Photoreceptor outer segment development: light and dark regulate the rate of membrane addition and loss

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The rate of membrane addition during outer segment development and the onset of membrane loss through shedding was evaluated for Xenopus laevis embryos reared at 23° C in constant light (LL), constant darkness (DD), and cyclic light (12 hr L/12 hr D; LD). Embryos were placed under these lighting conditions 1 day following fertilization at open neural plate stages and were sampled each day for 9 days. Rod and cone outer segments were present in each treatment group on day 3 of development. At the end of 10 days of development, rod outer segment volumes were 708 ± 195, 380 ± 40, and 297 ± 33 μm³ (mean ± S.D.) for LL, LD, and DD treatment groups, respectively, whereas mean cone outer segment volumes were 57 ± 8, 48 ± 8, and 35 ± 9 μm³ for these same treatment groups. The loss of outer segment material through shedding was assessed by monitoring the phagosome content of the pigment epithelium. Phagosomes were present in the pigment epithelium in DD embryos as early as 6 days of development, were present at day 5 only after the onset of the light cycle in LD embryos, and were rarely observed in LL embryos. The rate of outer segment membrane elaboration in rods (determined with ³H-leucine autoradiography) was virtually identical in LL and LD (100 and 98 μm³/day) but was greatly reduced in DD (70 μm³/day). These findings indicate that the relatively rapid rate of rod outer segment volume accumulation in LL embryos is due to a reduction in membrane loss through shedding and not to a higher rate of membrane addition as compared to animals reared in LD. In contrast, the reduced rate of rod outer segment development in DD embryos is the result of both a slower rate of membrane elaboration accompanied by the early onset and enhanced rate of membrane loss through shedding in darkness.

Key words: photoreceptor development, rods, cones, pigment epithelium, outer segment renewal, disc shedding
Differentiation of the light-sensitive photoreceptor cells in the vertebrate retina has been the subject of numerous studies. They are among the last retinal cells to stop mitotic activity. Morphological differentiation begins as the cells elongate to form an inner segment which protrudes from the outer retina toward the pigment epithelium (reviewed in refs. 5 and 6). Paired centrioles move into the apical end of the inner segment, and a cilium is elaborated. The plasma membrane of the cilium begins a repeated series of invaginations to form the first array of outer segment membrane discs. As outer segment disc formation continues, the majority of the rod discs detach from the plasma membrane, whereas in cones, disc membrane continuities with the plasma membrane are retained. Light-evoked electrical responses can first be recorded from the developing retina when this process of outer segment membrane formation begins.

As disc addition continues and adult outer segment lengths are attained, membrane addition does not stop but continues throughout life by a process referred to as outer segment membrane renewal. This renewal process is best understood for rod photoreceptors and was first described in the autoradiographic studies of Young and Young and Droz. When animals were injected with radioactive amino acids to label newly synthesized proteins, the first site of labeling was within the inner segment. Much of the newly synthesized protein was then transported to the rod outer segment base, where it formed a group of radioactive discs. As membrane addition continued, the radioactive discs were displaced along the length of the rod outer segment to the tip, where they were shed as a packet of membrane debris. The detached disc packet was then phagocytized and degraded by the pigment epithelium.

Recent evidence indicates that light plays an important role in regulating the rate of outer segment renewal. In animals maintained under conditions of cyclic lighting (LD), rod shedding occurs shortly after the onset of the light cycle, whereas in cones, shedding commences shortly after the beginning of the dark cycle. In constant darkness (DD) rod shedding follows a circadian pattern, but in constant light (LL) shedding is abolished.

It has also been recently established that the rate of disc addition to rod outer segments varies widely under different lighting conditions. In LD animals, 75% of the daily increment of disc addition occurs during the first 8 hr of the day. Furthermore, membrane addition proceeds more rapidly in LL than in LD animals and is greatly reduced in DD.

All these recent studies have been directed at assessing the effects of environmental lighting on the outer segment renewal process in larval and adult animals. In this study, we report the effects of different conditions of environmental lighting on the rate of membrane addition and the onset of membrane loss through shedding during development of photoreceptor outer segments in the toad Xenopus laevis.

