Axial Gradients of Rhodopsin in Light-exposed Retinal Rods of the Toad

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ABSTRACT Exposure of an intact vertebrate eye to light bleaches the rhodopsin in the photoreceptor outer segments in spatially nonuniform patterns. Some axial bleaching patterns produced in toad rods were determined using microspectrophotometric techniques. More rhodopsin was bleached at the base of the outer segment than at the distal tip. The shape of the bleaching gradient varied with the extent of bleach and with the spectral content of the illuminant. Monochromatic light at the lambda max of the rhodopsin gave rise to the steepest bleaching gradients and induced the greatest changes in the form of the gradient with increasing extent of bleach. These results were consistent with a mathematical model for pigment bleaching in an unstirred sample. The model did not fit bleaching patterns resulting from special lighting conditions that promoted the photoregeneration of rhodopsin from the intermediates of bleaching. Prolonged light adaptation of toads could also produce axial rhodopsin gradients that were not fit by the bleaching model. Under certain conditions the axial gradient of rhodopsin in a rod outer segment reversed with time in the light: the rhodopsin content became highest at the base. This result could be explained by an interaction between the pattern of bleaching and the intracellular topography of regeneration.

INTRODUCTION

Many properties of the vertebrate visual system trace back to the level of the photoreceptors, where light is converted into an electrical signal. Transduction occurs at the outer segment of the photoreceptor cell, where the light is absorbed in particular spatiotemporal patterns. The effects of the axial pattern of light absorption on photoreceptor function have been difficult to evaluate because of a lack of experimental information. We have approached this problem by measuring the axial distributions of rhodopsin in light-exposed toad rods using microspectrophotometry. The results obtained after brief light exposures were compared with a model for bleaching.

It was assumed that light propagates axially through the rods in the intact eye. Support for this assumption is provided by the observation that at all locations in the...
retina the long axes of the photoreceptor outer segments point toward the entrance pupil of the eye (Laties et al., 1968; Laties, 1969; Laties and Enoch, 1971; Webb, 1977). As light passes through an outer segment, it encounters a tightly packed array of pigment-laden membranes (Nilsson, 1964, 1965). Consequently, the initial attenuation of the light is exponential along the length of the outer segment and is described by the familiar Beer–Lambert law.

Light causes the visual pigment to bleach, however, which changes the pattern of attenuation along the outer segment as a function of time in the light. In rods the disks are not interconnected, nor do rhodopsin molecules "hop" from one disk to another (Liebman and Entine, 1974; Poo and Cone, 1974), so the resultant axial patterns of rhodopsin bleaching cannot dissipate via longitudinal diffusion. Ignoring the absorption of light by the photoproducts and assuming regeneration to be negligible, the distribution of unbleached rhodopsin remaining after illumination with monochromatic light will be (Rabinovitch, 1973; Hodgkin and O'Bryan, 1977):

$$
\frac{c_I}{c_o} = \frac{\exp \left[ a(\lambda)c_o I \right]}{\exp \left[ a(\lambda)c_o I \right] + \exp \left[ a(\lambda)\gamma I_o t \right] - 1}
$$

where $c_o$ is the dark-adapted rhodopsin concentration, $c_I$ is the rhodopsin concentration at distance $l$ from the base of the outer segment (the base is the end of the outer segment nearest the inner segment), $a(\lambda)$ is the Napierian molar absorbance coefficient of rhodopsin (for unpolarized light of wavelength $\lambda$ incident normal to a planar layer of rhodopsin molecules randomly oriented in two dimensions, $l \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$), $\gamma$ is the quantum efficiency of bleaching (the probability of bleaching given photon absorption), $I_o$ is the photon flux at the base of the outer segment, and $t$ is the duration of the light exposure. For comparative purposes, it will be useful to normalize $l$ for rhodopsin content: distance from the base will be expressed in terms of $\xi = a_{\text{max}} c_o I$, where $a_{\text{max}}$ is the absorbance coefficient at the wavelength of maximal absorbance. Eq. 1 predicts a remarkable change in the distribution of rhodopsin remaining after the presentation of different photon dosages (Fig. 1).

In time, bleached rhodopsin is regenerated; its spectral properties are restored. Regeneration occurs in the light as well as in darkness (Kuhne, 1879; Zewi, 1939; Hall and Bok, 1974). The regenerative process does not proceed uniformly along the length of the rod outer segment in all animals. In toad red rods rhodopsin is regenerated fastest at the base of the outer segment (ROS) in vivo (Williams and Penn, 1985). Thus, after exposure to light the final intracellular distribution of rhodopsin reflects the interaction between the topographies of bleaching and regeneration, the net result being determined by the relative rates of the two processes. In a dark-adapted rod exposed briefly to light the effects of bleaching predominate. However, with light exposures of longer duration the effects of regeneration become significant, producing "bleaching and regeneration" patterns. The bleaching and regeneration rhodopsin distribution may be an important parameter in setting the adaptation level of a rod.

