Exp. Eye Res. (1980) **30,** 545–557

Rod and Cone Disc Shedding in Light-Entrained Tree Squirrels

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(*Received 31 July 1979, New York*)

Adult tree squirrels (*Sciurus carolinensis*) entrained to a daily lighting cycle of 12L:12D, display a biphasic rhythm of disc shedding. One peak occurs 1–2 hr after light onset. The second peak occurs during the middle of the dark period, about three-quarters of a cycle out of phase with the morning peak. Rod disc shedding predominates in the morning, and cone shedding predominates during the dark, but both cell types shed discs throughout the cycle. These findings indicate that rod shedding in this species is temporally linked to light onset in a way consistent with earlier reports on a variety of other species. On the other hand, cone shedding is not temporally related to the onset of darkness in the same way that rod shedding is related to the onset of light.

Key words: rods; cones; pigment epithelium; phagocytosis; retina; tree squirrel.

1. Introduction

The cyclic nature of vertebrate photoreceptor renewal was first suggested by LaVail (1976) who reported that a synchronized burst of disc shedding occurs within the first 2 hr of light onset in albino rats entrained to 12L:12D. The burst persists in rats kept in constant darkness and is, therefore, a circadian rhythm (LaVail, 1976; Tamai, Teirstein, Goldman, O'Brien and Chader, 1978). A similar burst of shedding occurs in amphibian retinas soon after light onset, but species differences are apparent in this class of vertebrates regarding the circadian nature of this event. Besharse, Hollyfield and Rayborn (1977) have shown that rod shedding is a circadian rhythm in *Xenopus laevis* tadpoles maintained under conditions of constant darkness. However, light onset is required for a synchronized burst of rod shedding in adult *Rana pipiens*, although sporadic shedding occurs in prolonged darkness (Basinger, Hoffman and Matthes, 1976; Hollyfield, Besharse and Rayborn, 1976).

Cyclic variations in cone disc shedding have been reported for several non-mammalian species. In the duplex retinas of chick and goldfish (Young, 1978a; O'Day and Young, 1978), a peak of cone disc shedding occurs during the early part of the dark period in light-entrained animals. Disc shedding in the all-cone retina of Western fence lizards (*Sceloporus occidentalis*) occurs at a comparable time under similar conditions (Young, 1977).

We have examined the duplex retinas of tree squirrels entrained to a lighting cycle of 12L:12D. Our original hypothesis was that two peaks of disc shedding activity should be apparent in a mammal with a duplex retina possessing nearly equal numbers of rods and cones (Cohen, 1964; Anderson and Fisher, 1976), that is, a peak of rod shedding should take place immediately after light onset, and a peak of cone shedding should occur within the first few hours of the dark period. Our results indicate that the temporal pattern of cone shedding in tree squirrels is different than in other

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0014–4835/80/050545+13 $01.00/0

species, and is not related to light offset in the same way that rod shedding is related to light onset.

2. Methods and Materials

Animals

Twenty-one adult Eastern gray squirrels (*Sciurus carolinensis*; Starling Squirrel Ranch, Neches, Texas) of both sexes were used in this study. The animals were maintained in a controlled temperature room (21°C) under conditions of cyclic lighting (12L:12D) for periods ranging from 2 weeks to several months. Room illumination (350 lx) was provided by overhead fluorescent lights. Food and water were available at all times; the diet consisted of conventional lab chow supplemented with peanuts, apples and oranges.

One animal was fixed at each of the following times [measured from the time of lights-on, which is 0000]: 0030 (30 min following light onset); 0100 (1 hr following light onset); 0200; 0300; 0400; 060Q; 0800; 1000; 1200 (lights-off); 1230 (30 min after lights off); 1400; 1500; 1600; 1700; 1800; 2000; 2200; 2300. Two animals were fixed at 1300 and 1900.

Fixation

A single method of fixation was employed throughout this study. All animals were anesthetized with a lethal dose of sodium pentobarbital (Nembutal, 50 mg/ml, Abbott) 5–10 min prior to the specified time of killing. Animals were killed by intracardiac perfusion of an aldehyde mixture consisting of 1% paraformaldehyde and 1% glutaraldehyde in 0·086 M-phosphate buffer (pH 7·1) containing 0·05% calcium chloride. Each animal was perfused with 300–400 ml of perfusing medium at a pressure of 80–90 Torr. Following perfusion, both eyes were removed and the anterior one-half of the globe was cut away. The eyecups were immersed in fresh fixative for 4–12 hr at 4°C. The tissue was washed briefly in isotonic buffer, and then postfixed for 90 min in 2% OsO_4 in veronal acetate buffer (pH 7·4). The tissue was washed briefly in distilled water, and dehydrated through a graded ethanol–water series and propylene oxide. The eyecups were cut into quadrants, infiltrated with propylene oxide and Araldite (1:1), and embedded in pure Araldite (Cargille 6005).

Animals killed during the dark period (1230–2300) were anesthetized in the dark under dim red light (Wratten no. 2 filter, Kodak). The eyes were shielded from light by a light-tight hood placed over the animals' head before being brought into the light; the hood was not removed until perfusion was complete. The animal killed at 1200 (lights off) was anesthetized in the light at 1150; the hood was placed over the animal's head at the beginning of perfusion, at 1200.

Microscopy

Sections were cut either on a Porter–Blum MT-2B Ultramicrotome or an LKB Ultratome III. Thick sections (1·0 μM) for phagosome counts were taken from well-aligned blocks oriented in the longitudinal plane of the photoreceptor outer segments. Sections were placed on glass slides and stained with either toluidine blue or a mixture of methylene blue and azure II, in 1% sodium borate. Thin sections (500–700 Å) were taken from the same blocks used for the phagosome counts. The sections were placed on 75×300 mesh grids, stained with uranyl acetate (20 min) followed by lead citrate (10 min), and examined in a Siemens Elmiskop 1A electron microscope.

