The daily rhythm of shedding and degradation of rod and cone outer segment membranes in the chick retina

Richard W. Young

Newly hatched chickens were maintained on a daily light cycle of 12 hr of light and 12 hr of darkness for 12 days. The pigment epithelium was then examined by electron microscopy at different times of day. Shortly after the beginning of the light period, the rods discarded groups of outer segment membranes. During the remainder of the light period, the membranes were degraded by the pigment epithelium. Early in the dark period, the cones shed membranes, which were digested by the pigment epithelial cells during the subsequent hours of darkness. Available evidence suggests that at least some of the chemical activities of the visual cells and pigment epithelium oscillate with a daily rhythm, which is synchronized with the daily fluctuation of light in the environment.

Key words: visual cells, daily rhythms, renewal, retina, membranes

The continual renewal of the light-sensitive membranes of rod outer segments has been amply documented in a wide selection of vertebrate animals. In fact, the process has been observed wherever it has been sought—in amphibia, reptiles, birds, fish, and mammals. It seems to be a fundamental and universal characteristic of rod visual cells.1

The basic mechanism of the renewal process in rods is "membrane replacement," which has two components: formation of new membranes and disposal of old ones.2 The new membrane constituents are produced in the inner segment of the cell, then transported to the outer segment for the final steps of membrane assembly. Groups of old membranes are shed intermittently from the tips of the rods. This tends to occur early in the morning, in animals maintained on a cycle of 12 hr of light and 12 hr of darkness.3 The adjacent pigment epithelial cells, which envelop the ends of the outer segments, ingest and degrade the detached membranes.4

In contrast, progress in our understanding of the mechanism by which the other class of visual cells, the cones, renew their outer segments had been frustratingly slow. This deficiency is even more disturbing when we acknowledge that cones play a more important role in meeting human visual needs than do rods.

At one time, it seemed as if cones did not produce new membranes, but the autoradiographic evidence on which this conclusion was based later was found to have been misinterpreted.1, 5 Similarly, it appeared at first that cones did not shed membranes. This too proved to be incorrect, when evidence of disc shedding by cones...
was detected in humans and squirrels. Nevertheless, no synthesis emerged to tie the sparse bits of information into a coherent hypothesis of cone outer segment renewal.

Recently, in considering the implications of LaVail's report that rat rods shed membranes early in the daily light period, it occurred to me that cones might also dispose of outer segment membranes at a certain time of day. Accordingly, O'Day and I looked for evidence of this process in goldfish which were on a daily cycle of 12 hr of light and 12 hr of darkness.

Shortly after the beginning of the light period, the rods shed packets of membranes from the ends of their outer segments, thereby confirming in a fish a rhythmic process previously documented in rats and frogs. Later in the day, soon after the onset of the dark period, a comparable burst of membrane detachment from the ends of the cones took place. If the cones dispose of membranes on a regular basis, they must also repeatedly form new membranes. Otherwise, their outer segments would soon disappear.

This suggested a simple hypothesis: Rods and cones both renew their outer segments by membrane replacement. The process follows a daily rhythm, and the rhythms of rods and cones are separated in time by approximately 12 hr.

To learn if renewal by membrane replacement was unique to goldfish cones or was a more fundamental process which also occurs in other vertebrate animals, I repeated the experiment in a diurnal lizard which contained only cones in its retina. The results were unequivocal. Throughout the entire 12 hr light period, there was not the slightest evidence of any membrane detachment from the ends of the cones. However, less than an hour after the beginning of the dark period, there was a brief phase of membrane shedding from the tips of the cone outer segments. Before the dark period was over, the membranes had been fully degraded by the pigment epithelium.
The highly reproducible demonstration of a rhythmic daily disposal of membranes by cones in a fish and a reptile strongly supported the proposal that this was a common characteristic of vertebrate cones. However, there are some unusual features in these animals. Both are "cold-blooded" (ectothermic). The lizards are unusual among vertebrates in that they lack rods entirely. Furthermore, the pigment epithelium in the goldfish is atypical, being filled with tapetal granules, which seem to limit its phagocytic and degradative capacities, so that these are supplemented by the activities of ameboid cells.

Therefore I repeated the experiment in another class of vertebrate animals—a class which is homeothermic, with a duplex retina and a more conventional pigment epithelium. The animal selected was a bird, the chicken. A report of the results of this experiment is given below.

