darkroom with double doors and environmental conditions similar to those in the animal room. They were in the light for less than 4 minutes before perfusion.

14. The rats were anesthetized with ether and killed by vascular perfusion of a mixture of 2 percent formaldehyde and 2.5 percent glutaraldehyde in 0.1*M* phosphate buffer, *p*H 7.3 to 7.4, at room temperature (22°C). The eyes of the rats were processed, embedded in an Epon-Araldite mixture, and sectioned along the vertical meridian as described elsewhere [M. M. LaVail and B.-A. Battelle, *Exp. Eye Res.* **21,** 167 (1975)].
15. Young and Bok (*2*) established that in plastic-embedded tissues stained with toluidine blue, the intensely staining inclusion bodies within pigment epithelial cells are the phagocytized packets of outer segment disks. Of those phagosomes located in the pigment cell processes in the present study, only those that stained more intensely than outer segments were counted. It was assumed that they had begun to compress their membranes, had recently detached from the rod outer segments, and were already within the pigment epithelial cells (phagosomes), but this could not be resolved by light microscopy.
16. Outer segment length in Fischer rats is about 25 μm. Total outer segment renewal time in the rat is about 10 days (*1*).
17. The long dimension of the first 100 large phagosomes encountered in the pigment epithelial cell processes and of the first 100 in the pigment cell somas was measured in sections from four rats killed during the peak disk shedding period (about 25 measured from each animal) and from four rats killed later in the day, 5 to 10 hours after the onset of light. A phagosome was defined as being in the processes if any part of it was located there. The mean size of phagosomes during the peak disk shedding period was 1.73 ± 0.05 μm (mean ± standard error of the mean) in the processes and 1.32 ± 0.05 μm in the pigment cell somas. Phagosomes present later in the day measured 1.54 ± 0.03 μm in the processes and 1.13 ± 0.03 μm in the somas.
18. The change in phagosome size may involve rearrangement of membrane rather than (or in addition to) chemical degradation and probably represents only the early phases of disk digestion. Later phases may proceed more slowly [N. F. Johnson, *Exp. Eye Res.* **20,** 97 (1975)].
19. At about 1 hour before the dark phase of the lighting cycle, either one eyelid was sutured (one rat) or one eyelid was sutured and that side of the head was painted black with a felt-tip marker (three rats). Three other rats were anesthetized with chloral hydrate about 1 hour before the onset of light, by using dim red light (BCJ ruby bulb, General Electric Co.); a piece of a standard ophthalmologic eye patch was placed over one eye, this was painted black with a felt-tip marker, and the anesthetized rats were placed on their sides with the patched side of the head buried in cage bedding until they were perfused.
20. It is generally accepted that for rhythms to be termed circadian, they must complete a cycle in about a day's length and persist in the absence of lighting cues; these are distinguished from daily rhythms that are totally dependent on environmental cues of lighting transitions (*22*).
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Photoreceptor Shedding Is Initiated by Light in the Frog Retina

Abstract. *Frogs maintained on a diurnal light-dark cycle (14 hours light and 10 hours darkness) shed their rod photoreceptor outer segment tips shortly after the onset of light. Shedding is synchronous and occurs in about 25 percent of the rod photoreceptors each day. Prolonged exposure to total darkness decreases the amount of shedding, after which exposure to light results in a large burst of synchronous shedding. Thus, in the frog retina, the synchronous shedding of rod outer segment tips is shown to be directly related to light stimulation.*

Many biological events are induced by the onset of light, for example, the migration of retinal screening pigment in teleosts and amphibians (*1*) and increases in serotonin concentrations in vertebrates (*2*). Recently, LaVail (*3*) has shown that rat rod photoreceptors shed their outer segment tips according to a circadian rhythm. We have observed a similar, although not circadian, phenomenon in the frog retina, and this report describes our observations concerning the induction of photoreceptor shedding by light.

Photoreceptor rod outer segments (ROS) in vertebrate retinas are renewed through the assembly of new membrane disks at the base of the ROS. Through this process, older disks are displaced toward the apical end of the cell (*4*). Small packages of disks are shed from the tip of the ROS, thereby maintaining the outer segment at a constant length. The shed tips are engulfed by the retinal pigment epithelium (PE), forming inclusion bodies called phagosomes, which are subsequently digested and eliminated from the PE (*5, 6*). The shedding and phagocytosis of ROS tips has been described previously (*5, 6*), but until now very little was known about the initiation or regulation of these processes. We have studied the light induction of rod photoreceptor shedding in frogs previously adapted for at least 2 months to a diurnal cycle of 14 hours light and 10 hours dark.

