# Autophagy in visual cells and pigment epithelium

*Charlotte E. Remé*

*In the process of autophagy, parts of the cell's own cytoplasm are enclosed by membranes and digested by enzymes. The phenomenon of intracellular degradation is well known to occur in various tissues under different conditions. Autophagy in vertebrate visual cells was observed by Remé and Young, in the retina of hibernating ground squirrels, but has not been documented in vertebrate visual cells under normal conditions. The following study presents evidence of autophagy in visual cells and pigment epithelium in normal animals from five different species (cat, rat, frog, goldfish, ground squirrel). Autophagic vacuoles in visual cells are mainly located in the myoid region. The number of autophagic vacuoles containing degraded materials exceeds the number of vacuoles containing recognizable constituents. The ubiquitous presence of autophagy in visual cells and pigment epithelium, and the similarity of their morphological characteristics in several species, lead to the assumption that autophagy is part of a basic cellular process with a widespread distribution. Presumably, autophagy participates in the process of renewal by destroying cytoplasmic constituents which are continually synthesized by visual cells and pigment epithelium.*

**Key words:** retina, visual cells, turnover of cytoplasmic constituents, autophagy, intracellular digestion, pigment epithelium.

In the process of autophagy, parts of the cell's own cytoplasm are enclosed by membranes and then digested by enzymes. Newly formed autophagic vacuoles may contain all sorts of cytoplasmic constituents, including rough or smooth ER, ground plasma, or mitochondria. Intracellular degradation by autophagy in various tissues under different conditions has been reported by several investigators.[1, 2, 4-17]

Autophagy in vertebrate visual cells was first noted by Remé and Young[16] in the retina of hibernating ground squirrels. Occurrence of autophagy has not been previously reported in the retina of vertebrate species under normal conditions. Therefore the findings in hibernating ground squirrels raised the question of whether autophagy was unique to the visual cells of those animals, and was expressed only during hibernation, or whether autophagy may have a general significance and may be encountered in the eyes of other animals as well. Accordingly, investigations were undertaken to seek evidence for autophagy in visual cells and pigment epithelium in a wide variety of vertebrate animals. The following report demonstrates the ubiquitous presence of autophagic vacuoles in visual cells and pigment epithelium in nor-

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mal adult animals of five different species.

## Materials and methods

Adult, normal animals of the following species or strains were used in this study: cat *(Felis domesticus)*, rat (Wistar strain), ground squirrel *(Citellus tridecemlineatus)*, frog *(Rana pipiens)*, and goldfish *(Carassius auratus)*.

***Cats.*** Four cats, each weighing about 2 kg., were anesthetized (0.1 ml. of ketamine hydrochloride intramuscularly and 0.4 to 0.6 ml. of a 4 percent Surital sodium solution intravenously) and then killed by intracardiac administration of fixation at 100 mm. Hg pressure. The fixative consisted of a mixture of 4 percent glutaraldehyde and 5 percent paraformaldehyde, buffered with 0.1M cacodylate buffer to a final pH of 7.4. The eyes were then enucleated; the posterior halves were removed and remained in the same fixative overnight.

***Rats.*** Twelve rats, each weighing about 200 gm., were anesthetized with 0.5 ml of a 4 percent Surital sodium solution intraperitoneally and then killed by intracardiac perfusion of fixative consisting of 2.5 per cent glutaraldehyde buffered with 0.1M cacodylate buffer to a final pH of 7.4. The eyes were then enucleated; the posterior halves were removed and remained in the same fixative overnight.

***Ground squirrels.*** Three ground squirrels, each weighing about 200 gm., were anesthetized with 0.5 ml. of a 1.25 percent Nembutal sodium solution intraperitoneally and then killed by intracardiac perfusion of fixative consisting of a mixture of 1 percent glutaraldehyde and 1 percent paraformaldehyde in Millonig buffer at a final pH of 7.2. The eyes were then enucleated; the posterior halves were removed and remained in the same fixative overnight.

***Frogs and goldfish.*** Three frogs, each weighing about 80 gm., and three goldfish, about 10 cm. long, were killed by decapitation. The eyes were enucleated and immediately placed into fixative consisting of 2.5 percent glutaraldehyde in cacodylate buffer at a final pH of 7.4. The posterior halves were removed and remained in the same fixative overnight.