**Methods**

Twenty-six male chicks (Gallus domesticus, white Leghorn, XL-link variety) were obtained from Pace/Setter Products, Inc., Cucamonga, Calif., the day after hatching. They were maintained in a photographic darkroom in three metal cages, each measuring 30 by 45 by 60 cm, with a wire mesh floor. An electric heating pad, set at "medium," was lodged against the back wall of each cage to raise the temperature slightly above that of the darkroom, which was regulated at 24° to 25° C. Each cage was illuminated with a 60-watt incandescent bulb mounted in a standard goose-neck desk lamp. The bulb was situated 10 cm above and 10 cm away from the front of the cage, yielding 32 foot-candles (345 lu/m2) of light in the center of the cage. The chicks were placed on a cycle of 12 hr of light and 12 hr of darkness for 12 days. Chicken feed and water were available at all times.

On the thirteenth day, the animals were sacrificed by decapitation at the following times (hours: minutes) after the beginning of the light period: 0:15, 0:30, 0:45, 1, 2, 4:35, 6:35, 8:35, 10:10, 11:10, 11:40 (lights off at 12:00), 12:10, 12:20, 12:30, 12:40, 12:50, 13, 13:30, 14, 15, 16, 18, 22, 23, 23:55. Those taken during the dark period were quiet, apparently sleeping, and were gently removed from the cage without disturbing the remaining chicks. The heads were decapitated into ice-cold fixative in the dark, then brought into the light for further dissection. The eyes were enucleated, and the front half of the globe trimmed off. The eye cup was then fixed for 1 hr in 1% osmium tetroxide in the darkroom, which was regulated at 24° to 25° C. Each cage was illuminated with a 60-watt incandescent bulb mounted in a standard goose-neck desk lamp. The bulb was situated 10 cm above and 10 cm away from the front of the cage, yielding 32 foot-candles (345 lu/m2) of light in the center of the cage. The chicks were placed on a cycle of 12 hr of light and 12 hr of darkness for 12 days. Chicken feed and water were available at all times.

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Table 1. Number of phagosomes/100 visual cells in the central region of the retina at different times of day.
Fig. 2. Phagosomes within the cytoplasm of the chick pigment epithelium containing clusters of membranes detached from the tips of cone outer segments. A to C, "Fresh" phagosomes, which are not yet noticeably degraded (Stage I). D to F, Stage II phagosomes, in which signs of degradation are visible but the membranous structure still predominates. (A, ×48,600; B, ×39,000; C, ×40,000; D, ×38,000; E, ×52,600; F, ×45,000.)
Fig. 3. Phagosomes containing degraded membranes derived from cone outer segments. A to C, Stage III phagosomes, in which there is only sparse evidence of the original membranous structure. D to F, Stage IV phagosomes. The outer segment membrane structure is no longer detectable, although the predominantly reticular residues may contain regions with a layered orientation. Frequently, parallel filaments which appear to have the same structure as those forming the framework of melanosomes (cf. Fig. 6, A and B) were deposited on the surface of stage IV phagosomes (arrows, D and E). Two partially melanized premelanosomes which seem to be attached to the surface of a phagosome (x) are visible in F. (A, ×45,300; B, ×35,800; C, ×28,500; D, ×35,600; E, ×51,300; F, ×37,000.)
Fig. 4. For legend see opposite page.
uranyl acetate and lead citrate. Most of the blocks were oriented so that sections were cut parallel with the long axis of the visual cells. The analysis was largely restricted to the central region of the retina. However, a few transverse sections and a few specimens of the peripheral retina were also examined.

Quantitative analysis. In specimens from the central area of the retina, sectioned longitudinally for electron microscope examination, I recorded the number of phagosomes in the pigment epithelial cells (Fig. 1, Table I). The phagosomes were subdivided into four classes, which are depicted in Figs. 2 and 3. Stage I included those which were not perceptibly degraded, although they usually showed increased density. Stage II comprised phagosomes which were partially degraded, but consisted primarily of identifiable outer segment membranes. Stage III phagosomes contained some recognizable outer segment membranes, but most of the contents were digested beyond recognition. Stage IV included the final stages of degradation, in which outer segment membranes could no longer be identified. In each region of cell body analyzed, the number of associated rod and cone outer segments was recorded, so that the counts of phagosomes could be related to a known area (defined by the number of associated visual cell outer segments). Whenever the orientation and preservation of stage I phagosomes permitted, I counted the number of outer segment discs (doubled membranes) in the packet. These quantitative assessments were made on the fluorescent screen of the electron microscope.

