

# Intracellular Topography of Rhodopsin Regeneration in Vertebrate Rods

THEODORE P. WILLIAMS and JOHN S. PENN

From the Institute of Molecular Biophysics, Florida State University, Tallahassee, Florida 32306

**ABSTRACT** The vertebrate visual pigment of rods, rhodopsin, bleaches in light and regenerates in darkness. When the bleaching and regeneration are carried out *in vivo*, it is found that the regeneration takes place at nonuniform rates along the rod outer segment (ROS): toads and frogs regenerate rhodopsin faster in the proximal ends of the ROS than in the distal ends. Rats do the reverse. These patterns of regeneration persist whether the bleaching is done with flashes or with steady light. They are also independent of the extent to which the retinal pigment epithelium contains melanin. Furthermore, the dichotomy of patterns (proximal faster vs. distal faster) does not seem to depend upon the presence of an excess of stored retinoid in the eye. Instead, it is suggested that the villous processes of the epithelial cells may play an important role in the regeneration patterns. These processes in amphibia extend nearly to the rod inner segment but in the rat they surround only the apical end of the outer segment. If they "funnel" the retinoids back to the ROS, their location and morphology could explain the two different kinds of patterns seen.

## INTRODUCTION

Rhodopsin, the visual pigment of vertebrate rod cells, is a complex conjugate of a protein (opsin) and its chromophore (11-*cis* retinal). Upon absorption of light, the rhodopsin "bleaches" and, within a few seconds or minutes, the retinal is hydrolyzed from its chromophoric site (cf. Wald, 1968). In order for the rod cell to continue its function after a substantial bleach, the bleached rhodopsin must be "regenerated." The major aspects of regeneration—especially those that are relevant to the present work—can be summarized as follows: (a) once hydrolyzed from opsin, retinal is reduced to retinol while still inside the rod outer segment (ROS); (b) the retinol, by means presently unknown, leaves the ROS and enters the retinal pigment epithelium (RPE); (c) much of this retinol is esterified in the RPE and stored there during light exposure; (d) in the dark, the retinol is "mobilized" (this includes de-esterification) and re-enters the ROS; (e) retinol is oxidized to retinal and combines with opsin to regenerate the visual pigment.

Many of the details of rhodopsin regeneration are known and the reader is referred to two reviews on this and related topics (Bridges et al., 1983; Chader

Address reprint requests to Dr. Theodore P. Williams, Institute of Molecular Biophysics, Florida State University, Tallahassee, FL 32306. Dr. Penn's present address in Cullen Eye Institute, Baylor College of Medicine, Houston, TX 77030.

et al., 1983). However, the present article addresses itself to an aspect of regeneration about which nothing is known: namely, when regeneration occurs *in vivo*, does it do so uniformly along the ROS or are there regions within the ROS that regenerate faster than others? A reasonable hypothesis is that the regeneration topography within an ROS should be nonuniform. Such an hypothesis has its basis in the observation made by many investigators, and mentioned above, that the RPE stores the retinoid during light adaptation (Jancso and Jancso, 1936; Dowling, 1960; Zimmerman, 1974; Bridges, 1975). Thus, it might be expected that the distal end (tip) should regenerate faster than the proximal end (base) because the former is closer to the RPE than is the latter. This is to say that the retinoid, leaving the RPE, would be expected to encounter the ROS tips first and cause the tips to regenerate faster than the bases—all else being equal.

In this article, we report that the regeneration topographies within the rods of several species are indeed quite nonuniform along the ROS. However, whether the regeneration rate is higher in the tip or in the base depends upon which species is being examined.

#### MATERIALS AND METHODS

The ROS of rat, rabbit, toad, and frog were studied. Both pigmented (Long-Evans) and albino (Sprague-Dawley) rats were used. The former were purchased directly from Charles River Breeding Laboratories (Wilmington, MA), while the latter were born of Charles River stock in our colony room and were raised from birth in an ambient illuminance of  $3 \pm 2$  lx, cycled 12 h/12 h. The pigmented rats were adapted to our colony room for at least 1 wk before being used. One albino rabbit (N. Florida Rabbitry, Salem, FL) was also studied. It was housed in a room with an ambient illuminance of 150 lx, cycled 12 h/12 h.