Phagosome counts

Phagosomes were identified using a combination of light and electron microscopy. All phagosome counts were made by one of us (G A T) in the light microscope using a ×100 oil immersion objective. A mean number of phagosome/mm of retinal pigment epithelium

(RPE) was obtained for each animal by counting a 2 mm length of RPE in each of 20 well-aligned, 1·0 μm sections; thus, a total of 40 mm of RPE was examined for each animal. Each section used for counting was separated from its nearest neighbor by at least 3–4 μM to avoid duplication of the counts. In most cases, counts were made on portions of the RPE taken from within 2 mm of the posterior pole of the eyecup, but occasionally, counts were taken from more peripheral regions. All phagosomes were counted as a single population since no clearcut differences based on size were apparent in any of the animals.

3. Results

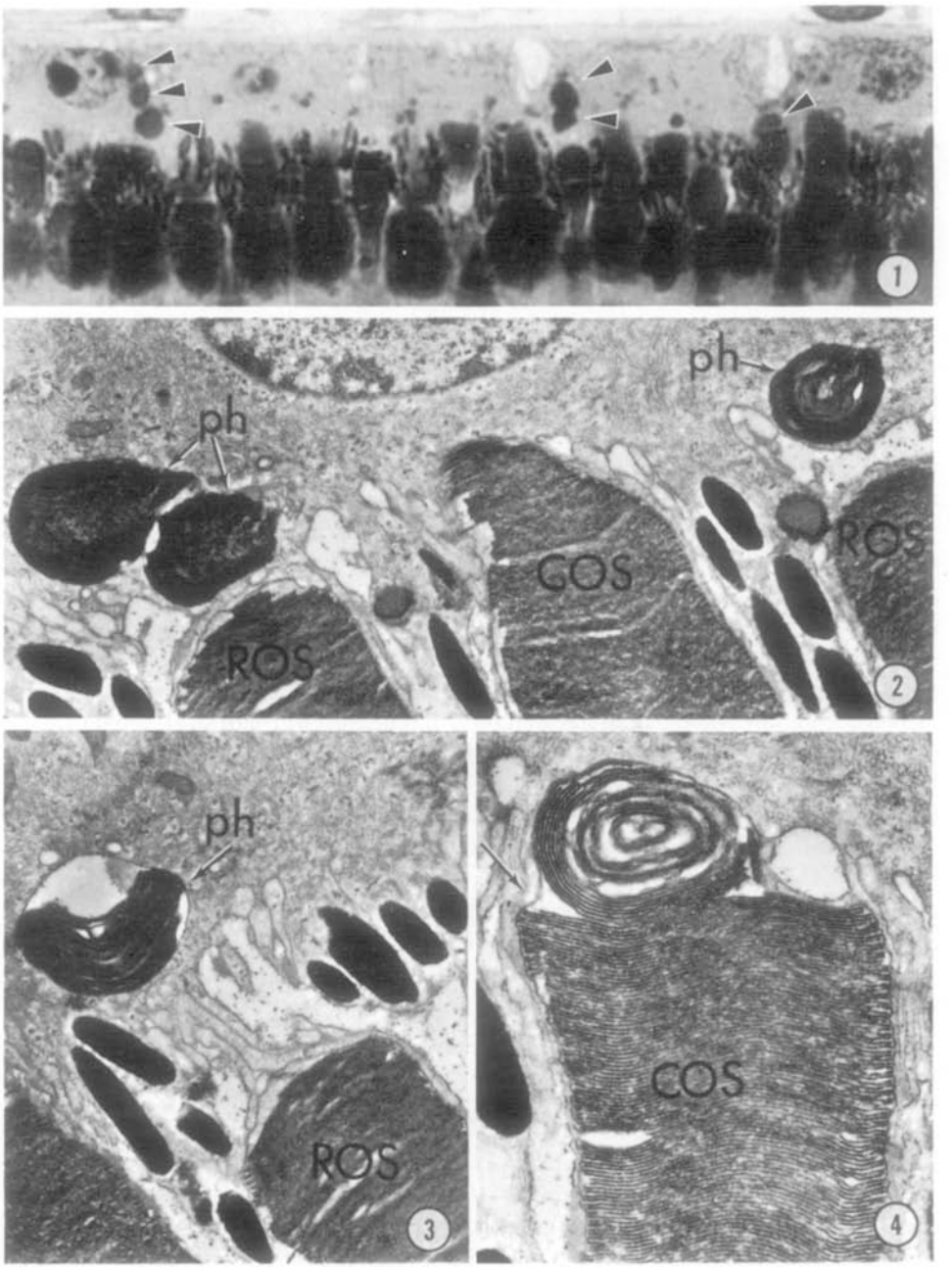
Rods and cones in the tree squirrel retina are clearly distinguishable in the light microscope. The inner segments are arranged in two tiers, with rod inner segments being vitreally displaced with respect to cone inner segments (see Cohen, 1964; Anderson and Fisher, 1976). The RPE appears very similar in animals killed during the middle of the light period or the early part of the dark period. Premelanosomes, melanosomes, melanolysosomes, and residual bodies are among the inclusions commonly found. Phagosomes occasionally seen at these fixation times are located in the apical, midregion, or basal RPE. Phagosomes can easily be distinguished from other RPE inclusions using a combination of light and electron microscopy. In the light microscope, phagosomes appear as 1–2 μM diameter, roughly spherical or oblong structures, which show a similar or slightly increased affinity for the basic stains than do the outer segments. Melanolysosomes appear as large, spherical accumulations of melanin granules. Premelanosomes show a reddish-brown, elliptical profile in the light microscope, and are somewhat larger than mature melanin granules. Residual bodies are small (0·75–1·0 μM), irregularly-shaped structures containing one or more dark granules, (examples of these inclusions are shown in Figs 1, 5, 8, 9 and 12).

