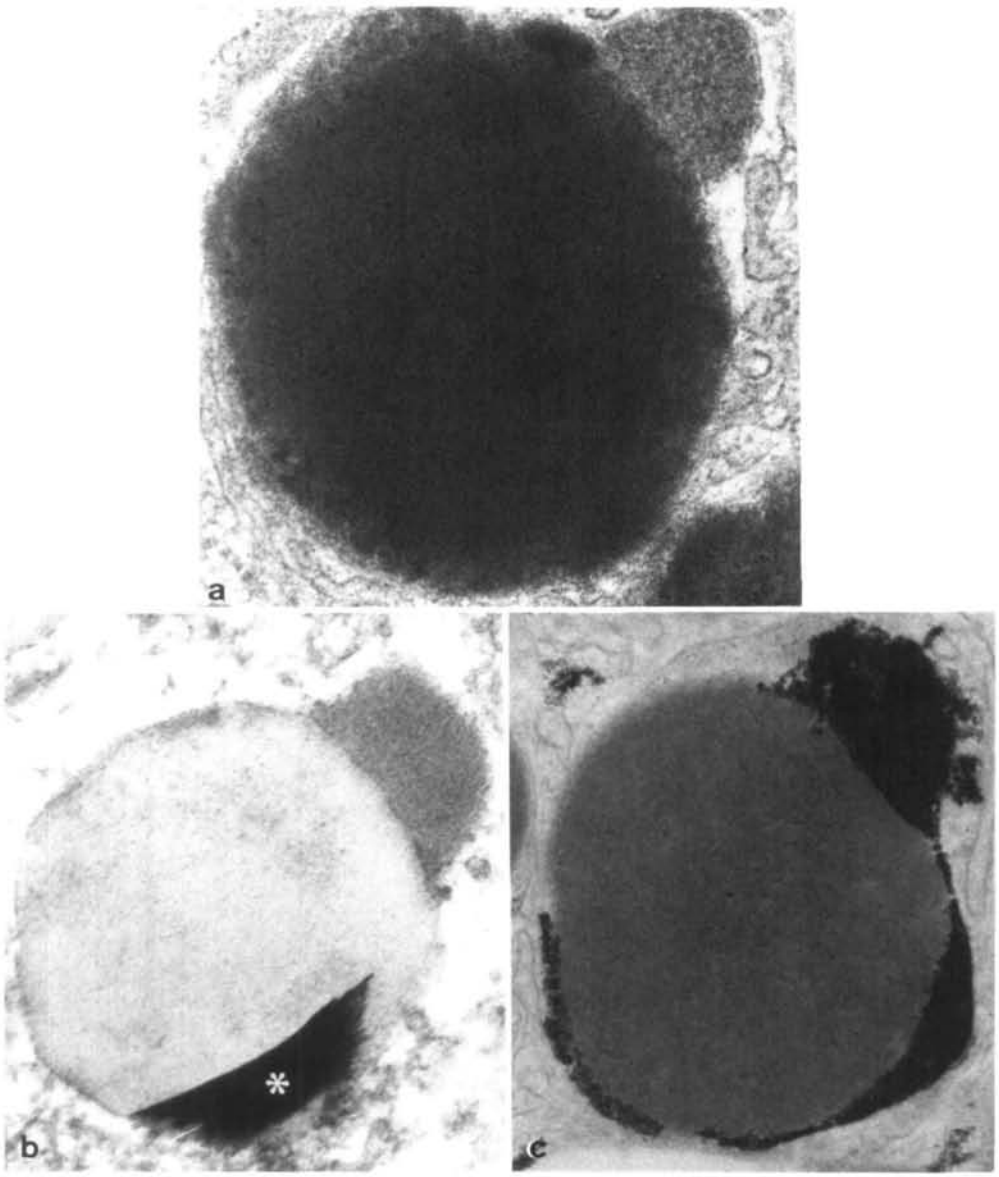

Fig. 7. Lipofuscin granules. **a,** Routine fixation (aldehyde and osmic acid) showing osmiophilic lipofuscin and a bleb of less osmiophilic material at the surface. (×90,000.) **b,** Lipid-extracted lipofuscin granule. The bleb contents are not extracted by lipid solvents. Asterisk indicates artifact of lead staining. (×90,000.) **c,** Acid phosphatase reaction. The lipofuscin granule has two blebs at the surface which contain enzyme reaction product, thereby identifying them as lysosomes. (×90,000.)

