

# RHYTHMIC DAILY SHEDDING OF OUTER-SEGMENT MEMBRANES BY VISUAL CELLS IN THE GOLDFISH

WILLIAM T. O'DAY and RICHARD W. YOUNG

From the Department of Anatomy and Jules Stein Eye Institute, University of California at Los Angeles, Los Angeles, California 90024

## ABSTRACT

Goldfish were placed on a daily light cycle of 12 h light and 12 h darkness for 18 days or longer. The visual cells and pigment epithelium of the retina were then examined by microscopy at many intervals throughout the cycle.

Goldfish rods and cones follow a rhythmic pattern in eliminating packets of photosensitive membranes from their outer segments. Rods shed membranes early in the light period. The detached membranes are ingested by pigment epithelial cells or by ameboid phagocytes, which degrade them during the remainder of the light period. Cones discard membranes from the ends of their outer segments early in the dark period. During the next several hours, this debris is digested by the pigment epithelium or by ameboid phagocytes. Thus, the disposal phase of the outer-segment renewal process is similar in rods and cones, but is displaced in time by about 12 h.

There is evidence that this daily rhythm of membrane disposal in rods and cones is a general property of vertebrate visual cells.

**KEY WORDS** retina · rods and cones · phagocytosis · daily rhythm

Visual cells of the vertebrate eye maintain their integrity for life, avoiding senescence through continuous replacement of their cellular constituents (16). The two classes of photoreceptor cells, the rods and cones, contain their light-sensitive pigments in a stack of disk-shaped membranes. These, together with the associated plasma membrane, form the outer segment of the cell (Fig. 1).

Rods repeatedly produce new membranous disks at the base of the outer segment, and periodically eliminate clusters of old ones from the apex (14, 15, 19, 20). The detached disks are then phagocytized and digested by the pigment epithelium. The equilibrium between formation and degradation of membranes maintains the rod

outer segment at a relatively constant length. The periodic shedding of membranes from the tips of rod cells has been observed without exception in every species of vertebrate animal which has been examined, including the human. Renewal of the outer segment by membrane replacement is therefore considered to be a universal characteristic of rod visual cells (16).

In contrast, evidence of disk shedding by cones is very rare, and has not been shown to be a regularly recurring process. It has been reported only in humans (3, 10) and squirrels (1). The mechanism of renewal of cone outer segments consequently has remained obscure.

The discovery that membrane shedding by rods occurs at a certain time of day suggests a new approach to the study of renewal in cones. LaVail (4) observed that in rats which have been maintained on a daily light cycle (12 h light, 12 h

darkness) the rods preferentially shed the tips of their outer segments about 1 h after the beginning of the daily light period. This finding led Young to suspect that cone shedding might also be cyclic: since rods shed membranes soon after the end of the dark period, then cones might shed membranes soon after the end of the light period. This schedule would allow each class of receptor to eliminate old disk membranes shortly after it had ceased to mediate vision, and would explain the rarity of previous observations of shedding by cones.

Histological examination of retinas from goldfish killed at regular intervals throughout the day and night has confirmed that hypothesis: rods shed membranes early in the light period; cones shed membranes early in the dark period.

## MATERIALS AND METHODS

### *Experimental Protocol*

Comet goldfish (*Carassius auratus* L.) from Ozark Fisheries (Stoutland, Mo.) were obtained through a local dealer. Their length ranged from 30 to 55 mm (snout to base of tail), their weight from 1 to 5 g. The fish were maintained with regular feeding in filtered, aerated water at room temperature ( $24^{\circ} \pm 2^{\circ}\text{C}$ ) for 18 days or longer on a photoperiod of 12 h light followed by 12 h darkness. The aquaria were located in a darkroom, where an automatic timer regulated the light cycle. Fluorescent lighting provided an intensity of approx. 700 lx (65 foot candles) at the center of the tanks.

A total of 120 fish were used in two experiments, an initial one covering the dark period and a later one spanning the full 24 h cycle. In the first experiment, three fish were sacrificed every 2 h, starting 1 h before the lights went off and ending 1 h after the lights went on. In the second experiment, 96 fish were sacrificed, four fish at each of 24 intervals. These intervals were 2 h apart, except during the 1st h of the light and dark periods, when they were 10 min apart.

### *Tissue Preparation*

Eyes were fixed in ice-cold 1% formaldehyde-2% glutaraldehyde in 0.080 M phosphate buffer, pH 7.2. After enucleation from living or freshly decapitated fish, they were immersed in cold fixative while the cornea was trimmed away and the lens removed. The eyecups were transferred to fresh, cold fixative, and were gently agitated for about 2 h, before storage at  $4^{\circ}\text{C}$ . During the closely spaced intervals, fish were decapitated into cold fixative in quick succession and their eyes then enucleated.

To facilitate their capture in total darkness, fish were placed in perforated containers of translucent white plastic, fitted with airstones. These containers were

withdrawn from the aquarium as needed. Three fish per interval were killed in total darkness by quickly decapitating them into cold fixative. The eyes were then enucleated in the light and dissected as previously described. During the dark period of the second experiment, one additional fish per interval was brought live into the lighted laboratory, where an eye was enucleated, opened, and fixed. This immediate dissection of the eye made a slight improvement in the quality of the fixation.

After fixation for several hours at  $4^{\circ}\text{C}$ , the eyecups were dissected further. Central to mid-peripheral retina from the dorsal half of the eye was cut into 2-3-mm pieces. The tissues were then brought to room temperature, rinsed in buffer (0.086 M phosphate, pH 7.2) and fixed for 1 h in 1% osmium tetroxide in the same buffer. They were rapidly dehydrated in an ascending series of ethanol-water mixtures, transferred to propylene oxide, and infiltrated with Araldite 502. The specimens were then embedded in fresh Araldite.

