Pathology of the Pigment Epithelium and Retina in Rabbits Poisoned with Lead

W. Franklin Hughes, PhD and P. S. Coogan, MD

Multifocal lesions of the retinal pigment epithelium were observed in rabbits fed a diet contaning 0.5% lead subacetate for periods of up to 2 years. Groups of pigment epithelial cells became congested with a lipofuscin pigment which was apparently derived from phagosomes of rod outer segments. Lipofuscin granules displaced melanin granules from the apical surface of the retinal pigment epithelial cells and resulted in conspicuous brown pigmentation of these cells in albino animals. Migration of macrophages and pigment epithelial cells into the subretinal space was common in affected areas. This pathology was not observed in the pigment epithelium of the ora serrata, or ciliary body. At the latest time periods, the abnormal lipofuscin pigmentation subsided, and degeneration of photoreceptors occurred. The pathogenesis of the lesions is discussed (Am J Pathol 77:237–254, 1974).

THE RETINAL PIGMENT EPITHELIUM has several well-known roles related to the structure and function of the outer retina. It serves as an absorptive surface which reduces light scatter, it constitutes a blood-retinal barrier, and it contributes to the maintenance of the photoreceptor. The pigment epithelium supplies metabolites of vitamin A and fatty acid components utilized in the turnover of photoreceptor outer segments.[1,2] As rods continue to synthesize new discs at the base of the outer segment, the oldest discs detach from the tip and are phagocytized and degraded by the pigment epithelium.[3–5] Damage to the closely integrated functions of the pigment epithelium–photoreceptor complex can result from genetic defects,[6–8] nutritional deficiency[9,10] or light exposure,[11–13] as well as agents with selective toxicity for the retina and pigment epithelium.[14]

A lesion of the retinal pigment epithelium characterized by swelling of the cells with an abnormal pigment was reported by Hass *et al*[15] in lead poisoned rabbits. Closer scrutiny of this lesion seemed warranted in light of the metabolic interrelationships of the pigment epithelium

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and photoreceptor. This descriptive study provides information about the nature and source of the abnormal pigment. The long-term progress of the pigment damage was evaluated with special attention to possible effects on the photoreceptor cell and the retina.

Materials and Methods

Eleven albino (New Zealand) and 3 pigmented rabbits were fed a standard pellet diet containing 0.5% lead subacetate. These animals were sacrificed at intervals ranging from 3 months to 26 months. Ten albino rabbits which were fed the standard diet without lead served as controls. Four of these were sacrificed at 6-month intervals during the same 26-month period as the experimental rabbits, while the remaining six were sacrificed at 2 to 4 years to evaluate any age-related changes. Eyes were immersed in 1% paraformaldehyde-2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) after removal of the anterior segment, or the animals were fixed by intravascular perfusion with the same fixative. Samples of the eyes were taken for: a) flat mounts of the pigment epithelium, b) light microscopic examination and special stains, and c) electron microscopy.

Paraffin sections were stained by several methods to determine the nature of the abnormal pigment. These stains included: hematoxylin and eosin, periodic acid–Schiff (PAS) methylene blue, Ziehl-Neelsen, Schmorl's ferricyanide, Fontana-Masson, and the method of Nassar *et al* [16] for lipofuscin. Unstained paraffin sections were cleared, mounted in Permount, and examined for autofluorescence with an ultraviolet microscope.

Some samples were prepared for electron microscopy. The aldehyde-fixed material was postfixed in 1% osmium tetroxide in the 0.1 M cacodylate (pH 7.4), stained *en bloc* with uranyl acetate, dehydrated in acetone, and embedded in Epon. One-micron sections were cut from these blocks and were stained with toluidine blue-azure II for light microscopy. Thin sections were stained with lead citrate and viewed with a Philips 300 microscope.

Results

Pigment Epithelial Changes

Focal lesions began to appear in the pigment epithelium of both pigmented and albino rabbits which had been on the lead diet for several months. Most of the observations were made on albino animals where the abnormal pigmentation was better visualized in the absence of melanin. In these animals, the lesions appeared as light brown specks in the eyecup once the retina had been stripped away. Flat preparations of the pigment epithelium showed that the lesions are comprised of groups of cells laden with cytoplasmic granules that stain intensely with methylene blue (Figures 1 and 2). In sections, pigment epithelial cells contained variable numbers of abnormal granules, from a few located mainly at the apical surface to large numbers that completely filled the cell causing it to bulge conspicuously among the outer seg-

ments (Figure 3). This abnormal pigmentation was not observed in the pigment epithelium of either the ora serrata or the ciliary body.

The staining properties of the granules indicated that they contained lipofuscin pigment.[17] In addition to staining with methylene blue, they were PAS and ferricyanide positive, acid fast, argyrophilic (Figure 4) and exhibited strong yellow-green autofluorescence under ultraviolet light (approximately 360 mμ). As seen with the electron microscope, the cytoplasmic granules are membrane bound, with contents suggesting more or less advanced stages in the degradation of outer segment discs. These contents included the homogeneous osmiophilic material seen most commonly, myelin-figures, membranous debris, and identifiable rod discs (Figure 5A–C). The latter rod phagosomes were located predominantly in the apical portions of the affected cells and probably accounted for the more intense staining of superficial granules with methylene blue or PAS. The homogeneous granules were of variable size, and in many cases coalesced to form large aggregates of pigment (Figure 5D). In pigmented animals the lipofuscin granules displaced melanin granules from their usual apical position (Figure 6).

Nuclei and mitochondria appeared normal in cells which contained enormous quantities of the lipochrome pigment. Affected cells did, however, show abnormalities of both the apical and the basal cell surfaces. Apical microvilli disappeared (Figure 7) or were displaced to the lateral cell borders, disrupting the regular pattern of microvillous interdigitations with photoreceptor outer segments seen in normal animals. The basal infoldings adjacent to Bruch's membrane also were absent in certain areas where the plasmalemma lay flattened along its basal lamina (Figure 8). These changes in surface morphology reduce the surface:volume ratio of the enlarged cells and suggest functional modification of the cell surface. The lack of apical microvilli did appear to have adverse effects on adhesion of the retina to the pigment epithelium since leaded animals showed a marked tendency for artifactual detachment during histologic preparations.