Normally pigmented *Bufo marinus*, *Rana catesbeiana*, and *Rana pipiens* were purchased from Sullivan Co. (Nashville, TN). Near-albino bullfrogs, *R. catesbeiana*, a gift from Dr. E. Frieden (Florida State University, Tallahassee, FL), were also used. Although these animals appeared to be albinos and had pink eyes, high-magnification light microscopy disclosed a few light-brown melanosomes in the RPE (unstained sections). All of the amphibia were kept in a tank with running well-water and were fed a varied diet of small fish, crayfish, and crickets. The ambient illuminance in the holding tanks was 20 lx, cycled 12 h/12 h.

To begin an experiment, an animal (or group of animals) was dark-adapted overnight. The following morning, the animal was light-adapted either with 1,000 lx of fluorescent lighting for 1–2 h or with 10–25 flashes from a xenon-type photographic flash gun. The flash gun (Strobonar 700, Honeywell Corp., Minneapolis, MN) was equipped with a yellow-orange filter (either Corning 3-68 or 3-71, Corning Glass Works, Corning, NY) in order to minimize photoreversal of bleaching, and each flash from this gun was capable of bleaching 50–70% of the rhodopsin present or remaining in the rods at or near the posterior pole of each species' eye. These flash or steady-light exposures were chosen because they completely bleach the rods surrounding the optic disk, the rods to be studied here. The irises of the pigmented rats were dilated with 1% atropine sulfate before the animals were exposed to the bleaching lights. Also, to ensure complete bleaching of these pigmented animals, we gave them 25 flashes per eye. (The albino rats needed only 10–12 flashes per eye for complete bleaching.)

For purposes of dark adaptation after the full bleach, rodents were put into a completely darkened cubicle capable of housing as many as eight animals. Amphibia were put into a completely darkened constant-temperature bath held at 30°C, and were immersed in such a way that they were free to move about, but no more than their eyes, nostrils, and skulls were out of the water. This assured that the body temperature of the animals was as close to bath temperature as possible and that any temperature variation from one animal to the next was minimal. (Temperature has a substantial effect on whole-retina rhodopsin regeneration rates; cf. Ratzlaff, 1975.)

After a prescribed time in the dark, an animal was removed from its dark-adaptation chamber and killed. This and all of the following procedures were carried out in darkness or in dim red light.

Rat eyes were quickly enucleated and dropped into cold fixative, 2.5% glutaraldehyde in 0.1 M PIPES. This procedure quenched regeneration immediately. After 30 min, the cornea was removed and the eyeball was returned to the fixative overnight at 4°C. The next morning, the lens was removed and the eyecup was washed in PIPES and trimmed. It was then quick-frozen in Freon 12 at the temperature of liquid nitrogen and mounted on the cutting stage of a cryomicrotome with Ames OCT fluid (Ames Co., Elkhart, IN). Sections through the optic disk in the vertical meridian were cut 10  $\mu\text{m}$  thick and deposited on No. 1 coverslips, warmed to room temperature, covered with a drop of Ringer's solution, and "sandwiched" with another coverslip. Groups of rat ROS within this sandwich were then ready for examination in the microspectrophotometer (MSP). The precision and accuracy of this frozen-section method are described elsewhere (Penn and Williams, 1984).

Single amphibian ROS are thick (5–7  $\mu\text{m}$ ) and can be examined individually. Consequently, preparing these ROS for the MSP required a different technique than was used with the rat. After the prescribed period of darkness, an animal was quickly decapitated and the head was immersed in an ice bath. Eyes were enucleated while still in the ice bath and then eyecups were prepared. These were dropped into Ringer's solution containing 50 mM  $\text{NH}_2\text{OH}$ , which quenched regeneration. Then the eyecup was cut into pieces and a region ( $\sim 3 \times 3$  mm) that surrounded the optic disk was isolated and the retina was teased away from the epithelium. This bit of retina was dabbed onto a No. 1 coverslip, depositing ROS and a drop of the  $\text{NH}_2\text{OH}$ -Ringer's. The coverslip had previously been coated with polylysine, which acts to "glue" cells to glass surfaces (Mazia et al., 1975). A coverslip sandwich was then prepared and the ROS were ready for MSP examination.

The MSP is a single-photon-counting, single-beam instrument (MacNichol, 1978). It is computer-assisted and uses an infrared-sensitive television system (IR-TV) for visualizing the preparations. The micromasuring beam had dimensions of  $\sim 5 \times 1.5$   $\mu\text{m}$  in the plane of focus and was capable of resolving the visual pigment in the ROS tip (distal end) from that either in the base (proximal end) or at a midpoint along the ROS.

