Cell Tissue Res (1985) 239:667–675

Cell and Tissue Research

Cone photoreceptor shedding in the tree shrew (*Tupaia belangerii*)

J.H. Immel and S.K. Fisher

Department of Physiology, University of California, San Francisco; Neurosciences Research Program and Department of Biological Sciences, University of California, Santa Barbara, USA

Summary. Tree shrews were sacrificed at various times during a 12 h light–12 h dark cycle and the retinal pigment epithelium (RPE) was examined for phagosomes. Analysis of photoreceptor densities showed that the tree-shrew retina consists of approximately 96% cone photoreceptors. Therefore, phagosomes in the RPE were assumed to be mostly those of cones. A peak in the number of RPE phagosomes was found about one hour after the onset of light. The number of phagosomes/mm RPE during the light cycle varied from 17.02 at the peak to 2.49 ten hours after light onset. During the dark cycle, values ranged from 0.10 to 0.61 phagosomes/mm RPE. Size profiles of phagosomes showed that large phagosomes peak in number $^1/_2$ h after light onset, while smaller sizes peak at about 1 h after light onset. This may indicate that maximal shedding and phagocytotic activity occurs sometime before the peak in the total number of phagosomes is reached. Statistical corrections for phagosome size, section thickness and phagosomal degradation time were applied to the data in order to assess outer segment renewal time for tree shrew cones.

Key words: Cone – Photoreceptor – Phagocytosis – Shedding – RPE – Tree shrew (*Tupaia belangerii*)

Cyclic shedding of photoreceptor outer segments was first described by LaVail (1976) who reported that rod photoreceptors in albino rats maximally shed disc packets (phagosomes) shortly after the onset of light. Subsequent studies have shown that a burst of rod shedding occurs shortly after light onset in *Rana* (Hollyfield et al. 1976), *Xenopus* (Basinger et al. 1977), chicks (Young 1977a), goldfish (O'Day and Young 1978), gray squirrels (Tabor et al. 1980), opossums (Herman and Steinberg 1982), and cats (Fisher et al. 1983).

Cyclic patterns of *cone* disc shedding show variation across species. In studies of cone shedding in goldfish (O'Day and Young 1978), chicks (Young 1977a), lizards (Young 1977b), and gray squirrels (Tabor et al. 1980), the peak of cone disc shedding occurs in the dark portion of the lighting cycle. In the retinae of lizards and chicks, this peak occurs 2 h after dark onset, while in goldfish it occurs 4 h into the dark period. Gray squirrels show the greatest disparity in dark shedding with a maximum of cone disc shedding occurring over several hours in the middle of the dark period, 7 h after dark onset. In studies of cat photoreceptor shedding, it has been shown that cones as well as rods shed about 1 h after light onset (Fisher et al. 1983).

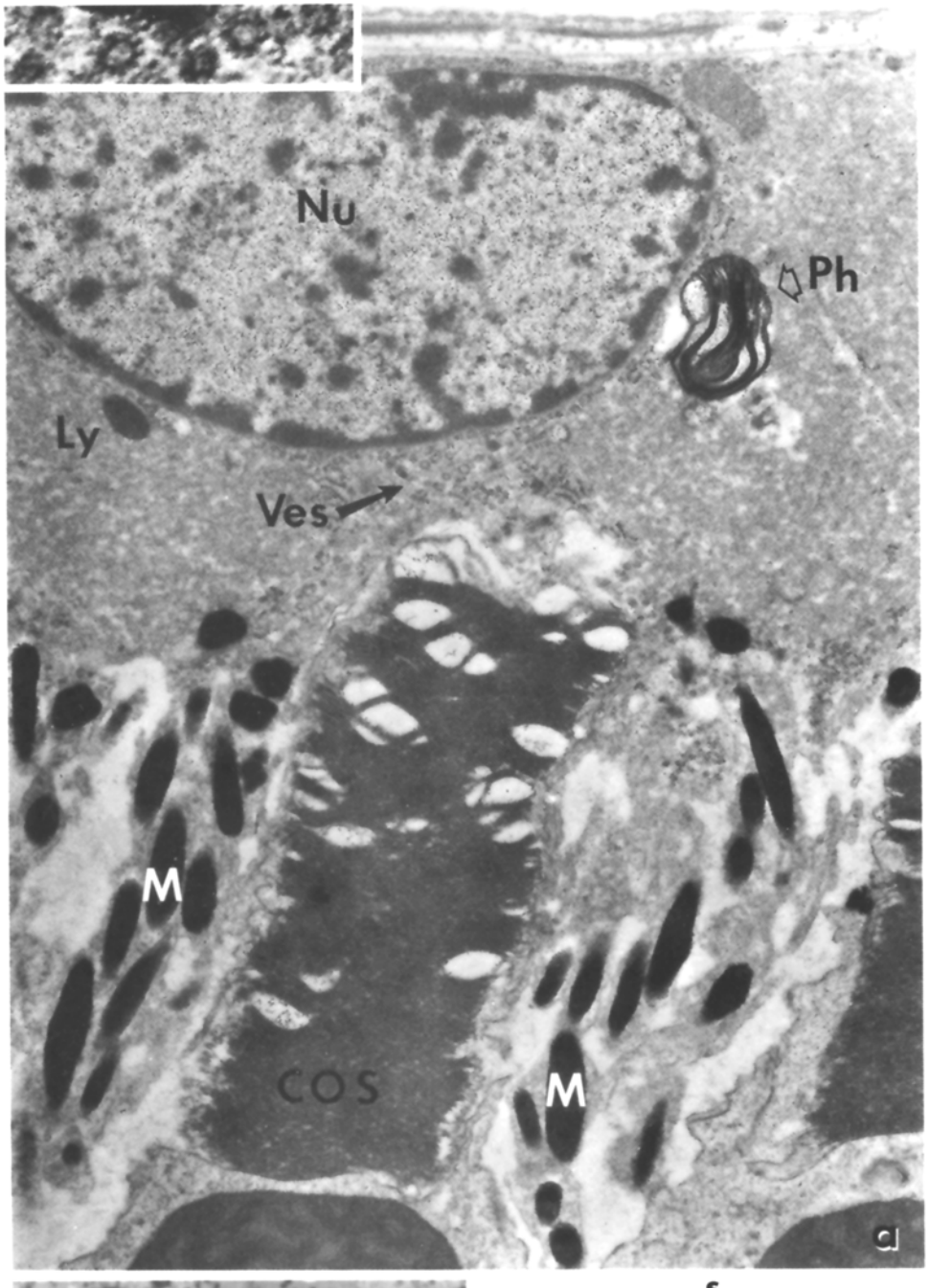
This study was initiated to further define the cyclic pattern of cone disc shedding. Since tree shrew photoreceptors are almost all cones (Rohen 1962; Tigges 1963; Rohen and Castenholtz 1967; Samorajski et al. 1966), rods contribute little to the numbers of effete disc packets found in the retinal pigment epithelium (RPE). Our results show a maximal response occurring shortly after the onset of light in the tree shrew.