Prominent lesions were further accented by the accumulation of pigment in adjacent pigment epithelial cells and in cells which migrated onto the vitread surface of the pigment epithelium (Figure 9). Some of these lipofuscin-laden cells on the surface appeared to be macrophages (Figure 10); however, certain of these cells contained an abundance of smooth endoplasmic reticulum, melanin granules in pigmented animals, abundant microvilli, and desmosomal connections with underlying pigment epithelial cells which suggested that they were pigment epithelial cells that had detached from Bruch's membrane (Figure 11).

In spite of potential holes produced by detachment or death of pigment epithelial cells, the epithelium seemed to remain intact. Remnants of degenerating pigment epithelial cells could be found occasionally in Bruch's membrane or the stroma of the adjacent choroid (Figure 12). Defects in epithelial continuity were not conspicuous in our sampling for electron microscopy and were evidently repaired by the migration of adjacent pigment epithelial cells over or under degenerating cells. The absence of basal infoldings noted above may indicate areas where pigment epithelial cells have reduced adhesion to Bruch's membrane, and where the cell border is stretched out by such movement.

The cells most swollen with granules progressed to a hydropic degeneration characterized by the appearance of large membrane-limited vacuoles, autolysis, and extrusion of cellular contents into the subretinal space (Figure 13). Large (5 μ) mineralized deposits were occasionally observed in vacuoles.

Photoreceptor Degeneration

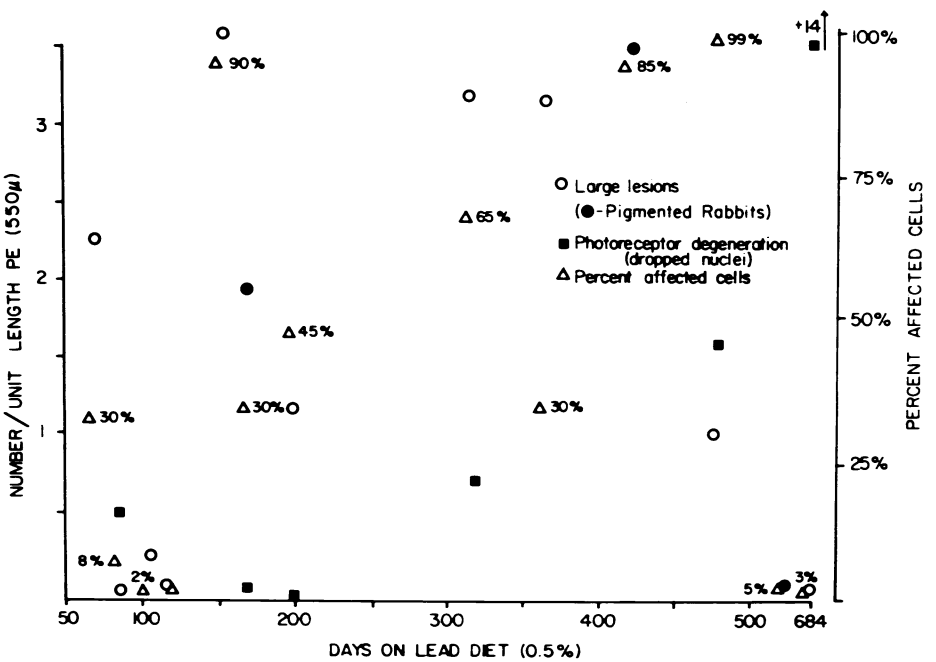
In most of the animals there was no overt degeneration of photoreceptors. Disorientation of outer segments and disruption of disc membranes was apparent over the largest pigment epithelial lesions along with displacement and sometimes a loss of nuclei from the outer nuclear layer (Figures 3 and 13). In the oldest animals, however, degeneration of photoreceptors was observed that was not necessarily associated with pigment epithelial lesions. These degenerating cells were characterized by nuclear pyknosis and increased cytoplasmic density throughout the inner segments, soma and synaptic spherules (Figures 14–16). Sometimes the degeneration was associated with the migration of the nucleus into the inner segment. This was particularly prominent in the oldest animals which also showed reduction in the thickness of the outer nuclear layer (Figure 17). Although fragmentation and disorganization of outer segment discs could be observed in degenerate photoreceptors, the outer limbs remained remarkably intact at apparently late stages of degeneration. The agonal changes, therefore, probably occur rapidly in relation to outer segment turnover.

The severity of pigment epithelial changes increased after several months, so that by about 1 year, most pigment epithelial cells contained some lipofuscin and the greatest number of swollen, multicellular foci were apparent. A surprising contrast was found in 2 animals sacrificed after 2 years in an effort to extrapolate the long-term effects of pigment epithelial damage. The pigment epithelium in these rabbits was of normal appearance except for large aggregated granules in a few cells,

but there was extensive photoreceptor degeneration. Those animals on normal diet sampled at various ages over the 2-year period or between 2 to 4 years of age did not show these pathologic effects. The results for animals on the lead regimen are summarized in Text-figure 1.

Discussion

Affected pigment epithelial cells accumulate granules which contain, according to their staining properties,[17] a lipofuscin pigment similar to that found in normal human pigment epithelium.[18] The following observations suggest that this pigment is derived from incomplete lysosomal degradation of rod discs shed from the overlying photoreceptors: a) The pigment epithelium of the ora serrata and the ciliary body which are continuous with the retinal pigment epithelium have no overlying photoreceptors and did not show the abnormal pigmentation. b) Apical distribution of lipofuscin granules is observed in moderately affected cells, and in those more severely swollen with pigment an unusual abundance of rod phagosomes are present at the apical surface. In



TEXT-FIG 1—Points recorded for each rabbit show numbers of severely congested cells per unit length (*circles*), the approximate percentage of cells showing lipofuscin pigmentation (*triangles*) and photoreceptor degeneration per unit length in instances where it was conspicuous (*squares*).

pigmented animals the melanin granules are dispersed from their normal apical location because of accumulation of the abnormal material. c) Granules contain partially degraded membranous contents so that one can visualize a likely transformation of outer segment disc membranes within these heterophagosomes to the homogeneous lipochrome pigment. d) In instances where the pigment epithelium has cells on its surface, those cells adjacent to the photoreceptor are commonly most swollen with pigment. Either unaffected cells have migrated beneath affected ones, or underlying cells are shielded from accumulating more debris from outer segments by the overlying cells which assume the phagocytic load. e) Finally, in several animals whose retinas were detached [19] prior to putting them on the lead diet, few large lesions developed in the pigment epithelium beneath detached areas.