Results

The chick retina contains one type of rod and several types of cones. The outer segments of all the cones are similar but differ from those of the rods. The cone outer segment discs are continuous with the outer membrane and with one another, forming a continuous membrane system. In rods, only the discs at the base of the outer segment retain such continuities. The rod outer segments are larger in diameter (about 2.0 μm) than those of the cones (1.5 μm). This difference is greater in the periphery, where the rod outer segments are longer, bulkier, and more numerous. (Rods comprise 33% of all visual cells in the periphery compared to 14% in the center.) Rod and cone membranes also reacted differently to the fixative. The cone outer segments were not well preserved, although other structures, including rod outer segments and phagosomes derived from the cones were satisfactorily stabilized. The rods underwent photomechanical movements, extending in the light and contracting in the dark, but the cones were inanimate. Each pigment epithelial cell is associated with 16 to 18 visual cells in the center and 11 to 12 visual cells in the periphery of the retina.

The hexagonal pigment epithelial cells have the usual features of this type of cell, including infoldings of the basal surface, lateral junctional complexes; and long, apical, melanosome-containing processes which envelop the visual cell outer segments. The cell body contains small Golgi zones, multivesicular bodies, autophagic vacuoles, small granules (like those described in the lizard), melanosomes, mitochondria, myeloid bodies free polysomes, very sparse smooth and rough endoplasmic reticulum, and phagosomes. The phagosomes vary in number and structure, depending on the time of day, as will now be described.

The light period. During the first few minutes of the light period, the rods began their photomechanical movement, pushing their outer segments deeper into the pigment epithelium, until their tips were on a level with the ends of the stationary cone outer segments, or slightly beyond. After 15 min there was no evidence of membrane shedding, although a few phagosomes remained in the pigment epithelium in late stages of degradation from an earlier shedding event (Table I). Fifteen minutes later,
Fig. 5. For legend see opposite page.
the displacement of the rods was complete, and the shedding of membranes was vigorously underway (Figs. 4 and 5, A). All or practically all of the rods appeared to discard membranes. Rarely did a section through a rod fail to contain a large phagosome near the tip. The vast majority of the membranes were detached in a single packet. However, occasionally two groups of discs appeared to have been derived from the same rod (Figs. 4, B, and 5, A). A few new (stage I) phagosomes containing cone membranes were detected. These were rare, but unequivocal (Fig. 4, A). The number of membranous discs was recorded in 25 rod phagosomes. The average was 68 discs/phagosome (range 52 to 86). The shedding of membranes was completed by 1 hr, and by the mid-point of the light period, the degradation of the ingested membranes was in its terminal stages (Table I and Fig. 1). Twenty minutes before the end of the light period, a few cone-derived phagosomes were detected.

The dark period. Ten minutes into the dark period, the rods had initiated their photomechanical contraction which, when complete about 20 min later, would result in their tips being on a level near the base of the cone outer segments. At 10 min, the cones were already actively discarding membranes. As had been the case with the rods during the light period, the detachment of membranes from the ends of the cones was largely completed within an hour. However, a small fraction of the cones continued to shed batches of membranes throughout the dark period, and as noted above, a few continued to do so as late as 1 hr into the light period. As a result, phagosomes in various stages of degradation were present within the pigment epithelial cells throughout the entire dark period (Table I and Fig. 1).

The number of discs contained in 32 cone-derived phagosomes was determined. The range was 11 to 72, and the average was 37 discs/packet. The vast majority occurred singly, but in a few cases the membranes were shed from one cone in two separate clusters.

The structural changes which accompany the degradation of rod and cone phagosomes were similar. In the latter stages of digestion, when it was no longer possible to discern visual cell membranes in the digestive vacuole, the contents commonly appeared reticulated and were frequently associated with oriented filaments which were in layers on the outer surface of the phagosome. These filaments were very similar to those which form the framework of melanosomes (Figs. 3, D to F, and 6, A to C). There is also a close association between the residues of phagosome digestion and melanosome formation in the pigment epithelium of the lizard.11

Changes in the appearance of mitochondria in the pigment epithelium were noted (Fig. 5, B and C). Increased polymorphism seemed to predominate in the middle of the light period and again early in the dark period. Sequestering and degradation of melanosomes along the apical edge of the pigment epithelial cell body (Fig. 6, D and E) appeared to reach a maximum early in the light period, although it was not restricted to that interval.