***Preparation for electron microscopic observation.*** After dissection of central parts of the retina into small specimens and postfixation in osmium tetroxide (in S-collidine buffer at a pH of 7.2) for 1 hr., followed by alcoholic dehydration, all tissues were embedded in Epon 812 and oriented, so that longitudinal (vitreal-sceral) semithin and thin sections could be cut. Semithin sections were stained with methylene blue. Thin sections, stained with uranyl acetate and lead citrate, were observed with a Philips 300 electron microscope.

***Quantitative analysis.*** In three species (frog, goldfish, cat) the total number of autophagic vacuoles was counted and their localization defined in 200 visual cells of each group of animals. The counts were performed under direct observation on the electron microscope screen at a magnification of 14,000. Only those visual cells were included whose longitudinal orientation permitted a complete view of the inner segment, including ellipsoid, myoid, and perinuclear area. Besides that, no other kind of selection of cells for quantification was done, and rods and cones were combined. In order to reduce the possibility of counting the same vacuoles several times, every sixth thin section of a series was mounted on the grid for observation. Two main groups of autophagic vacuoles were selected according to their morphological characteristics and counted separately: (1) vacuoles containing recognizable cytoplasmic constituents bounded by two or more membranes and (2) vacuoles containing unidentified or hardly recognizable materials bounded by one membrane. In very few instances, autophagic vacuoles were seen bounded by two membranes but containing unrecognizable materials or bounded by one membrane but revealing identifiable contents. Since those two types of autophagic vacuoles appeared to be extremely rare, they were not included in the quantifications. The localization of autophagic vacuoles in visual cell inner segments was defined by the following criteria. Autophagic vacuoles found in transitional zones between ellipsoid and myoid were considered to be situated in the myoid. The perinuclear area comprised the region from the outer limiting membrane to the vitreal end of the nucleus. The number of autophagic vacuoles situated in the myoid, the perinuclear area, or the ellipsoid was registered, and the total number of autophagic vacuoles in each visual cell was counted. Because of the scarcity of autophagic vacuoles occurring in the connecting fiber or the synaptic body of visual cells, those areas were not included in the quantification.

## Results

Autophagic vacuoles always showed essentially the same morphological characteristics in visual cells and pigment epithelium of all species examined. Therefore all observations are described together, and no attempt was made to illustrate with figures all types of autophagic vacuoles in every species.

***Localization of autophagic vacuoles.*** Autophagic vacuoles were found in the myoid portion of visual cells, in the ellipsoid portion, and in the perinuclear area.

Occasionally, there was an autophagic vacuole in the connecting fiber between nucleus and synaptic body or in the synaptic body itself. In the pigment epithelium, autophagic vacuoles were scattered throughout the cytoplasm. They were not encountered in basal infoldings or apical cell processes (Fig. 1).

***Morphological characteristics of autophagic vacuoles***

*Autophagic vacuoles with recognizable contents.* Identifiable cytoplasmic constituents were separated from their surroundings by a more or less distinct cleft. Both sides of the cleft were bounded by membranes. Occasionally, whorls of membranes appeared in the cleft or among the enclosed cytoplasmic organelles. The vacuoles contained all sorts of cytoplasmic constituents, including ground plasma, membrances of smooth or rough ER, clusters of ribosomes, Golgi-derived vesicles and membranes, and mitochondria. The sizes of the vacuoles varied considerably (Fig. 2, *A* to *C*).

*Autophagic vacuoles with unrecognizable contents.* In the course of degradation, the cytoplasmic constituents inside the autophagic vacuoles gradually became unidentifiable. Different appearances of vacuoles, which were mostly bounded by one membrane, were distinguished. Some vacuoles contained almost homogeneous, moderately dense materials, others were transformed into clumped, very dense masses, others comprised optically empty space and scattered particles. In some transitional forms of vacuoles, parts of cytoplasmic organelles were still recognizable (Fig. 2, *A*, *D*, and *F*).