Shortly after light onset, there is a striking increase in the number of phagosomes located in the RPE apical cytoplasm (Figs 1, 2). They show little evidence of enzymatic breakdown. The disc membranes are easily recognized although an increase in electron density is sometimes apparent (Figs 2, 3). Occasionally, phagosomes that have been recently engulfed show little or no lamellar organization, but in most cases, this is due to the plane of section (Fig. 2).

The animal fixed at 0100 (1 hr following the onset of light) showed the maximum of phagosomes during the light period (Fig. 5). The majority of phagosomes was found in the apical RPE above the tips of rod outer segments (ROS) (Figs 5, 6). In well-aligned sections, an increase in the distance between the RPE apical border and ROS tips due to detachment of a disc packet can be seen (Figs 3, 5).

Rod phagosomes usually contain 35–40 discs (Figs 2, 3, 6). The discs may be partially or fully curled (Figs 2, 3), or they may be oriented similarly to the outer segment discs (Fig. 6). An electron-lucent zone containing extracellular-like material is apparent in many newly-ingested rod phagosomes (Figs 3, 5). It is common to find multiple phagosomes located above a single ROS tip. When this occurs, each phagosome contains the usual complement of disc membranes (Figs 1, 2). Occasionally, phagosomes are found at deeper locations in the RPE at these early fixation times (Figs 1, 5).

Cones are observed in various stages of disc shedding during the peak of shedding in the morning, but the number is low compared to rods. An early stage of cone disc shedding is seen in Fig. 4; RPE microvillous processes follow the curvature of the



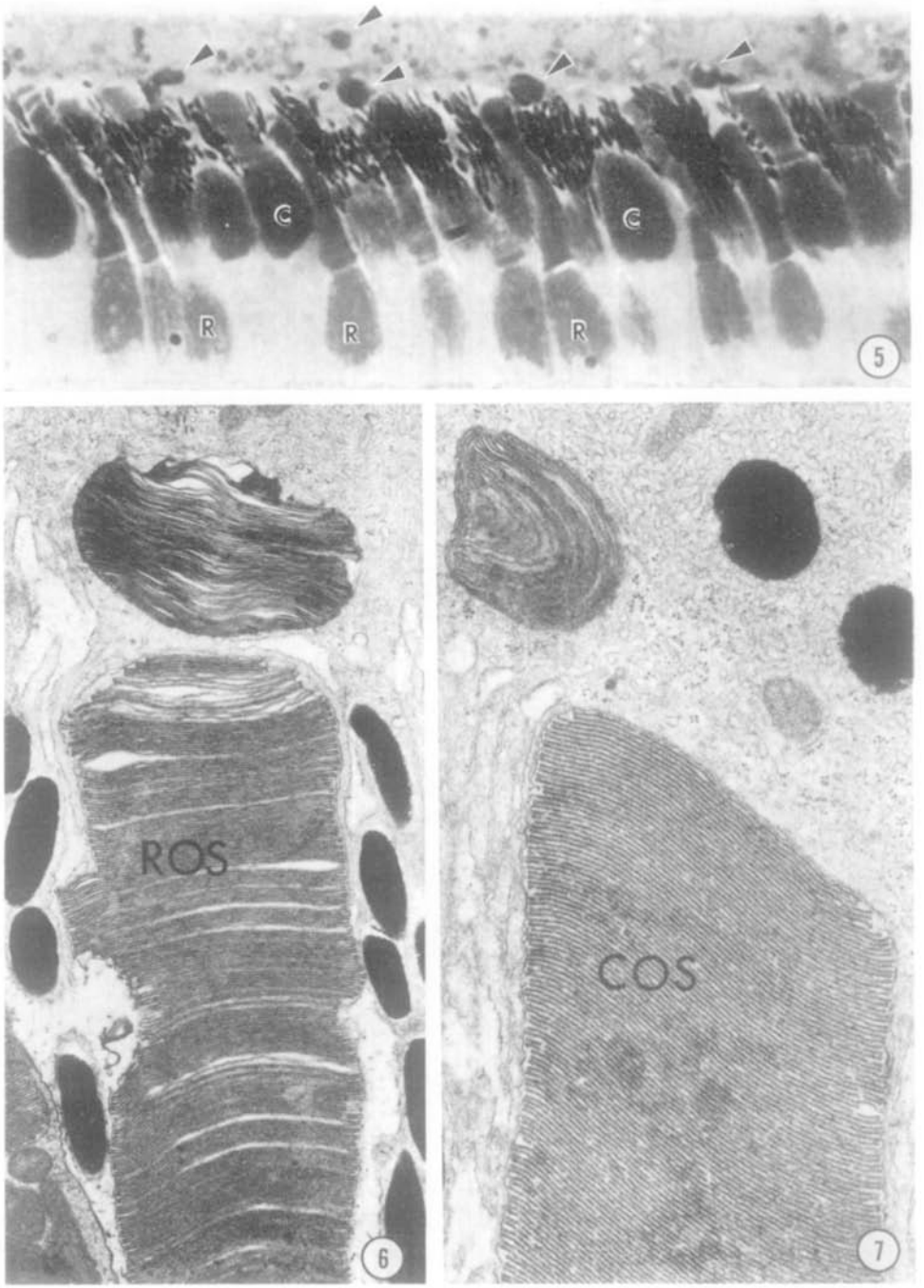
FIGS 1–4. Light and electron micrographs of the animal killed at 0030.