In this study some of these axial patterns of bleaching have been measured. Toad rods were exposed to light through the intact optics of the eye under conditions in which regeneration was inhibited. Subsequent microspectrophotometric measure-
ments were made of the rhodopsin remaining in single rod outer segments (ROSs) as a function of $l$ to ascertain the axial profiles of bleaching. The observed profiles were then compared with the theoretical model, as stated by Eq. 1. In other experiments toads were exposed to light for prolonged periods of time without inhibiting regeneration. The rhodopsin in single ROSs was measured microspectrophotometrically. Alternatively, the rhodopsin content of the whole retina was determined by taking difference spectra of rhodopsin extracts.

**METHODS**

*Animal Model*

Toads, *Bufo marinus*, were housed in a container with slowly running water. The toads were fed crickets dipped in a vitamin supplement (Multiprime vitamin supplement [Burroughs-Wellcome, Research Triangle Park, NC] + vitamin E, Nat Brand + vitamins A and D, Injacom) and baby mice when available. Lights were cycled on a 12:12 light:dark schedule. Before an experiment a toad was dark-adapted for a minimum of 12 h.

For most of the experiments in which regeneration was inhibited, the toad was partially immersed in a 10% aqueous solution of MS-222 (tricaine methanesulphonate; Sandoz Ltd., Basel, Switzerland) for 30 min (Hoffman and Basinger, 1977; Rapp and Basinger, 1982). The toad was then decapitated, the brain and spinal cord were pithed, and the eyes were enucleated in dim red light. One eye was placed in Ringer's, covered, and refrigerated. The Ringer's contained 90 mM NaCl, 11.9 mM NaHCO$_3$, 3.3 mM NaH$_2$PO$_4$, 2.5 mM KCl, 3 mM MgCl$_2$, and 10 mM D-glucose. The pH was adjusted to 7.8 with NaOH. The other eye was mounted on an indented test tube stopper and exposed to light.

In all other experiments one eye of an unanesthetized, dark-adapted toad was covered with black tape, and the other eye was exposed to light. The toad was decapitated and pithed and the eyes were enucleated immediately after the light exposure.
Light Exposures

The bleaching source was a 150-W xenon arc lamp, connected to a regulated power supply. For bleaching with regeneration inhibited, the beam was passed through a heat filter and brought to focus at the rear of an integrating sphere constructed from a ping-pong ball wrapped in aluminum foil. One of several interference filters (546, 500, 487, or 435 nm; Ealing Electro-optics Inc., Holliston, MA) or a yellow glass cutoff filter (Kodak #21, 50% cutoff at 550 nm) was inserted into the light path. An electronically controlled shutter delivered a light pulse of given duration to the isolated eye. The longest exposure duration was 20 min. For short duration exposures (90% of the energy delivered in 2 ms) a Strobonar flash gun (Honeywell Inc., Pleasantville, NY) was used either with the yellow glass cutoff filter and a neutral density filter attached to its front surface or with no filters at all. For unfiltered flashes the flash gun was pointed directly at one eye of an unanesthetized toad. For yellow flashes a hemisected ping-pong ball was interposed. Bleaching was carried out at room temperature, 17–21°C.

Possible screening effects of photointermediates were investigated in two ways. In the first method the flash gun was used without filters. This maximized the probability of screening by the metarhodopsin I (MI) and metarhodopsin II (MII) photointermediates. In the second method a dark-adapted, unanesthetized toad was placed in a pan of water. Ice was slowly added over a 2-h period, bringing the toad's oral temperature to 6°C. One eye was covered while the other eye was exposed to the xenon arc lamp shone through the 487-nm filter and a hemisected ping-pong ball for 10 min. These conditions were chosen to promote screening by the metarhodopsin III (MIII) photointermediate.