The following procedures were used to determine the intracellular topography of the rhodopsin that had regenerated during the dark. For the rat, a retinal section was examined with the IR-TV system in order to locate the optic disk. Then a group of ROS within  $\sim 1$  mm of the disk was selected for measuring. The absorbance spectrum was determined at both ends of the ROS and then at mid-ROS. In the case of the amphibia, a single ROS with a bit of inner segment still attached would be sought, and spectra were measured beginning at one end or the other as with the rat. 30 scans of the spectrum were run and averaged for each part of the ROS. These scans did not cause any detectable bleaching; in fact, bleaching was not detectable even after 120 scans through the same part of ROS. Thus, the regeneration topographies were not distorted by the spectral measurements.

## RESULTS

Fig. 1 shows the time course of complete regeneration of albino rat rhodopsin in groups of ROS near the optic disk. Note that the regeneration is fastest in the distal tips of the ROS and slowest in the proximal bases; the mid-ROS positions

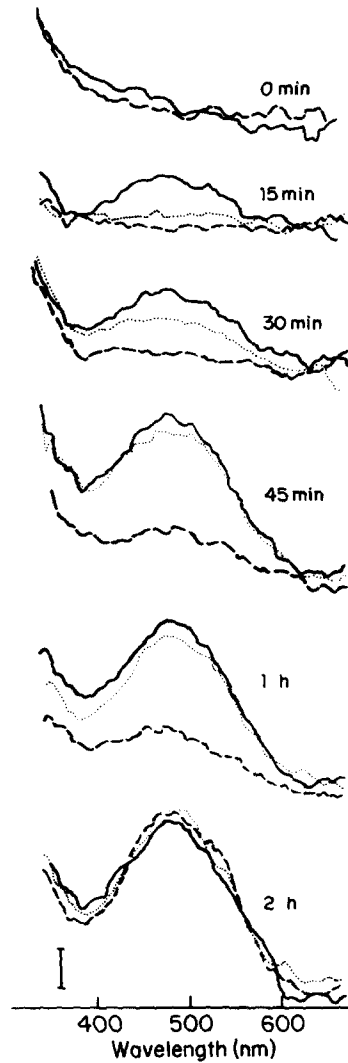


FIGURE 1. Time course and topography of rhodopsin regeneration in rat rods. Solid lines are spectra obtained from distal ends of ROS; broken lines are from proximal ends; dotted lines are from mid-ROS regions. Note that bleaching was complete (0-min spectra indicate no rhodopsin present); also note that the distal ends regenerate fastest and are virtually completely regenerated by 45 min, even though the proximal ends have regenerated very little by this time. The mid-ROS regions regenerate with intermediate rates. The calibration bar is 0.02 absorbance units.

regenerate with intermediate rates. The tips of the ROS are virtually completely regenerated before regeneration of the bases even begins. Thus, there is a large gradient of pigment along the ROS during the early stages of normal *in vivo* regeneration. It was more difficult to obtain such complete results with pigmented rats because the melanin of the RPE villous processes obscured the extreme tips of the ROS. Nevertheless, two especially well-oriented sections were obtained from two different pigmented rats, each of which had been completely bleached and then dark-adapted for 30 min. These ROS showed the same pattern of regeneration as did those from the albinos: distal regions of the ROS regenerated faster than the bases. In all, seven measurements were made on these sections and the ratio of absorbances of the melanin-free, distal-most accessible regions of the ROS to those of the bases varied from ~2:1 to 3.5:1.

One mature albino rabbit was studied in the same way as were the rats, i.e., frozen sections were prepared and groups of ROS were examined near the optic disk. These experiments showed that the ROS of the rabbit retina regenerated with a topography that resembled the ones seen in rats: tips regenerated faster than bases.

Fig. 2 shows an approximate time course of regeneration of toad rhodopsin, measured within individual ROS. The pattern was the reverse of that seen in the rat: the base regenerated faster than the tip. Notice that the absorbance of the 1-h dark-adapted ROS is greater than that of the 3-h one, despite the fact that regeneration is more nearly complete in the latter. This is because individual toad ROS ( $N = 180$ ) vary by as much as 21% in their transverse absorbances, even if completely dark-adapted (e.g., overnight) (Johnson, 1984). This kind of variability makes it difficult to determine the exact time course of regeneration in the toad ROS. Another sort of variability was found in the toad: the gradients of regeneration after a fixed time in the dark were not always of the same magnitude from one ROS to another. Nevertheless, except for two ROS, measured when we were still unskilled in these techniques, all ROS (>80) showed faster regeneration in the base than in the tip. In those two aberrant ROS, the rhodopsin absorbance was approximately equal in the two ends after 1 h of darkness.