The first appearance of isolated pigment epithelial cells bulging with pigment indicated that certain cells in the pigment epithelial population are especially vulnerable. With time, the eyes become more severely affected, showing mild pigmentation in the majority of cells and groups of heavily pigmented cells in the largest lesions. These clusters of abnormally pigmented cells suggest that congested pigment epithelial cells have a limited capacity to phagocytize additional material and that adjacent cells and macrophages must assume the phagocytic overload in these foci. Since many severely swollen cells appear to be degenerating, the eventual outcome is probably cell death, and migration of adjacent pigment epithelial cells would be necessary to maintain the integrity of the membrane. Such reparative responses are observed after pigment epithelial injury.[20–22] Replacement of degenerating cells by pigment epithelial cells which are less susceptible could explain the decrease in numbers of lesions in animals poisoned for the longest period of time. Such an adaptive phenomenon is observed in renal tubular epithelium during lead poisoning where there is regression of an initially progressive pathology that involves pigment granulation, intranuclear inclusions, and giant cells.[15] In spite of extensive lesions of pigment epithelial cells in our animals, it was remarkable during long periods of lead ingestion that processes of repair or adaptation enabled these animals to maintain an apparently normal electroretinogram (ERG) and electrooculogram (EOG).[23] This lack of obvious functional defect might be accounted for by the persistence of normal photoreceptors and the integrity of the pigment epithelial membrane.

In rabbits poisoned with lead, macrophages or pigment epithelial cells moved readily into the subretinal space to remove debris from congested, degenerating pigment epithelial cells and retinal separation

from the pigment epithelium did not occur. Excrescences extruded via Bruch's membrane did not accumulate to form significant subepithelial deposits. This efficient mobilization of degenerating material is in distinction to other pathologic conditions. In inherited retinal dystrophies in rats, rod outer segment debris is not phagocytized by the pigment epithelium (or macrophages), and accumulates in the subretinal space to result in retinal detachment and degeneration.[7] In certain older human eyes, Bruch's membrane evidently constitutes a barrier to removal by the subepithelial route of cell and lytic products which accumulate as drusen.[24]

The mechanisms by which lead intoxication produces pigmented epithelial lesions and photoreceptor degeneration can only be a matter for speculation at this point. Hass *et al* [15] originally suggested that lead might inhibit zinc-dependent enzyme systems. The high chorioretinal zinc content of the rabbit as well as other species [25] could predispose this site to such an inhibitory action of lead; *eg*, on the zinc-dependent alcohol dehydrogenase involved in the conversion of vitamin A alcohol to its aldehyde (retinal). Although such a local or even a systemic synthetic block might generate the abnormal pigmentation by accumulations of an intermediary metabolite, anatomic evidence suggests that the lipochrome pigment is derived from ingested outer segment material. This might accumulate because of overload from increased turnover of rod discs, some modification of the disc membranes which renders them indigestible or, most likely, impairment of their enzymatic degradation, recycling, or disposal by the pigment epithelial cell. A primary action on the degradative functions of the pigment epithelium rather than on metabolic or synthetic activity important to photoreceptor maintenance is also supported by the lack of early, prominent effects on photoreceptors. This contrasts with the more extensive retinopathies produced by vitamin A deficiency,[9,10] iodoacetate [26,27] and iron [28,29] where photoreceptor degeneration first becomes apparent in outer segment abnormalities. The pigment epithelial changes, however, do share swelling and irregularities of the pigment epithelium seen in these retinopathies, and particularly in lesions produced by diaminodiphenoxyalkanes [21,30] which have a primary effect on the pigment epithelium. The pathologic effects of lead on pigment epithelium, along with known retinotoxic actions of diphenylthiocarbazone chelation [31,32] and siderosis [28,29] suggest important implications of heavy metals for the functional and anatomic integrity of these tissues. The mechanisms of damage by lead ingestion may relate to a species peculiarity of rabbit

since we have been unable to produce the lesion in guinea pigs, rats or monkeys maintained on a similar diet.

Although the specific mechanisms which may be operative in the rabbit remain obscure, lead intoxication produces a graphic abnormality in the pigment epithelium–photoreceptor complex. Pigment epithelial cells become congested by lipochrome pigment derived from residue of phagocytized outer segments, many of these cells die, the remaining epithelium begins to show less evidence of lipofuscin pigmentation, and a degenerative retinopathy develops.

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Acknowledgments

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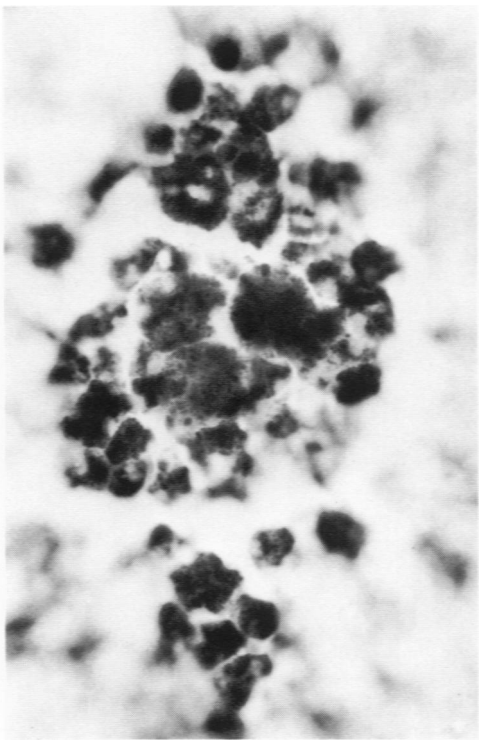
Legends for Figures

Fig 1—Flat mount of pigment epithelium and choroid (albino rabbit) shows clumps of affected cells which contain lipofuscin granules (Methylene blue, × 285).

Fig 2—A flat section (1 μ) of albino pigment epithelium demonstrates relatively unaffected cells and others swollen more extensively with lipofuscin granules *(arrows)* (Epon, toluidine blue-azure II, × 450).

Fig 3—Cross-section (1 μ) shows albino pigment epithelial cells swollen with lipofuscin pigment *(L)*. Note apical distribution of pigment in lightly affected cells *(arrows)*, as well as displacement of photoreceptor outer segments above lesions (Epon, toluidine blue-azure II, × 610).