For monochromatic light experiments with regeneration allowed, the xenon arc lamp was focused onto a hemisected ping-pong ball through a heat filter and a 500-nm interference filter. Two lighting levels were used, 2–3 or 25–35 lx. These levels will hereafter be referred to as low intensity and high intensity, respectively, although 25–35 lx is not typically considered a high intensity. The low intensity level corresponded to 2–3 x 10⁴ photons · μm⁻² · s⁻¹ at the cornea, and the high intensity level corresponded to 3–4 x 10⁵ photons · μm⁻² · s⁻¹ at the cornea. White light exposures were obtained by placing toads inside a specially constructed light box designed to provide uniform illumination from all directions. The lighting was provided by cool-white fluorescent lamps (General Electric, Westinghouse) attenuated to 20–30 lx with black screens. Expressed in terms of 500-nm photons, 20–30 lux corresponds to 2–3 x 10⁴ photons · μm⁻² · s⁻¹. Pupil size was not controlled; in preliminary experiments topical application of either atropine or noradrenaline (Armstrong and Bell, 1968) failed to prevent pupillary responses. The experiments were conducted at room temperature, 17–23°C.

After the light exposure, the anterior segment of the eye and the lens were quickly removed, and 15 ml of 50-mM NH₄OH in Ringer's was poured into the eyecup. After a few minutes the eyecup was immersed in a solution of 10 mM NH₄OH in Ringer's and refrigerated until ready for use. All steps subsequent to the light exposure were performed in darkness or in dim red light.

Microspectrophotometry

Small pieces of retina were cut free and dabbed onto a polylysine-coated coverslip (Mazia et al., 1975). This broke off many outer segments at the connecting cilium, but some cells detached at the myoid region retaining a portion of their inner segments. The perimeter of the coverslip was painted with silicone oil and another, uncoated coverslip was placed on top. This preparation was mounted onto a single beam, photon counting microspectrophotometer (MSP) similar to that described by MacNicoll (1978). Two modifications were quartz optics and a piezoelectric autofocusing system. After locating a suitable ROS from a red rod, the
polarized measuring beam (nominally 1 x 4 µm for experiments with regeneration inhibited, 1 x 3 µm for experiments with regeneration allowed) was aligned so that its electric vector was perpendicular to the long axis of the ROS. Transverse absorbance measurements were made between 350 and 650 nm. ROS dimensions were measured using a television monitor connected to an infrared-sensitive camera.

It was not possible to determine the rhodopsin content of a given rod before and after bleaching in situ. To estimate $c_i$, for a light-exposed ROS, measurements were made on ROSs from the other eye (which was not exposed to light). Specific absorbance varied with ROS diameter in dark-adapted rods, but the linear relationship described by Johnson (1984) was not consistently observed. Additional variability in specific absorbance arose from differences in the rhodopsin alignment in different rods, as indicated by their dichroic ratios, 3.8 ± 0.6 (mean ± SD, n = 6). To minimize these two sources of error in estimating $c_i$, mean specific absorbances were determined for various diameter classes of ROSs. A new set of specific absorbances was measured for each toad to control for animal to animal variation in rhodopsin concentration and dichroism. The rhodopsin content of a light-exposed rod was then expressed in terms of $c_i/c_o$, the fraction of the dark-adapted rhodopsin concentration remaining at normalized distance from the base, $\xi$. $\xi$ was set equal to the product: (2.303) (transverse specific absorbance) (distance from the base). Experimental results were fitted by the bleaching model using a Simplex curve-fitting routine (Caceci and Cacheris, 1984) for the monochromatic light experiments and by numerical analysis (see Appendix) for the nonmonochromatic light experiments, where the photon dosage was a free parameter of the fit. In calculating the photon dosage from $r$, it was assumed that $\gamma = 0.67$ (Dartnall, 1968) and $\alpha_{max} = (2.303) (1.35) (42,000 l \cdot mol^{-1} \cdot cm^{-1})$. The factor 2.303 converts to Napierian exponents. The factor 1.35 corrects for dichroism and noncollimated condenser conditions (Harosi, 1975).

The total amount of rhodopsin remaining in a rod after a light exposure was estimated by assuming the concentration of rhodopsin remaining to be constant in a cylindrical segment of the ROS surrounding the point of measurement. When two adjacent measurements differed, the intracellular rhodopsin concentration was assumed to vary in a stepwise fashion midway between measurement loci.

**Control Experiments**

Several control experiments were conducted to test the efficacy of the procedures used to prevent rhodopsin regeneration and to check for bleaching artifacts caused by the use of the chemical agents. Dark-adapted ROSs were examined microspectrophotometrically after being bathed in 5% MS-222 in Ringer’s in vitro. A small absorbance band appeared at 320–340 nm, but the amplitude of the band was low and its location spectrally distant from the wavelengths of light used for bleaching. Therefore, screening effects of the MS-222 were negligible. MS-222 did not appear to cause chemical bleaching of the rhodopsin either. Finally, illumination of the rods on the stage of the MSP did not appear to lead to the formation of any unusual long-lived photoproducts in the presence of MS-222.