Results with *R. pipiens* and normal and near-albino bullfrogs were very similar to those with the toad. The rate of regeneration was not uniform along the ROS, with bases regenerating faster than tips. Neither the species of *Rana* nor the degree to which the RPE was pigmented with melanin changed the pattern of regeneration found in these amphibia. Fig. 3 presents examples of these results.

The diametrically opposite patterns of regeneration in rats and toads were observed whether the bleaching was done with flashes or with steady light. However, the rates of regeneration and the steepness of the gradients along the ROS were somewhat affected by the method of bleaching: rates were slower and gradients steeper with prolonged steady-light bleaches.

In one experiment, "conditioning" bleaches were given to a toad. These bleaches consisted of two sets of 20 strobe flashes delivered to each eye of the animal, the sets of flashes being separated from each other by 2 h of darkness at 30°C. Then, after an additional 2 h of darkness, a final bleach (20 more flashes

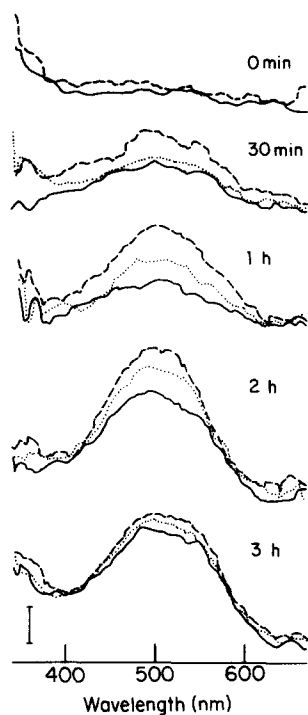


FIGURE 2. Approximate time course and topography of rhodopsin regeneration in toad rods. Solid lines are spectra obtained from distal ends of ROS; broken lines are from proximal ends; dotted lines are from mid-ROS regions. Note that bleaching was complete and that regeneration rates were greatest in the proximal ends of the ROS. This “proximal-first” pattern is the reverse of that found in rats (Fig. 1). The calibration bar is 0.02 absorbance units.

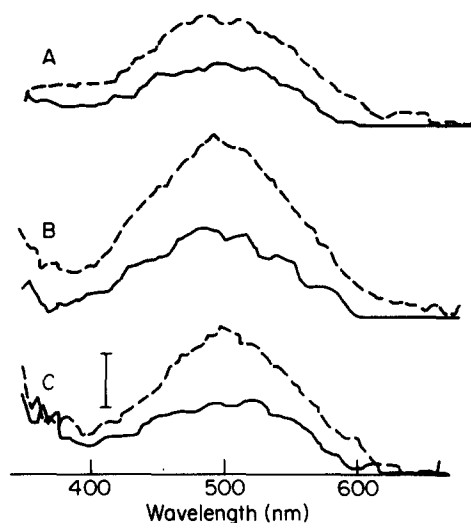


FIGURE 3. Examples of base-first patterns of rhodopsin regeneration in *R. pipiens* (A), *R. catesbeiana* (B), and near-albino *R. catesbeiana* (C). Broken lines are spectra obtained from proximal ends of ROS; solid lines are from distal ends. Spectra were recorded after 30 min of darkness after a complete bleach. The calibration bar is 0.02 absorbance units.

per eye) was given and the animal was dark-adapted for another hour (30°C) and killed. The ROS, examined in the usual way, showed the typical toad regeneration topography: the base had regenerated faster than the tip. Thus, “conditioning” bleaches had no effect on the observed patterns.

#### DISCUSSION

The results presented here demonstrate an interesting phenomenon: intracellular topographies of rhodopsin regeneration *in vivo* are not uniform along the ROS. Moreover, amphibian and rat ROS have opposite patterns of regeneration rates. It may be that lagomorphs, also, regenerate pigment faster in ROS tips than bases because the one rabbit we examined displayed a rat-like pattern.

It seems highly unlikely that the differences in the two types of regeneration topographies can be caused by the degree to which the RPE contains melanin; the patterns were species-specific, not pigmentation-specific. However, the retinomotor phenomenon, usually associated with melanin pigmentation, deserves further consideration because amphibia show strong retinomotor responses but rats show only weak ones (Burnside and Nagle, 1983; Kuwabara, 1979). Measuring the topography in amphibian ROS that have regenerated in a living eye with retinomotor responses blocked (cf. Burnside and Nagle, 1983) might disclose a pattern's dependence upon a functional retinomotor response.