### *Light Microscopy*

Sections,  $0.5 \mu\text{m}$  thick, cut parallel with the long axis of the visual cells, were stained with 1% toluidine blue 0 in 1% sodium borate, after removal of the embedding matrix by incubation in a solution of sodium hydroxide in absolute ethanol. Measurements were made with an ocular micrometer, previously calibrated against a stage micrometer. The length of retina in the section was measured, without correcting for curvature. The number of phagosomes (packets of shed, outer-segment membranes) was then counted. All phagosomes larger than  $1 \mu\text{m}$  in any dimension were recorded. For each time-point, the mean number of rod or cone phagosomes per millimeter retina was then determined.

In goldfish, photomechanical movements segregate the outer segments of rods and cones at different levels within the pigment epithelium (Fig. 1). This facilitated the study of phagocytic activity associated with each class of receptor, as did the extensive separation in time of the major periods of shedding by the rods and cones.

### *Electron Microscopy*

Blocks of tissue representing different times of day and night were selected for electron microscopy on the basis of previous study with the light microscope. Sections showing silver interference colors were cut, supported on uncoated 200-mesh grids, and stained with uranyl acetate (2.5% in 50% ethanol) and Reynolds' lead citrate.

## RESULTS

The goldfish retina contains a single type of rod and seven types of cone, whose relative numbers do not vary systematically in different regions of the retina (11). We found an average of 5.6 rods per cone in 10 goldfish 30-45 mm long. The rods and cones are readily distinguished in the light microscope (Fig. 1). Because qualitative observa-

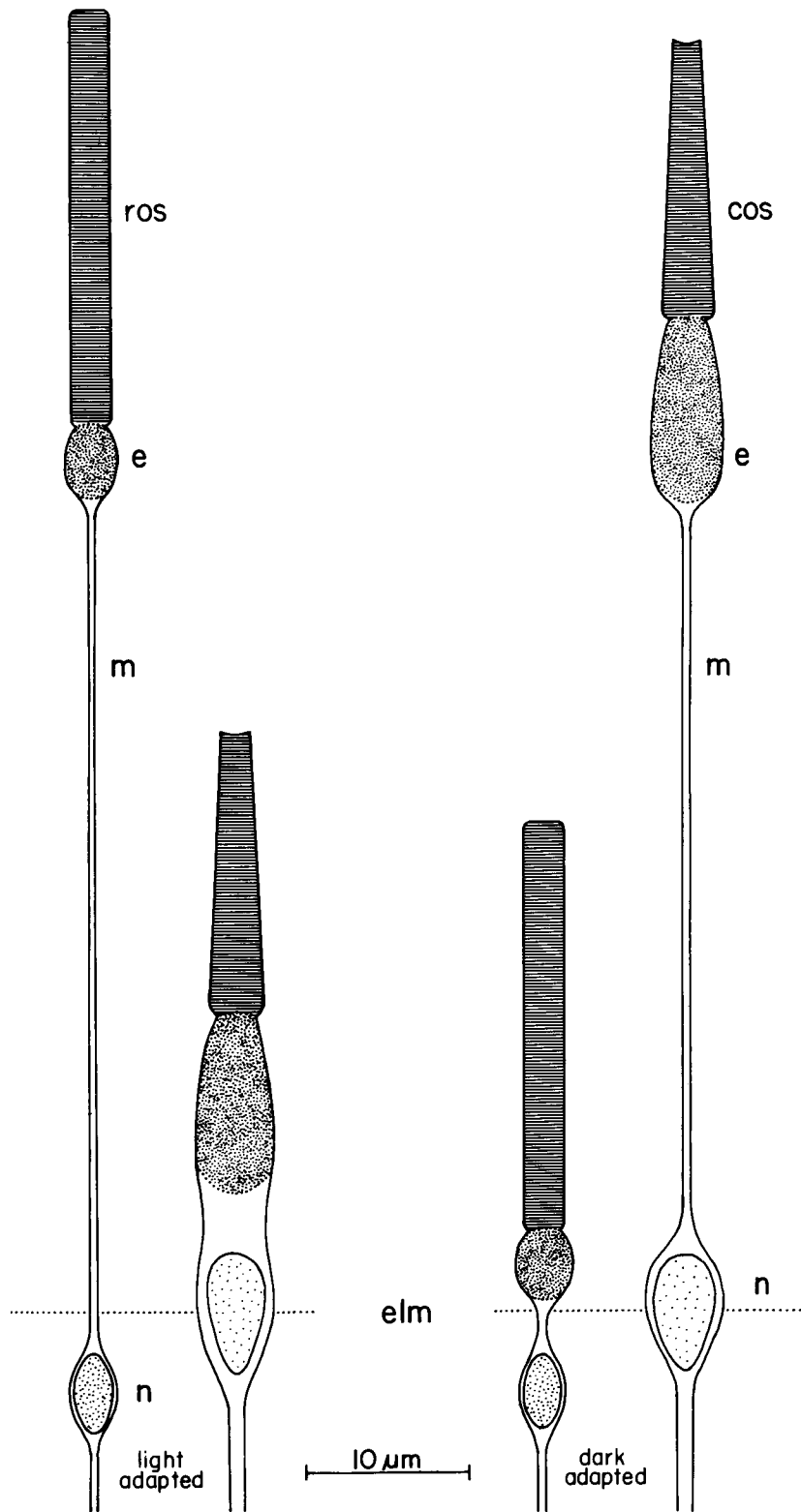


FIGURE 1 Effects of photomechanical movement on a rod and cone of the goldfish. In the light-adapted state, the rod outer segment (*ros*) is displaced from its nucleus (*n*) by extension of the myoid (*m*). In the dark-adapted state, the cone outer segment (*cos*) is similarly displaced. *e*, ellipsoid; *elm*, external limiting membrane.

tions revealed no difference in their membrane-shedding behavior, we did not subdivide the cones into their several types. In addition to the photomechanical movements of the visual cells (Fig. 1), the melanosomes of the pigment epithelium also migrate with the state of light adaptation. They move within the apical cell processes, away from the cell bodies of the pigment epithelium in the light and towards them in the dark. The epithelial cells contain numerous spherical inclusions, thought to be lipid droplets, which function as a light-reflecting tapetum (6). The tapetal droplets are absent from most of the ventral retina (11). The greater height of the pigment epithelial cells in the dorsal retina, associated with the tapetal inclusions, allows a greater separation of rods and cones through photomechanical movement, facilitating the separate analysis of rod and cone phagosomes. A final distinctive feature of the goldfish retina is the presence of a heterogeneous population of mobile cells within the pigment epithelium. These cells apparently have not been reported previously. Because they participate in the ingestion and degradation of shed membranes, we shall refer to them as ameboid phagocytes.

