

Reports

Prevention of Rod Disk Shedding by Detachment From the Retinal Pigment Epithelium

David S. Williams*† and Steven K. Fisher*

The authors tested whether or not rod outer segment (ROS) disks are shed when the neural retina is detached from the retinal pigment epithelium (RPE). Adult *Xenopus* were injected with ³H-L-leucine. Later, when the distal disks of their ROSs were labeled with a band of ³H-leucine, their eyes were enucleated. Intact eyecups, eyecups with partially detached retinae, and retinae that were peeled completely away from the RPE were incubated in culture medium. Disk shedding was stimulated by changes in lighting, or the addition of 0.5 mM ouabain. Where the retina was attached, phagosomes in the RPE, and not the ROSs, contained most of the radiolabel. Where there was retinal detachment, ROSs were still heavily radiolabeled near their distal ends. It was concluded that mechanical retinal detachment prevents ROS disk shedding. *Invest Ophthalmol Vis Sci* 28:184–187, 1987

In most arthropods, the photoreceptors themselves are responsible for the uptake and digestion of their photoreceptor membrane that is shed as part of its turnover.¹ Direct endocytosis into the photoreceptor cell is the most common means of uptake; although, in at least one case (that of tipulid flies), portions of the photoreceptive microvilli are first shed into extracellular space, and then phagocytosed by the photoreceptors.² In contrast, disposal of the photoreceptive outer segment disks during turnover in the vertebrate retina involves phagocytosis of the terminal disks by another type of cell, the retinal pigment epithelium (RPE),³ or (in fish) the RPE and wandering phagocytes.⁴ Therefore, in vertebrates, a primary question in understanding the control of photoreceptor membrane shedding concerns the relative roles played by two different types of cell.

Many researchers have assumed that the disks are actively shed by the photoreceptors prior to any participation by the RPE. Young⁵ presented morphological evidence in support of this process. On the other hand, Spitznas and Hogan⁶ interpreted their morphological studies to indicate that the RPE cells actively phagocytose, or “bite off” outer segment disks. More recently, Bok and Young⁷ and Besharse⁸ reported observing disk shedding from detached frog retinae (i.e.,

without the RPE) in vitro. Besharse⁸ mentioned that rod outer segments (ROSs) of detached *Xenopus* retinae shortened during incubation in light. This effect was reported to be enhanced in the presence of colchicine.⁸ Nevertheless, the presence of shed packets of disks in the extracellular space has not been clearly shown.

We have reexamined the ability of ROSs to shed their terminal disks after detachment from the RPE. By radiolabeling these disks prior to experimentation, we have been able to assess unequivocally whether they have or have not been discarded.

Materials and Methods. Adult *Xenopus laevis*, 2.5–3 cm long, were purchased and kept in aquaria on a 12 hr dark/12 hr light cycle at 24°C for a few weeks before use. During the light phase, cold fluorescent lighting provided illuminance at the water surface of 500 lux. Every time an experiment was run, each of 4–6 animals was injected subcutaneously with 1 mCi ³H-L-leucine (specific activity: 61 Ci/mmol) in 0.2 ml water, and returned to its aquarium. Seventeen days later, some animals were transferred to constant light (500 lux, as above) for 4 days, and then killed, still in the light. The others were maintained on cyclic lighting, and were killed under infrared or dim red (15 W bulb, number 1 filter from Kodak, Rochester, NY) light, just before light onset on day 18. A preliminary experiment had determined that, 18 days after an injection of ³H-leucine, the ROSs of animals, maintained under cyclic lighting at 24°C, were each labeled with a band of radioactivity near their distal ends (see also ref. 9). Animals were handled according to the ARVO Resolution on the Use of Animals in Research. They were killed by decapitation, and then their spinal cords and brains were immediately pithed.

Immediately after the animals were killed, their eyes were enucleated and dissected under the same lighting. The cornea and lens of each enucleated eye was discarded, and the remaining posterior segment was treated as follows.