(melanin) (Fig. 2). At higher magnification individual fluorescent granules measuring about 1.7 μm in diameter were apparent (Fig. 2, inset; Fig. 5 *a*).

Electron microscopy of the RPE of the 1-year-old eye seen in Fig. 1 showed melanosomes in several stages of development but no typical lipofuscin granules (Fig. 3). A few secondary lysosomes (membrane-limited bodies with heterogeneous contents) containing amorphous lipoidal material were the apparent source of the fluorescence seen by flu-

orescence microscopy. The cytoplasm of the adult RPE seen in Fig. 2, when examined by electron microscopy, showed a predominance of lipofuscin granules (Fig. 4). These membrane-bound granules characteristically lay in the basal half of the cell, measured 1 to 1.2 μm in diameter, and had somewhat undulated or scalloped outlines (Figs. 4 and 6). Their electron density generally differed from that of melanin, being in some cases darker than melanin and in some cases lighter, depending apparently on human variation rather than any alterations in preparatory techniques. The typical lipofuscin granule of old age was homogeneously osmiophilic, although almost every RPE cell of adult eyes contained some lipofuscin granules with heterogeneous contents. The most common inclusion of the lipofuscin granule was a melanosome (Fig. 5, *c* and *d*) (see below); crystalline proteinaceous material and other osmiophilic densities were also seen (Fig. 4, arrow; Fig. 6, *d*).

An RPE cell photographed by fluorescence microscopy (Fig. 5, *a*) and subsequently thin-sectioned for electron microscopy is shown in Fig. 5, *c*. The majority of the fluorescent granules were identified as typical lipofuscin granules by electron microscopy. A second class of granule having a fluorescent rim and a nonfluorescent core (Fig. 5, *a*, arrows) was identified ultrastructurally as melanolysosomes (Fig. 5, *a*).[18, 20] Note that both the fluorescence and the electron density of the rim are less intense than that of lipofuscin. The encased melanosomes in this particular cell were all structurally similar to melanosomes of ontological stage three, i.e., the melanoprotein lamellae are not tightly compacted.

Lipid extraction. In order to determine the effect of lipid extraction of RPE on fluorescence, both fresh and aldehyde-fixed cells from a given eye were examined before and after extraction with various solvents. Twelve eyes, 37 to 80 years of age, were examined. Considerable variation was found from one individual to another in the effect of lipid solvents on fluorescence. The most effective reduction of fluorescence is shown in Fig. 5, *a* and *b*, a 46-year-old aldehyde-fixed RPE extracted for 1 hr with ethyl ether. In this specimen the brilliant granules in the cytoplasm were gone; however, considerable background fluorescence remained, some of which appeared to emanate from granule membranes. Changes in fluorescence following treatment with ethanol and chloroform:methanol were more subtle, but again, variation from one individual to another was apparent. Fluorescent granules of the younger eyes seemed to be more readily extracted than those of the older eyes. Unfixed, solvent-extracted RPE also showed retention of fluorescence, but the morphology of these cells was too poor for photomicrography.

RPE, fixed in aldehyde fixative and then extracted with chloroform:methanol, ethyl ether, or ethanol prior to osmication and processing, showed by electron microscopy loss of variable amounts of osmiophilic material within lipofuscin granules (Fig. 5, *d*). Also, RPE fixed in standard aldehyde fixative, then processed without osmication, showed almost complete absence of electron-dense contents in the lipofuscin granules (Fig. 6, *b*); all that remained was the granule membrane, a fine filamentous network, and an over-all homogeneous background density. These data are summarized in Table I.

Enzyme cytochemistry and lipofuscin granules. Lipofuscin granules frequently had one or more blebs of nonlipid material at the periphery of the granule (Fig. 7, *a* and *b*). The morphology of the bleb resembled a vesicle the size of a primary lysosome (about 0.2 μm) fusing with the lipofuscin granule. Acid phosphatase and aryl sulfatase methods showed heavy deposition of reaction product at the site of the bleb and often as a rim around the granule (Figs. 6, *c*, and 7, *c*). Phagosomes, Golgi cisternae, and secondary lysosomes were also positive, as has been reported by others.[6]

Melanin-containing complex granules. As already noted in our earlier study,[3] melanin is associated with or incorporated in lipofuscin granules. Typical forms of melanolipofuscin granules are shown in Fig. 8; extraction of lipid components allows the melanin