#### *Shedding of Membranes by Rods*

Disk shedding by rods occurs soon after the lights go on. At later times in the 24-h cycle, it becomes increasingly rare (Table I). Fig. 2 shows the time-course of shedding in one experiment. Immediately before the beginning of the light period, no rod phagosomes were visible. After the lights went on, packets of detached rod membranes began to appear within 10 min, and then rapidly increased in number, reaching a peak at 50 min (Figs. 3-6). During the second half of the light period, very few fresh phagosomes were observed (Fig. 2). (Such phagosomes scarcely differ in shape and staining properties from the tips of intact outer segments.) Instead, the par-

tially digested remains of earlier shedding events predominated during this portion of the rod curve.

During the 1st h after the lights went off, as the photomechanical movements of the visual cells took place, a small proportion of the rods shed membranes while their tips were still above the ends of some cone outer segments. These rods were usually closely associated with ameboid phagocytes. By 2200 h, the rods were situated well below the cones, and no additional membrane shedding by rods was observed during the remaining hours of the dark period.

#### *Shedding of Membranes by Cones*

The time-course of disk shedding by cones is complementary to that of rods (Fig. 2 and Table I). During the light period, it approached 0. However, during 1-4 h after the lights went off, there was a burst of membrane shedding from the cone outer segments. The number of cone phagosomes then declined steadily until, at 0600 h, when the lights went on, it once again was near 0. Most of the fresh phagosomes were seen in the first few hours of darkness (Figs. 7-9). During the latter stages of the dark period, the shed clusters of membranes gradually decreased in size as digestion proceeded. Since the frequency of rod shedding during the night was negligible, these partially degraded phagosomes were presumed to be remnants of the earlier burst of cone shedding. The time of peak cone shedding was somewhat variable. In the experiment just described, it occurred about 4 h into the dark period. In another experiment, it took place 1 h after the lights went off.

#### *Ameboid Phagocytes*

The disposal of rod and cone apical membranous disks was achieved in two different ways. In some cases, groups of disks were detached from the tips of the outer segments, then degraded

TABLE I  
*Membrane shedding by rods and cones at different times of day*

Time	Length of retina examined	Mean inclusions from rods per millimeter retina*	Mean inclusions from cones per millimeter retina*
	<i>mm</i>		
1 h after the lights have gone on (0700 h)	8.9	245 ± 26	0
10 h after the lights have gone on (1600 h)	8.6	23 ± 6	0.1 ± 0.1
4 h after the lights have gone off (2200 h)	5.3	0	75 ± 9
10 h after the lights have gone off (0400 h)	6.8	0	10 ± 3

\* The values shown are means ± one standard error. Data from four fish were averaged at each time.

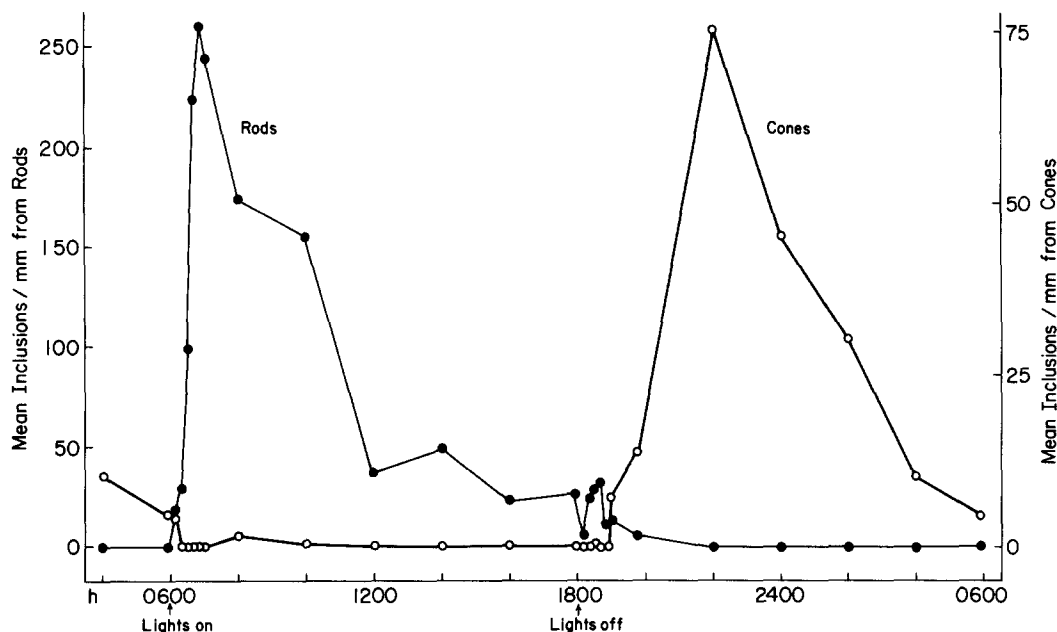


FIGURE 2 Mean number of inclusions (phagosomes) derived from outer segment membranes at different times of day. Each point represents the mean of counts from four fish, including all phagosomes larger than  $1 \mu\text{m}$  in the pigment epithelium and ameboid phagocytes. Ordinates have been adjusted in height to compensate for the different numbers of rods and cones per millimeter of retina.

within the cytoplasm of the pigment epithelium (Figs. 3, 5, and 9). This is the "conventional" mechanism, described in all previous reports of membrane shedding by visual cells. The second method, which has not been described before now, involves the participation of ameboid phagocytes, which remove and digest rod and cone photoreceptive membranes (Figs. 4, 6, 7, and 8).