In the first experiment, which was repeated twice, the retina of one eye from each animal was completely

detached by peeling it away from the RPE and removing it from the eyecup (in *Xenopus*, interdigitation between the ROSs and apical processes of the RPE is not very extensive, so that its retinae, even when light-adapted, can be detached relatively easily by this means). The posterior segment of the second eye was left as an intact eyecup. Each detached retina and intact eyecup were then placed together in Wolf and Quimby amphibian culture medium (GIBCO, Grand Island, NY), with NaHCO_3 increased to 35 mM.¹⁰ The culture medium was maintained at 24°C and gassed with a humidified mixture of 95% O_2 + 5% CO_2 . After collection in culture medium, retinae and eyecups from the animals that were exposed to 17 days of cyclic lighting plus 4 days of constant light were incubated for 30 min, still in the light, then in darkness for 60 min, and finally in light again for 120 min, before being fixed. Retinae and eyecups from the animals that were maintained entirely under cyclic lighting were incubated for a further 10 min under dim red or infrared light, and then for 150 min under fluorescent lighting (as above), either with or without 0.5 mM ouabain added to the culture medium at all times.

In a second experiment, which was repeated four times, the same procedure was followed, except that the detached retinae were only partially detached. Anywhere from 10–90% of the retina was peeled away from the RPE, and then left to lie in the eyecup—still connected to the attached part of the retina.

Tissues were fixed in 2% glutaraldehyde + 2% formaldehyde in phosphate buffer, postfixed in buffered 1% OsO_4 , dehydrated in ethanol, and embedded in Araldite. Semi-thin sections (1 μm) were cut along the long axes of the rods and collected on glass slides for light microscopical autoradiography. They were dipped in 50% Kodak NBT-2 emulsion. After suitable exposure, they were developed in full strength Kodak D-19, fixed, and finally stained with Azure II. Ultrathin sections were cut from the same blocks and collected on formvar-coated slot grids for electron microscopy. They were stained with uranyl acetate and lead citrate.

Results. The first experiment compared completely detached retinae with retinae in intact eyecups. In the intact eyecups, ROS disk shedding was manifest by phagosomes in the RPE. Many of the phagosomes were radiolabeled. In eyecups that were incubated with ouabain, or were from animals exposed to 4 days of constant light, phagosomes were particularly numerous, and only a very few ROSs exhibited a band of radiolabel (Figs. 1A, 2A). By contrast, in all the detached retinae, a band of radiolabel was evident near the distal ends of practically every ROS (Figs. 1B, 2A).

In one detached retina, which was from an animal exposed to 4 days of constant light (and then incubated