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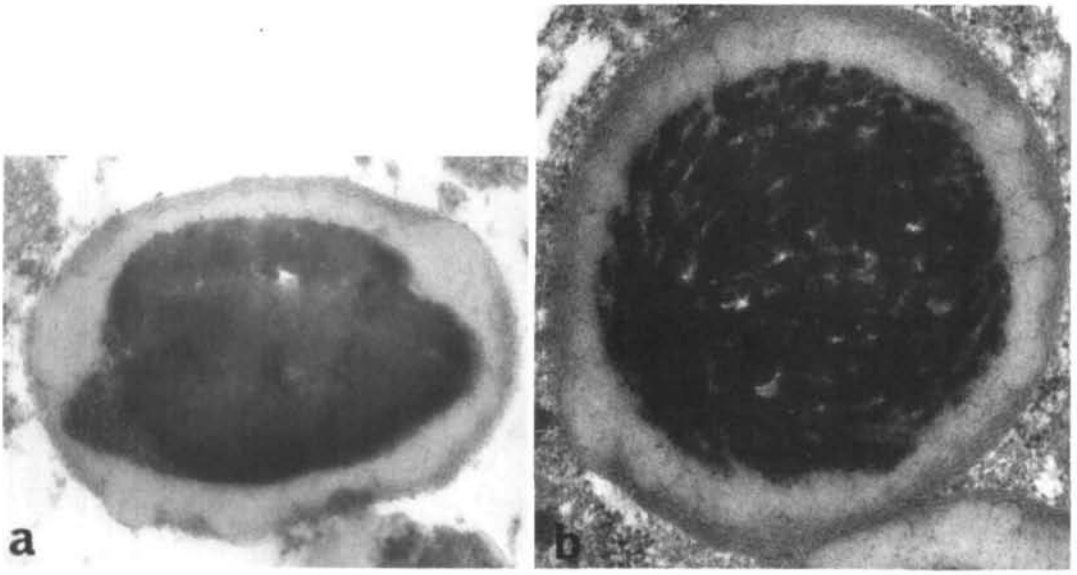

Fig. 8, a-b. Lipid-extracted melanolipofuscin granules of RPE of older individuals (**a** and **b**, 60 years old). **a**, Mature melanin granules within lipofuscin granules. (×35,000.) **b**, Melanin granule showing loose melanoprotein cords. (×35,000.)

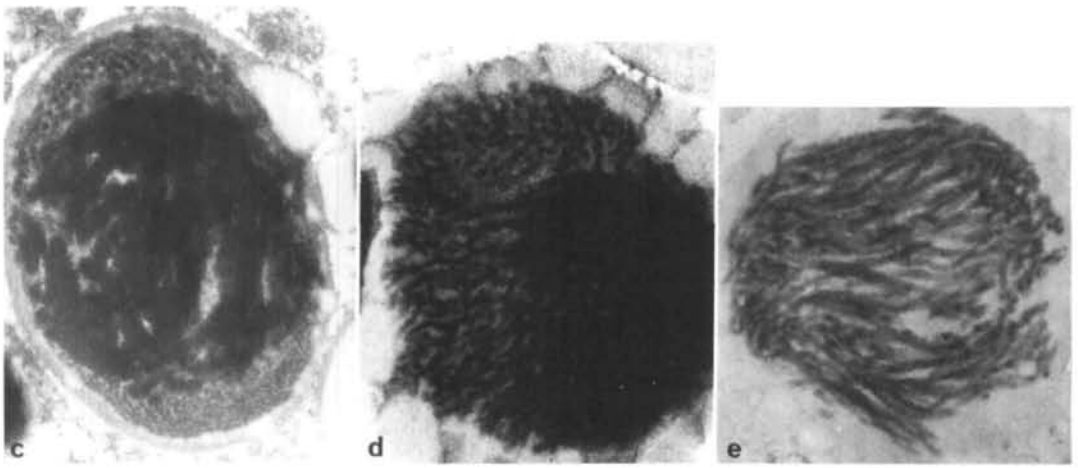

Fig. 8, c-e. See legend to Fig. 8, **a-b.** (**c, d,** and **e,** 56 years old). **c,** Granule showing three degrees of melanization. (×65,000.) **d,** Loose cords of melanoprotein at periphery of the lipofuscin-enclosed melanin granule. (×60,000.) **e,** Splayed cords of melanoprotein. (×45,000.)

to be seen clearly. The lipid-extracted spaces often had a lobular outline, and nonlipid material lined the lobules (Fig. 8, *b*, and d, arrows). The melanin enclosed in these granules showed (Fig. 8, *c*) various degrees of compaction of the layers of melanopolymer.