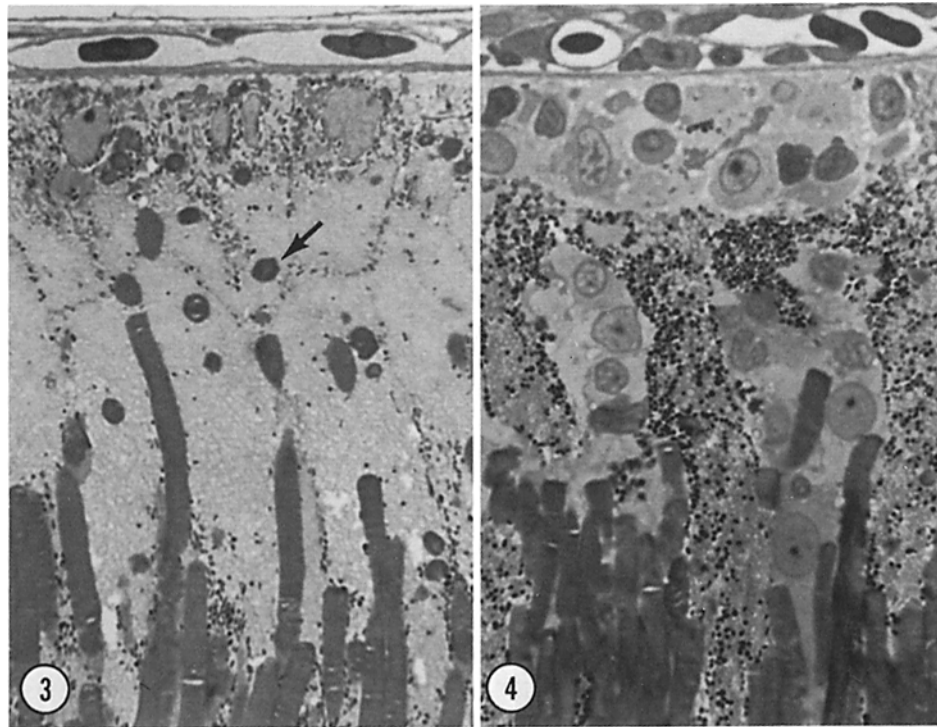
The population of ameboid cells includes several morphologically different types, which vary in size, cytoplasmic appearance and volume, nuclear configuration, chromatin pattern, and staining properties (Figs. 9 and 10). Except when engaged in membrane removal, the ameboid cells are generally inconspicuous, and lie close to the basal lamina of the pigment epithelium, singly or in small clumps. Their cytoplasm is usually devoid of inclusions larger than  $1 \mu\text{m}$  during these intervals.

During periods of peak shedding, the cells move towards the rods and cones, until they contact the outermost layer of outer segments. The phagocytes may contact only the tips of the outer segments, or they may envelop nearly their entire length. Some ameboid cells extend cytoplasmic processes to the outer segments, without migrating bodily to that level. Rarely, a phagocyte may

penetrate beyond the outer tier of outer segments to the inner layer of visual cells. They appear capable of moving freely among the pigment epithelial cells and their processes.

Phagosomes in various stages of degradation soon appear in the cytoplasm of the ameboid phagocytes. The fresh phagosomes within the phagocytes vary widely in size. Some of them represent as much as 25% of an intact outer segment. At progressively later intervals after the periods of peak shedding, the inclusions in the phagocytes decline in size and number. The phagocytized membranes stain intensely with toluidine blue, and the membranous disks become more disordered, compacted, and opaque as digestion proceeds.

The ameboid phagocytes appear to be derived from two sources. They can multiply within the retina, as revealed by the presence of mitotic figures. However, their major source seems to lie outside the retina. Cells resembling the phagocytes may be observed in the capillaries and connective tissue of the adjacent choroid layer. Moreover, occasional phagocytes can be found penetrating the complex basal lamina (Bruch's membrane) which separates the retina from the



FIGURES 3-4 Light micrographs of rod outer segments 1 h after the lights have gone on. In Fig. 3, several phagosomes (arrow) appear in the pigment epithelium. In Fig. 4, aggregations of ameboid phagocytes contact rod outer segments.  $\times 1,260$ .

choroid. Some of these cells contain phagosomes (Fig. 10).