Materials and methods

Animals and treatments. All embryos used in this study were from a single clutch of eggs obtained through induced breeding of adult X. laevis by the injection of human chorionic gonadotropin (Ayerst Laboratories, New York, N.Y.). Following ovulation, groups of 20 to 30 fertile eggs were placed in individual fingerbowls in two constant temperature incubators. Each incubator was equipped with two 15 w incandescent bulbs which delivered 200 to 250 lx/m² at the level of animal containers. Lighting in one incubator was on a diurnal cycle of 12 hr light and 12 hr darkness per day (LD). The interior of the second incubator was under continuous illumination (LL). Conditions of continuous darkness (DD) were provided by placing fingerbowls of embryos in black, lightproof sacks made from double-thickness darkroom cloth. These shrouded bowls were placed in the LL incubator. Water temperature in LD, LL, and DD treatment groups was monitored daily and was consistently 23° C. From the sixth through the ninth day of development, a suspension of commercial frog food (Nasco) was added to each of the animal containers. Animals to be used for autoradiography received intraperitoneal injections of 1 to 0.5 µl of L-[N 4, 5-3H] leucine with a specific activity of 60 Ci/mM (New England Nuclear, Bos-
Fig. 1. Section through the center of an eye from stage 37/38 *Xenopus laevis* embryo fixed 3 days after fertilization. The rectangular area near the posterior pole of the retina is shown in Fig. 2. (Calibration bar, 100 μm.)

Fig. 2. Higher magnification of the area indicated in Fig. 1. Photoreceptor layer (PL), pigment epithelium (PE), and developing choroid (C) are shown. The large, deeply staining oval structures, some of which have been circled, are yolk platelets, not phagosomes. Several photoreceptors contain short outer segments. Rod outer segments indicated with the large oblique arrows; cone outer segments indicated with the smaller horizontal arrows. Note that nuclei of cone photoreceptors are more sclerally located than the nuclei of rod photoreceptors. (Calibration bar, 10 μm.)

Microscopy. All material was fixed overnight with a mixture of 3% glutaraldehyde and 3% freshly prepared formaldehyde in 0.087M phosphate buffer (pH 7.2) containing 0.05% CaCl₂. For the first 3 days of fixation, embryo heads were severed in the fixative, and the corneas were gently punctured with a steel needle to allow fixative penetration. On subsequent days, eyes were dissected from the heads and were punctured. DD animals were fixed in the dark with only a dim
Fig. 3. Electron micrograph of the developing photoreceptor–pigment epithelium complex. The electron-dense, spherical to oval-shaped granules across the center of the micrograph are melanosomes in the apical border of the pigment epithelium. Three developing rod photoreceptors containing 20 to 30 outer segment membrane discs (OS) are present. Two of the photoreceptors contain prominent oil-droplets (OD). A trilobular yolk platelet (YP) is present in the pigment epithelium. The inset, upper right, shows the paracrystallin ultrastructure of the yolk platelet, easily distinguished from phagosomes which appear later. (Calibration bar, 2 μm; inset ×78,000.)

red light (Wratten No. 2 filter, 15 w bulb). Postfixation was for 90 min with 1% osmium tetroxide buffered as above. Tissues were then quickly dehydrated through graded ethanol and were embedded in a mixture of Araldite and Epon, with propylene oxide used as an infiltrating agent.

Sections 1 μm thick were cut along the dorso-ventral axis of eyes from three or more animals at each sample time. Sections for analysis were from the center of eyes near the optic nerve head. They were stained directly with toluidine blue or following removal of the plastic.24

Sections from 3H-leucine–injected embryos were placed on glass microscope slides and were dipped in Kodak Nuclear Track Emulsion (NTB2) diluted 1:1 with distilled water at 40° C. After drying, slides were stored in air and light-tight
Bakelite slide boxes containing a desiccant (anhydrous CaSO₄). After exposure for 1 to 2 months at 4° C, the slides were developed in Kodak Dektol diluted 1:1 with distilled water at 15° C. After a distilled water rinse, they were fixed with Kodak fixer for 5 min. Sections were stained for 10 to 15 sec. with 1% toluidine blue in 0.087M phosphate buffer (pH 7.2) at 60% C. After being rinsed in distilled water, the sections were dried, and cover glasses were applied with immersion oil.

Selected blocks were thin-sectioned for electron microscopy. Thin sections with silver to grey diffraction colors were placed on single-hole, parlodion-coated grids. Sections stained with uranyl acetate and lead citrate were observed with a Siemens Elmiskop 1A.