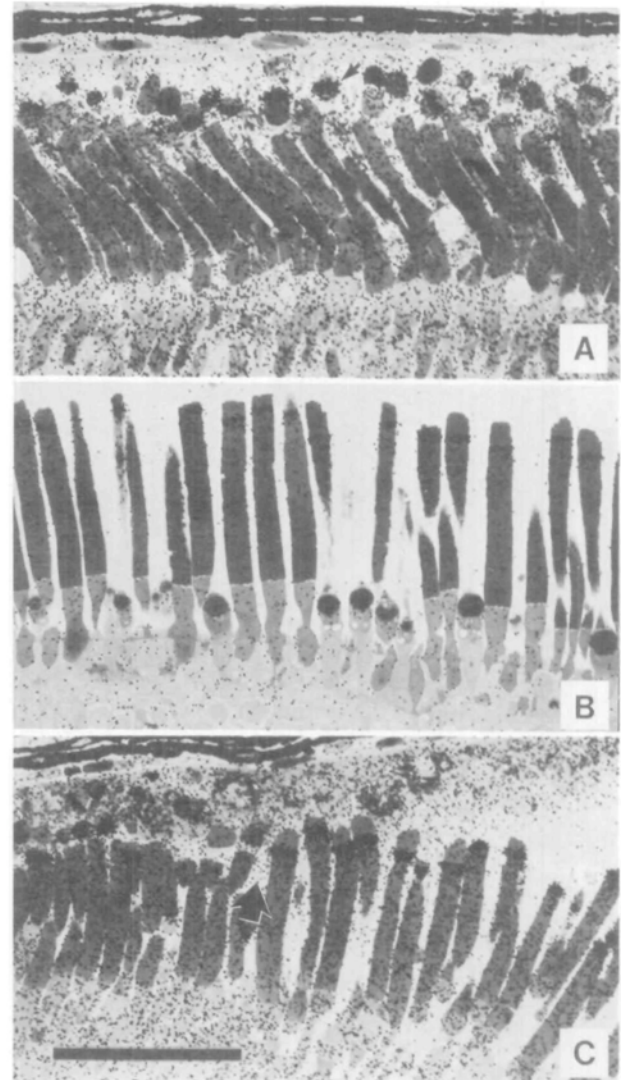


Fig. 1. Light microscopical autoradiographs of retinae that were: **A**, left attached; **B**, completely detached; and **C**, partially detached. All retinae were from animals that were exposed to 4 days of constant light, with the retinae subsequently exposed to 1 hr dark + 2 hr light in culture. **A** and **B** are from different eyes of the same animal. In **B**, a band of label is evident near the distal end of each ROS. In **A**, as a result of disk shedding, dense label is found instead in phagosomes (e.g., arrow). In **C**, where the retina is attached (left), label is found mostly in phagosomes, indicating that disk shedding has occurred. Where the retina is detached (right), the ROSs are still labeled, indicating that the distal disks have not been shed. An arrow marks the boundary between regions of shedding and no shedding. **A**, **B**, and **C** are of the same magnification; scale bar = 50 μm .

in dark + light), most ROSs appeared, with light microscopy, to have a lightly-staining band about 5 μm from its distal end. In the electron microscope, this band was found to consist of vesiculated disk membrane, like that shown in previously published micrographs by several others.^{8,11,12} However, the distal disks

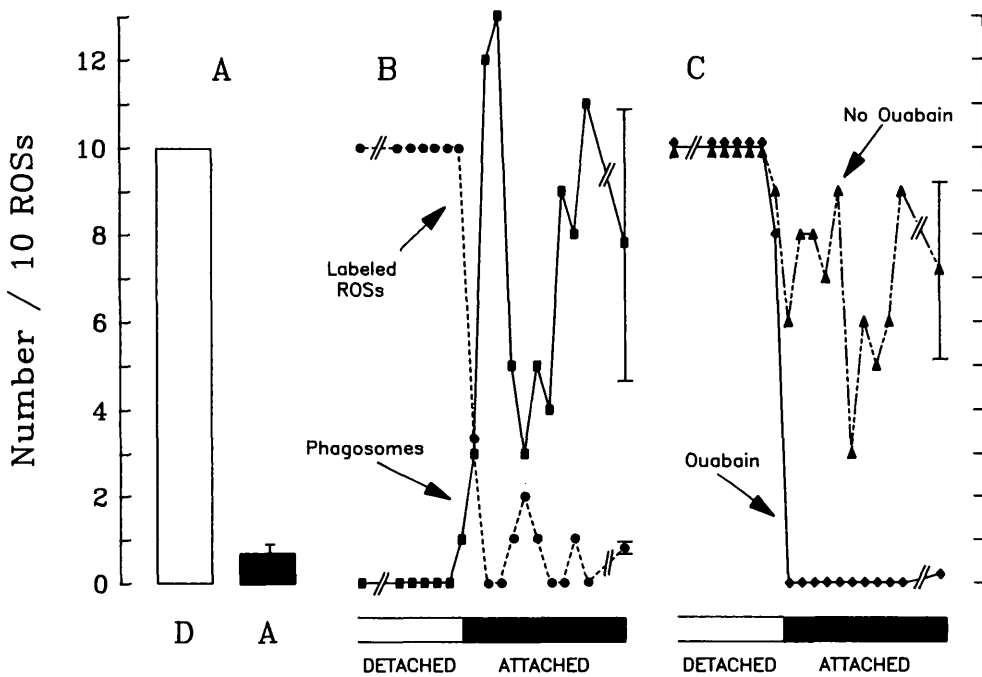


Fig. 2. Comparison of ROS labeling (and phagosome numbers) between detached and attached retinas. Data are from retinal sections in which the ROSs were aligned along their long axes (like those in Fig. 1). The sections were divided into sequential sets of 10 ROSs, so that the 10 ROSs closest to one retinal margin were in the first set, the next closest in the second set, and so on across the section. The number of ROSs labeled by a band of radioactivity (and, in **B**, the number of phagosomes) were counted for each set of 10 ROSs. Error bars represent ± 1 standard deviation; where they are absent, error is too small to be illustrated. **A**, The number of labeled ROSs/10 ROSs from