Other melanin-containing granules in the RPE cytoplasm resembled these melanolipofuscin granules, but the cortical material was not extracted by lipid solvents (Fig. 9, *a*). The cortex of these granules appeared to be protein in nature (see Table I), and the possibility that it constitutes lysosomal enzyme was investigated by means of cytochemical meth-

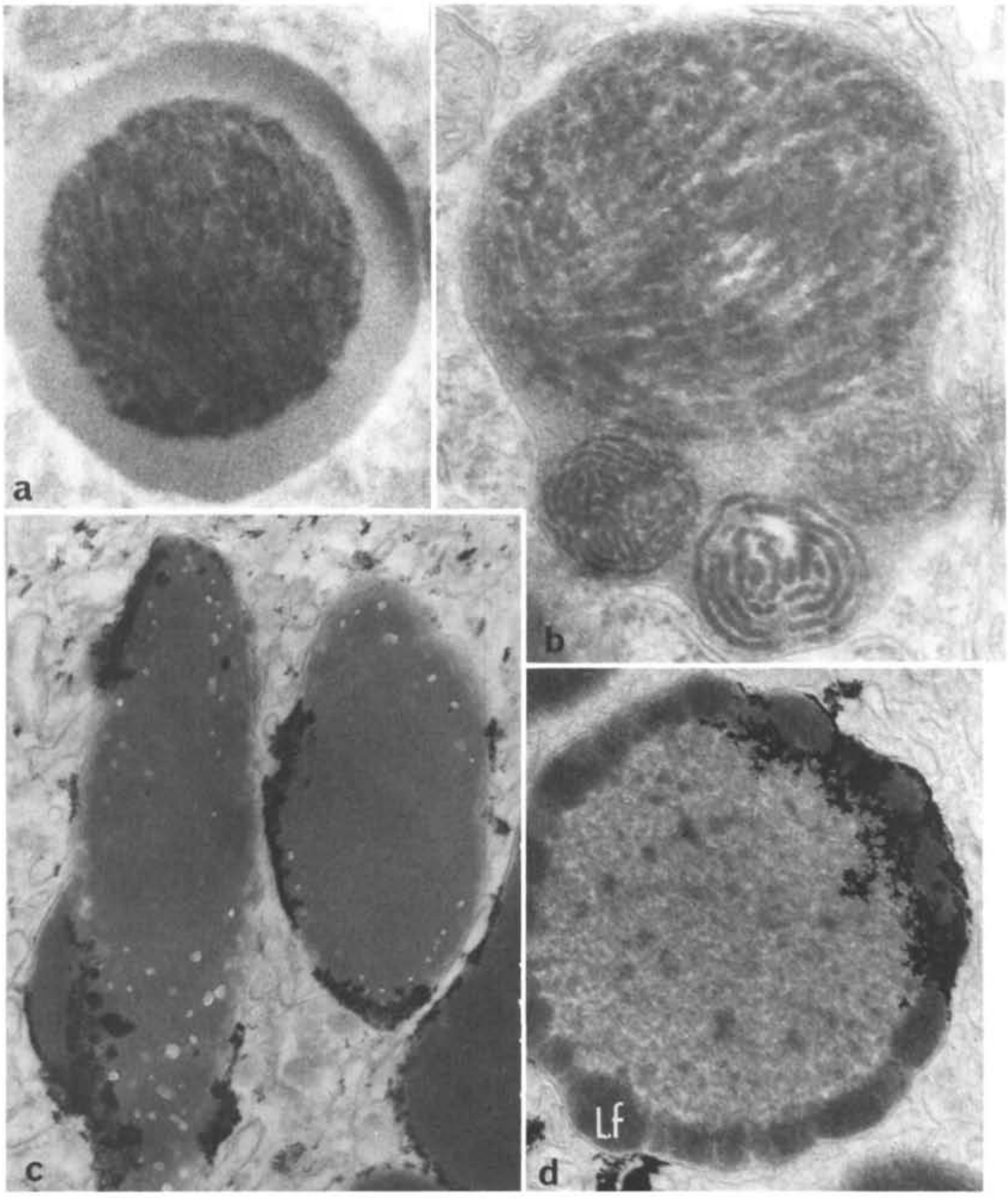

Fig. 9. Melanolysosomes of human RPE (<60 years old). **a,** Chloroform:methanol–extracted specimen showing the nonlipid nature of the cortical material. (×37,000.) **b,** Four melanin granules enclosed within a lysosome-like matrix. Note the various degrees of melanin compaction. (×50,000.) **c,** Acid phosphatase reaction at periphery of mature melanin granule. Note the bleb of reactive material and compare with Fig. 7. (×55,000.) **d,** Positive aryl sulfatase reaction in part of the cortical layer. *Lf,* Lipofuscin. (×60,000.)

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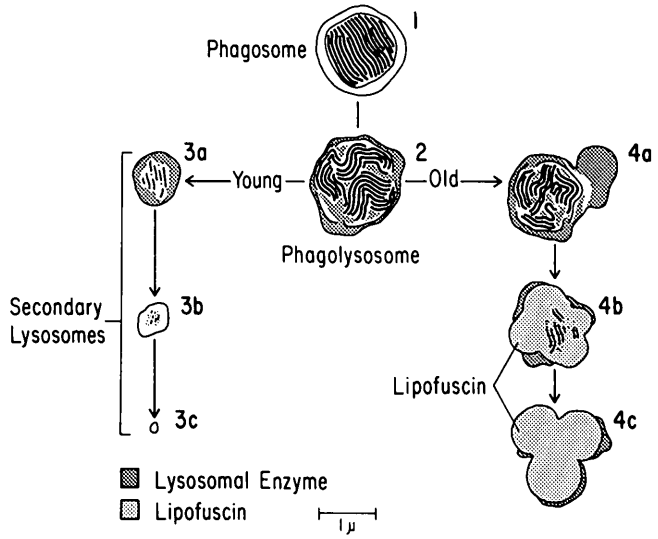

Fig. 10. Sequence of formation of fluorescent granules in human RPE. *1*, Phagocytized outer segment disks; fluorescent properties unknown. *2*, Phagolysosome or large secondary lysosome; fluorescence seems to begin here. *3a* to *3c*, Sequential diminution in size of fluorescent secondary lysosomes in young eyes. *4*, Sequence in old eyes (all fluorescent): *4a*, fusion of phagolysosomes with pre-existing lipofuscin; *4b*, fusion of primary lysosomes with lipofuscin; *4c*, fusion of multiple lipofuscin granules and continued fusion of primary lysosomes.

ods (see below). Multiple granules enclosed in membrane-bound structures resembling autophagic vacuoles were occasionally seen (Fig. 9, *b*).