All quantitative data from light microscopy was obtained with a 100X oil-immersion objective and 10x ocular lenses. One ocular was equipped with a micrometer with 0.9 μm divisions. Outer segments of both rods and cones during early development are tapered, with widths of the base exceeding widths of the tip. Their shapes closely approximate the frustum of a cone. Their volumes (V) were calculated by the formula:

\[ V = \frac{\pi h}{3}(r_1^2 + r_1 r_2 + r_2^2) \]

where h is the length of the outer segment from the base to the tip and r₁ and r₂ are the radii of the outer segment tip and base, respectively. Volumes of 20 rod and 10 cone outer segments in the posterior pole of the retina were determined in each eye. Quantitative data were taken from three eyes from three different animals at each treatment and sampling time. Shapes of both yolk platelets and phagosomes in the pigment epithelium varied from circular and oval profiles through highly irregular contours. Because of these irregular shapes, all measurements of these structures were of the largest dimension, i.e., length of elongate structures, diameter of circular profiles.

Twenty rod measurements per eye were made in the autoradiographic experiments. The above formula was used to calculate the volume of rod outer segment material under a band of radioactivity from the ³H-leucine-injected animals except that r₁ in this case was the radius of the outer segment at the level of the ³H-band.

**Results**

Previous studies of developing *Xenopus* photoreceptors have established that as outer segments first appear and begin to elongate, the initial shape of these components in both rods and cones is conical. In addition, both rod and cone inner segments during these early stages possessed a prominent oil droplet (Figs. 1 and 2), a feature characteristic of only cones at later stages. Despite these similarities during early development, morphological differences were present which made it relatively easy to identify these two cell types. At the time when outer segments were first observe on day 3 of development, the cone nuclei were more sclerally positioned in the outer nuclear layer than were the nuclei of rods (Fig. 2). As development proceeded, the conical outer segment shape in cones persisted and the cones remained relatively short, whereas in rods a cylindrical shape was assumed and the rods continued to increase in length throughout premetamorphic development and beyond.
Figs. 5 to 10. Light micrographs of the photoreceptor-pigment epithelium complex in animals that were prepared for microscopy on day 10 of development following the treatment indicated below. Rod outer segments are broad-based at this stage, and their nuclei and perikaryon cytoplasm are more darkly stained than equivalent regions in cones. The cone outer segments are short and thin as compared to rods, and the cone inner segments contain a prominent oil droplet. (All micrographs at same magnification; calibration bar, 20 μm.)

Fig. 5. Retina from the LD group fixed at 0800 hours in the dark just prior to the onset of the light cycle. One phagosome is present in the pigment epithelium (arrow).

Fig. 6. Section through an eye fixed at 1100 hours, 3 hr after the onset of the light cycle. Several phagosomes are now present in the pigment epithelium (arrows).

Fig. 7. Eye from an animal fixed at 1100 hours which had developed for 10 days in LL. Note the long, tapered rod outer segments which protrude into the pigment epithelium. Several circular myeloid bodies are present in the pigment epithelium, but phagosomes are absent.

Fig. 8. Eye from an animal developing for 10 days in LL which was placed in the dark for 1 hr beginning at 0800 hours and was returned to LL at 0900 hours. The eye was fixed for microscopy at 1100 hours. Note that many of the rod outer segments are shorter than those shown in Fig. 7 and that some of these have lost their conical shape. Phagosomes are present in the pigment epithelium (arrows).

Fig. 9. Eye from an animal fixed at 1100 hours which developed for 10 days in DD. Note the short outer segments as compared with those in Fig. 7. A phagosome is present in the pigment epithelium (arrow).

Fig. 10. Eye from an animal developing 10 days in DD which was brought into the light at 0800 hours and was fixed 3 hrs later at 1100 hours. Note the numerous phagosomes present in the pigment epithelium (arrows).
Fig. 11. Electron micrograph of the photoreceptor-pigment epithelium complex from an animal developing for 10 days in LD. Three rod outer segments (ROS) and one cone outer segment (COS) are present in the lower border of the illustration. A large phagosome (P) is present within the pigment epithelium. This material was prepared for microscopy 3 hrs after the onset of the light cycle. The inset at the upper right shows the membranous composition of a phagosome. (Calibration bar, 2 μm; inset ×33,000.)