#### DISCUSSION

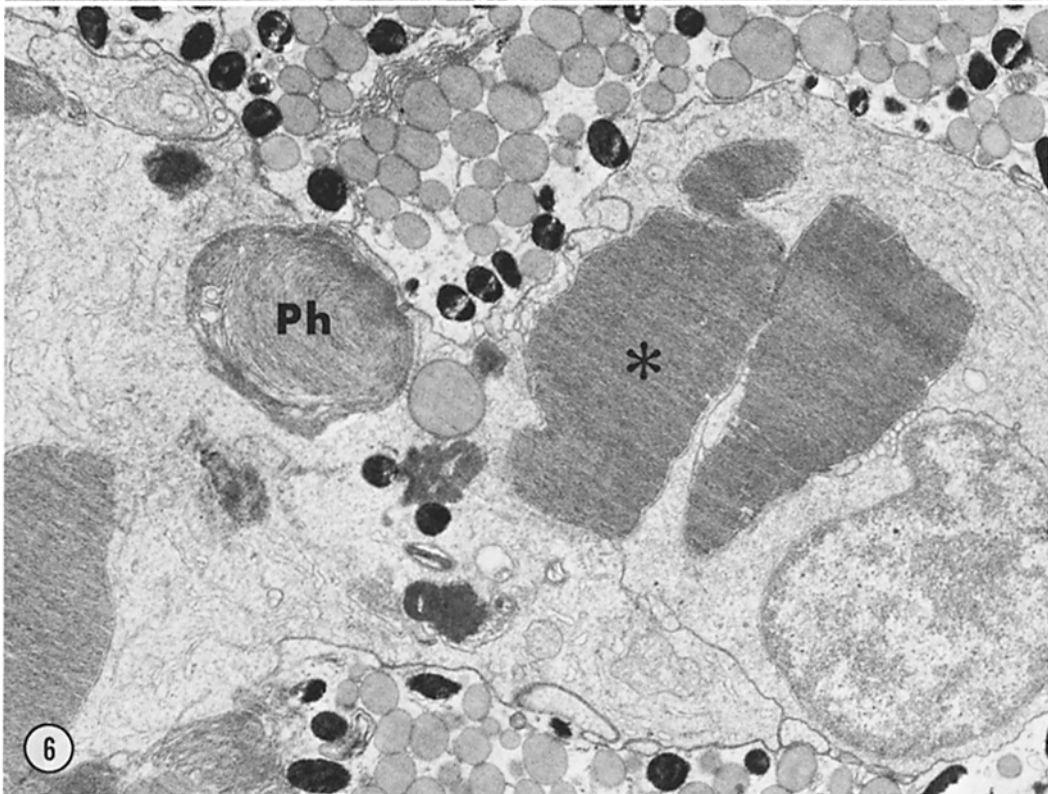
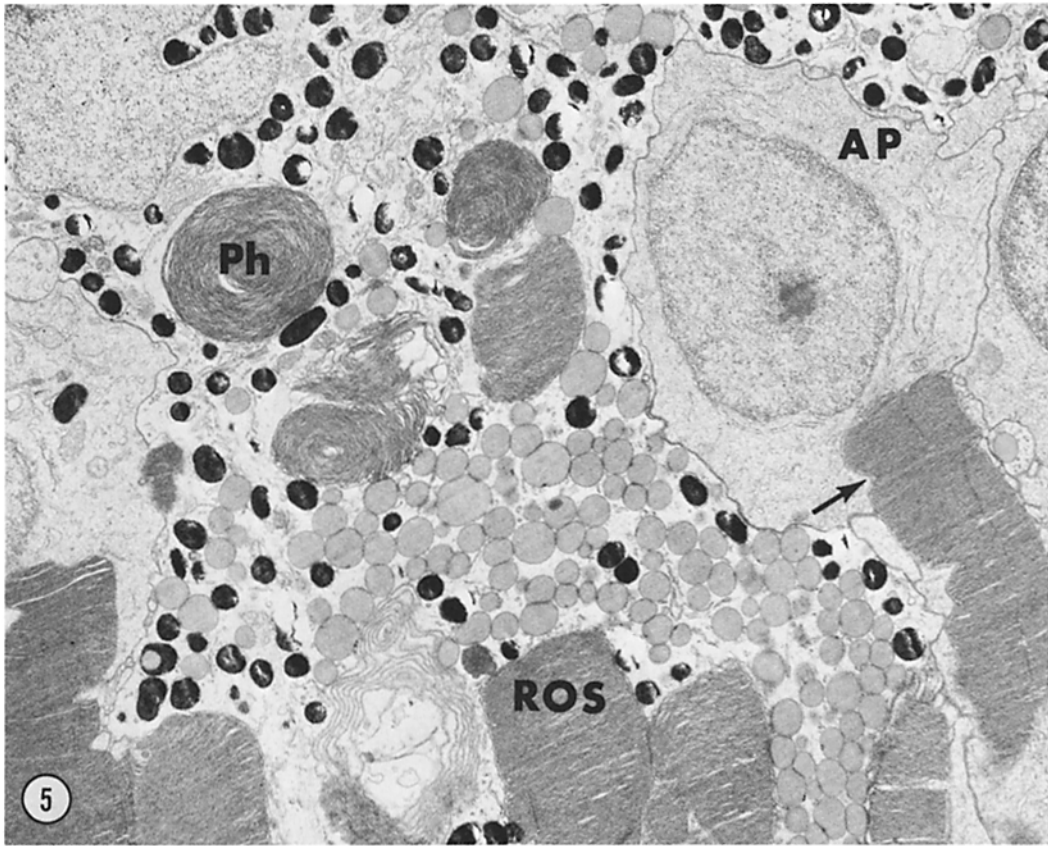
Both rods and cones in the goldfish regularly eliminate groups of photosensitive membranes from the ends of their outer segments. There seems to be no fundamental difference between the two classes of visual cells in the mechanism of membrane disposal. However, there is a significant difference in the time of day when these events take place. The membrane disposal process of rods and cones is separated in time by approx.