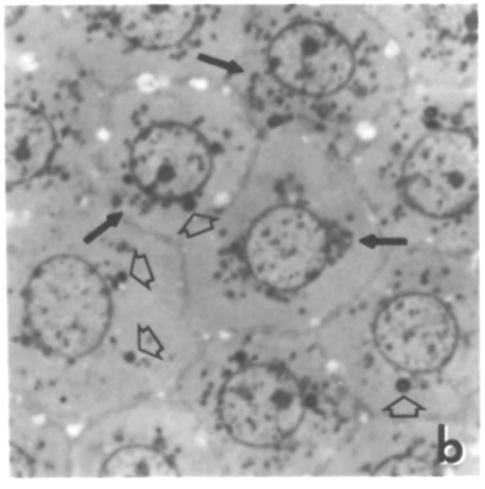
Send offprint requests to: Joe Immel, Department of Physiology S-762, University of California, San Francisco, CA 94143, USA

Materials and methods

Adult tree shrews (*Tupaia belangerii*) of approximately the same size and weight (about 120 g) and of both sexes were obtained from Primate Imports (New York, NY) or were littermates bred in captivity. The animals bred in captivity were sacrificed at 00:30, 06:00, and 10:00. Since there was a limited number of animals available for this study, they were sacrificed at timepoints shown to be important in other studies of mammalian cone disc shedding – 1 h after light onset and one to 8 h after light offset. One animal was sacrificed at each timepoint. Animals were kept under controlled conditions of temperature (27° C), humidity (rh 80%), and illumination for at least three months prior to sacrifice. The lighting regimen was 12 h of light (provided by overhead fluorescent lights) followed by 12 h of complete darkness. Food and water were available at all times. The animals were minimally stressed and access to their room was strictly controlled.

Procedure. Animals were anaesthetized with 0.5 to 1.0 ml Nembutal (50 mg sodium pentobarbital per ml USP; Abbott Laboratories). All perfusates were warmed to room temperature before use. Before perfusion of saline, 0.5 ml heparin (10000 units per ml USP; Riker Laboratories) was injected into the left ventricle. After 1 min the animal was perfused with 0.9% NaCl containing heparin (10 units per ml) for three to 5 min or until the fluid from the punctured right ventricle appeared clear. The perfusate was then changed to fixative (2.5% glutaraldehyde, 1% dimethyl sulfoxide and 0.05% calcium chloride in 0.065 M sodium cacodylate buffer, pH 7.4) and the animal was perfused until fixative flowed from the right ventricle and the body was rigid. The pressure of perfusates was maintained at





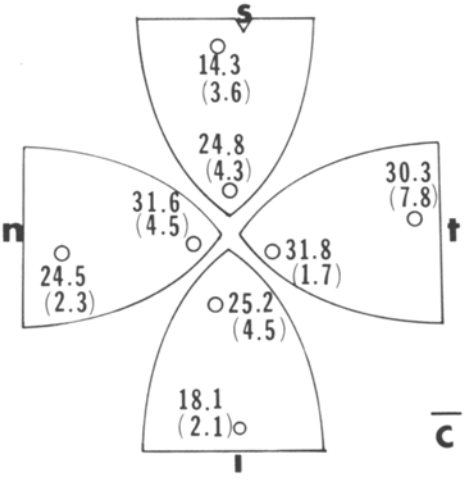


Fig. 1. a Electron micrograph of a portion of a RPE cell at 01:15 showing a recently ingested phagosome and coated vesicles. *Inset:* Higher magnification of some coated vesicles. *Ph* phagosome; *Ves* coated vesicles; *Nu* nucleus; *COS* cone outer segment; *M* melanin granule; *Ly* lysosome. ×13750 (Inset ×67000)
b Light micrograph of a tangential section through the RPE about midway through the RPE cell nucleus. Each nucleus is surrounded by phagosomes (e.g., *open arrowheads*), and lysosome-like and residual bodies (e.g., *closed arrowhead*). The peripheral areas of the cells are mostly clear of dark-staining material. ×1000
c Diagram depicting the density of photoreceptors at various areas of the tree shrew retina. *Upper numbers* are thousands of photoreceptors/mm^2. *Numbers in parentheses* are the percentage of photoreceptors at each location that are rods. *Circles* are locations sampled and show the approximate size of the sample area (0.2 mm^2)

80 mm Hg. The eye was enucleated and the anterior structures removed, but no attempt was made to remove the vitreous since the retina may detach with only slight manipulation. The eyecup was immersed in fixative and refrigerated for about 12 h.