In studies of the rate of rod outer segment development in Xenopus, Kinney and Fisher²⁵²⁷ have established that the length of these structures increases rapidly during the early conical shape phase but slows as membranes with wider expanses are added to the outer segment base. By taking into account the shape changes from the conical to cylindrical configuration and expressing outer segment growth as volume of material added with time, they observed a constant rate of membrane addition throughout all embryonic and larval stages. In this study, the volume analysis of Kinney and Fisher²⁷ was used to follow the rate of outer segment development under different lighting conditions. In this same material we have monitored the pigment epithelium for phagosomes as an indication of the time of onset of outer segment shedding.

One of the difficulties encountered in our analysis of phagosomes was that during the early stages studied, cells throughout the embryo, including the retina, pigment epithelium, and choroid, contained numerous yolk platelets (Fig. 2). These structures had staining characteristics similar to phagosomes. Electron microscopic analysis of selected early stages (Fig. 3) established that these densely staining bodies have an ultrastructure consisting of a paracrystalline lattice which has been well documented as
Fig. 12. Changes in the volume of outer segments in rod and cone photoreceptors in relationship to the phagosome content of the pigment epithelium during development in LD. Closed circles, Animals fixed in the dark at 0800 hours on each recovery day; Open circle, animals fixed at 1100 hours, 3 hr after the onset of the light cycle. Rod outer segments show a near-linear increase in volume through 8 days of development. On day 9 and day 10 significant reduction in outer segment volume occur following the onset of the light cycle. Cone outer segment increase in volume during the period of observation, but no significant changes in their volume are apparent. Small numbers of phagosomes are present in the pigment epithelium as early as day 5 of development in animals fixed after the onset of the light cycle. As will be reported below, they were present only in eyes that had undergone recent lighting transitions and were both larger than the 5-day yolk platelets and of a different shape. These differences made it possible to distinguish between the two inclusion bodies on day 5 when both yolk platelets and phagosomes were present in the pigment epithelium. Large numbers of phagosomes did not appear until day 8 or 9 of development, 2 days after the disappearance of yolk platelets from the pigment epithelium. Selected light micrographs from animals on the tenth day of development showing numerous phagosomes are presented in Figs. 5 to 10. An electron micrograph of outer segments and the pigment epithelium showing the membranous composition of a phagosome is presented in Fig. 11.

Development in LD animals. Two recoveries were made each day during development in the LD group. One sample was taken just prior to the onset of the light cycle at 0800 hours, and the second sample was taken 3 hr into the light cycle at 1100. The quantitative data of rod and cone volume and the analysis of phagosomes in the pigment epithelium from this material are presented in Fig. 12.

Rod outer segments were not present until the third day of development. The initial outer segment volumes at this time were $13 \pm 3$ and $11 \pm 2 \mu m^2$ for the 0800 and 1100 hour samples, respectively. Rod outer segment volume increased thereafter in a near-linear fashion until the 1100 hour sample on day 9 of development, when a sig-
significant reduction in the rod outer segment volume occurred following the lighting transition on that day. On the tenth day of development a similar reduction in outer segment volume occurred between the 0800 and 1100 hour samples. The final volume of rod outer segment at the end of the period of observation was $380 \pm 40 \mu m^3$. This reflects an average rate of volume accumulation of $54 \mu m^3$/day during the 7 days of development after outer segments first make their appearance on day 3.

Cone outer segments were also first observed on the third day of development, but their volumes were small compared to rods. The initial cone outer segment volumes were $1 \pm 0$ and $3 \pm 3 \mu m^3$ in the 0800 and 1100 hour samples. There was a gradual increase in volume through the remaining sample periods, and the final mean volume of cone outer segments at the 1100 hour sample on the tenth day of development was $44 \pm 3 \mu m^3$, reflecting an average rate of cone outer segment volume accumulation of $6.3 \mu m^3$/day. No significant differences in cone outer segment volumes were apparent following the light transitions on any of the recovery days.

Phagosomes were first present in the pigment epithelium in the 1100 hour sample on the fifth day of development. At this time one phagosome was present in one of the three eyes examined, and it was $5 \mu m$ long, larger than the yolk platelets present in the 5-day material recovered at 0800 hour (Fig. 5, stage 44). The shape of this phagosome was rectangular as compared to the oval shape of the smaller yolk platelets. On the sixth and seventh days of development in the 1100 hour sample, one phagosome was also present on each of these days. On the eighth through the tenth days of development there was a progressive increase in the number of phagosomes in the pigment epithelium in the samples fixed at 1100 hour. Photomicrographs of material recovered at 0800 and 1100 hours on the tenth day of development are presented in Figs. 5 and 6.