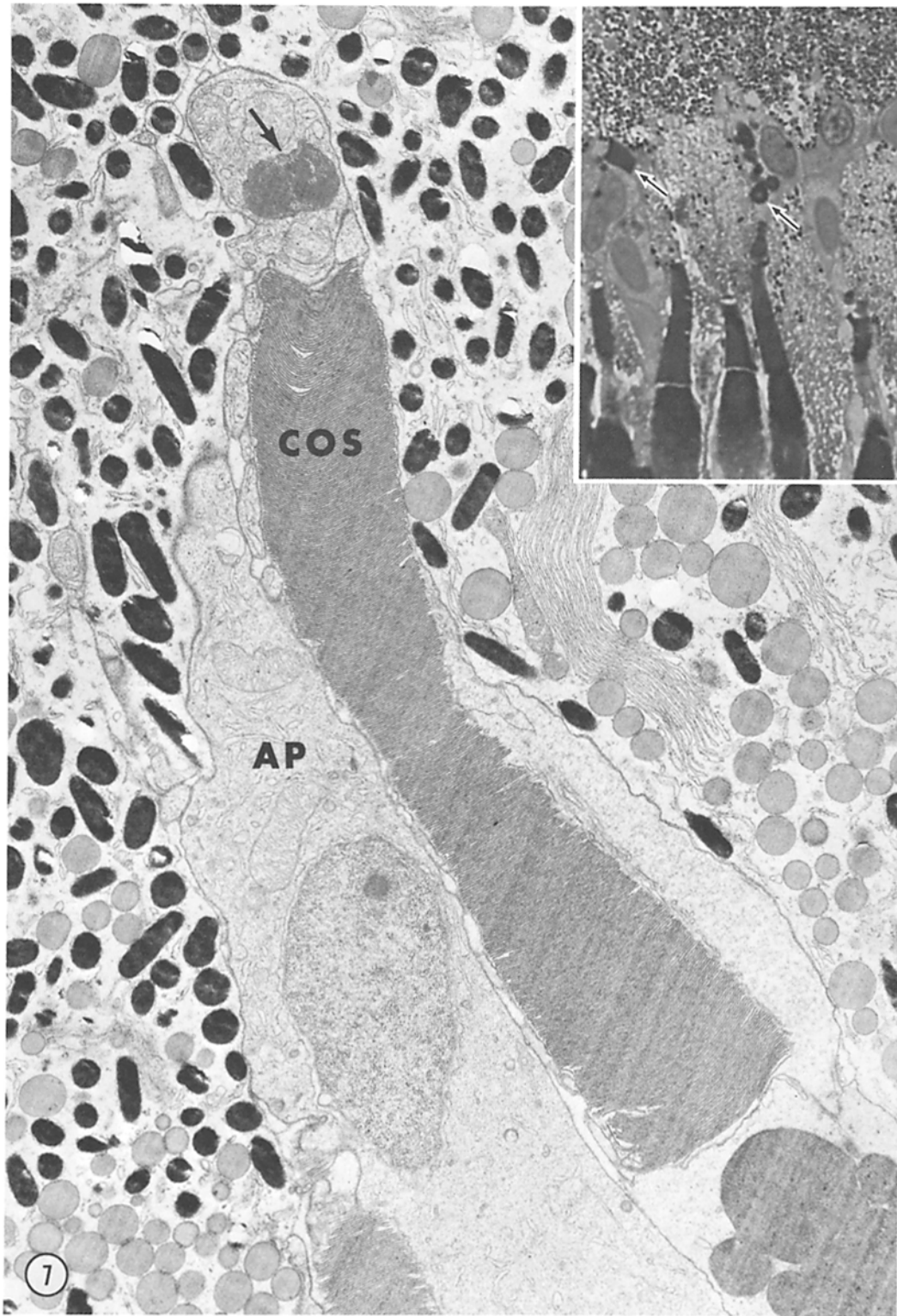
12 h. It occurs with a daily rhythm, and the rhythms of rods and cones are out of phase by one-half day. The origin of this difference may be traced to Schultze's century-old generalization (9) which associated rods with vision during the dim light of the night and cones with vision during the bright light of daytime. Our observations on goldfish photoreceptors reveal that rods shed membranes at the beginning of the day, whereas cones shed membranes early in the night. The two kinds of visual cells discard membranes shortly after they have ceased to mediate vision. Thus, the major degradative phase of the outer segment

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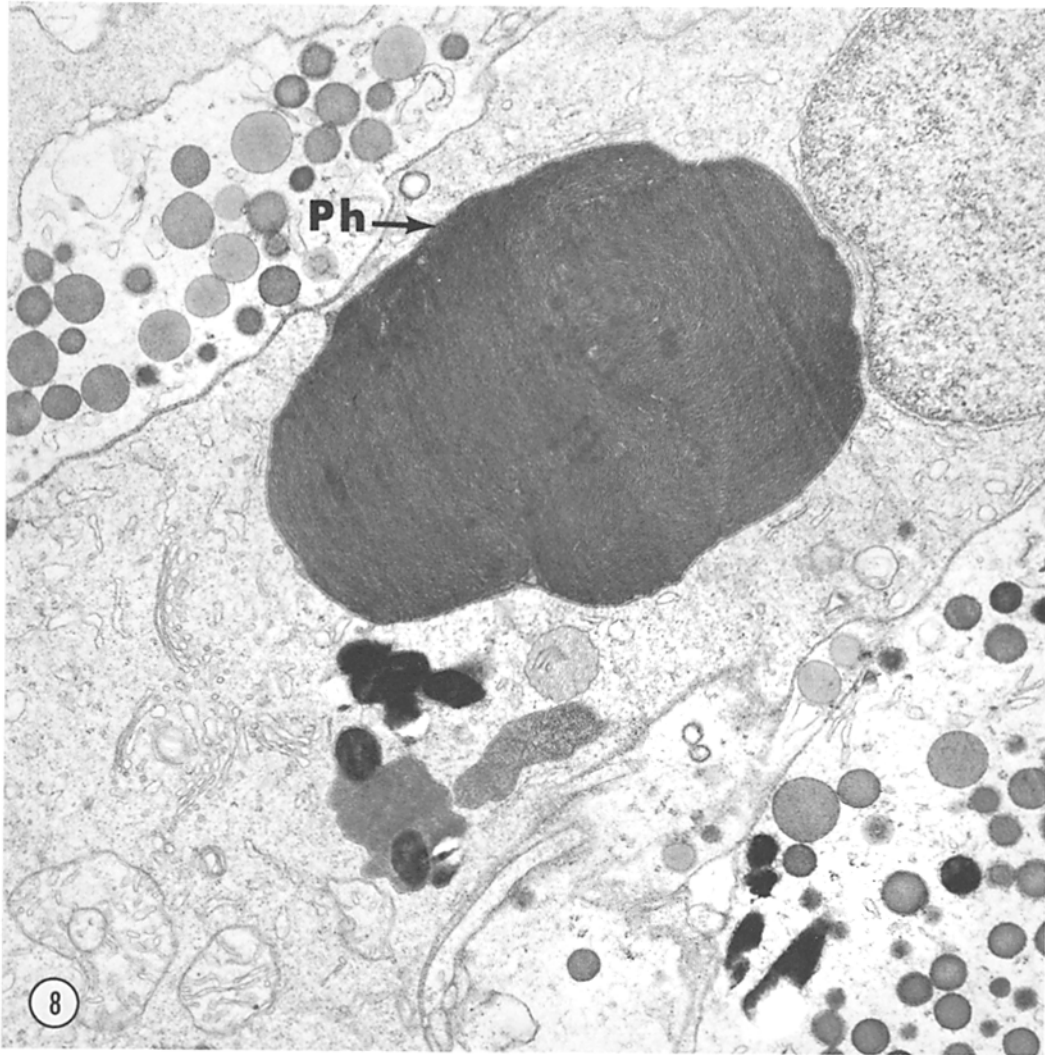
FIGURE 5 Phagosomes (*Ph*) near the tips of rod outer segments (*ROS*) 1 h after the lights have gone on. The cytoplasm of the pigment epithelial cell which encloses the phagosomes also contains numerous melanin granules and spherical tapetal inclusions. An ameboid phagocyte (*AP*) enfolds the tip of a rod outer segment, whose apical disks appear to be in an initial stage of detachment (arrow).  $\times 6,100$ .