FIG. 1. Light micrograph of the photoreceptors and RPE showing numerous phagosomes (arrowheads) located primarily in the apical region of the RPE cytoplasm (×1170).

FIG. 2. Electron micrograph of a region of the RPE adjacent to the previous figure. Phagosomes (ph) that have been recently engulfed lie in the apical RPE above the tips of rod outer segments (ROS). The rod at the left of the figure has given rise to two phagosomes. (×10 800).

FIG. 3. A phagosome containing intact disc membranes lies in the apical cytoplasm of the RPE above a ROS tip. An electron-lucent zone is apparent within the phagosome and is probably due to engulfment of extracellular material during phagocytosis of the disc packet (×10 800).

FIG. 4. In this electron micrograph, a packet of curled discs is seen at the tip of a COS. The arrow marks an apical RPE process which follows the invaginating plasma membrane of the COS (×18 000).



FIGS 5–7. Light and electron micrographs of the animal killed at 0100.

FIG. 5. Light micrograph of the photoreceptors and RPE showing numerous apically located phagosomes (arrowheads). Most of the phagosomes overlie rod outer segments (R, ROS) tips: the phagosome at the far left of the figure is probably derived from a cone outer segment (C, COS) which is only partially in the plane of section (×1160).

FIG. 6. Electron micrograph of a recently engulfed phagosome containing well-preserved discs. The phagosome is located in the apical cytoplasm of the RPE above the tip of an ROS. Other than a slight increase in electron density, the ingested discs appear identical to the outer segment discs (×14 400).

FIG. 7. A recently ingested phagosome derived from a COS at 0100 (×21 600).

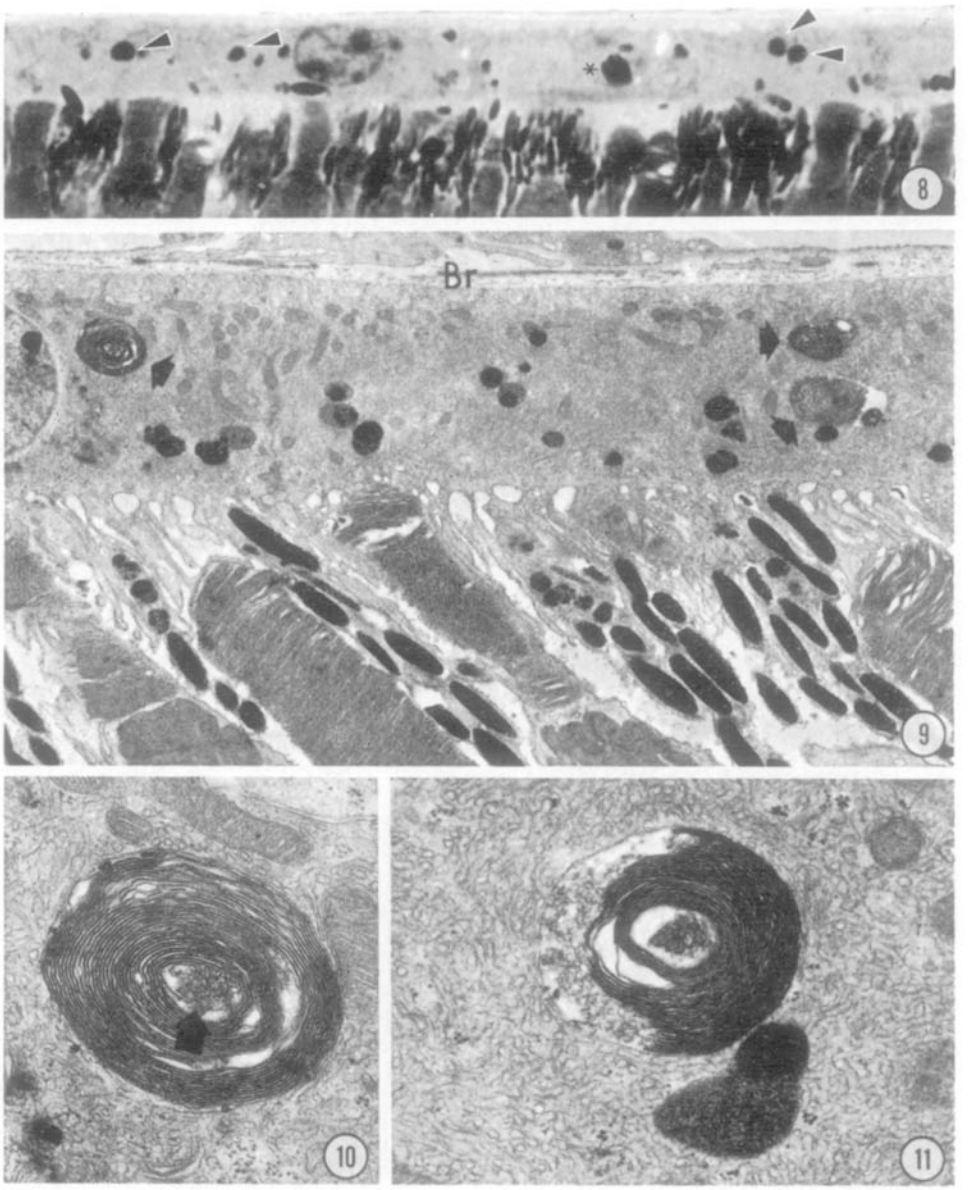


FIG. 8. Light micrograph of photoreceptors and RPE at 0400. The phagosomes (arrowheads) are all located in the mid to basal RPE, and are smaller than those found earlier in the light period. A melanolysosome (asterisk) containing 5–6 melanin granules is present (×2000).