**Development in LL embryos.** Two recoveries were made each day from the em-

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**Fig. 13.** Changes in the volume of outer segments in rod and cone photoreceptors in relationship to the phagosome content of the pigment epithelium during development in LL. Open circles, Animals fixed in the light at 1100 hours on each of the sampling days; closed circles, animals which had developed in LL up to 0800 hours on each of the sampling days, been placed in darkness for 1 hr, been returned to light, and were fixed at 1100 hours. Note the large outer segment volume achieved at the end of 10 days treatment as compared to LD animals presented in Fig. 12. A significant reduction in mean outer segment volume occurs on day 10 of development in animals given 1 hr of darkness followed by 2 hr of light. Cone outer segment volume increased during the period studied but showed no significant change in volume on any of the days studied following dark-light manipulation. Phagosomes are present in the pigment epithelium on day 8 in the LL material. A few phagosomes are present in the dark-light manipulated material on day 7 and 8, and larger numbers are present on day 9 and 10. Vertical bars as in Fig. 12.
Development in DD embryos. Two re-

rod and cone photoreceptors in relationship to the phagosome content of the pigment epithelium during development in DD. Closed circles, Animals fixed in the dark at 1100 hours on each recovery day; Open circles, animals brought into the light at 0800 hours and fixed in the light, 3 hr later. Note the slow rate of volume accumulation in rod outer segments in DD as compared to rod volume accumulation in LL and LD material. Cone outer segments increase during the period of analysis but show no significant volume changes on any of the recovery days following exposure to light. Phagosomes are present as early as 5 days of development in numbers higher than were observed in either LD or LL groups. Larger numbers of phagosomes are present in the pigment epithelium following 3 hr of exposure to light on day 5, 7, and 10. Vertical bars as in Fig. 12.

Development in DD embryos. Two re-

development outer segment shedding could be initiated by a short period of darkness. Previous experiments with adult frogs indicate that conditions of LL delay shedding for periods as long as 10 days. When LL frogs are given as little as 30 min of darkness and are then returned to light, rod shedding proceeds within 1 to 2 hr. The quantitative data for rod and cone volumes and the analysis of phagosomes in the pigment epithelium in this material are presented in Fig. 13.

Rod outer segments were first present on the third day of development in LL and mean volume at this time was 11 ± 2 μm³. Their volume increased during subsequent developmental days to achieve a final volume of 708 ± 190 μm³. This reflects an average rate of volume accumulation of 101 μm³/day during the 7 days of development in LL after outer segments first made their appearance. Significant reduction in rod outer segment volume in the animals given 1 hr D + 2 hr L occurred only on the tenth day of development.

Cone outer segments were also present on the third day of development in LL animals, and their mean volume at this time was 5 ± 3 μm³. Their volumes increased slowly through the tenth day of development to achieve a final volume of 57 ± 12 μm³. This reflects a rate of volume accumulation of 8.1 μm³/day. No significant difference in outer segment volumes were apparent between animals fixed in LL and those given 1 hr D + 2 hr L before fixation.

Phagosomes were first observed in the LL animals on the eighth day of development. Phagosomes were present in only two of the three eyes examined at this time, one phagosome in one eye and two in the other. On the ninth day of development, one phagosome was present in one of the three eyes examined. On the tenth day, phagosomes were present in each of the three eyes examined, with a high of 5/eye and a low of 3. In the LL group given 1 hr D + 2 hr L, significantly more phagosomes were present on the ninth and tenth day of development in this material than were in the LL material recovered at corresponding times.

Development in DD embryos. Two re-
Fig. 15. Histograms showing the pooled size-frequency distribution of phagosomes in the pigment epithelium on the ninth and tenth day of development in all treatment groups. Note that in the LD animals fixed at 0800 hours and the LL animals, relatively few phagosomes are present as compared to DD animals (upper three histograms). In contrast, abundant phagosomes are present in the pigment epithelium 3 hr after the onset of the light cycle in LD animals and in LL animals which were dark-light manipulated and DD animals which had received 3 hr of light prior to fixation. The dimensions of the phagosomes in the LD and LL material are relatively uniform. In DD, phagosomes with dimensions larger than encountered in the other two treatment groups are present.

recoveries were made each day from animals in the DD group. One sample was fixed under dim red light at 1100 hours on each of the recovery days. Another sample was also taken at 1100 hours each day, but these animals had been exposed to light since 0800 hours on the same morning. This latter group was included to determine when outer segments developing in DD could be stimulated to shed following exposure to light. Previous experiments in larval and adult frogs have shown that in animals maintained for several days in DD, a few hours’ exposure to light is sufficient to induce a maximum outer segment shedding response. Photomicrographs of DD material from the tenth day of development are presented in Figs. 9 and 10. The quantitative data from the DD reared animals is presented in Fig. 14.