FIGURE 6 Portions of two ameboid phagocytes 1 h after the lights have gone on. A partially digested phagosome (*Ph*) lies in the cytoplasm of one ameboid phagocyte. The more recently detached tips of two rod outer segments, which have undergone little change in the regular arrangement of their membranous disks (\*), are being ingested by another ameboid phagocyte.  $\times 8,100$ .









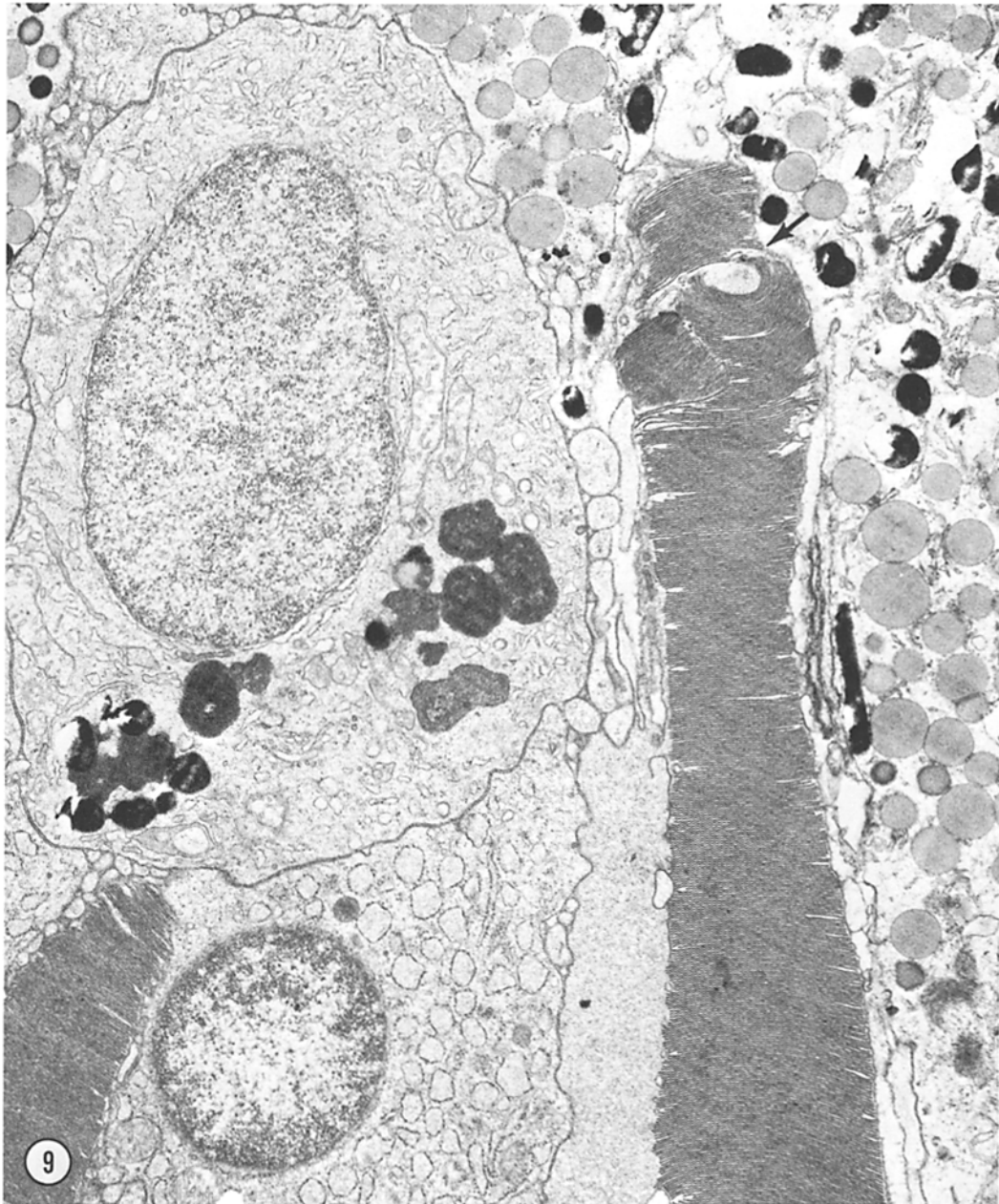
**FIGURE 8** A partially degraded cone phagosome (*Ph*) within the cytoplasm of an ameboid phagocyte, 1 h after the lights have gone off.  $\times 13,500$ .

renewal process in each of the two classes of visual cell complements its normal period of visual function.

In goldfish, the ameboid phagocytes play an important, perhaps essential role in the membrane renewal process of rods and cones. Like the pigment epithelium, they ingest and degrade

membranous disks. They differ from the pigment epithelial cells in their mobility, heterogeneous cellular composition, ability to penetrate Bruch's membrane, and apparently transient residency in the retina. The engorgement of the pigment epithelial cells with tapetal droplets may have reduced their ability to fulfill their normal phago-

**FIGURE 7** A cone outer segment (*COS*) adjacent to an ameboid phagocyte (*AP*). A well-digested phagosome (arrow) appears in the cytoplasm of an ameboid phagocyte above the tip of the cone outer segment. Time: 2400 h.  $\times 16,300$ . *Inset*: appearance of cones, ameboid phagocytes, and phagosomes (arrows) in the light microscope. Time: 1900 h.  $\times 1,260$ .