Frogs (*Rana pipiens*, northern variety) were kept at room temperature (22° to 23°C) in a large Plexiglas chamber with a supply of constantly running water. Illumination was provided by ceiling fluorescent lamps and was at a level of 60 footcandles (645 lu/m^2) on the floor of the frog chamber. Automatic timers were used to turn room lights on at 8 a.m. and off at 10 p.m. Animals were killed at regular intervals during the diurnal cycle, and longitudinal sections through the photoreceptors and pigment epithelium were examined for the presence of phagosomes (*7*).

Just before the onset of light (7:50 a.m., Fig. 1a), no newly shed phagosomes are observed in the PE (*8*). Only small phagosomes (< 2.0 μm), presumably in the terminal stages of digestion, are seen. As is typical of dark-adapted amphibians, the granules of melanin are aggregated at the apical border of the PE cell. One hour after the onset of light (9 a.m., Fig. 1b), many newly shed phagosomes (5.0 to 6.5 μm) are seen above the tips of the ROS just inside the apical border of the PE. Most of the phagosomes are still rectangular, suggesting that they have just been shed from the ROS tips, and their dark staining appearance indicates that their degredation has already begun (*5, 9*). The outer segments of rods that have shed their tips are slightly shorter (about 10 percent) than the outer segments of those that have not, and some elongated ROS appear deeply inserted into the PE. Extensive examination of longitudinal sections at this time reveals that about 25 percent of the ROS shed their tips within the first hour after the onset of light. Ten hours into the diurnal cycle (6 p.m., Fig. 1c), the phagosomes shed after the onset of light are considerably smaller (2.0 to 4.0 μm) and are displaced toward the basal border of the PE. No new phagosomes are present at this time. At later times during the diurnal cycle (16 hours and 20 hours, not shown) only very small phagosomes with dimensions less than 2.0 μm are seen.