Rod outer segments were present on the third day of development in DD, and their volumes at this time were 17 ± 2 μm³, increasing in subsequent days of development to achieve a final volume of 297 ± 33 μm³, an average rate of volume accumulation of 43 μm³/day. In the animals exposed to light for 3 hr, significant reductions in rod outer segment volumes occurred only on the ninth day of development.

Cone outer segments were first observed on day 3 of development in DD. Their mean volumes at this time were 3 ± 1 μm³. On subsequent days of development the volume increased to a final volume of 35 ± 9 μm³, an average rate of volume accumulation of 5 μm³/day.

Phagosomes were more abundant in the pigment epithelium of the DD-maintained animals at earlier developmental stages than were observed in either the LD or LL material. More phagosomes were present in the material fixed 3 hr after exposure to light on day 5, 7, and 10 than were observed in the DD group. Photomicrographs showing phagosomes in the pigment epithelium in DD and DD + 3 hr L from the tenth day of development are presented in Figs. 9 and 10.

In addition to the differences in phagosome numbers and time of their appearance observed in these three treatment groups, there were also striking differences in the size of phagosomes in animals developing under the three conditions. Histograms showing the size-frequency distribution of
Figs. 16 and 17. Autoradiographs of the retina from *X. laevis* reared in LD following injections of \(^{3}H\)-leucine at stage 41 on day 4 of development. The autoradiograph in Fig. 16 was prepared 1 day after labeling, whereas Fig. 17 was prepared 3 days following labeling. Discrete bands of radioactivity are present across the outer segments (arrows). The volume of the outer segment material between the labeled bands and the outer segment base was calculated to determine the rate of membrane production under the different lighting conditions. See text for further explanation. (Both photomicrographs at the same magnification; calibration bar, 20 \( \mu m \).

Phagosomes from all animals recovered on the ninth and tenth day of development are presented in Fig. 15. As can be seen in this figure, phagosomes with similar dimensions are present in both LD and LL material. In contrast, phagosomes with larger dimensions were present in the pigment epithelium of animals maintained in DD.

The volume of outer segment material present at the end of our analysis represents only the net amount accumulated during this period. The differences in outer segment dimensions could be due to dissimilarities in the rate of membrane addition, the rate of membrane loss through shedding, or a combination of these two factors. It is possible to
directly assess the rate of membrane addition in rod photoreceptors with autoradiography following 3H-leucine injection. This label is incorporated into newly synthesized membrane precursors, some of which will then be transported to the base of the outer segment and assembled into a band of radioactive membrane discs. These bands of radioactive discs are displaced away from the base of the outer segment as the process of membrane addition continues. The rates of membrane accumulation under the 3H-discs in animals maintained under different lighting conditions can then be measured by the same method used for total volume determinations, except in this case the radius of the outer segment at the level of the radioactive band is substituted for the measurement of the radius of the outer segment tip. This method cannot be used to study cone development, since cone outer segments are diffusely labeled and do not form a band of radioactive discs following 3H-leucine injection.13

3H-leucine was injected into 20 stage 41 embryos at 1000 hours on the fourth day of development in LD. They were retained for an additional 24 hr in LD. At this time, three animals were prepared for autoradiography to determine the volume of outer segment material produced during this initial 24 hr period. The remaining animals were divided into three groups. One was retained under LD, and the others were placed in DD or LL. After two additional days (day 7 of development, stage 45) all were prepared for autoradiography. The rate of volume accumulation under these conditions was then measured by assessing the difference between outer segment volumes present under the 3H-bands on day 5 and day 7 (see Figs. 16 and 17). This procedure eliminates the period following injection when 3H-leucine was incorporated, transported, and assembled into radioactive discs. By eliminating this lag period, the method provides a more accurate assessment of the rate of volume increase. It is evident from this autoradiographic data (presented in Table I) that under LL and LD, the addition of membrane material to rod outer segments proceeds at virtually identical rates. In DD however, the rate of outer segment volume increase proceeds at a rate which is reduced by approximately 30% from that observed in LL or LD.