***Associated membranes and vesicles.*** Occasionally, membranes of the Golgi complex or membranes of smooth or rough ER were found to be partially surrounding or entirely enclosing parts of the cytoplasm (Fig. 2, *E*). Sometimes autophagic vacuoles were found in close apposition to or in direct contact with Golgi-derived membranes or vesicles, or membranes of smooth or rough ER (Fig. 2, *F*). Autophagic vacuoles were encountered in cells containing lysosome-like dense bodies, like the pigment epithelium of the rat (Fig. 3, *A* and *B*) or cat (Fig. 3, *C*), as well as in cells which apparently did not contain any lysosome-like structures, like the pigment epithelium of ground squirrels (Fig. 3, *D*).

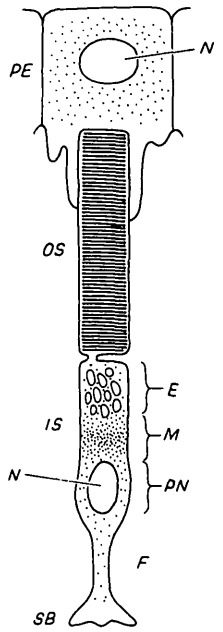


Fig. 1. Schematic drawing in which the distribution of dots demonstrates the localization of autophagic vacuoles in visual cells and pigment epithelium. In the pigment epithelium *(PE)*, autophagic vacuoles are scattered through the cytoplasm; they are not found in basal infoldings and cytoplasmic processes surrounding the tips of visual cell outer segments. In the inner segment of visual cells, autophagic vacuoles are found more often in the myoid part *(M)* than in any other part of the cell. Less frequently they are encountered in the perinuclear area *(PN)* and the ellipsoid *(E)*, rarely in the connecting fiber *(F)* or in the synaptic body *(SB)*. They were never observed in the nucleus *(N)* or outer segment *(OS)*.

***Quantitative analysis.*** In three species, the number of autophagic vacuoles occurring in inner segments of visual cells was quantified (Table I). In 200 frog inner segments, 57 autophagic vacuoles were counted; in 200 goldfish inner segments, 193 autophagic vacuoles were observed; and in 200 cat visual cells, 27 autophagic vacuoles were recorded. In all animals, the