After fixation, the tissue was washed with cold buffer (0.065 M cacodylate buffer, 0.05% calcium chloride and 4.5% sucrose). Post-fixation was for one h with 2% OsO_4 in veronal acetate buffer, followed by a wash in distilled water. The tissue was then dehydrated in a graded ethanol series and placed in propylene oxide. Araldite 6005 or Epon 812 was used to embed the tissue for light and electron microscopy.

Microscopy. Eyes were cut into quadrants and the central portion of each quadrant was sampled. One-micron sections were cut in longitudinal orientation on an LKB Ultratome V. For phagosome counts and measurements, every fifth section was collected for light microscopy and stained for two min with a combination of 0.25% azure II, 0.25% methylene blue and 0.5% toluidine blue with 0.5% sodium borate and for five min with 0.5% basic fucsin in 5.25% ethanol. Sections for electron microscopy were stained with uranyl acetate followed by lead citrate. A 0.8 μm cutoff was adopted for the smallest objects to be considered clearly discernible as phagosomes, although smaller phagosomes or portions of phagosomes were often seen. In about half of the sections examined, the maximum diameter of each phagosome was recorded.

Animals in this report are identified by the time of fixation. The 24-h period of the lighting cycle is designated in hours:minutes. The lights came on at 00:00 and went off at 12:00 remaining off until 00:00 (24:00). Both light and dark periods began abruptly by turning on or off all fluorescent lights at once.

Phagosome counts. Phagosomes were identified as dark-staining, magenta inclusions larger than 0.8 μm within the RPE cell body. Dark brown inclusions or melanin, usually found only in the apical processes during the light period, were not counted. Lysosome-like inclusions and residual bodies were easily differentiated from phagosomes by light microscopy using the stains described. Sections averaging about 2.5 mm in length and containing well-aligned outer segments and no retinal detachment were used for phagosome counts and collecting size data. The variation in the numbers of phagosomes from section to section at each timepoint was calculated (standard error and standard deviation).

In a preliminary double-blind test, identities of tissue specimens and light-microscope sections were encoded and concealed from the experimenters. All of the animals sacrificed during the dark cycle and two animals from the beginning of the light cycle (01:15 and 02:00) were part of this study.

Photoreceptor density. One eye not used in the shedding study was carefully oriented and sectioned into quadrants in order to sample the density of photoreceptors across the retina. Tangential sections were cut from the choroid until the cone photoreceptor inner segments were uncovered. At this level, rod outer segments were easily identified. The numbers of rods and cones per square mm were found for each quadrant at the central tip and two-thirds of the distance to the ora serata in the periphery.

Outer segment turnover. In order to assess the frequency of cone outer segment shedding, two factors must be determined: 1) the average amount of outer segment material included in each phagosome; and 2) the total number of phagosomes shed during the light period. An estimate of the number of discs per phagosome was obtained from electron micrographs of 11 large phagosomes from 01:15 in which *all* of the discs were clearly discernible. (It should be mentioned that large phagosomes with completely intact discs are rare even at the time of peak shedding.) In the same sections the number of discs per outer segment was obtained from eight cones in which all of the discs along the length of the outer segment were distinguishable. The total number of phagosomes was derived using the method of Abercrombie 1946 (see also Dubin 1970). Because the size of phagosomes is comparable to the thickness of the sections in which they are counted, raw counts of phagosomes do not reflect accurate numbers and are overestimates. Corrected numbers of phagosomes counted were derived by applying the following equations (from Dubin 1970):

$$N=\frac{Nc\,T}{T+D-2k} \tag{1}$$

$$k=\frac{D-\sqrt{D^2+Dm^2}}{2} \tag{2}$$

where N is the corrected count, Nc is the actual number counted, T is the section thickness (1 μm), D is the average diameter, and Dm is the minimum diameter of the counted phagosomes (0.8 μm). The value k is the correction factor.

If phagosomes disappear before they are counted, the

Table 1. Numbers of RPE phagosomes during the lighting cycle

Circadian time	Light period					Dark period					
	00:30	01:15	02:00	06:00	10:00	14:00	16:30	17:00	18:45	20:45	21:15
Phagosomes/mm RPE (Blind experiment)	–	17.28	5.30	–	–	0.50	0.12	0.14	0.00	0.42	0.33
Phagosomes/mm RPE	6.47	17.02	5.67	4.60	2.49	0.61	0.20	0.16	0.10	0.18	0.29
Standard error	0.64	0.94	0.54	0.51	0.33	0.08	0.09	0.10	0.06	0.11	0.10
Standard deviation	2.11	4.08	1.94	1.84	0.88	0.21	0.25	0.28	0.18	0.30	0.30
Number of sections	11	19	13	13	7	8	8	7	8	7	8
mm RPE counted	23.81	28.77	31.58	29.06	16.96	25.35	26.49	24.17	19.87	17.80	24.36
Phagosomes/mm RPE (Corrected values)	1.61	4.12	1.57	1.34	0.64	0.19	0.07	0.05	0.02	0.03	0.11

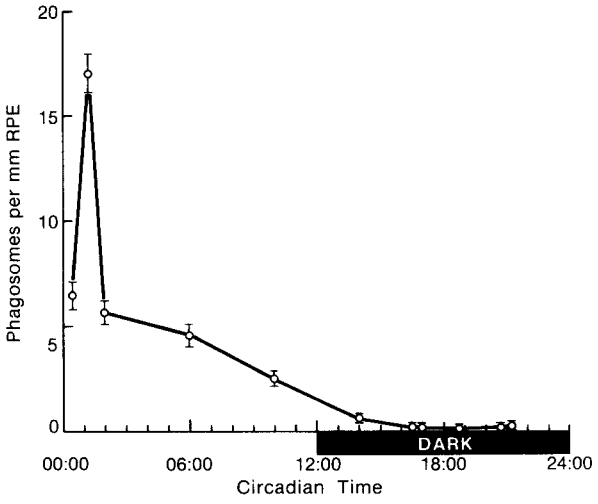


Fig. 2. Graph depicting numbers of phagosomes found in tree shrew RPE cells during a complete circadian lighting cycle. A large peak in the number of phagosomes is seen at 01:15. Error bars show variation (±SD) from section to section at each timepoint