Enzyme cytochemistry of complex granules. Many, but not all, of the complex granules showed enzyme activity. Reaction product may lie in all or a portion of the cortical material (Fig. 9, *c* and *d*). The melanin granules enclosed by the proteinaceous material may be in a variety of forms, from mature solid granules (Fig. 9, *c*) to more "immature" forms (Fig. 9, *d*). Mature granules often showed reaction product around them, with little or no evidence of a layer of cortical protein.

Age-related changes in melanin and lipofuscin. During dissection of the human eyes the RPE of many older individuals showed a distinctly golden color when viewed in situ with reflected light, in contrast to that of younger eyes where a brown color was apparent. This held true regardless of iris color. These observations suggested that in addition to the accumulation of golden lipofuscin, there might also be a concomitant gradual loss of brown melanin with age; this is a logical expectation given the demonstration of numerous melanin granules in the lysosomal compartment of the RPE cells. Therefore a rough estimate of the relative quantities of melanin and lipofuscin was made by tabulation of granules in 10 electron micrographs of RPE from each of the 26 specimens. RPE from comparable areas of retina could not be evaluated, however. These counts showed that typical lipofuscin granules were not present in the three individuals under 10 years of age, but between 12 and 40 years (nine individuals) there was a sharp increase, after which the numbers remained fairly stable (13 individuals). Melanin content did not show a significant change with age in this population sample.