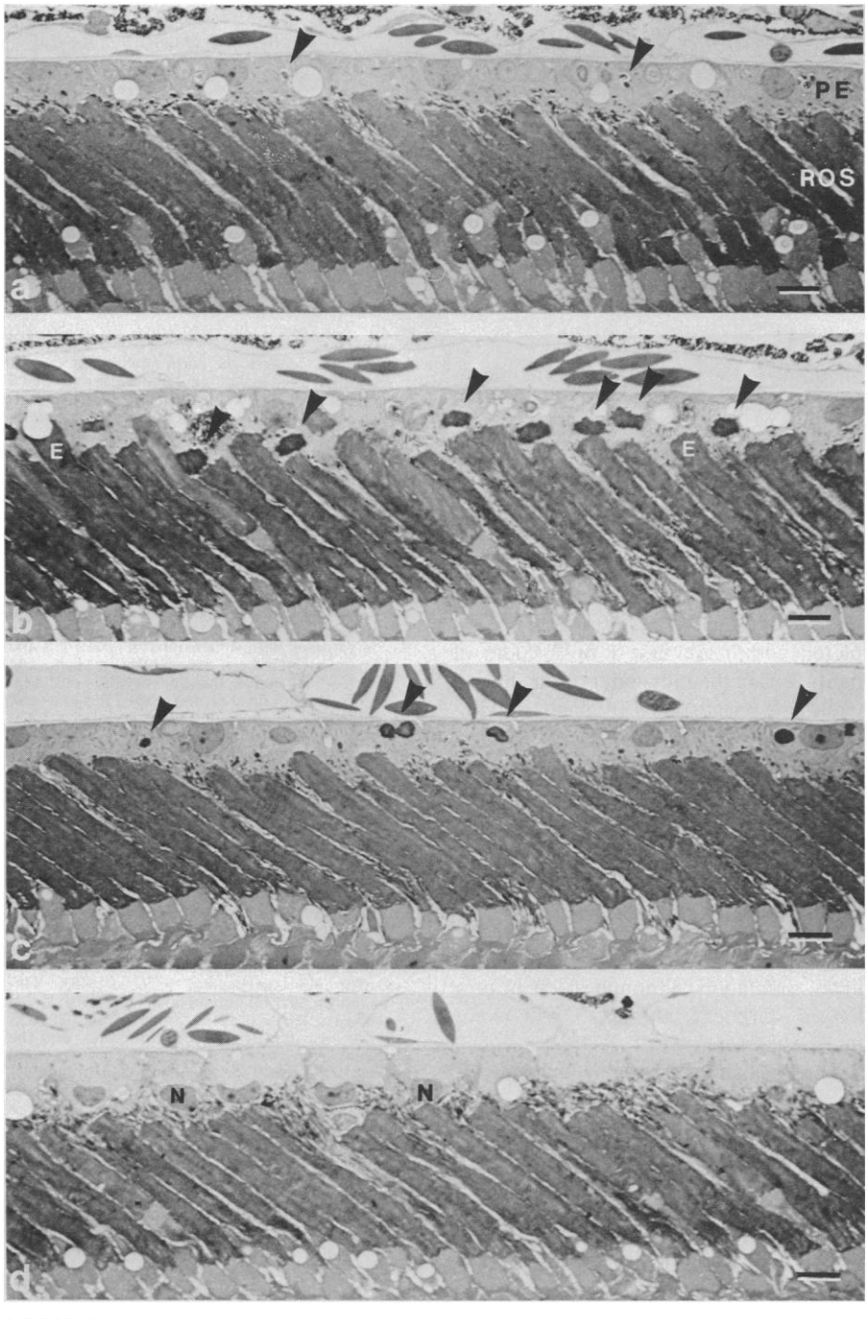
Figure 2 shows the time course of the appearance of new phagosomes during one complete diurnal cycle. To illustrate the light-induced synchrony of the shedding process, only rectangular phagosomes located just distal to the tips of the outer segments were counted (*8*). This temporal sequence suggests that shedding is synchronously initiated in about 25 percent of the ROS by the onset of light and is completed within the first 2 hours of the diurnal cycle, but the new phagosomes shed during the first 2 hours are, in most cases, completely digest-

ed by the beginning of the next cycle.

To confirm that illumination was the inducer for the synchronous shedding observed in Fig 1b, a group of frogs was kept in the dark for the first 2 hours of the light phase of the diurnal cycle. Examination of these retinas (10 a.m., Fig. 1d) reveals no new phagosomes, although the PE nuclei have migrated to the apical border of the PE cells. Retinas from frogs kept in the dark for 6 and 14 hours after the normal onset of light (not shown) also show no new phagosomes. This indicates that synchronous shedding of ROS tips in the frog retina is induced by the onset of light and does not follow a circadian rhythm as seen in the rat (*3*).

Red ROS from the frogs used in these experiments measure about 6 by 45 μm, and phagosomes examined at the 1-hour time point measure about 10 percent of this length. Frogs maintained at room temperature (22°C) completely renew their ROS about every 40 days (*4*). Shedding a phagosome equivalent to 10 percent of the ROS length once every 4 days would serve to keep the ROS at a relatively constant length. This implies that on a particular day about 25 percent of the ROS should shed, which is supported by our experimental observations (Fig. 2). It appears that at the beginning of the diurnal cycle, certain ROS (about 25 percent) become primed to shed; then shedding is initiated by the onset of light. These primed ROS complete the shedding process within the first 2 hours, and virtually no further shedding occurs during the rest of the diurnal cycle. Unprimed ROS do not shed even upon the onset of light.

To determine if light exposure was absolutely required for shedding to occur, a group of frogs was kept in total darkness