FIG. 9. Electron micrograph from the animal killed at 0200. The phagosomes (arrows) lie in the mid to basal RPE and show ultrastructural evidence of enzymatic digestion. Numerous residual bodies are scattered throughout the RPE. Br, Bruch's membrane (×5850).

FIG. 10. The phagosome seen at the far left of the preceding figure is enlarged in this electron micrograph. A small amount of granular material is seen in the center of the phagosome and is indicative of breakdown of the ingested discs (arrow) (×27 000).

FIG. 11. A later stage of phagosome digestion is seen in this electron micrograph taken from the 0400 animal. It appears that the ingested membranes eventually are replaced by the granular material. A small residual body containing a single dark granule is closely associated with the phagosome (× 27 000).

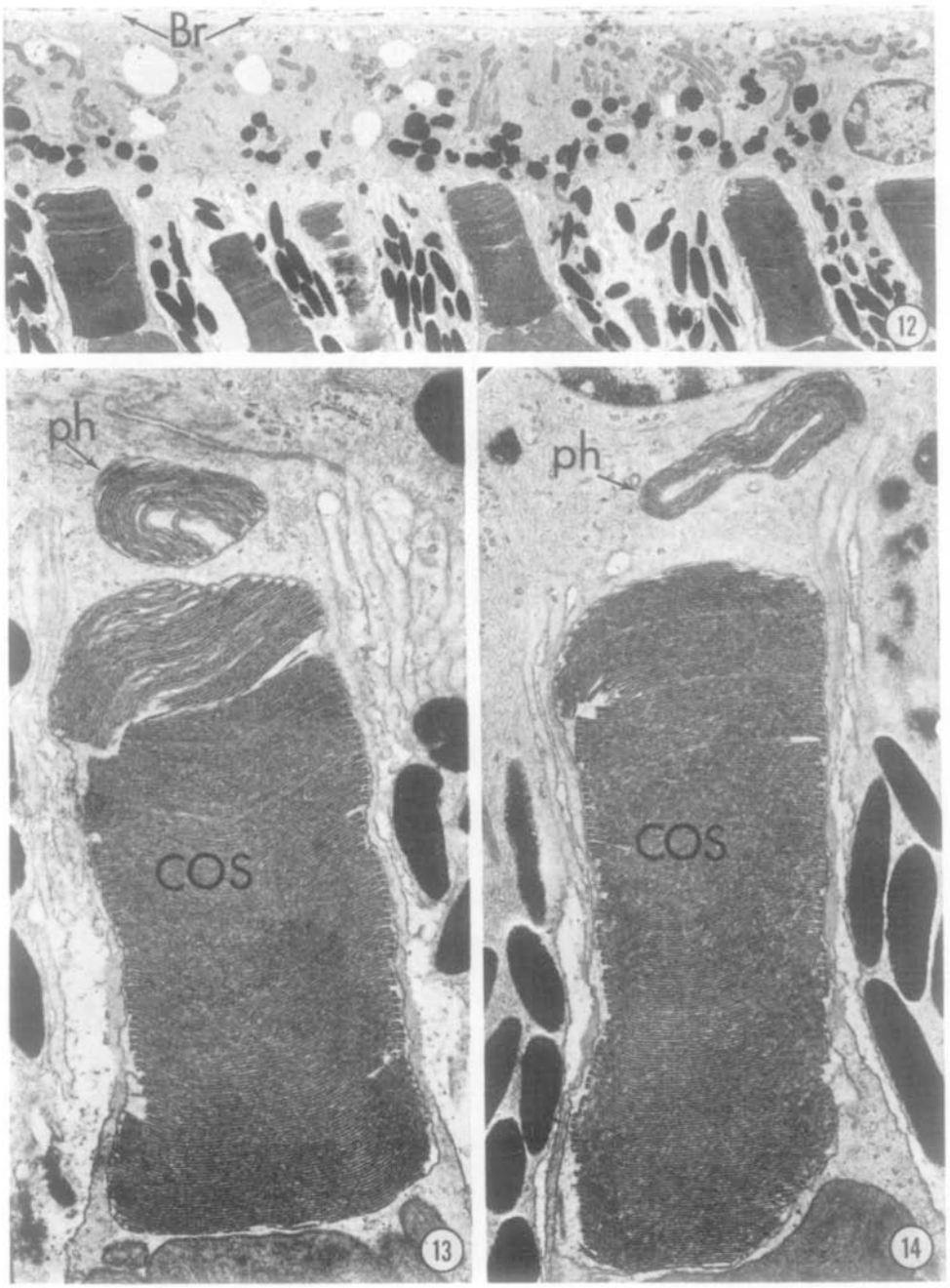


FIG. 12. A low power electron micrograph of the RPE 1 hr following the onset of the dark period (1300). The RPE contains numerous electron-dense inclusions including residual bodies, melanin granules, and melanolysosomes, but is devoid of phagosomes. Br, Bruch's membrane (×3510).

FIG. 13. An occasional phagosome (ph) is seen during the non-peak hours of the shedding cycle (0600–1500). An electron micrograph of a cone outer segment (COS) which has recently shed the disc packet located in the apical cytoplasm of the RPE (×15 750).

FIG. 14. The first evidence of increased cone disc shedding is seen at 1600. This is an electron micrograph showing a phagosome derived from a COS shortly after its engulfment by the RPE. Virtually all of the phagosomes at this time were found above COS tips (×13 500).