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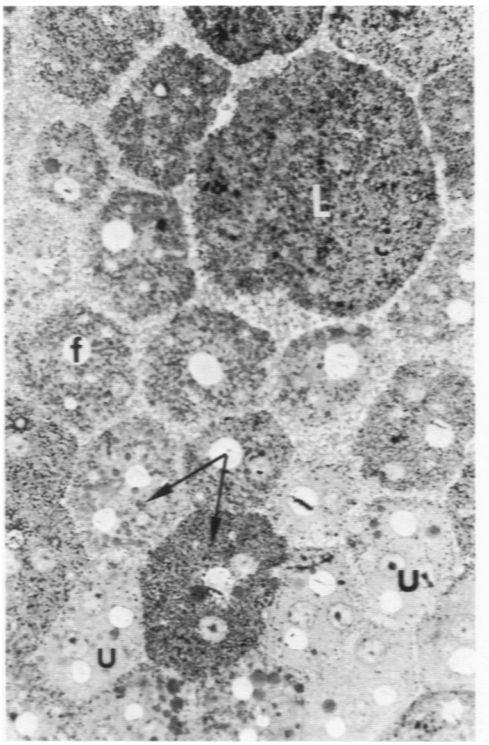

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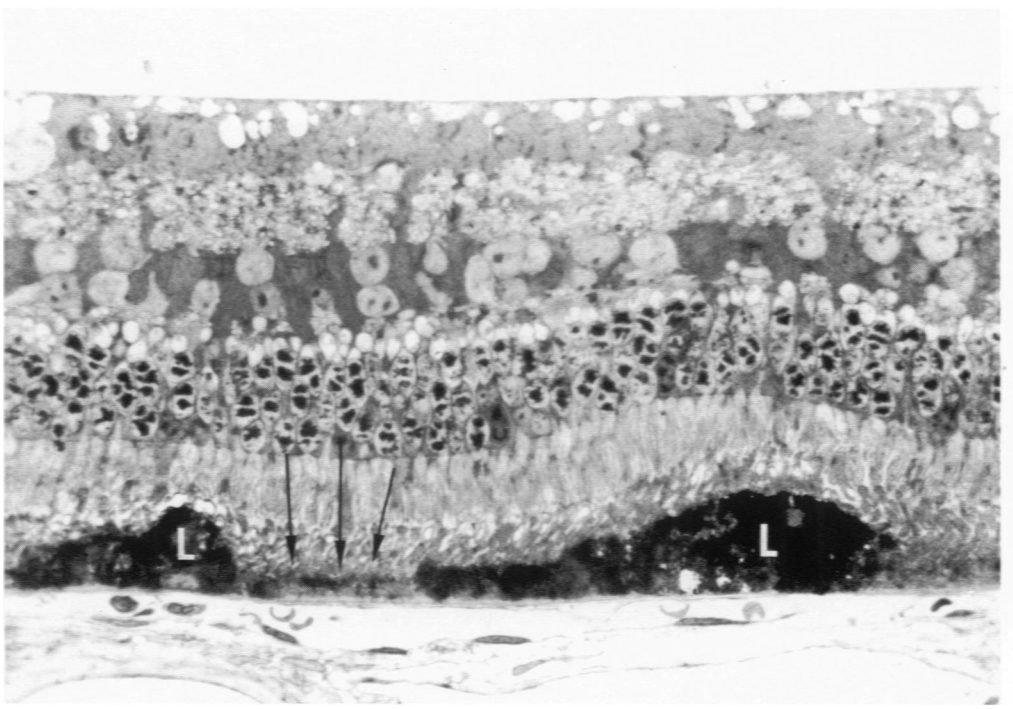

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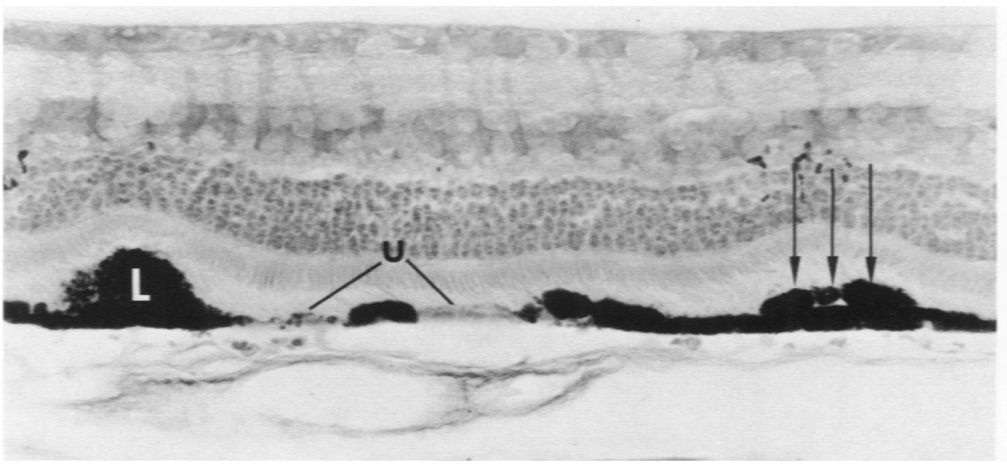
Fig 4—Lipofuscin granules in albino pigment epithelial lesions (*L*) stain intensely with silver. Unaffected cells (*u*) and cells on vitread surface of PE (*arrows*) are shown (Fontana-Masson, × 400).

Fig 5A—Disc membrane (*arrow*) in a rod phagosome seen commonly in lesioned cells (Uranyl acetate and lead citrate, × 65,000). **B**—Group of phagosomes at the surface of an affected pigment epithelial cell (Uranyl acetate and lead citrate, × 15,000). **C**—Field of homogeneous lipofuscin granules from a pigment epithelial lesion (Uranyl acetate and lead citrate, × 13,500). **D**—Anastamosing lipofuscin granules from large droplets. *Ser*=smooth endoplasmic reticulum. (Uranyl acetate and lead citrate, × 17,000).