Discussion

The experimental animals formed a remarkably homogeneous group. They were all males from an inbred stock, hatched the same day, and raised together in the same environment. The experimental design was of utmost simplicity, since the only known variable was a light cycle of 12 hr of illumination and 12 hr of darkness. Nevertheless, dramatic changes were recorded in the pigment epithelium and visual cells which were strictly correlated with time of day. These

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Fig. 5. A, Three dense phagosomes slightly displaced from the ends of two rod outer segments are visible in this field. Essentially all the rods appear to shed membranes early in the light period, as indicated by the presence of phagosomes near their tips. The rods do not discard membranes at any other time of day. (×7,400.) B and C, Fluctuation between the rounded, regular appearance of mitochondria in the pigment epithelium (B) and the polymorphic state (C) occurred at different times of day. (B, ×19,000; C, ×23,500.)
Fig. 6. Melanosomes are continually renewed in the chick pigment epithelium. A to C, Premelanosomes in three stages of development. D, At certain times of day—especially, early in the light period—groups of melanosomes are sequestered and degraded. E, This process takes place in the apical part of the cytoplasm, near the visual cell outer segments. The arrow indicates a residual body resulting from melanosome degradation. (A, x65,000; B, x65,600; C, x48,800; D, x12,000; E, x17,300.)
events must have been synchronized by the light cycle—a conclusion fully consistent with an established biological principle: Daily rhythms are entrained by the daily fluctuations in light energy.\textsuperscript{15-17}

In these chicks, the rods shed groups of membranes from the ends of their outer segments early in the light period. Then, one-half day later, the cones discarded packets of membranes from their tips early in the dark period. These results perfectly complement those obtained earlier in a fish\textsuperscript{8} and a reptile,\textsuperscript{11} thereby adding strong support to the hypothesis stated in the introduction, that rods and cones renew their outer segments by membrane replacement according to daily rhythms which are separated in time by approximately 12 hr.

All the rods appear to shed membranes daily. This conclusion is based upon the observations that (1) 30 to 45 min into the light period a phagosome was associated with the tip of practically every rod and (2) the sum of all stages of phagosomes was about 15 per 100 visual cells in the central retina (Table I), which is similar to the proportion of rods (14\%) in that part of the retina. It was difficult to determine whether all the cones shed daily, since the event was less restricted in time, occurring mainly at the beginning of the dark period but taking place sporadically throughout the 12 hr of darkness. In none of the chicks did the sum of all phagosome stages exceed 36\% (Table I). However, even if it is assumed that all the cones discarded membranes daily, the average number of discs per phagosome was only slightly more than half that recorded for rod phagosomes (37 compared to 68). These chicks were examined 13 days after hatching, when outer segment growth is essentially complete.\textsuperscript{14} Therefore, in this steady-state condition, where disc formation is balanced by disc shedding, the rate of membrane renewal is less in cones than it is in rods. During the prehatching stage, Godfrey\textsuperscript{14} found that when the outer segments are still increasing in length, the rods produce about 120 discs/day and the cones about 90/day, adding support to the conclusion that rods produce membranes faster than cones in the chicken.

At 13 days after hatching, rod outer segments contain about 575 discs and cones about 860 discs in the central retina.\textsuperscript{14} These are based on daytime counts, when the rods are somewhat shorter. If we use 600 discs as a rough estimate for the rods, a renewal time of about 9 days (600 ÷ 68/day) is obtained. For cones, the estimated renewal time for the outer segment would be on the order of 23 days (860 ÷ 37), on the assumption that they all shed daily, and even longer if they don’t. These are only crude estimates, but they do suggest that outer segment membrane replacement occurs at a slower pace in chick cones than it does in chick rods.

In Fig. 1, the undegraded (stage I) phagosomes are separated from phagosomes in various states of digestion. This distinguishes two different pigment epithelial cell activities—phagocytosis of the membranes and digestion of the phagocytized material. Unlike the lizard, with its pure-cone retina, which manifests a single interval of phagocytosis and digestion during the dark period,\textsuperscript{11} there are two such daily events in the duplex retinas of the chick and goldfish.\textsuperscript{8} The degradative phase overlaps the phagocytic phase and lasts longer. In fact, degradation continues throughout most of the day, with two broad maxima. In the lizard pigment epithelium, in addition to the phase of phagocytosis and digestion, a phase of accumulation of small granules and a phase of autophagy have been described.\textsuperscript{11} These also overlap in time, and their maxima and minima do not coincide. The daily rhythm of metabolism may therefore be conceived as a regulated succession of numerous overlapping maxima and minima producing continuously changing chemical conditions in the cell in a sequence which is cyclic; that is, it repeats itself.

In recent years, the awareness has grown that the metabolism of the visual cells and pigment epithelium is closely intertwined. The study of daily rhythms adds another dimension to this interrelationship—the time dimension. At least some, and probably all,\textsuperscript{18} of the chemical activities of these cells proceed according to daily rhythms which are synchronized by light.
The assistance of Mirdza Lasmanis, Zoja Trirogoff, Myunghee Chun, Margarita Woo, and Dr. William T. O’Day is gratefully acknowledged.

REFERENCES

Erratum
In the December issue of Investigative Ophthalmology & Visual Science, p. 1142, the name of the author in reference 15 and the first author in reference 23 should be Lovekin.