Discussion

Fluorescent granules in human RPE are identical ultrastructurally with lipofuscin granules and other heterogenous secondary lysosomes presumed to be intermediates in lipo-

fuscin formation. In young eyes, the few small fluorescent granules are identified in the electron microscope as secondary lysosomes; there are no typical lipofuscin granules in these cells. With increasing age the quantity of fluorescent granules increases. This appears to occur through progressive accumulation of osmiophilic lipid complexes within secondary lysosomes (Figs. 3 and 4). By the fifth decade of life RPE cells are strikingly fluorescent, and by electron microscopy lipofuscin granules predominate in the cytoplasm. The progressive accumulation of sudanophilic granules in the RPE of human eyes was shown by Streeten[1]; the present study confirms and extends her findings. The postulated cellular mechanism for this cumulative process is shown in Fig. 10.

The specificity of the fluorescence demonstrated in this study deserves comment, because of the overlapping fluorescence spectra of lipofuscin and vitamin A and the known involvement of RPE cells in vitamin A metabolism. The maximal excitation and emission peaks of lipofuscin (365 and 440 nm)[21] are well separated from those of vitamin A (328 and 500 nm).[22] Moreover, the crown glass lenses of the microscope absorb radiation below 330 nm,[23] and most of the radiation transmitted by the UG1 filter is between 340 and 370 nm; thus it seems unlikely that sites of vitamin A storage contribute to the brilliant fluorescence seen here. Furthermore, preparations of fresh dark-adapted bovine ROS, containing vitamin A (retinaldehyde), show only weak transient yellow fluorescence (<30 sec) under these illumination conditions, whereas slides of RPE cells retain granule fluorescence for months, if not years. The possibility that retinoyl-cholesterol complexes contribute to the fluorescence seen in fresh or aldehyde-fixed human RPE is not ruled out, however (see Nelson and Halley[24] for discussion of this controversy).

Lipid extraction studies. A certain fraction of the autofluorescence of the RPE is extractable by ethyl ether, ethanol, chloroform: methanol, and acetone/propylene oxide (Table I, top). Electron microscopy indicates that these lipid solvents remove intensely osmiophilic components of the lipofuscin granules. Since osmium reacts mainly with unsaturated lipids,[25] it appears that some extractable unsaturated lipids contribute to the autofluorescence of the RPE cells in general and the lipofuscin granules in particular. Despite the "empty" appearance of most of the granules in the electron microscope, a significant amount of fluorescence remained in the RPE cells after solvent extraction. This fluorescence could emanate from insoluble peroxidized phospholipid[13] or various aldehyde cross-linked macromolecules[12] *within* the lipofuscin granules; alternatively, it could emanate from similar compounds in the limiting membrane of each granule as well as from other cytoplasmic membranes (Fig. 5, *b*). Some of the residual material in solvent-extracted lipofuscin granules appears to be lysosomal enzyme (Figs. 5, *d*, 7, *a*, *b*, and *c*). It is unclear at this time if lysosomes, per se,

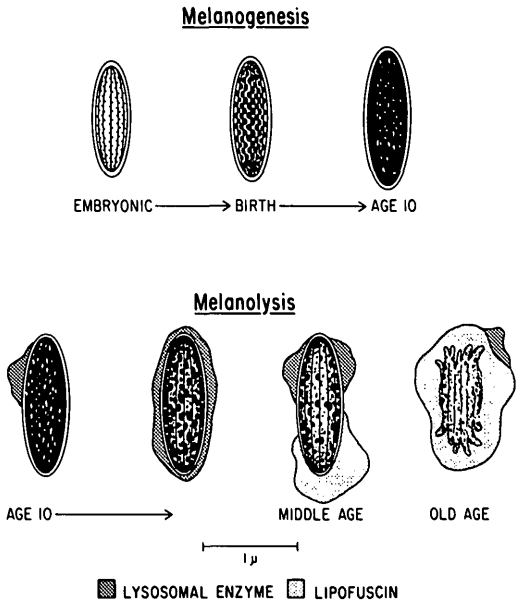

Fig. 11. Formation of melanin (above) and the postulated dissolution of melanin (below). Primary lysosomes fuse with fully melanized granule, forming a melanolysosome. As dissolution begins, lipofuscin granules also fuse, forming a melanolipofuscin granule with a fluorescent rim.

are autofluorescent in the unfixed condition or if fixation induces fluorescence by cross-linking certain macromolecules.[12] The stage at which fluorescence develops in the phagolysosome (Fig. 11) is under investigation.