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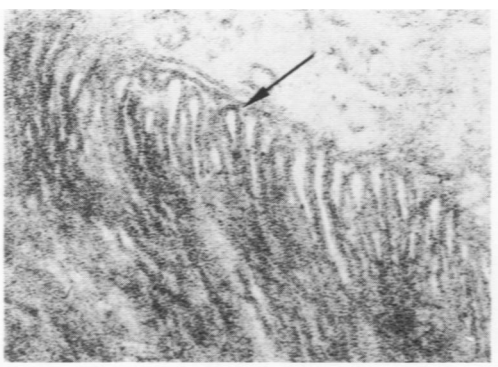

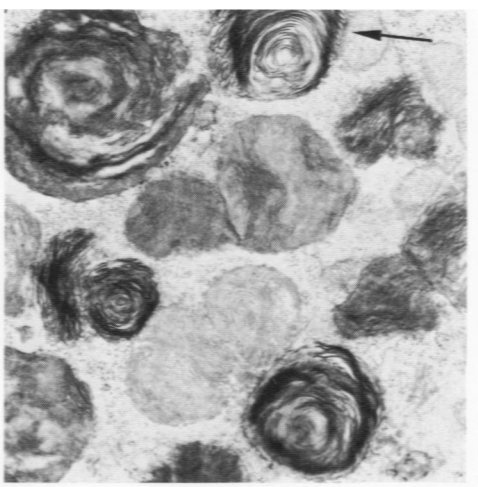

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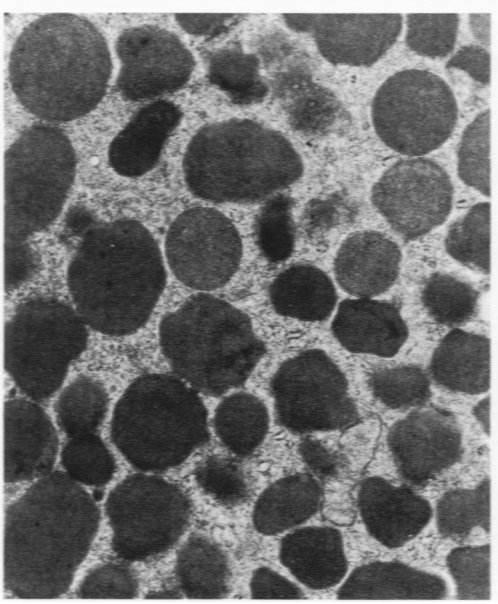

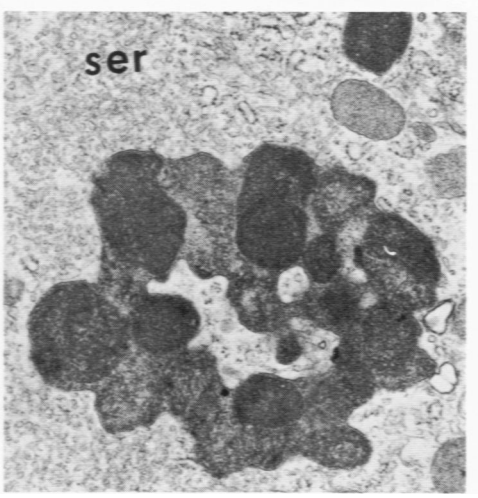

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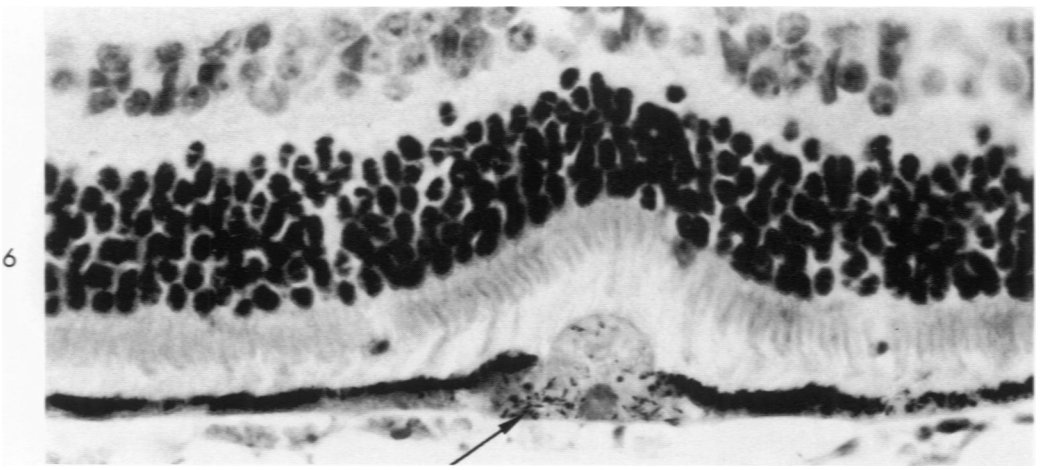


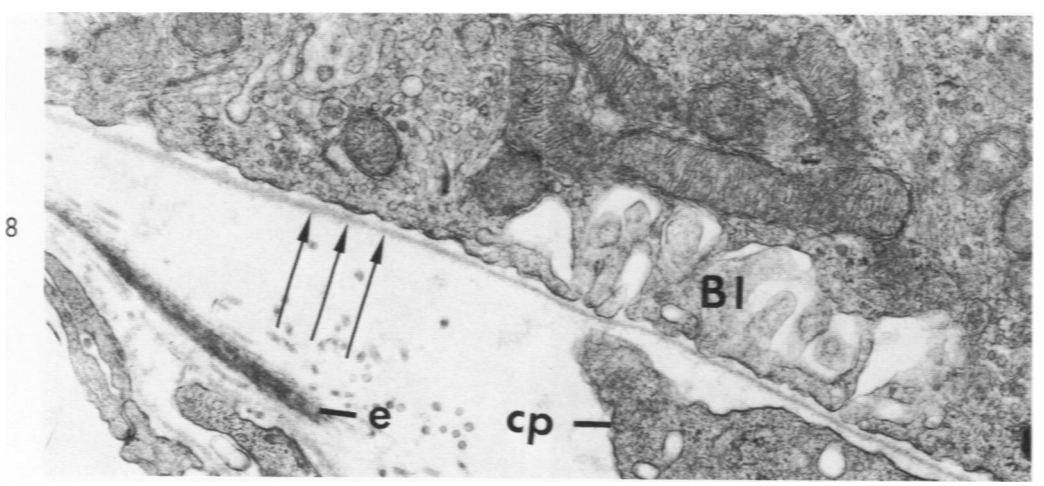
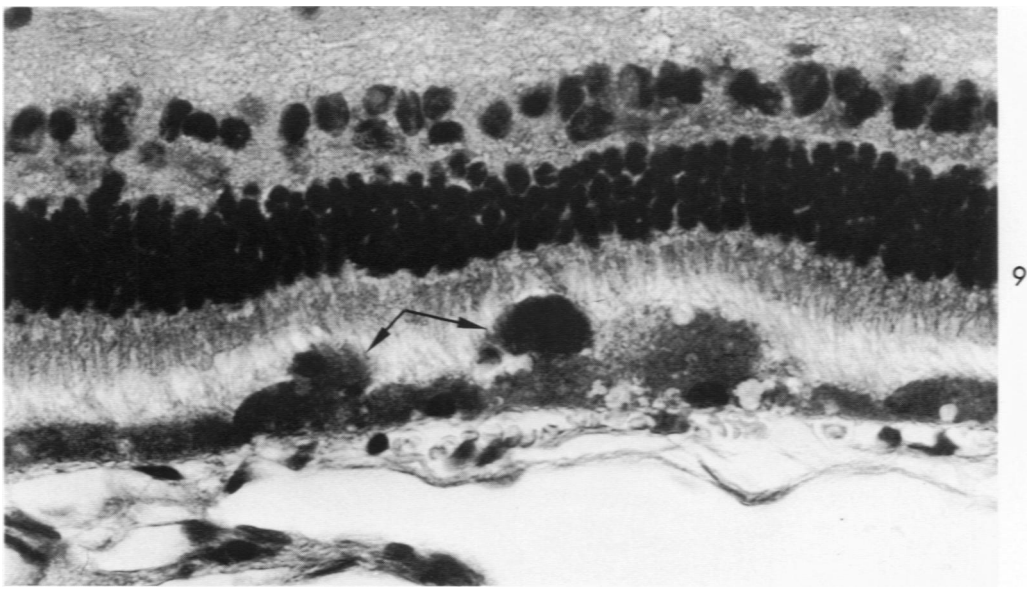