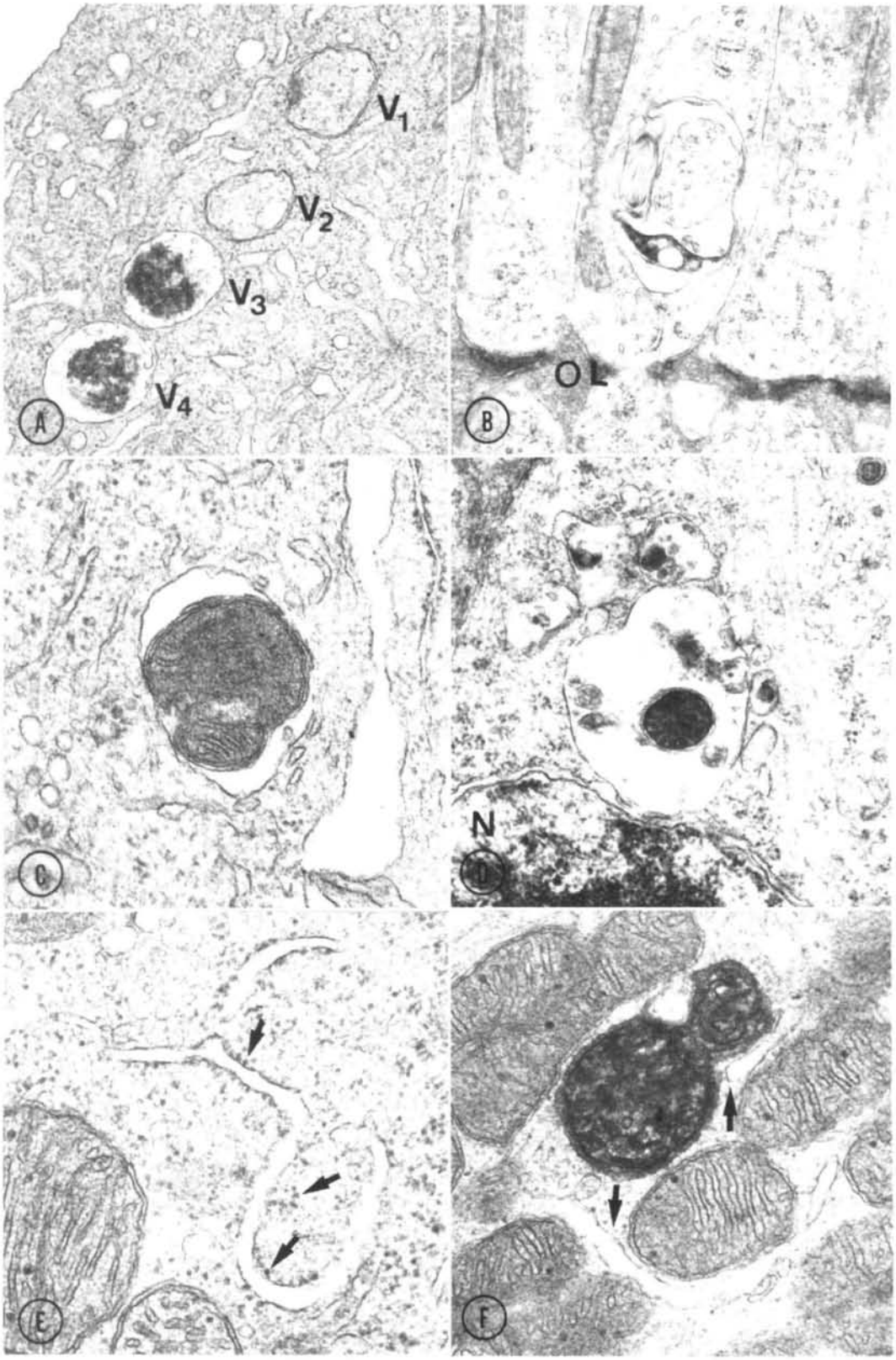


**Fig. 2.** For legend see opposite page.

**Table I.** Number, location, and appearance of autophagic vacuoles (AV) in visual cells from three species

| Animal | No. of visual cells | No. of AV vacuoles | | | | AV with identifiable contents (%) |
|---|---|---|---|---|---|---|
| | | Ellipsoid | Myoid | Perinuclear | Total | |
| Frog | 200 | 8 | 44 | 5 | 57 | 50 |
| Goldfish | 200 | 3 | 172 | 18 | 193 | 13 |
| Cat | 200 | 3 | 19 | 5 | 27 | 13 |

number of vacuoles containing unidentifiable materials was considerably higher than the number of vacuoles comprising recognizable cytoplasmic constituents. Autophagic vacuoles were encountered more often in the myoid portion than in any other part of the cell (Table I). They were seen less frequently in the perinuclear area or the ellipsoid portion.

## Discussion

Autophagy was observed in the visual cells and pigment epithelium of all five vertebrate species whose retinas I examined. In addition, the autophagic vacuoles contained all of the morphologically distinguishable types of cytoplasmic constituents, and always displayed the same range of morphological features without regard to cell type or species. These results suggest that autophagy is part of a basic process common to many, perhaps all, vertebrate visual cells and pigment epithelial cells. Furthermore, its amply documented occurrence in many other types of cells[1, 2, 4-17] indicates that it is a common process of fundamental importance in the metabolism of a very wide variety of vertebrate cells.[12]

In the process of autophagy, cell constituents isolated by the surrounding membranous wall of the autophagic vacuole are digested by enzymes. Degradation continues until the breakdown products reach a size which allows them to pass through the membrane into the surrounding cytoplasm, where they may be used in synthetic pathways. Cells may employ different methods for delivering digestive enzymes to the autophagic vacuoles, including Golgi vesicles and primary and secondary lysosomes.[12] No organelles or structures of this kind were uniformly observed in the vicinity of autophagic vacuoles in these speci-