raw counts will be underestimates. On the other hand, if the degradation time is longer than the counting interval, raw counts will be overestimates. To take into consideration the effects of degradation time, we derived the total number of phagosomes shed during the light period by arbitrarily choosing intervals for the phagosomes to degrade to undersized specimens (i.e., less than 0.8 μm). A curve was generated using values for counts obtained during the light period which were corrected using the appropriate correction factor (k), then the number of phagosomes for the chosen interval were counted on it. Thus, for a degradation time of 30 min we started at 00:30 and added the values found every 30 min along the curve drawn using corrected values. In this analysis we assume that phagosomes "counted" at 01:00 are all new phagosomes, since those phagocytosed at 00:30 would have degraded to uncountable sizes. The corrected number of phagosomes/mm shed during the light period is then taken to be the sum of the values found along the curve every 30 min. Dividing the number of phagosomes shed by the number of cones (158 cones/mm RPE; Rohen and Castenholtz 1967: Results) yields the percentage of cones shedding each day. Since each phagosome represents about 20% of the cone outer segment volume, the number of days required to shed 100% of the cone outer segment volume can be calculated.

The half-life of cone outer segments was defined as the time in which 50% of the outer segment is new material and 50% is old material. It was calculated by assuming that cone outer segment membrane is confluent and that 20% of the outer segment is shed at each event. Thus, the half-life is equal to the time required for a cone outer segment to undergo three shedding events (see Discussion).

Results

Figure 1a shows a portion of a tree-shrew RPE cell including apical and basal membranes and various organelles.

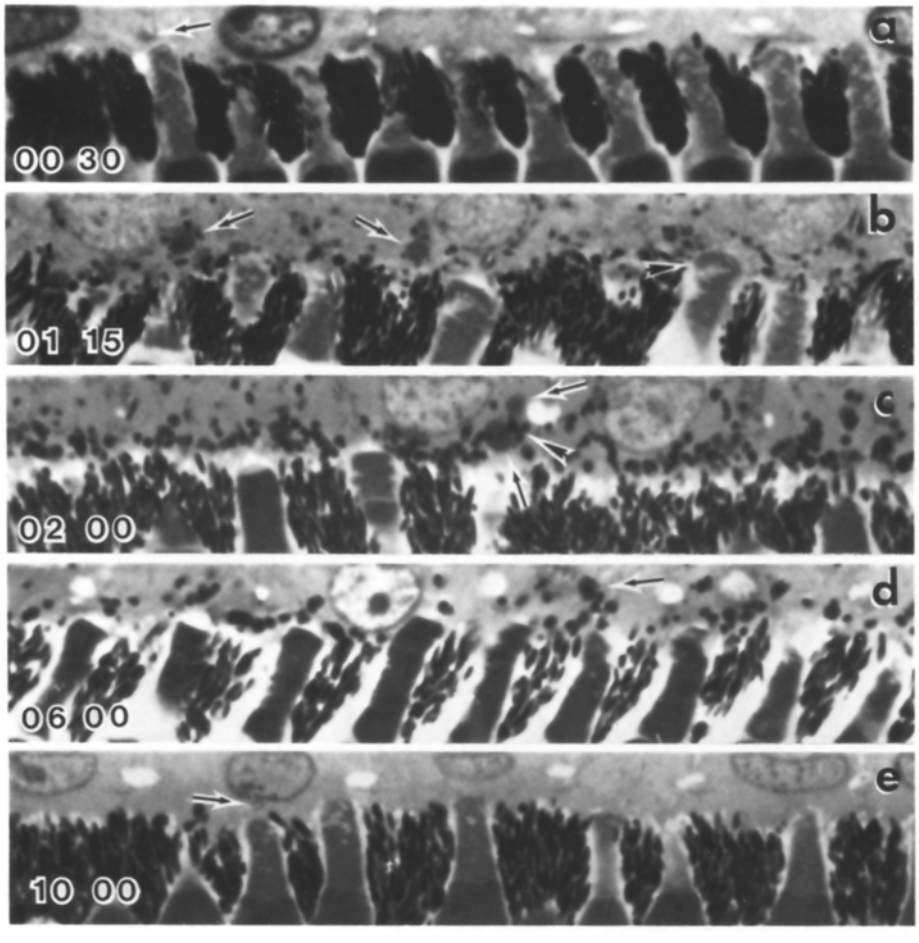


Fig. 3a–e. Light micrographs of the RPE at various times during the light period. ×1500
a Just after light onset the RPE is mostly free of inclusions. *Arrow* indicates a single phagosome.
b About an hour after light onset the RPE contains many phagosomes (*arrows*) and lysosome-like inclusions. *Arrowhead* indicates what may be a shedding disc packet.
c 45 min later than **b** there are many phagosomes (*arrows*), residual bodies (*arrowhead*) and lysosome-like inclusions.
d Midway through the light period there are many residual bodies (dark-staining inclusions) but few phagosomes (*arrow*) or lysosome-like inclusions.
e Near the end of the light period there are almost no inclusions in the RPE. *Arrow* indicates a small phagosome

The basal border of the RPE cell is flat, without invaginations. Numerous microvilli-containing pigment granules extend from the apical border to about 10 μm vitread, thus surrounding cone outer segments and filling most of the 4 μm space between cone outer segments. Melanin granules are spindle shaped, 0.2 μm to 0.5 μm in length. In tangential sections the RPE cells display a hexagonal outline with a centrally located nucleus (Fig. 1 b).