Fig 6—Lesion in pigment epithelium of a pigmented rabbit. *Arrow* shows displacement of melanin granules from their usual apical position seen in adjacent cells (H&E, × 730).

Fig 7—Absence of apical microvilli (*mv*) on the apical surface of lipofuscin-congested pigment epithelial cell (*PE*). *ROS*=rod outer segment (Uranyl acetate and lead citrate, × 25,000). **Fig 8**—*Arrows* illustrate a portion of an extensive area where basal infoldings (*BI*) are flattened along Bruch's membrane in affected cells. Note the cell process (*cp*) in Bruch's membrane. *e*=elastic layer (× 28,000).



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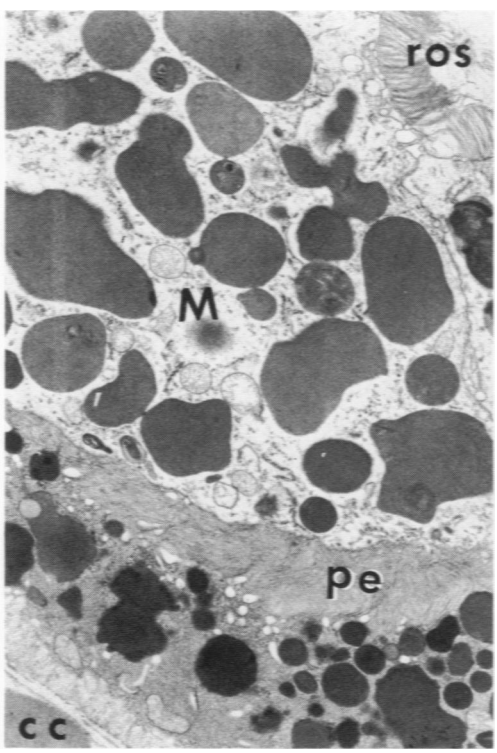

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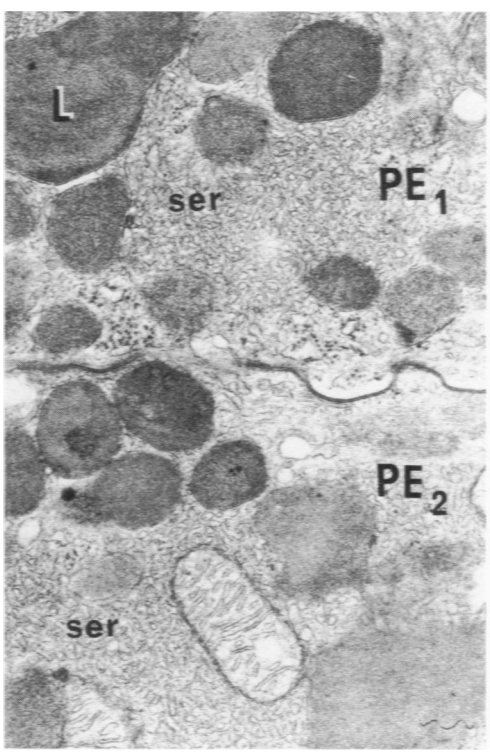

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Fig 9—Cells on the vitread surface of the pigment epithelium are indicated by *arrows* (H&E, × 700). **Fig 10**—A macrophage (*M*) containing pigment is inteposed between the pigment epithelium (*PE*) and rod outer segments (*ros*). *cc*=choriocapillaris (Uranyl acetate and lead citrate, × 7000). **Fig 11**—Pigment epithelial cells (PE_1) appear on the vitread surface of the pigment epithelium (PE_2). Both contain the abundant smooth endoplasmic reticulum, and junctional complexes are often (*arrow* indicates gap junction) seen between them (Uranyl acetate and lead citrate, × 17,500).