pairs of retinæ of which one was completely detached from the RPE (**D**) and the other left attached (**A**). Data were pooled from ouabain-treated retinæ and retinæ from constant light-treated animals. Six pairs of retinæ were sampled. **B**, The number of labeled ROSs and phagosomes/10 ROSs at the junction of attached and detached areas of a partially detached retina. Counted phagosomes were larger than $2 \mu\text{m}$ in their greatest diameter. The retina was from a constant light-treated animal. Sixteen sets of ten ROSs on either side of the junction are shown individually. The single points beyond the breaks represent the mean of the next sets of 10 ROSs (minimum of four sets). **C**, The number of labeled ROSs at the junction of attached and detached areas, as in **B**, but in two different partially detached retinæ. Both these retinæ were from animals maintained under cyclic lighting and dissected just before light onset. One retina was incubated in control medium, the other in the presence of ouabain.

of ten other detached retinæ which were examined by electron microscopy appeared unaltered.

In the second experiment, in which retinæ were only partially detached, we found that detachment again prevented discarding of the terminal disks. Where the retinæ were left attached, fewer ROSs were labeled by a band of radioactivity, and the RPE contained phagosomes, many of which were labeled. In the detached regions, the ROSs were radiolabeled by a band near their distal end, as above, and the nearby RPE cells contained no phagosomes. Because of massive shedding in the attached regions, this difference was especially pronounced in eyecups that were incubated with ouabain, or were from animals exposed to 4 days of constant light (Figs. 1C, 2B, C).

Discussion. Our results with intact eyecups are in agreement with previous studies that show that both 60 min of darkness plus 120 min of light after 4 days of constant light,¹⁰ and incubation with ouabain for 150 min after light onset¹³ induce a large amount of ROS disk shedding in *Xenopus* eyecups. However, even with these treatments, we found that the disks of detached retinæ did not shed. Moreover, most detached retinæ exhibited unaltered distal disk structure, so that

there was no consistent indication that shedding might have at least been initiated.

The observation of vesiculated distal disks in one of the detached retinæ warrants some comment, however. These disks appeared the same as those described from occasional observations of frog retinæ by others.^{8,11,12} Matsumoto and Besharse¹⁴ found that disks in the same distal position in *Xenopus* retinæ stained selectively with lucifer yellow, especially after priming for massive shedding by exposure to 4 days of constant light plus 60 min of darkness plus 60–120 min of light. Given their distal location, it seems that this lucifer yellow staining and the disk vesiculation might both be manifestations of an early change in the process of disk shedding, initiated within the rods. A problem with this suggestion is that the disk vesiculation has been observed only irregularly from retina to retina and without significant correlation to any shedding stimulus (in addition to the present results, cf. ref 12). However, all observations of this phenomenon, including the present one, have been made using similar conventional processing procedures for microscopy. Perhaps the inconsistency with which it has been observed is because conventional microscopy might per-

mit one to see only the occasional extreme case of an alteration that can be detected more readily when lucifer yellow is employed as an extracellular probe.

In conclusion, our results show that mechanical disruption of the intact relationship between the ROSs and the RPE and/or interphotoreceptor matrix prevents the abscission of the terminal disks under conditions in which they would normally be shed. Particularly in view of the above discussion, this finding does not necessarily mean that the RPE actively bites off the ends of ROSs by "piecemeal phagocytosis" without any participation by the rods, but it does indicate that the neural retina does not have autonomous control over the shedding of its ROS disks.

Key words: photoreceptor outer segments, rod disk, retinal pigment epithelium, membrane turnover, retinal detachment

Acknowledgments. We gratefully acknowledge Dean Bok's critical comments on a draft of the manuscript and Steve Bernstein's preparation of Figure 2.

From the *Neuroscience Research Program and Department of Biological Sciences, University of California, Santa Barbara, the †Jules Stein Eye Institute, UCLA School of Medicine, Los Angeles, and the ‡Department of Visual Science, Indiana University, Bloomington. Supported by NSF Grant BNS-8420242 and NIH Grant EY-00888. Submitted for publication: June 27, 1985. Reprint requests: David S. Williams, Department of Visual Sciences, School of Optometry, Indiana University, Bloomington, IN 47405.