At certain times during the lighting cycle, phagosomes and lysosome-like bodies are observed in varying numbers. In electron micrographs lysosome-like inclusions appear as amorphous, osmophilic bodies typically 0.2 μm by 0.5 μm, but not exceeding 1.0 μm. At the time of peak shedding (01:15), many coated vesicles were found at the apical border associated with phagocytosis-related inclusions (Fig. 1a, inset). In light micrographs phagosomes range from light-staining, round inclusions seen most frequently during the early part of the light period, to dark-staining, convoluted inclusions seen most frequently later during the light period. Electron micrographs (Fig. 1 a) show that large phagosomes look like portions of photoreceptor outer segments. The discs may be clearly discernible, but are usually compacted and contorted. Most of the phagosomes observed at 01:15 and at later times during the light period were found near the RPE cell nucleus. Phagosomes not in the immediate vicinity of the nucleus were generally found at the apical border of the cell. At 00:30 most phagosomes were at the apical border. There was a tendency for the phagosomes to surround the apical half of the nucleus, forming a cup of material. In tangential sections of RPE cells taken midway through the light period, this cup appears as a ring of residual bodies around the nucleus (Fig. 1 b). The average number of discs per phagosome was found to be 36.5 (SD = 9.8). The number of discs per cone outer segment was 182.4 (SD = 29.2). Thus, a rough estimate of phagosome size is 20% of the cone outer segment length.

The distribution of photoreceptors across the retina is shown in Fig. 1 c. The density of rods in the central areas (where phagosomes were counted) averages less than 4% of the total photoreceptor density. In the periphery, the photoreceptor density is less than that found in the central areas, except for the peripheral temporal area which has relatively high numbers of rods and cones. The average density of photoreceptors was found to be 25104.5/mm^2. Expressed in linear terms (in order to compare tangential to longitudinal sections) this equals 158.4 photoreceptors/mm retina.

Pattern of shedding and phagocytosis

This investigation was undertaken initially in anticipation of a gradual peak of cone phagocytosis similar to that found in another study from this laboratory (Tabor et al. 1980). However, in the preliminary double-blind test, timepoints in the light period corresponded with significant numbers of RPE phagosomes (Table 1).

We then added more timepoints during the light period and found that, soon after the onset of light, the number of phagosomes observed in the RPE increased dramatically to a peak value and fell off rapidly within 2 h (Table 1 and Fig. 2). At the peak, the number of phagosomes (01:15; 17.02/mm RPE) is approximately three times that seen 45 min before (00:30; 6.47/mm RPE) and three times that seen 45 min after (02:00; 5.67/mm RPE). The number of phagosomes decreases at a steady rate from 02:00 to minimum values during the dark period. At the beginning of the dark period the number of phagosomes (14:00; 0.61/mm RPE) is only 3% of the peak value and 13% of the number found in the middle of the light period (06:00; 4.60/mm RPE). Later in the dark portion of the lighting cycle, the number of phagosomes is even smaller (e.g., 21:15; 0.29/mm RPE).

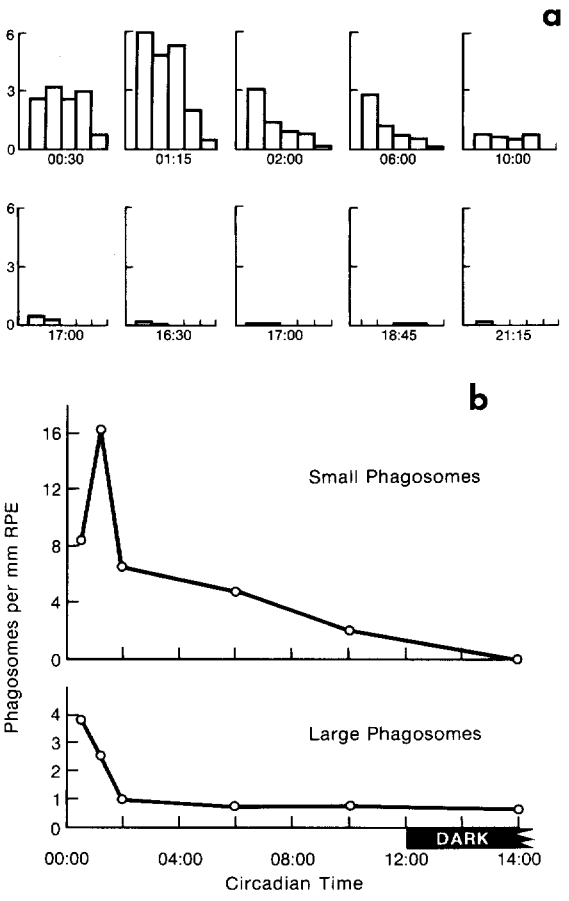


Fig. 4. a Histograms of phagosomal size distributions throughout the lighting cycle. Bins are 0.4 μm starting at 0.8 μm. 17.8 mm of RPE were examined at 20:45 but no phagosomes were found and a histogram is not included. **b** Graphs depicting the numbers of large (greater than 1.8 μm) and small (less than 1.8 μm) phagosomes during the lighting cycle. Note that large phagosomes peak at 00:30 while small phagosomes peak at 01:15

Fig. 3 is a series of light micrographs that demonstrate the changing pattern of RPE inclusions during the light period. At 00:30 there are almost no dark-staining inclusions. Phagosomes observed at this time are usually large and light-staining (see Fig. 3a). At 01:15 the RPE cell bodies contain some large phagosomes, and many small light-staining, lysosome-like bodies appear in the apical half of the cell. By 02:00 this portion is filled with many phagosomes of various sizes, as well as possible residual bodies and lysosomes. Putative residual bodies aggregate, appearing to consolidate into larger residual bodies, at 06:00 (Fig. 3d, arrow). Fewer phagosomes are seen at this time and large phagosomes are very rare (Fig. 4a). The RPE cytoplasm is free of phagocytic inclusions by 10:00 and appears very much like it did at 00:30.