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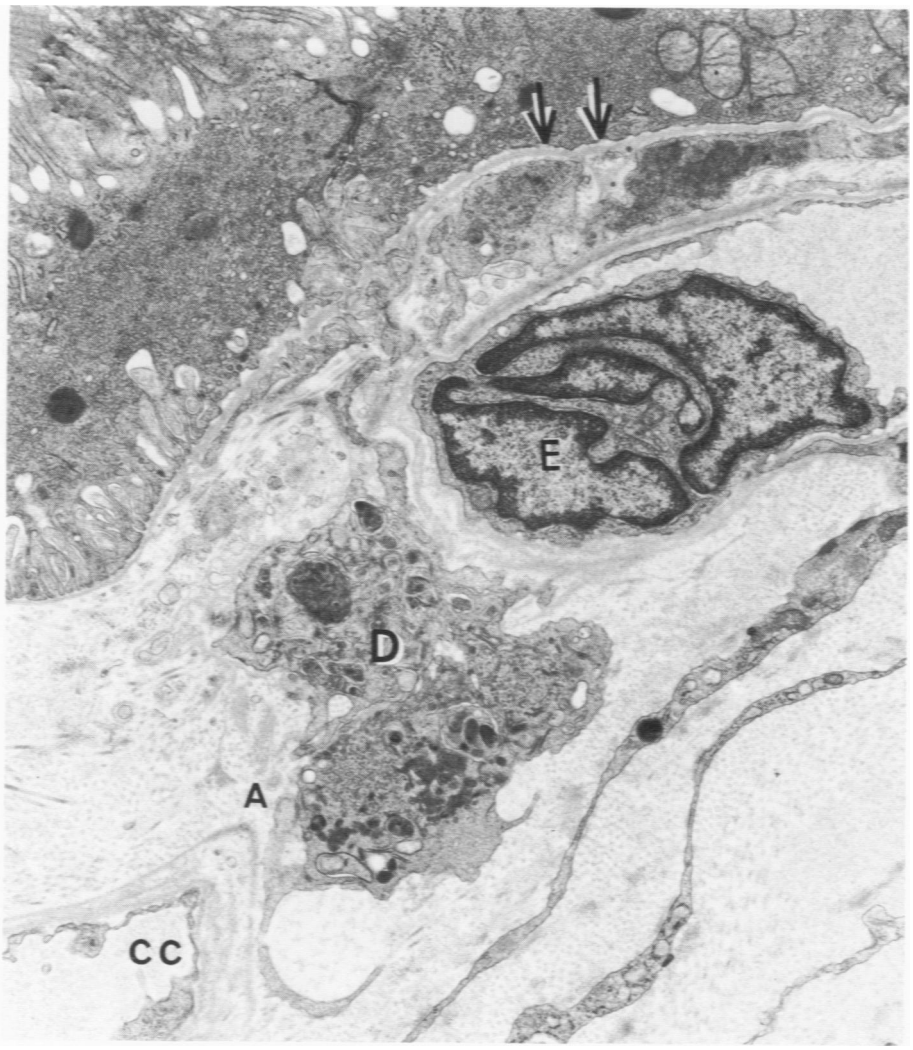

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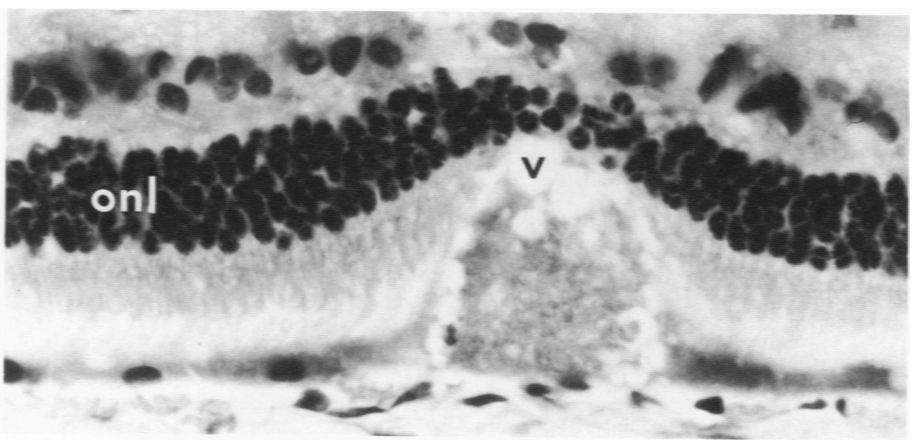

Fig 12—Degenerate cell (*D*) extruded via Bruch's membrane into the choroid. A cell process is seen in Bruch's membrane between flattened basal infoldings of a pigment epithelial cell (*arrows*) and an endothelial cell (*E*) of the choriocapillaris (Urany acetate and lead citrate, × 11,500). **Fig 13**—Degenerating pigment epithelial cell shows vacuoles (*V*) and watery autolytic contents. Noted reduced thickness of the outer nuclear layer (*ONL*) (H&E, × 690).