There was a twofold purpose to the experiment in which “conditioning” bleaches were used. The first purpose has to do with the fact that frogs have a small but significant store of nonvisual pigment retinoid in their neural retinas (Bridges, 1975). It has been suggested that this store might be used for the daily synthesis of new rhodopsin in the basal disks (Bridges, 1975). If the store is situated near or in the base of the outer segment and if it can be used for purposes of regeneration, this might explain the “base-first” pattern in frogs (and toads). “Conditioning” bleaches could conceivably deplete this store and change the regeneration pattern, perhaps even reverse it. However, no change in the patterns was observed when such bleaches were used. Thus, either the stores within the neural retina are not the cause of base-first regeneration or such stores are very quickly replenished so that conditioning bleaches do not deplete them.

The second purpose behind using conditioning bleaches also deals with a store of retinoid, but specifically, in this case, 11-*cis* retinoids. It has been shown (Hubbard and Dowling, 1962; Bridges, 1976a) that a limited store of 11-*cis* retinoids exists in the retinas of thoroughly dark-adapted animals and that this store can be depleted by successive bleaches of the visual pigment. After such bleaches, it takes several hours to reaccumulate these 11-*cis* precursors of rhodopsin. It is possible that these *cis* retinoids react with opsin faster than do *trans* isomers. If this is true and if the limited store of *cis* precursors is situated in or near the base of the ROS, it might produce a base-first regeneration pattern. However, we used two conditioning bleaches and were unable to alter the regeneration pattern. Therefore, it is our tentative conclusion that stored 11-*cis* retinoids are not the cause of base-first regeneration in frogs and toads.

The main reason we examined the rabbit was that this animal is known to have an excess of retinoid stored in its RPE (Bridges, 1976b; Alvarez et al.,

1980). In this regard, rabbits are similar to frogs but are unlike rats (which have almost no excess retinoid stored in any retinal compartment; cf. Bridges et al., 1983). The rabbit exhibited the same "tip-first" pattern as did the rats. Therefore, we conclude that the pattern of regeneration found in the rods of an animal does not depend upon whether the RPE contains an excess of retinoid.

Amphibia differ from rats in a way that may help explain the different topographies of regeneration: the RPE villous processes in toads and frogs extend all the way to the rod inner segment, whereas in rats these processes surround only the distal tips of the ROS (Kuwabara, 1979). If, during regeneration, the retinoids are "funneled" through these processes and exit from the apical tips of the processes, the result would be that the retinoids would first encounter the ROS base in amphibia and the ROS tip in rats. This would result in concentration gradients along the ROS that would be in opposite directions in the two classes of animals and these gradients would thus be in the correct direction to explain our observations. Limited support for this idea derives from the work of Bunt-Milam and Saari (1983), who showed that a retinoid-binding protein (IRBP) exists in high levels along the apical border of rat (and other mammalian) RPE. If IRBP is the carrier of retinoid between ROS and RPE, tip-first regeneration might be expected (post hoc) in the rat.

Aside from this suggestion that the RPE villous process may guide the retinoids to the ROS, we have no satisfactory explanation for the intracellular patterns of regeneration. Nevertheless, the mere demonstration that such patterns exist in vivo may have implications for others who work on regeneration and/or visual adaptation. For example, it has now been established that light adaptation in rods is more or less localized to the region that has absorbed the light (Jagger, 1979; Baylor and Lamb, 1982; Cornwall et al., 1983). The experiments that have demonstrated this have all been done with superfused preparations of rods. Because these preparations do not regenerate rhodopsin, it is not known whether, as living rods regenerate pigment, the regions of the ROS to regenerate first are also the ones to become responsive first. One way to examine this possibility is to thoroughly bleach a toad eye in vivo, let the animal begin to regenerate the pigment, kill it before regeneration is complete, and measure the threshold (or responsivity) of the isolated rods at various places along their lengths. If it is true, as seems likely, that heavily regenerated regions are the most sensitive or responsive, new questions will be raised: Why should those responsive regions of the ROS be at opposite ends for amphibia and rodents? Do responses generated at the ROS tip and base produce the same visual effects in these animals? Is there a survival benefit for rodents and amphibia reflected in these patterns of regeneration?

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