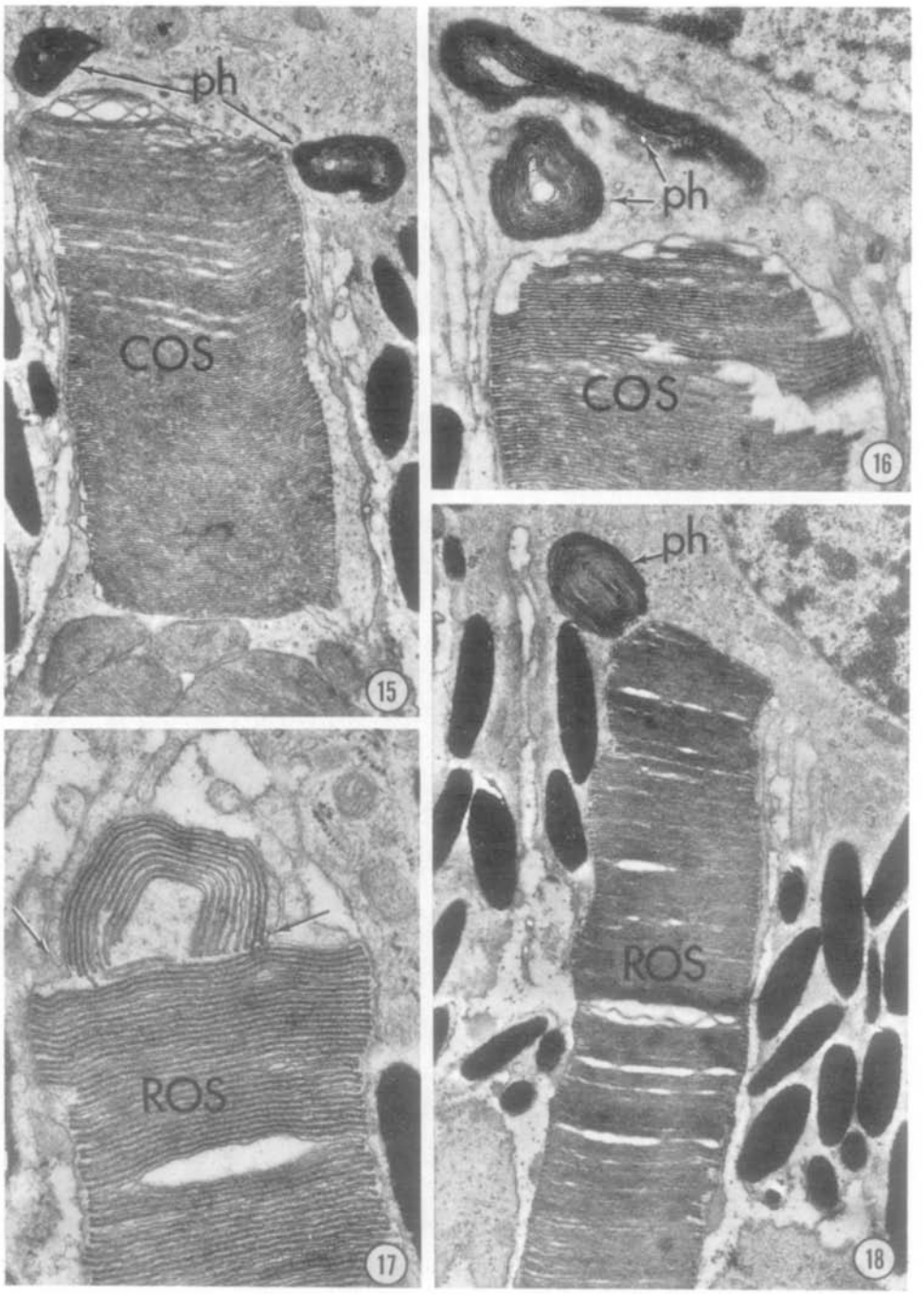
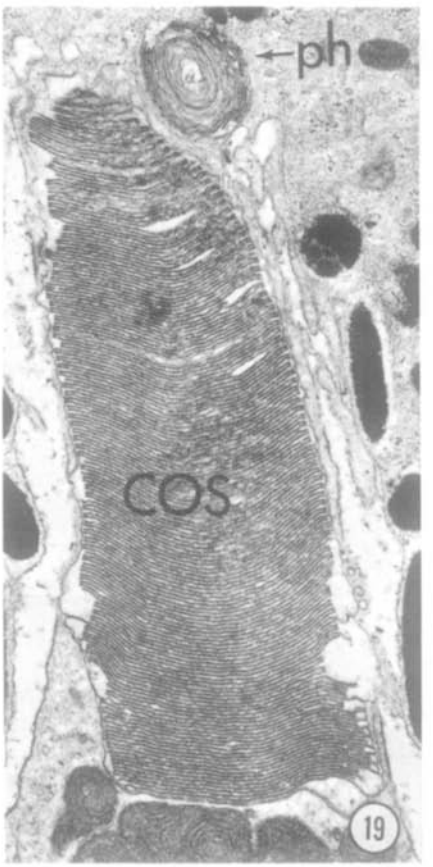


FIG. 15. Most of the cones shedding discs at the peak of shedding during the dark give rise to more than one phagosome as shown in this electron micrograph from the 1900 animal. Two phagosomes (ph) are located in the apical RPE above a single cone tip (×13 500).

FIG. 16. Phagosomes derived from cones are more irregularly shaped than rod phagosomes. This electron micrograph shows two dissimilarly shaped phagosomes overlying a single COS tip (×18 000).

FIG. 17. A packet of curled discs at the tip of a ROS from the 1900 animal. Apical RPE processes follow the curvature of the invaginating plasma membrane of the outer segment (arrows) (×30 700).

FIG. 18. A recently ingested phagosome lies above a ROS tip. Rods are often observed shedding discs during the dark period (×10 800).