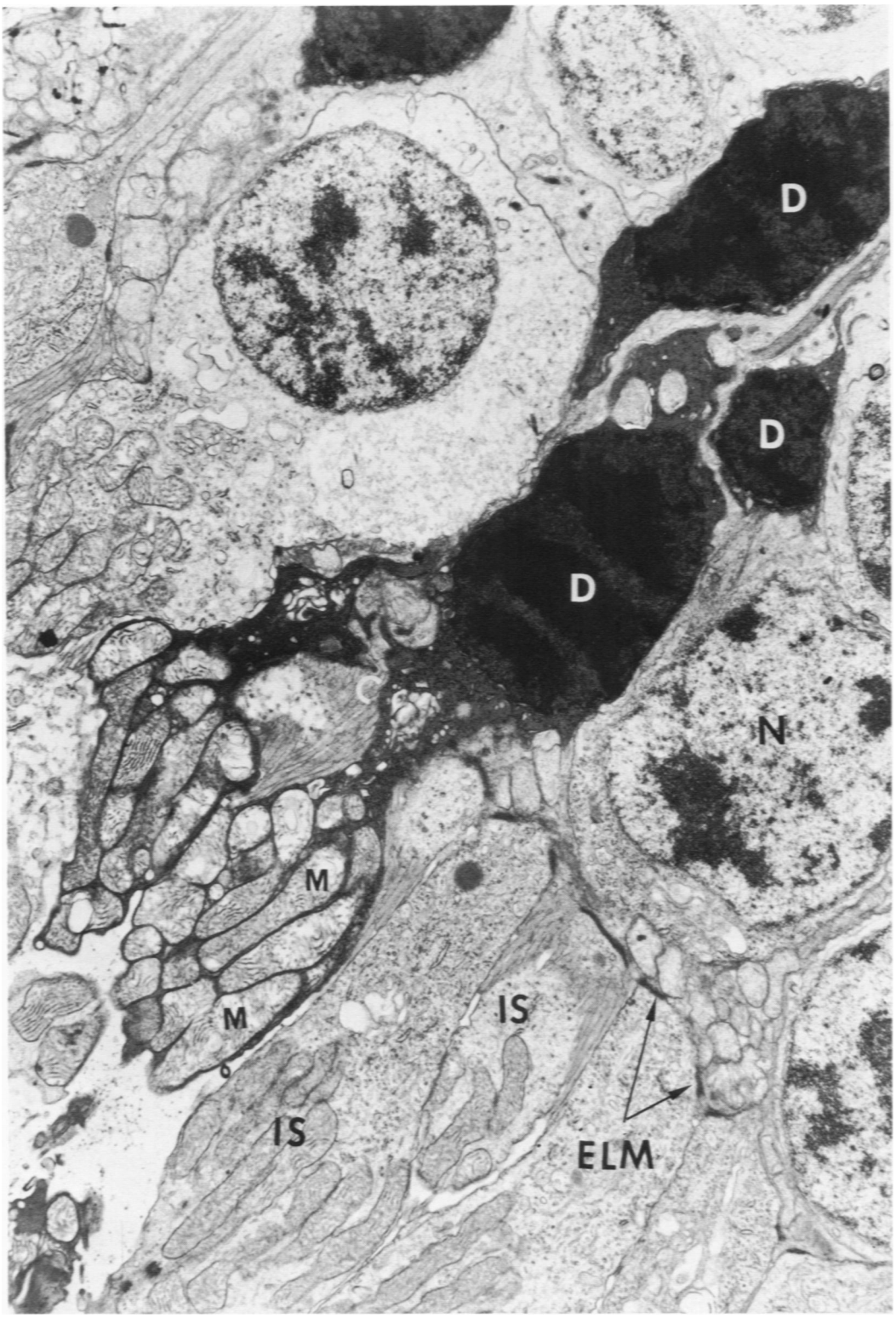

Fig 14—Degenerating photoreceptors (*D*). Compare pyknotic nuclei, dark cytoplasm, and swollen mitochondria (*m*) with nuclei (*N*) and inner segments (*IS*) of adjacent photoreceptors. *ELM*=external limiting membrane (Uranyl acetate and lead citrate, × 14,000).

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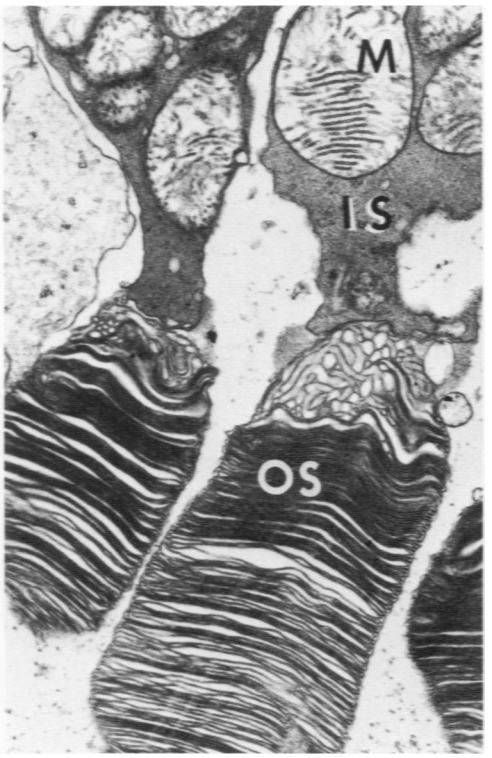

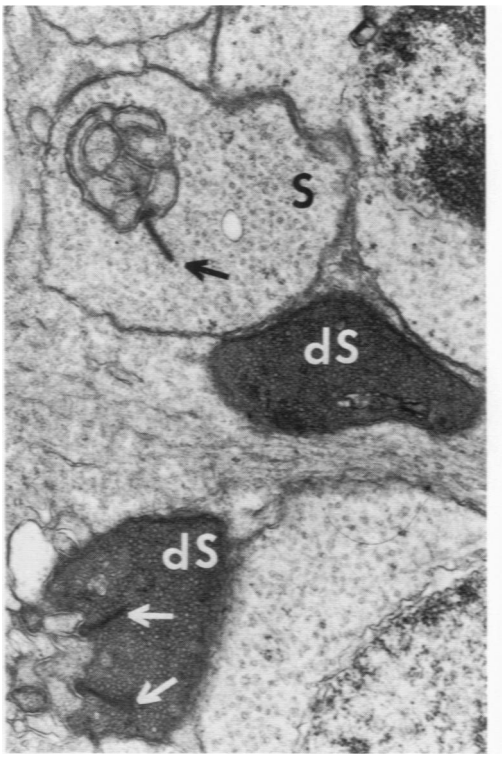

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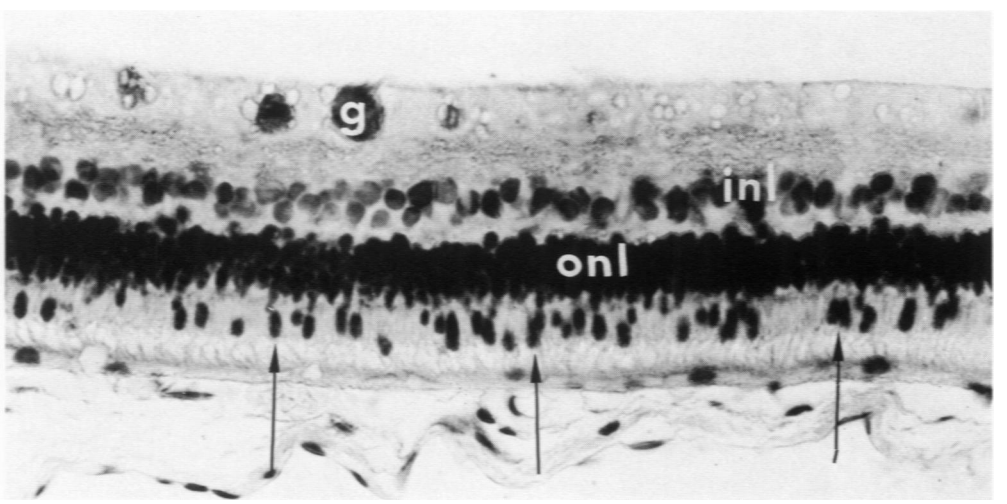

Fig 15—Outer segments (*OS*) of two degenerate photoreceptors appear relatively intact. Some vesiculation of proximal discs is apparent, and swelling of mitochondria (*m*) (Uranyl acetate and lead citrate, × 16,500). **Fig 16**—Degenerating synaptic spherules (*dS*) contrast with normal terminals (*S*). *Arrows* designate synaptic ribbons (Uranyl acetate and lead citrate, × 12,000). **Fig 17**—Photoreceptor degeneration in an old lead-treated rabbit shows abundant "dropping down" of nuclei from the outer nuclear layer (*ONL*) into inner segments (*arrows*). *INL*=inner nuclear layer, *g*=ganglion cell. Note absence of pigment epithelial lesions (H&E, × 410).