**Fig. 2.** Electron micrographs depicting autophagic vacuoles in visual cells of various species. *A*, Four autophagic vacuoles lined up in the myoid of a frog visual cell. Two of them contain still clearly recognizable ground plasma, ribosomes, and a membrane of smooth ER ($V_1$, $V_2$). The other two vacuoles are bounded by a single membrane and enclose dense materials among optically empty space ($V_3$, $V_4$). (×28,800.) *B*, Autophagic vacuole in the myoid portion of a cat visual cell containing ground plasma and clusters of ribosomes. The space between the inner and outer membranes is filled with membranous whorls and electron-dense materials. *OL*, Outer limiting membrane. (×16,800.) *C*, Autophagic vacuole in the myoid of a ground squirrel cone, containing a densified but still identifiable mitochondrion. A gap between the inner and outer membranes of the vacuole is visible. (×34,500.) *D*, A group of autophagic vacuoles consisting of one large and several small vesicles in the perinuclear area of a cat visual cell. The large vacuole contains some dense, homogeneous material and some vesicular materials scattered throughout the vacuole. All vacuoles are bounded by a single membrane. *N*, Nucleus. (×41,400.) *E*, Membranes of rough ER in the process of wrapping themselves around parts of the cytoplasm in the myoid of a frog visual cell. Ribosomes in the ground plasma and along the membranes of ER are identifiable (arrow). (×47,800.) *F*, Autophagic vacuole in the ellipsoid of a frog visual cell, consisting of fairly homogeneous dense material. Note the membranes of smooth ER (arrow) close to the vacuole and close to some nearby mitochondria. (×40,150.) All electron micrographs: uranyl acetate and lead citrate.