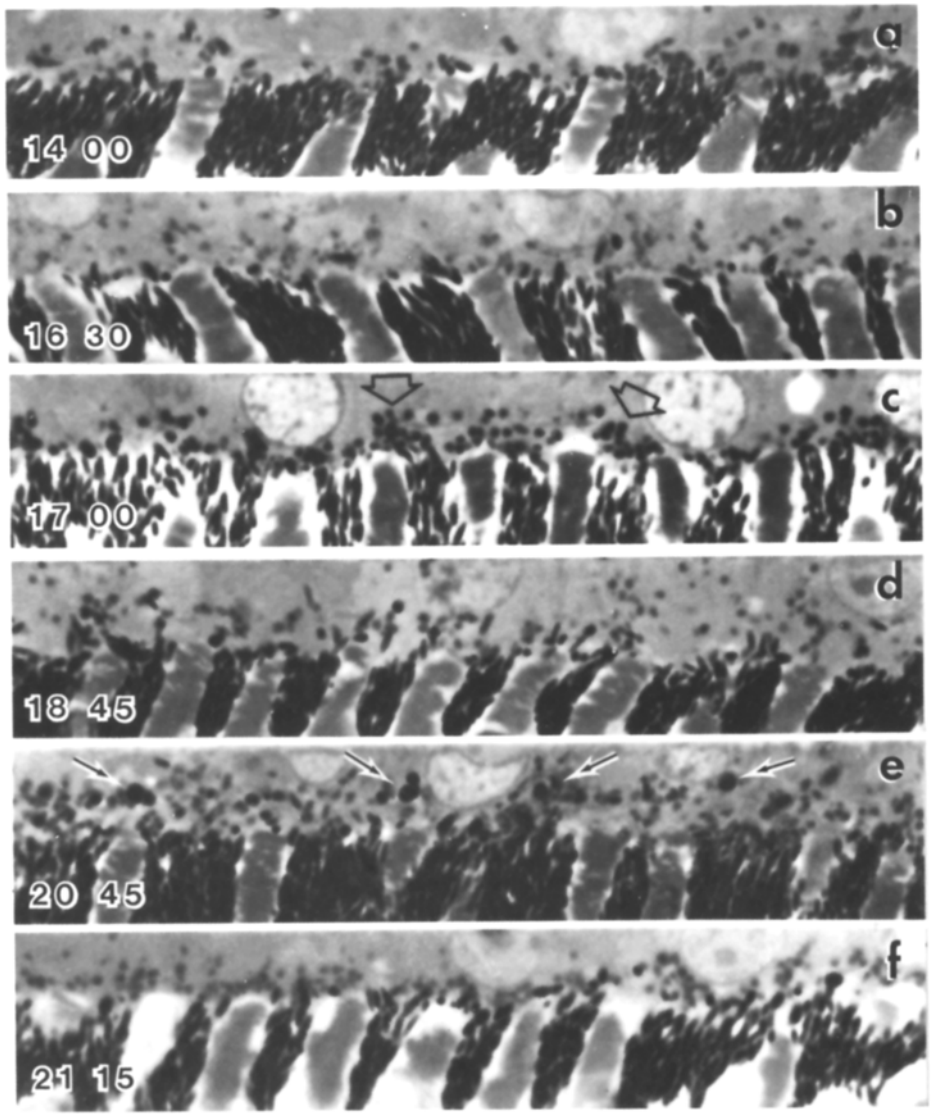


Fig. 5a–f. Light micrographs of the RPE at various times during the dark period. ×1500
a–b Early in the dark period there are few inclusions in the RPE.
c–d Midway through the dark period aggregations of dark-staining inclusions are found (*arrowheads*).
e Large dark-staining inclusions (*arrows*) are found among other aggregated, dark-staining inclusions.
f The RPE has few inclusions near the end of the dark period

During the lighting cycle, the profile of RPE phagosome sizes changes markedly. At light onset there are approximately the same number of phagosomes in each size class except for the largest (Fig. 4a). At the peak (01:15), many small phagosomes are found. Large phagosomes are more numerous at 00:30 and 01:15 than at other times during the lighting cycle, but always number less than small phagosomes (Fig. 4a, b). The distributions of phagosome sizes at 02:00, 06:00, and 10:00 are clearly biased towards increasingly smaller size classes (Fig. 4a). The gradual, almost linear falloff in the number of phagosomes from 02:00 to 14:00 is concomitant with the gradual reduction in the total number of small phagosomes and the rapid disappearance of large phagosomes (Fig. 4b).

Other RPE inclusions

Dark-staining inclusions resembling melanin show a variable pattern of distribution in the RPE cell body throughout the *dark* period (Fig. 5). At the beginning of the dark period (14:00) some melanin granules appear beyond the apical border of the RPE cell in the cytoplasm of the apical third of the cell. A few light-staining inclusions, possibly lysosomes, are also found in this region. During the dark period more melanin granules, residual bodies and light-staining inclusions are observed above the apical border of the RPE cell in the cytoplasm (16:30, 17:00 and 18:45; Fig. 5b, c, d). At 20:45 many large aggregates of dark-staining (but not brown) residual bodies are also found among other inclusions (Fig. 5e). Large residual bodies are evident in Fig. 5d and e. Ball-shaped aggregates of brown-colored melanin granules are also found in some RPE cells. At 21:15 (Fig. 5f), the RPE cell contains only a few small, light-staining bodies which occupy the lower third of the cell body. No change in the morphology of the apical processes is apparent at any time during the light or dark portions of the lighting cycle.