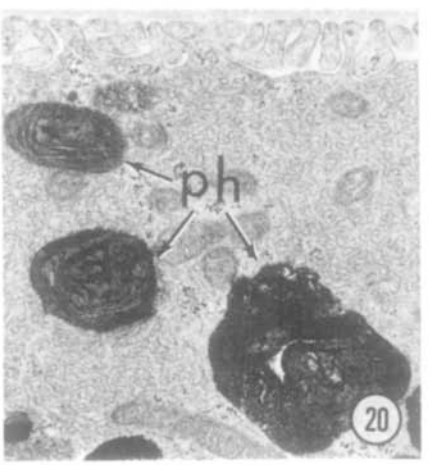


FIG. 19. Apical phagosomes are infrequent during the dark period following the nightime peak. When present, they are primarily above COS tips such as the one shown in this electron micrograph from the 2300 animal (×15 000).

FIG. 20. The majority of phagosomes in the 2300 animal appear to be degraded. In this micrograph, the phagosomes contain highly distorted discs which are electron dense (×17 500).

invaginating plasma membrane of the outer segment. In Fig. 7, a phagosome can be seen above a cone tip.

The majority of phagosomes in the animals killed from 0200–0400 are preferentially located in the mid- to basal RPE. Phagosomes at these times are 1·0–1·5 μM in diameter, and appear in the light microscope as more regularly spherical, densely staining bodies (Fig. 8). Most phagosomes at these fixation times show ultrastructural evidence of enzymatic breakdown (Figs 9–11). Most of the phagosomes present in the animal killed at 0600 are in the late stages of degradation, and are located deep (more basally) within the RPE. Small numbers of rods and cones are observed shedding discs throughout the remainder of the light period, and into the early hours of the dark period. Approximately equal numbers of intact and partially digested phagosomes are present in these animals.

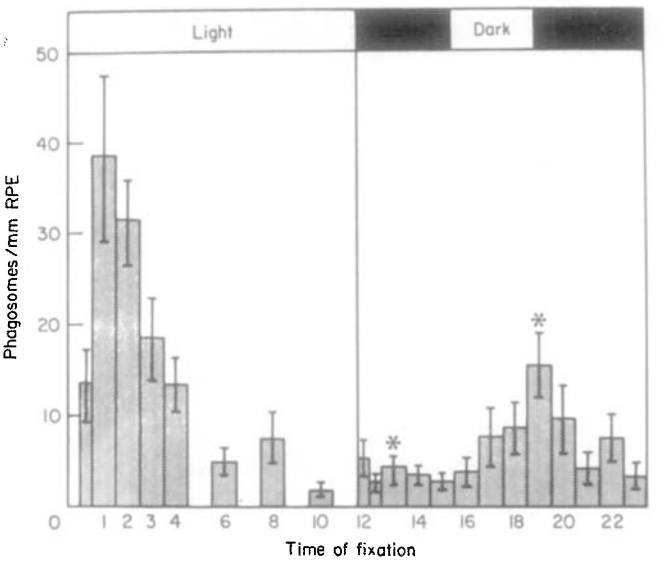


FIG. 21. Histogram showing the pattern of photoreceptor disc shedding in light-entrained tree-squirrels over a 24 hr light–dark period. Each large vertical bar represents the mean total phagosomes per mm RPE obtained from counts of a single animal killed at hourly intervals except for half-width bars which designate animals killed at 30 min intervals. Error bars = 1 standard deviation of the mean. (*) Two animals killed at each of these times.

No increase in disc shedding is observed in animals killed during the first few hours of darkness. Several animals killed from 30 min to 3 hr after lights-off (1230–1500), about one-half cycle out of phase with the morning peak, show accumulations of residual bodies, presumably derived from earlier shedding events, and/or autophagy (Fig. 12). Occasionally, recently ingested phagosomes are present (Fig. 13), but it is equally likely to find partially degraded phagosomes lying deep in the RPE.

The first indication of a second increase in disc shedding is observed in the animal killed 4 hr following the onset of darkness (1600). In this animal, the majority of phagosomes lie above COS tips and appear to have been recently engulfed (Fig. 14). Phagosomes during the dark period are 1·0–2·0 μM in diameter, more irregularly shaped, and contain fewer discs (15–20) than those seen during the light period.

The maximum number of phagosomes during the dark period occurs at 1900. Interestingly, many of the cones shed more than one disc packet (Figs 15, 16).

Whether or not this occurs, the number of discs shed by individual cones appears roughly equivalent. Invariably, a few rods are also observed shedding discs during the dark period. Different stages in rod shedding are seen in Figs 17 and 18, taken from the animal fixed at 1900.

Fewer phagosomes are seen in the animals fixed after the night-time peak, from 2000–2300. Recently shed phagosomes, although infrequent, are usually found above COS tips (Fig. 19). Most of the remaining phagosomes lie in the basal region of the RPE. They appear to be in the final stage of breakdown, with little recognizable disc structure remaining (Fig. 20).

Figure 21 shows the temporal pattern of disc shedding throughout one 24 hr cycle. The phagosome content of the RPE reaches two peaks. A relatively stable baseline level of shedding occurs during the latter part of the light period, and extends into the first few hours of darkness. The morning peak of shedding is nearly 10-fold greater than the baseline phagosome concentration; the night-time peak is smaller, reaching about a 5–6-fold increase over baseline. The two peaks are separated in time by approximately 18 hr, or about 3/4 of a cycle.