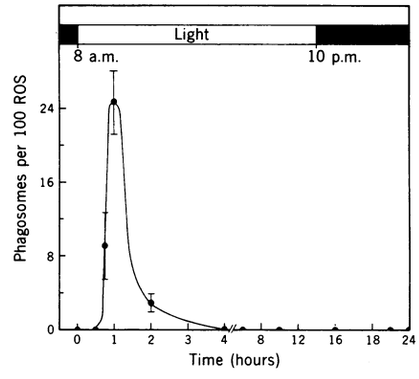


Fig. 1 (left). Longitudinal section through the ROS and PE of a frog retina. (a) Ten minutes before the onset of light (7:50 a.m.). No newly shed phagosomes are present in the PE; there are only small inclusions (arrows) representing phagosomes in the final stages of digestion. (b) One hour after the onset of light (9 a.m.). Numerous new phagosomes (arrows) have been synchronously shed into the PE. A few elongated ROS (*E*) appear deeply inserted into the PE. (c) Ten hours after the onset of light (6 p.m.). Phagosomes in the process of digestion (arrows) are present in the PE. They have migrated toward the basal border of the cell, and no new phagosomes appear in the PE. (d) Control retina kept in the dark for 2 hours after the normal onset of light (10 a.m.). In the absence of light stimulation, no new phagosomes have appeared in the PE. The nuclei (*N*) of the PE have migrated to the tips of the photoreceptor cells. Scale markers, 10 μm. Fig. 2 (above). Time course of ROS shedding in the frog retina. With well-oriented longitudinal sections taken from frogs at the indicated times (•), the number of newly shed phagosomes was counted and expressed as number of phagosomes per 100 ROS [see (*8*) for details]. Synchronous shedding is observed in about 25 percent of the ROS within the first 2 hours of the diurnal cycle. The bar at the top shows the light and dark phases of the diurnal cycle (14 hours light and 10 hours darkness).

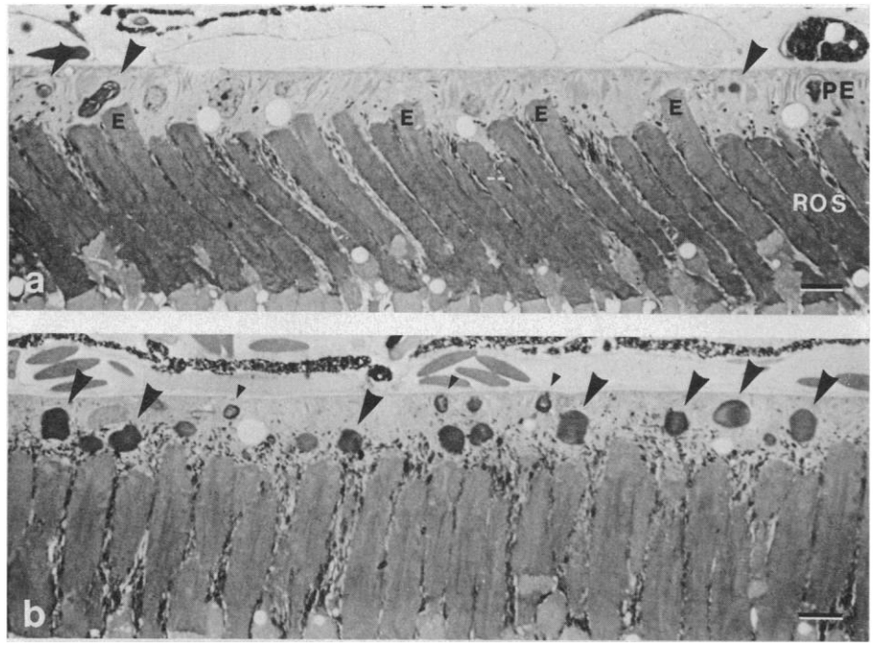


Fig. 3. Longitudinal sections through the ROS and PE of frogs kept in total darkness through five complete diurnal cycles. (a) Control retina, not exposed to light. A few phagosomes (arrows) in various stages of digestion are present in the PE. Elongated ROS (*E*) appear deeply inserted in the PE. (b) Two hours after the onset of light. Numerous new phagosomes (large arrows) are present in the PE. Some of the phagosomes are larger than usual (compare to Fig. 1b) and a few phagosomes shed before the beginning of the light period (small arrows) are also present. Scale markers, 10 μm.

through five complete diurnal cycles. At the beginning of the sixth cycle (8 a.m.), some animals were exposed to 2 hours of light while the remainder were kept in the dark. Figure 3a shows the retina of a frog kept in total darkness. Even in the prolonged absence of light, some shedding and phagocytosis has occurred. The phagosomes in the PE are in various stages of digestion, indicating that shedding has not occurred synchronously during the 6 days of total darkness. Additionally, the ROS of these animals are significantly longer (12.5 percent, $2P < 0.001$) than those of frogs at an equivalent time point on the normal diurnal cycle (*10*). Animals exposed to 2 hours of light after prolonged darkness (Fig. 3b) demonstrate a large burst of nearly synchronous shedding. These retinas contain a higher percentage of phagosomes (54 percent) than the normal 25 percent seen in animals maintained on the diurnal cycle, suggesting that many ROS became primed during the prolonged exposure to total darkness. Some very large phagosomes are present, which suggests that some ROS may have been doubly primed. Thus, exposure to light is not an absolute requirement for shedding, but definitely serves as an initiator of synchronous shedding.