Rate of cone renewal

The total number of phagosomes shed at each timepoint during the light period was found by correcting raw counts of phagosomes according to their size distribution (see Methods). Corrected values are found at the bottom of Table 1 and correction factors are found in Table 2. Using a curve constructed from the corrected values (which is

Table 2. Average phagosome diameter and correction factor during the lighting cycle

Time	00:30	01:15	02:00	06:00	10:00	14:00	16:30	17:00	18:45	21:15
Ave. Diam. (μm)	1.52	1.38	1.25	1.21	1.46	1.00	0.93	1.00	1.80	0.80
Correction factor	0.44	0.47	0.51	0.53	0.45	–	–	–	–	–
N	94	112	69	67	25	7	3	2	2	2
mm RPE	23.8	28.8	31.6	29.1	16.9	25.4	26.5	24.2	19.0	24.4

Table 3. Calculation of cone outer segment renewal times adjusted for various rates of degradation

Degradation time (min)	Phagosomes/mm daily uncorrected	Phagosomes/mm daily corrected (N)	% Cones shedding/day (P)[a]	Days to shed 100% volume (T)[b]	Half-life in days ($T_{1/2}$)[c]
15	462.2	117.4	74.3	6.7	4.0
30	230.7	58.6	37.1	13.5	8.1
45	79.0	40.2	25.4	19.7	11.8
60	60.0	30.0	19.0	26.3	15.8
90	53.3	18.3	11.6	43.2	25.9

[a] P = N/158 cone outer segments per mm
[b] Phagosome volume = 20% outer segment volume, therefore in 5 days 100% is shed; T = 5 days/P
[c] Half-life at 20% shed per day is 3 days; $T_{1/2} = 3$ days/P

essentially the same as Fig. 2 using corrected values) the number of phagosomes shed during the light period was calculated for five possible rates of degradation. These data are summarized in Table 3 and are compared with values obtained from the uncorrected curve. The percentage of cone outer segments shed each day, the number of days to shed the volume equivalent to one cone outer segment, and the calculated outer segment half-life are listed for each of the possible degradation times considered.

For example, 30 min might be considered a likely time for phagosomes to degrade to sizes below the counting cut-off. In this case the number of phagosomes shed each day would be 58.6/mm RPE (230.7/mm uncorrected) and the percentage of cones shedding 37.1. Here the time to shed a complete outer segment is 13.5 days with a corresponding half-life of 8.1 days.

Discussion

Shedding and phagocytosis

The RPE of the tree shrew is a very convenient system in which to study cone photoreceptor shedding because there is little melanin in the main portion of the cell body and there are relatively few outer segments lying apposed to the RPE cells. For comparison, the RPE cells of cat and tree shrew are approximately the same size; however, there are 700 to 900 tightly packed outer segments per mm of RPE in the cat (Fisher et al. 1983), while the tree shrew possesses only about 158 outer segments per mm, on the average (Rohen and Castenholtz 1967; Results). Since rods on the average number only 4% the number of cones, there is probably little contamination of phagosome counts by rod phagosomes.

The pattern of shedding and phagocytosis is clearly evident from light micrographs of the RPE during the lighting cycle. At the beginning of the light period, the cytoplasm is at first free of phagosomes and lysosome-like inclusions. This is followed by the appearance of many phagosomes and lysosomes. Residual bodies appear as the number of phagosomes decreases. By the end of the light period the RPE cytoplasm is once again free of phagocytic inclusions. Counts of phagosomes at timepoints throughout the lighting cycle demonstrate that tree shrew cones shed outer segment material soon after light onset. This is similar to results obtained in cat (Fisher et al. 1983) where cones also shed during the light period. In another mammal, gray squirrel (Tabor et al. 1980), and in other vertebrates, cones shed during the dark period (see Young and Bok 1979).

The possibility of cone shedding during the dark might be raised since there are no data for the earliest part of the dark period, from 12:00 to 14:00 hours. Unfortunately, tree shrews became unavailable to us during the study and we were unable to fill in these timepoints. However, cone shedding during the dark portion of the lighting cycle can be discounted from our observations showing so few phagosomes and residual bodies at 14:00. The facts that residual bodies and low levels of shedding persist for some time after the light shedding peak, and that the number of phagosomes decreases steadily to low values in the dark, lend credence to the conclusion that there is no large burst of shedding at the onset of darkness in the tree shrew.

The shape of the curve representing shedding and phagocytosis is qualitatively similar to those derived from previous studies (Young and Bok 1979), with a sharp increase (peak) in the number of phagosomes followed by a gradual reduction in their number. Though each point in Fig. 2 represents counts from only one animal, two factors support our conclusions. First, the pattern is very much like that observed for cyclic cone shedding in the cat (Fisher et al. 1983) and second, the peak value is very distinct with the variation from section to section well beyond adjacent points. Thus, we tend to believe that the peak is not spurious. If the value at 01:15 is spurious, however, it will have a small effect on calculations of the total number of phagosomes shed (see Rate of cone renewal). By measuring the area under the curve in Fig. 2, it was determined that 60.6 phagosomes/mm RPE were shed during the light peri-

od. Elimination of the peak value yields 52.3 phagosomes/mm RPE, a difference of 13.7%.

Rate of cone renewal and frequency of shedding

The rate at which cone outer segment material is renewed is a difficult problem to address because the structure of cone outer segments, with discs that are confluent with the plasmalemma (Cohen 1970), precludes the use of radioactive labelling. Labelling of rod outer segments with a radioactive amino acid produces a clearly defined band of radioactively labelled protein that is displaced towards the RPE as new material is added to the base while cones are diffusely labelled since disc material is continuous with the plasmalemma and other discs (Young and Bok 1979). In the following discussion we use RPE phagosome data to estimate the rate of cone renewal.

In order to assess the frequency of cone outer segment shedding, two factors have been determined: 1) the average amount of outer segment material included in each phagosome; and 2) the total number of phagosomes shed during the light period. The rate at which phagosomes are degraded will also affect our counts. We presume that phagosomes are degraded even as shedding occurs. This follows from the observations that: (a) at the peak of shedding, 01:15, there are already many condensed, uncountable (i.e., less than 0.8 μm) phagosomes and residual bodies which have been shed and degraded in the 45 min prior to 01:15 (Fig. 3b); (b) large phagosomes show a peak at 00:30 while those in smaller size classes show a peak at 01:15 (Fig. 4), suggesting that the bulk of newly-shed phagosomes are shed around 00:30 and have undergone significant degradation; and (c) the number of large and small phagosomes decreases dramatically by 02:00. In view of these data we believe that the time for degradation of tree shrew phagosomes (to uncountable sizes) is probably 30 min or less as indicated by the rapid disappearance of countable phagosomes during the shedding peak. Thus, we would propose that most shedding occurs between 00:30 and 01:15.