Table I. Rod outer segment development in X. laevis

<table>
<thead>
<tr>
<th>Volume of ROS (μm³) below 3H-band (mean ± S.D.)</th>
<th>Rate of volume production (μm³/day)</th>
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</thead>
<tbody>
<tr>
<td>Day 5 (t₁)*</td>
<td>Day 7 (t₂)†</td>
</tr>
<tr>
<td>LD 77 ± 42</td>
<td>273 ± 82</td>
</tr>
<tr>
<td>LL§ 77 ± 42</td>
<td>276 ± 89</td>
</tr>
<tr>
<td>DD§ 77 ± 42</td>
<td>217 ± 17</td>
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</tbody>
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*Rod outer segment (ROS) volume on day 5 (t₁) of development (stage 44) was obtained from a group of 3 LD animals injected with 3H-leucine 24 hr earlier (stage 41).  †Values on day 7 (t₂) were obtained from LD maintained animals injected 3 days earlier.  ‡Rate (R) of volume production per day was calculated by the formula:

\[ R = \frac{V_{t₂} - V_{t₁}}{t₂ - t₁} \]

where V is the calculated volume of ROS material below the 3H-band and t is the elapsed time following injection. See text for further explanation.  §Values for LL and DD were obtained from LD animals injected on day 4, maintained for 24 hr in LD, and then placed in LL or DD and allowed to develop for 2 additional days.

Table II. Summary of rod outer segment membrane addition and loss (μm³/day) in X. laevis developing under different lighting conditions

<table>
<thead>
<tr>
<th>Rate of production (A) (autoradiography)</th>
<th>Rate of accumulation (B) (total volume)</th>
<th>ROS material loss (C) (A - B)</th>
<th>% loss (C/A x 100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LD 98</td>
<td>101</td>
<td>44 (-1)</td>
<td>40</td>
</tr>
<tr>
<td>LL 100</td>
<td>43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DD 70</td>
<td>27</td>
<td></td>
<td></td>
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</table>

It is possible to calculate the amount of rod outer segment material lost in each of the light treatment groups by subtracting the average volume accumulation per day from the rate of volume production per day as determined with autoradiography. It is apparent from this calculation (presented in Table II) that in LL the rate of outer segment production and rate of net volume accumulation are identical, indicating that little if any outer segment material was lost in LL during the period of analysis. In contrast, although the rate of outer segment production in LD is similar to the rate of production in LL, 44%
Discussion

Previous studies have found that light is not an absolute requirement for the development of photoreceptor outer segments.\textsuperscript{31–33} Our studies support these earlier observations. In both rods and cones, outer segments develop in the absence of light. The rate at which rod and cone outer segments develop, however, is dramatically different under different lighting conditions. As judged by the volume of outer segments present at the end of our sampling times, LL promotes the most rapid rate of development, DD the slowest rate, and LD an intermediate rate between the two extremes.

The autoradiographic data show a high rate of membrane addition to rod outer segments in LL embryos which is virtually identical to the rate of volume accumulation (Table II). We conclude from the similarity of these two independently derived values that membrane loss from the rod outer segments in the LL group did not occur to any appreciable extent. This is supported by the paucity of phagosomes present in the pigment epithelium in LL animals. Further evidence of the absence of shedding in the LL treatment group is the retention of the narrow apical outer segment membranes which were added during early stages of development (Fig. 7). In contrast, these narrow tips were lost through shedding from rod outer segments in both LD and DD treatment groups.

Although rod outer segment volumes in LD animals at the end of the period of observation were smaller than those present in LL animals, the autoradiographic data indicate that membrane addition proceeds at approximately the same rates under these two lighting conditions. We conclude from this comparison that the smaller volume present in LD animals was due to the loss of outer segment material. Most of this loss occurs on the ninth and tenth day of development following the onset of the light cycle, as evidenced by the reduction in mean rod outer segment volumes at these times. The reduction in volume was coincident with an increase in phagosome content of the pigment epithelium.