4. Discussion

Counts of RPE phagosomes in tree squirrels entrained to a daily lighting cycle of 12L:12D show that two peaks of disc shedding activity occur during a 24 hr period. One peak occurs within the first few hours following the onset of light. The other peak occurs around the middle of the dark period, more than 6 hr after lights-off. The timing of the peaks in the cycle cannot be established precisely since the data in Fig. 21 are based upon counts taken from one animal for each fixation point in the cycle. On the other hand, counts taken from two additional animals (one each at 1300 and 1900), confirmed that disc shedding is low 1 hr into the dark period and reaches a maximum around the middle of the dark period. The morning peak is due primarily to rod shedding; cone shedding predominates during the night-time peak, but both cell types shed discs at a low level throughout the cycle (Tabor, Fisher and Anderson, 1979). This evidence indicates that photoreceptor disc shedding in tree squirrels entrained to 12L:12D occurs as a biphasic rhythm superimposed upon a baseline level of rod and cone disc shedding occurring throughout the light–dark cycle.

The appearance of the RPE of animals killed soon after light onset strongly suggests that the morning peak is due to synchronized rod shedding. Within 30 min to 1 hr following light onset (0030–0100), the majority of phagosomes are found at the tips of ROS. An hour or two later, the majority of ingested packets show signs of enzymatic breakdown, and lie deeper in the RPE cell cytoplasm. There is a concomitant decrease in the number of recently formed phagosomes, and the total number of phagosomes drops from the peak value. The period of synchronous shedding occurring during the light period subsides about midday after which time disc shedding in tree squirrels is characterized by a baseline level of shedding involving both photoreceptor types.

The distribution of rod and cone phagosomes in animals killed from midday through the first few hours of the dark period is about equal. The low level of shedding occurring during this time may be analogous to the persistence of a stable population of small ($< 2\,\mu$M diameter) phagosomes seen in amphibian retinas (Hollyfield, Besharse and Rayborn, 1976; Besharse et al., 1977). They report that the population of small phagosomes is independent of cyclic changes in the lighting environment,

and that these phagosomes account for about 20% of the daily increment of rod outer segment shedding (Hollyfield et al., 1976). It is not clear what percentage of outer segment renewal can be attributed to baseline shedding in tree squirrels, since no size differences in this species were apparent between phagosomes found during the peaks of shedding and those found at other times of the cycle.

The temporal relationship between light onset and the morning peak of disc shedding in tree squirrels is consistent with findings in rat (LaVail, 1976; Tamai et al., 1978), frog (Basinger et al., 1976; Hollyfield et al., 1976; Besharse et al., 1977), chick (Young, 1978a), and goldfish (O'Day and Young, 1978). Similar results have been obtained in *Xenopus laevis* larvae (Besharse et al., 1977). Light onset is required for synchronized rod shedding in *Rana pipiens*, although a low level of rod shedding occurs in this species under conditions of prolonged darkness (Basinger et al., 1976; Hollyfield et al., 1976). We have recently found that the morning peak of shedding persists in tree squirrels under conditions of constant darkness and is, therefore, a circadian rhythm (Tabor, Fisher and Anderson, 1979).

A burst of cone disc shedding has been reported for the duplex retinas of chick and goldfish (Young, 1978a; O'Day and Young, 1978), and for the all-cone retina of a lizard, *Sceloporus occidentalis* (Young, 1977). In these animals, cone shedding is almost entirely restricted to the early hours of the dark period. In chick retina, cone disc shedding reaches a peak within one hour following the onset of darkness, about 1/2 cycle out of phase with the peak of rod shedding in the morning (Young, 1978a). The peak of cone shedding in goldfish is displaced from the rod peak by greater than 1/2 cycle; it reaches a maximum about 4 hr following lights-off (see O'Day and Young, 1978), whereas the rod peak occurs about 1 hr into the light period. The maximum concentration of cone phagosomes in lizard occurs about 2 hr into the dark period. Based on these findings, it has been proposed that a temporal relationship exists between cone disc shedding and the onset of darkness that is analogous to the temporal link between rod disc shedding and light onset, and that this is a general property of vertebrate visual cells (Young, 1977, 1978a,b; O'Day and Young, 1978).

In the tree squirrel, cone disc shedding is temporally related to the transition from light to dark in a *different* way than rod shedding is related to light onset (Fig. 21). Rod shedding reaches a peak within 2 hr after lights-on; at least 4 hr elapses between the time of lights-off and the first sign of increased cone shedding. Further, cone shedding in this species cannot be characterized as a synchronized "burst". Rather, the peak number of phagosomes reached during the dark period results from the gradual onset of a period of increased cone shedding which has no close temporal link with the light-to-dark transition. The peak number of cone phagosomes is reached around the middle of the dark period (Fig. 21). In comparison to the morning peak, the events of the dark peak appear to be less synchronous since all stages of phagosome formation and degradation are apparent in larger numbers at all times during the dark peak.

Our evidence suggests that in tree squirrel, the renewal rate for rods exceeds that for cones. First, the morning peak is more than double the night-time peak in amplitude (Fig. 21). Second, phagosomes found during the morning peak contain 2–3 times more discs than those found during the night-time peak. Taken together, these findings suggest that the number of discs degraded by the RPE during a 24 hr period is greater for rods than for cones.

By varying the time of light onset in light-entrained *Rana pipiens*, Basinger, Hoffman and Matthes (1976) have established a direct link between the onset of light

and the onset of rod disc shedding. No such evidence exists for an analogous relationship between cones and the transition from light to dark in any species so far examined. The peak of cone shedding found in chick, goldfish, lizard (Young, 1977, 1978a; O'Day and Young, 1978), and in the tree squirrel serves to establish that cone disc shedding occurs preferentially at specific intervals in the light–dark cycle. However, a direct relationship between cone shedding and the onset of darkness can be drawn only by showing that a consistent temporal relationship exists between the peak of cone shedding and the light–dark transition following changes in the time of dark onset. Until then, it cannot be ruled out that rhythmic cone shedding is entrained by light onset in the way that rod shedding is, but merely exhibits a longer latency.

ACKNOWLEDGMENTS

This research was supported by research grants EY 02082 and EY 00888 from the National Eye Institute.

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