Table 3 summarizes the calculation of cone renewal times for five different degradation times. Note that the time assumed for phagosomes to degrade has a tremendous effect on the number of phagosomes counted, given the curve generated using our sampling procedure. The difference between 30 and 60 min degradation times is almost 400%, which in turn has a great influence on the calculated values of cone outer segment renewal times.

Since cone membrane material is confluent throughout the cone outer segment (Cohen 1970), newly added membrane material should mix with old material by lateral diffusion. Thus, each shedding event should produce a phagosome containing some new material along with the old material (see Tabor et al. 1982; Fisher et al. 1983). Therefore, the renewal time for tree shrew cones is best expressed as the half-life of outer segment membrane material; i.e., from a given starting point, the amount of time required to produce an outer segment composed of 50% old and 50% new membrane. Since 20% of the cone outer segment is shed at each event, *three* shedding events are required to make 49% of the outer segment material new and 51% of it old (for our purposes, the half-life). The last column of Table 3 shows the number of days of shedding required to reach the half-life of cone outer segment material; for a 30 min degradation time this is 8.1 days.

Coated vesicles

A peculiar type of cytoplasmic feature found only near the RPE apical membrane at sites of phagocytosis during the peak of shedding consists of aggregations of coated vesicles (Fig. 1a, inset). Orzalesi et al. (1982) have reported coated vesicles at both the apical and basal membranes of RPE in rabbit and man, and have hypothesized that the vesicles are involved in pinocytosis and transport of extracellular material. In the tree shrew, we have observed coated vesicles only during the peak of phagocytosis in association with phagocytotic inclusions near the apical border. We have not determined the spatial distribution of the vesicles statistically in the manner of Orzalesi et al. A possible coincidence between the studies may lie in the time at which the vesicles appear. Their observations were made in the morning for human eyes and at 09:00 a.m. for all of the rabbit eyes. Although the exact time after onset of light is not stated, it is conceivable that these observations could have been made during times of cyclic photoreceptor shedding, which is consistent with our observations in tree shrews. If the coated vesicles are involved in pinocytosis at the time of shedding and phagocytosis, then they would contribute to the depletion of RPE apical membrane, which is at the same time being donated to surround engulfed phagosomes. If, on the other hand, these vesicles are fusing to the plasmalemma, they may be a source of membrane used to surround the outer segment fragments.

References

Abercrombie M (1946) Estimation of nuclear population from microtome sections. Anat Rec 94:239–247

Basinger J, Hollyfield JG, Rayborn ME (1977) Turnover of rod photoreceptor outer segments. II Membrane addition and loss in relation to light. J Cell Biol 75:507–527

Cohen AI (1970) Further studies on the question of the patency of saccules in outer segments of vertebrate photoreceptors. Vision Res 10:445–453

Dubin MW (1970) The inner plexiform layer of the vertebrate retina: A quantitative and comparative electron microscopic analysis. J Comp Neurol 140:479–506

Fisher SK, Pfeffer BA, Anderson DH (1983) Both rod and cone disc shedding are related to light onset in the cat. Invest Ophthalmol Vis Sci 24:844–856

Herman KG, Steinberg RH (1982) Phagosome movement and the diurnal pattern of phagocytosis in the tapetal retinal pigment epithelium of the opossum. Invest Ophthalmol Vis Sci 23:277–290

Hollyfield JG, Besharse JC, Rayborn ME (1976) The effect of light on the quantity of phagosomes in the pigment epithelium. Exp Eye Res 23:623–635

LaVail MM (1976) Rod outer segment disc shedding in rat retina: Relationship to cyclic shedding. Science 194:1071–1074

O'Day WT, Young RH (1978) Rhythmic daily shedding of outer segment membranes by visual cells in the goldfish. J Cell Biol 76:593–604

Orzalesi N, Fossarello M, Carta S, Del Fiacco G, Diaz D (1982) Identification and distribution of coated vesicles in the retinal pigment epithelium of man and rabbit. Invest Opthalmol Vis Sci 23:689–696

Rohen JW (1962) 'Sehorgan'. In: Primatologia 2:1210

Rohen JW, Castenholtz A (1967) Über die Zentralisation der Retina bei Primaten. Folia Primat 5:92–147

Samorajski T, Ordy JM, O'Keefe JR (1966) Structural organization of the retina in the tree shrew (*Tupaia glis*). J Cell Biol 28:489–504

Tabor GA, Fisher SK, Anderson DH (1980) Rod and cone disc shedding in light-entrained tree squirrels. Exp Eye Res 30:545–557

Tabor GA, Anderson DH, Fisher SK, Hollyfield JG (1982) Circadian rod and cone disc shedding in mammalian retina. In: Hollyfield JG (ed) The structure of the eye (Proceedings of the Fourth International Symposium). Elsevier, Amsterdam, pp 67–73

Tigges J (1963) Untersuchungen über den Farbensinn von *Tupaia glis*. Z Morphol Anthrop 53:109–123

Young RW (1977a) The daily rhythm of shedding and degradation of cone outer segment membranes in the chick retina. Invest Ophthalmol Vis Sci 17:105–116

Young RW (1977b) The daily rhythm of shedding and degradation of cone outer segment membranes in the lizard retina. J Ultrastruct Res 61:172–185

Young RW, Bok D (1979) Metabolism of the retinal pigment epithelium. In: Zinn KM, Marmor MF (eds) The retinal pigment epithelium. Harvard University Press, Cambridge, Mass, pp 103–403

Accepted November 6, 1984