References

- White RH: The effect of light and light deprivation upon the structure of the larval mosquito eye. III Multivesicular bodies and protein uptake. *J Exp Zool* 169:261, 1968.
- Williams DS and Blest AD: Extracellular shedding of photoreceptor membrane in the open rhabdom of a tipulid fly. *Cell Tissue Res* 205:423, 1980.
- Young RW and Bok D: Participation of the retinal pigment epithelium in the rod outer segment renewal process. *J Cell Biol* 42:392, 1969.
- O'Day WT and Young RW: Rhythmic daily shedding of outer segment membranes by visual cells in the goldfish. *J Cell Biol* 76:593, 1978.
- Young RW: Shedding of discs from rod outer segments in the Rhesus monkey. *J Ultrastruct Res* 34:190, 1971.
- Spitznas M and Hogan MJ: Outer segments of photoreceptors and the retinal pigment epithelium. *Arch Ophthalmol* 84:810, 1970.
- Bok D and Young RW: Phagocytic properties of the retinal pigment epithelium. In *The Retinal Pigment Epithelium*, Zinn KM and Marmor MF, editors. Cambridge, MA, Harvard University Press, 1979, pp. 148-174.
- Besharse JC: The daily light-dark cycle and rhythmic metabolism in the photoreceptor-pigment epithelial complex. *Prog Retina Res* 1:81, 1982.
- Hollyfield JG and Rayborn ME: Membrane assembly in photoreceptor outer segments: progressive increase in 'open' basal discs with increased temperature. *Exp Eye Res* 34:115, 1982.
- Besharse JC, Terrill RO, and Dunis DA: Light-evoked disc shedding by rod photoreceptors in vitro: relationship to medium bicarbonate concentration. *Invest Ophthalmol Vis Sci* 19:1512, 1980.
- Currie JR, Hollyfield JG, and Rayborn ME: Rod outer segments elongate in constant light: darkness is required for normal shedding. *Vision Res* 18:995, 1978.
- Heath AR and Basinger SF: Simple sugars inhibit rod outer segment disc shedding by the frog retina. *Vision Res* 23:1371, 1983.
- Williams DS, Wilson C, Linberg K, and Fisher S: Effects of low sodium, ouabain, and strophanthidin on the shedding of rod outer segment discs. *J Comp Physiol A* 154:763, 1984.
- Matsumoto B and Besharse JC: Light and temperature modulated staining of the rod outer segment distal tips with lucifer yellow. *Invest Ophthalmol Vis Sci* 26:628, 1985.

Normal Pattern Electroretinograms in Amblyopia

Irene Gottlob* and Lutz Welge-Lüssen†

Checkerboard reversal stimuli were used to evoke transient pattern electroretinograms (P-ERGs) from the eyes of 14 patients with amblyopia and 14 normal subjects. In the control group, and in normal eyes of patients, pattern electroretinograms were obtained with monocular central fixation. Amblyopic eyes were examined by monocular and binocular fixation, and the fixation point was shifted horizontally and/or vertically until the P-ERG reached its maximal amplitude. After adjusting visual fixation, there were no significant differences in amplitude between the normal and the amblyopic eyes. In addition, the differences between both eyes were compared with the right-left eye variability of the 14 normal subjects. In the amblyopic eyes, no P-ERG abnormality was observed. These results do not support previous reports of reduced P-ERG amplitudes and are in agreement with recent

findings obtained under steady-state conditions. *Invest Ophthalmol Vis Sci* 28:187-191, 1987

The question as to what extent the retina is affected in amblyopia has remained unanswered. In amblyopic eyes, several authors¹⁻³ have described a reduction of the first positive component of the transient pattern electroretinogram (P-ERG). Recently, Arden et al⁴ found in a large population of amblyopic children that the transient P-ERG amplitude was reduced in most amblyopic eyes at 2 Hz modulation. Occlusion at an earlier age reduces the P-ERG amplitude and orthoptic treatment increases it. Hess et al^{5,6} showed that with