In summary, our observations are consistent with the following picture of ROS shedding in the frog retina. At the beginning of the diurnal cycle, certain ROS (about 25 percent) have become primed to shed. The mechanism of priming is unknown, but it may be related to the length of the outer segment, in that only ROS that have undergone 4 days of renewal since last shedding are primed. The onset of light then induces a synchronous shedding of ROS tips, which is completed within the first 1 or 2 hours of light. During the remainder of the diurnal cycle, the phagosomes are digested within the PE, and no further shedding takes place until the onset of light at the beginning of the next cycle.

The work of LaVail (*3*) has shown that the shedding of ROS tips in the rat follows a circadian rhythm, occurring even without light stimulation. Our work suggests that the shedding process in the frog retina is not circadian, but requires light stimulation, although if left in prolonged darkness, the ROS will eventually shed (*11*).

The mechanism through which light induces ROS shedding is unknown. Control of this phenomenon may lie entirely within the retina; that is, light reception by photopigments in the retina may generate the shedding message. Alternately, the higher centers, particularly the pineal, could be involved (*2*). However, the shedding process must be linked in some way to the renewal process. If it were not, then shedding might proceed at a rate incompatible with renewal, resulting in shortening or elongation of ROS. The ROS renewal rate in frogs is temperature dependent (*4*), and we have observed increased shedding in frogs maintained at 36°C, which suggests that shedding of ROS tips is sensitive to the rate at which new outer segment disks are synthesized. It is unlikely that the renewal mechanism per se is responsible for priming ROS to shed, but it is clear that the overall process of shedding and phagocytosis occurs at a rate that is proportional to the overall renewal rate.

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7. After enucleation, the corneas were slit and the eyes immersed in 2 percent glutaraldehyde and 2 percent formaldehyde in 87 m*M* phosphate buffer, *p*H 7.2, at room temperature. After 30 minutes, the anterior portion was dissected away, and the eyecup was fixed for 4 to 6 additional hours at 4°C. Retinas from dark-adapted frogs were kept dark throughout fixation. Small pieces were cut from the posterior pole to the midperiphery, rinsed in phosphate buffer, postfixed in phosphate-buffered 1 percent OsO_4, dehydrated in graded ethanols, and then embedded in Araldite 502 (Electron Microscopy Sciences). Sections of 0.5 μm were cut, deplasticized, and stained with 1 percent toluidine blue for 10 minutes at room temperature.
8. Phagosomes with dimensions between 5.0 and 6.5 μm, which were rectangular in shape and located just above the tips of the outer segments, were defined as newly shed phagosomes. Only these phagosomes were counted for the data compiled in Fig. 2. At 4 hours (not shown) phagosomes measured 4.0 to 5.0 μm, were displaced from the ROS tips, and often stained irregularly. At 10 hours, phagosomes were displaced toward the basal border of the pigment epithelium, appearing as spherical, densely staining bodies measuring between 2.0 and 4.0 μm. By the end of the diurnal cycle, phagosomes were seen as small dense inclusions located in the distal half of the pigment epithelium with dimensions less than 2.0 μm.
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10. Outer segment length was measured with an ocular micrometer and a Zeiss ×63 planapochromatic oil immersion objective. Using well-oriented sections, a minimum of 100 ROS were measured from each time point, and the means were compared by using a two-tailed unpaired *t*-test. The mean (± standard deviation) ROS length of frogs kept in prolonged darkness (Fig. 3a) was 48.7 ± 3.5 μm, and on a regular diurnal cycle plus 1 hour of light (Fig. 1b) was 43.3 ± 3.9 μm.
11. Similar observations have been made with adult frogs by R. W. Young and with tadpoles by J. G. Hollyfield (personal communications).
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