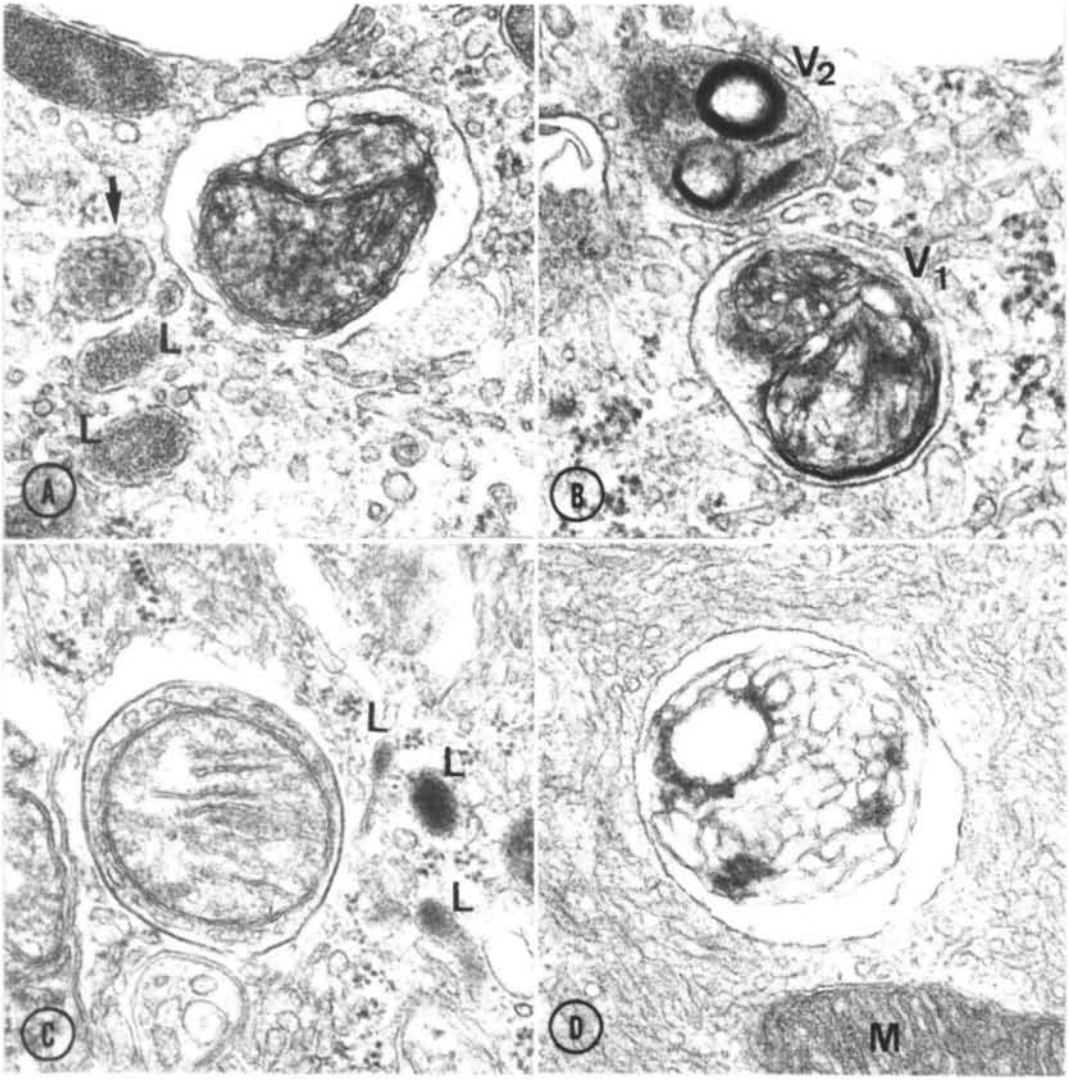


**Fig. 3.** Electron micrographs demonstrating autophagic vacuoles in the pigment epithelium of various species. *A*, Autophagic vacuole in the pigment epithelium of a rat eye comprising dense materials which are no longer clearly identifiable but could have been a mitochondrion. Close to the vacuole is a multivesicular body (arrow) and structures which may be lysosomes *(L)*. (×53,700.) *B*, Autophagic vacuole in the pigment epithelium of a rat eye, containing dense, almost unrecognizable material, probably derived from smooth ER ($V_1$). The vacuole is bounded by two membranes. Close to this vacuole another vacuole ($V_2$) is visible containing dense, partially membranous materials. It is not obvious whether the second vacuole is a heterophagosome or an autophagic vacuole. (×49,300.) *C*, Autophagic vacuole in the pigment epithelium of a cat eye, comprising a mitochondrion and a small amount of smooth ER. One limiting membrane is clearly visible. In the neighborhood there appear some lysosome-like bodies *(L)*. (×39,800.) *D*, Autophagic vacuole in the pigment epithelium of a ground squirrel eye. The contents of the vacuole, being bordered by two membranes, appear to be smooth ER, which underwent slight dilatations of its membranes and focal densifications. *M*, Mitochondrion. (×37,600.) All electron micrographs: uranyl acetate and lead citrate.

mens, so that mode of entry of degradative enzymes was not determined. Possibly the membranes surrounding the vacuoles themselves may in certain cases supply the enzymes.

It has been amply documented that all of the major components of visual cells are continuously renewed, and that most of the synthetic pathways are localized in the inner segments, particularly the myoid zone.[19, 20] Similarly, it has been demonstrated that all the major classes of molecules in the pigment epithelium undergo perpetual replacement.[22, 23] These conclusions were derived from experimental studies in which the continuous synthesis of new molecules was revealed by radioisotope methods in mature cells. However, apart from the intermittent shedding of groups of membranes from the tips of rod visual cells,[18, 19, 21] no degradative mechanism has ever been described which could account for the disappearance of any of the cell constituents known to be continuously formed.

The observations reported above, that autophagy is a process which takes place in the cytoplasm of the visual cells and pigment epithelium of every vertebrate species so far examined, and that this process is capable of digesting all of the morphologically distinguishable cytoplasmic constituents, suggest that autophagy may be one of the degradative mechanisms by which these cells maintain their balance between formation and degradation in the over-all process of renewal.[20]

Autophagy might also have an important regulative function at the cellular level of visual cells and pigment epithelium, disposing of apparently surplus cellular products. This appears to be the case in hibernating ground squirrels.[16] Also, during ontogeny of the pigment epithelium in albino mice, accumulated unmelanized premelanosomes were found to be destroyed by autophagy.[10] In the bovine pigment epithelium, "mistakenly" synthesized melanosomes in the intermediate cell type appeared to be removed by autophagic digestion.[2] A diurnal variation of the amount of autophagy in visual cell inner segments has been documented in this laboratory and will be reported in a subsequent publication.

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