Because cone outer segments did not undergo volume reductions, we must conclude that the phagosomes present in the pigment epithelium are of rod origin. Since each phagosome is the result of a single shedding event by a photoreceptor in the subjacent retina,\textsuperscript{17, 20, 30} from our mean phagosome count we estimate that in LD an average of 20% of the rods were shed on day 9 and 45% on day 10. This is based on counts of 49 ± 2 rod profiles below a 500 μm expanse of pigment epithelium. The latter value is higher than the daily rod shedding frequency of 22% to 30% reported for \textit{Xenopus} tadpoles maintained at 28° C.\textsuperscript{18}

The rod outer segments present in animals developing under DD had the smallest volumes of the three treatment groups. Our autoradiographic data indicate that the rate of membrane addition in the DD group is reduced by 30% from that observed in LL and LD groups. This slower rate of membrane addition does not account completely for the reduced volume of outer segment material present. The relatively abundant population of phagosomes some of which were larger than those present in LL or LD embryos (Fig. 15) indicates that animals in DD also lose considerable amounts of outer segment material through shedding. We estimate that of the total amount of rod outer segment material added, 40% of this volume is lost by the tenth day of development in DD (Table II).

In larval frogs a relationship exists in rods between the amount of outer segment material added and the frequency of shedding. An elevation in the rate of membrane addition results in an increase in the shedding rate.\textsuperscript{30} One might predict that shedding would first be initiated in developing rods, when the outer segment volume exceeded some critical limit. This, however, was not what we observed in the present study. If five or more phagosomes/500 μm expanse of pigment epithelium in any one animal per sampling time...
is considered as a significant shedding response (10% rod shedding), this level was reached in DD on day 6 when the mean outer segment volume was 224 ± 70 μm³, in LD on day 8 when the mean volume was 452 ± 46 μm³, and in LL on day 10 when mean volume was 708 ± 195 μm³. Although outer segment sizes were very different when shedding began, one striking correlation is that the onset of shedding occurred earliest in animals that were in darkness for the longest period of time.

The importance of darkness in the rod-shedding phenomenon is further indicated in the LL animals which were placed in the dark for 1 hr and returned to the light for 2 hr prior to fixation. This brief period of darkness was sufficient to support significant shedding on the ninth and tenth day of development. Although DD animals on day 5, 7, and 10 which were brought into the light for 3 hr before fixation showed enhanced shedding over dark-fixed controls, the similarity in phagosome numbers on days 6, 8, and 9 in the DD-fixed and DD animals exposed to light for 3 hr suggests that rod shedding had taken place in the dark and could not be further enhanced by light exposure.

What product of darkness mediates the rod shedding phenomenon? Although an answer to this question must await further analysis, in larval and adult animals close parallels exist between changes in the pineal output and blood levels of melatonin and the times of rod shedding. Shedding, however, will proceed in the absence of the pineal in both the frog and rat. This observation does not rule out the involvement of the melatonin pathway in the response, since the enzyme necessary for converting serotonin to melatonin is present within the eye and melatonin has been localized immunohistochemically in the photoreceptor layer in the rat. Furthermore, this enzyme is reported to appear during the development of the eye in Xenopus at stages when rod shedding begins.

Our measurements of cones did not reveal significant reductions in outer segment volumes following any of the lighting transitions provided. Although cone shedding may have occurred, the confidence limits around the mean volumes were so broad that we were unable to assess any significant changes. However, there were differences in the volumes of cone outer segments present at the end of our analysis among the three light treatment groups. The analysis indicates that cone outer segments develop in LL, LD, and DD at rates of 8.1, 6.3, and 5.0 μm³/day, respectively. These differences in the rate of cone development are similar to the pattern in our rod analysis in that rod volumes accumulate fastest in LL, slowest in DD, and at an intermediate rate in LD. Because cones are not amenable to the kinds of autoradiographic analysis possible with rods, we could not analyze independently the rates of membrane addition to and loss from their outer segments. However, the differences in rate of volume accumulation in cone outer segment under the three lighting conditions and the similarities of this pattern to that observed in rods suggest that light may also stimulate the rate of membrane addition to cone outer segments as well.

We conclude from this study that the development of rod and cone photoreceptor outer segments proceeds most rapidly in LL, slowest in DD, and at an intermediate rate in LD. With respect to rods, membrane addition to outer segments occurs at approximately the same rate in LL and LD but at a reduced rate in DD. Membrane loss from rods through shedding occurs earliest during development in DD, somewhat later in LD, and at a greatly reduced rate when embryos develop under LL.

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