**FIGURE 9** Disk shedding from the apex of a cone outer segment (arrow). There is no evident participation by the nearby ameboid phagocytic cells. Note the phagocytized material in the cytoplasm of one of the two phagocytes, and the differences in nuclear and cytoplasmic structure.  $\times 9,000$ .

cytic function, so that the ameboid phagocytes are required as a supplementary system for removing the large daily residue of membranous debris. Cells with phagocytic capacities have been observed to invade the mammalian retina in a variety

of pathological conditions involving outer segment degeneration (5, 7, 8, 12, 13). It is therefore conceivable that the ameboid cell system of the goldfish represents an elaboration of a mechanism which is more commonly reserved for emergen-

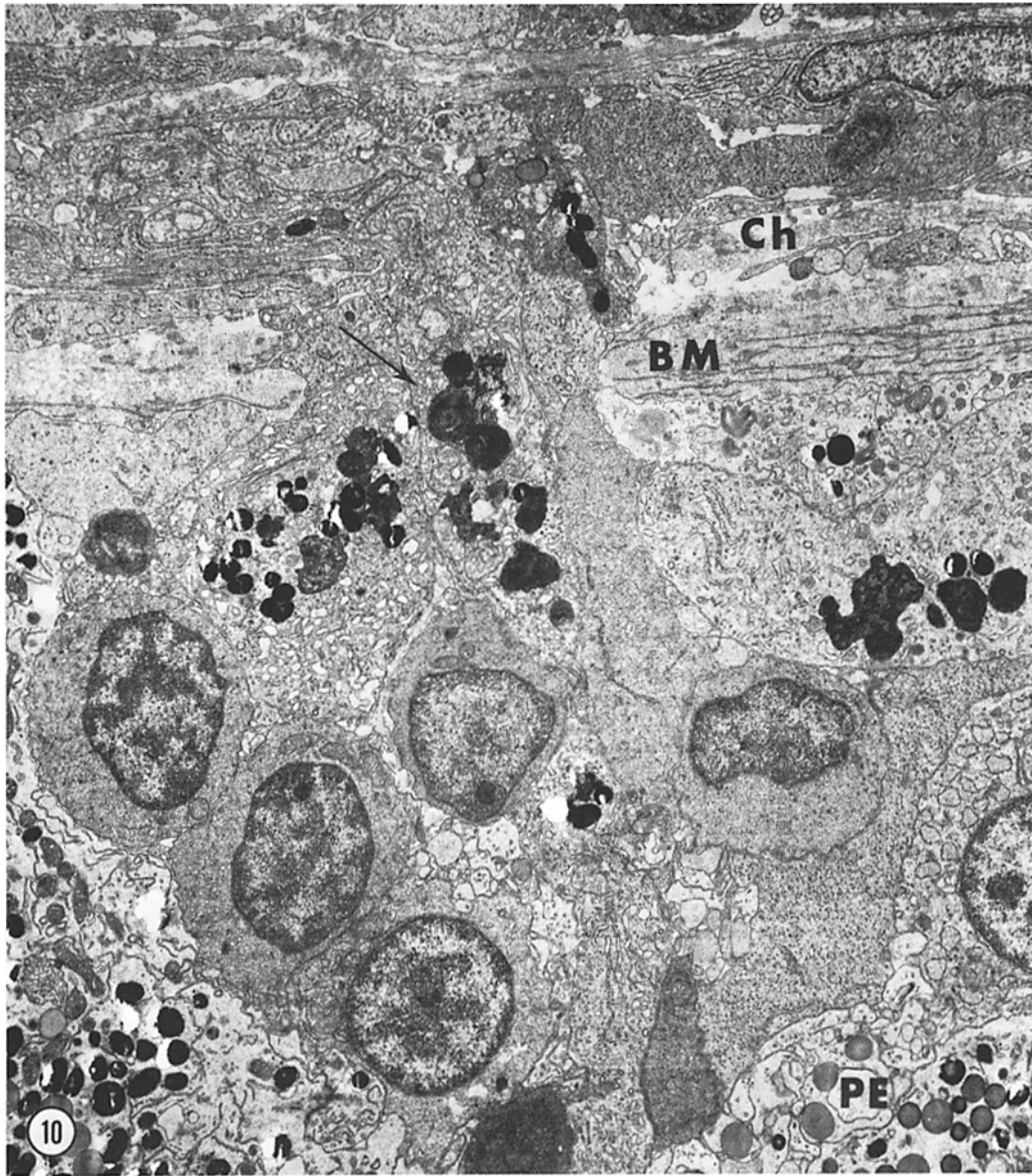


FIGURE 10 Aggregation of ameboid phagocytes penetrating Bruch's membrane (*BM*), which separates the choroid (*Ch*) from the pigment epithelium (*PE*). The presence of phagosomes (arrow) in some ameboid phagocytes suggests that these cells are leaving the retina.  $\times 5,400$ .

cies. The examination of retinas of other vertebrate species with tapetal inclusions in the pigment epithelium may disclose additional examples of regularly occurring ameboid phagocytes in the retina.

To learn whether rhythmic daily shedding of outer segment membranes by rods and cones is a common feature of the vertebrate retina, com-

parable experiments have been carried out in a reptile (a lizard with a retina containing only cones) and a bird (17, 18). The results fully confirm the generality of the observations first made in goldfish. In fish, reptiles, and birds, cones shed membranes early in the dark period. In fish, birds, mammals (the rat: reference 4), and amphibia (the frog: reference 2), rods shed mem-

branes early in the light period. No exceptions to these rules are known. Therefore, we suggest that the daily rhythm of membrane shedding by rods and cones, separated in time by one-half day (as is their participation in visual function), will prove to be a general feature of vertebrate visual cells.

We acknowledge gratefully the important assistance of Mirdza Lasmanis, Zoja Trirogoff, and Myunghee Chun.

This work was supported by grants EY 00095 and EY 00444 from the United States Public Health Service (USPHS), and a USPHS National Research Service Award to Dr. O'Day.

Received for publication 9 June 1977, and in revised form 25 October 1977.

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