The preliminary finding that fluorescence was more readily extracted from lipofuscin granules of 40- to 50-year-old eyes than from 70- to 80-year-old RPE suggests that the chemical composition of these granules is different in the older population. This individual variation suggests that there also may be differences in the degradative processes occurring in phagolysosomes of different individuals. Alternatively, there could be differences in the lipid composition of the phagocytized photoreceptor membranes. The lipids of lipofuscin granules originate mainly from the phospholipids of photoreceptor outer segment disks (Fig. 10), which are rich in polyunsaturated fatty acids.[20, 26] Chemical analysis has shown that the lipids of bovine RPE differ from those of ROS[27]; human RPE lipids have not been analyzed, to my knowledge. Since bovine and human RPE differ greatly in their content of lipofuscin when examined by fluorescence microscopy (personal observation) or by electron microscopy,[20] the lipid composition of lipofuscin-laden human RPE may be expected to differ considerably from that of bovine RPE. Finally, this observed differential extractibility of fluorescence with age, if it is verified in a larger sample, should serve as a caution to investigators using lipid solvents in the preparation of tissue for fluorescence quantitation of lipofuscin in individual eyes.[28]

Correlation of lipofuscin with RPE fluorescence was noted by Kolb and Gouras[29] in a case of retinitis pigmentosa. Fluorescence was present only in RPE of the macular region, where outer segments were presumably still being synthesized, detached, and phagocytized. Although the authors believed that the quantity of lipofuscin in the RPE was abnormally high in their 68-year-old patient compared to their three control subjects 60 years of age or older, the present study indicates that many apparently normal older individuals have RPE similarly engorged with lipofuscin and melanolipofuscin.

Enzyme cytochemistry. Small autofluorescent granules (secondary lysosomes) of young eyes contain extractable lipid and proteinaceous material (Fig. 6, *b*). Part of the proteinaceous material is lysosomal enzyme, as shown by the acid phosphatase and aryl sulfatase reactions, and presumably some is undigested opsin. The process of degradation of outer segment disks undoubtedly involves many complex processes, and the time required to accomplish complete digestion is not known. In the present study, it was apparent that by the third decade of life secondary lysosomes are fairly lipid rich and by the sixth decade granules filled entirely with lipid (lipofuscin) predominate. Observations on hundreds of granules suggest that primary lysosomes continue to bring lysosomal enzymes to lipofuscin granules (Fig. 7), i.e., the involvement of primary lysosomes is not confined to the initial phagosome-lysosome interaction (Fig. 10). Moreover, fusions between large secondary lysosomes and lipofuscin granules also occur. Thus the lipofuscin compartment, despite its residual body designation, is a dynamic intracellular compartment capable of many different interactions with other organelles in the RPE cytoplasm.

Significance of melanolysosomes and melanolipofuscin. The biology of melanin has been studied extensively in melanocytes of skin, where it is known that melanosomes are synthesized continually in one cell, the melanocyte, but are injected into or phagocytized by another cell, the keratinocyte.[14] Enzyme cytochemical studies show lysosomal enzymes associated with the melanosomes of the keratinocyte,[30] as might be expected since these melanosomes are exogenous phagocytized structures sequestered in the phagolysosomal system of the keratinocyte. Degradation of the melanin may or may not occur, but it is somewhat irrelevant since the keratinocyte sloughs from the surface anyway.

The functional significance of lysosomal enzymes within the melanosomes of adult human RPE is unknown. Large numbers of melanin granules are incorporated into the residua of the phagolysosomal system, there-

by forming melanolipofuscin granules; other granules have a cuff of lysosomal enzyme, the melanolysosomes. From these data it seems reasonable to postulate that melanin of the RPE is undergoing autophagic remodeling, if not actual degradation, during the human life-span. The postulated sequence is shown in Fig. 11. Melanin polymer, although highly insoluble, is linked to protein(s) which is amenable to enzymatic digestion.[31] Disassembly of melanin via dissolution of the protein matrix of the melanosome is suggested by profiles of melanin granules seen in Figs. 8 and 9.

Cursory analysis of the number of melanin granules in RPE cells of these eyes does not confirm or deny the postulated loss of melanin with aging. This is not surprising, given the small sample size and the variation in genetic makeup of the 30 individuals. Numerous genes affect the structure of melanin,[3, 16, 17] and it seems likely that the number of melanin granules in RPE is also under genetic control. The question of the possible loss of this pigment during the human life-span deserves further investigation because melanin plays a key role in protecting cells from light-generated free radicals (see discussion in Feeney and Berman[32]). Loss of melanin or change in the quality of melanin could lead to other so-called senile changes in the RPE.

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