To determine the extent of chemical bleaching by NH$_4$OH in these experiments, a dark-adapted retina was cut in half. ROSs from one half were examined on the MSP immediately. The other half was placed in 10 mM NH$_4$OH in Ringer’s and refrigerated for 9 h before MSP analysis. Although there was a small decrease (<4%) in the specific absorbances of diameter-matched ROSs, the change was not significant. In another experiment, dark-adapted ROSs were examined on the MSP before and after perfusion with 10 mM NH$_4$OH. A small (<5%) but significant decrease in rhodopsin content ($P < 0.02$) was observed over a 3-h period. This effect was less than that observed by Bowmaker and Loew (1976), probably because a lower hydroxylamine concentration was used in these experiments. To guard against
this slow chemical bleaching, new preparations were made from refrigerated tissue at intervals of <3 h.

Finally, a test was made for the possibility of regeneration during the interim period between light exposure and the addition of hydroxylamine to the eyecup. Two eyes from an MS-222-treated toad were flashed 110 times with a Strobonar flash gun (Honeywell Inc.) through a 550-nm cut-off filter. One eye was opened immediately and treated with hydroxylamine. The other eye was treated with hydroxylamine 25 min later (the longest interim period was <25 min). ROSs from the posterior pole of the two eyes were compared for rhodopsin content. There were no differences in the rhodopsin content of single ROSs at 8-, 25-, and 42-μm distances from the base. Gradients, estimated by subtracting the rhodopsin content at 8 μm from the base from that at 25 μm from the base, were also identical. Thus it appeared that the procedures adequately inhibited rhodopsin regeneration without introducing serious artifacts.

**Whole-Retina Rhodopsin Measurements**

Rhodopsin extractions were performed on some animals instead of microspectrophotometric analysis. After decapitating and pithing these animals, the enucleated eyes were opened and a 10-mm punch was taken from around the optic disk using a sharpened cork borer. The retinal punch (central retinal sample) and the remaining tissue (peripheral retinal sample) were placed in separate tubes containing 50 mM NH₄OH in phosphate buffer, pH = 5.5. The tubes were centrifuged at 12,000 g for 10 min. The supernatant was decanted and the tubes were inverted. Then the insides of the tubes were carefully wiped to remove any remaining supernatant. The central retinal samples were resuspended in 0.5 ml 15% Triton in phosphate buffer, pH = 7.0, the peripheral retinal samples were resuspended in 1.0 ml Triton. The samples were rotated at 20 rpm for 4 h, then centrifuged at 17,000 g for 15 min. Difference spectra were obtained on a Unicam SP1800 UV/Visible spectrophotometer. Rhodopsin contents were normalized by calculating $R_\text{c}$, the ratio of the light-exposed rhodopsin absorption to the dark-adapted rhodopsin absorption.

**RESULTS**

**Bleaching with Regeneration Inhibited: Monochromatic Light**

The absorbance spectra in Fig. 2 were recorded from a red rod bleached by 500-nm monochromatic light. There was less rhodopsin at the base than at the tip. This was the expected result, given that the light was incident at the base and attenuated as it traveled toward the tip. Eq. 1 provided a good description of the pattern of bleaching (Fig. 3). The patterns of bleaching in other ROSs exposed to different amounts of light varied considerably from each other, but nearly all conformed quite closely to Eq. 1. These findings confirm the results of an earlier report (Makino et al., 1987).

The axial patterns of bleaching were also affected by the wavelength of the bleaching light (Fig. 4). For a given extent of bleach in a ROS, the greater the difference between the wavelength of the bleaching light and the lambda max of the pigment, the greater the axial uniformity of bleaching. However, after varying the absorbance coefficient $\alpha(\lambda)$ in accordance with a Dartnall nomogram for a 503-nm pigment (Dartnall, 1953; Ebrey and Honig, 1977), Eq. 1 accurately described this decrease in the axial gradient of bleaching and in addition predicted a decrease in
the total amount of rhodopsin bleached per photon dosage. Lowering the temperature to ~6°C did not significantly affect the results (data not shown).

Although the majority of the observed bleaching gradients adhered quite closely to Eq. 1, a few were slightly steeper than predicted. The incidence of a steep gradient did not appear to be wavelength dependent. In some minimally bleached ROSs with steep gradients, the calculated fraction of rhodopsin remaining at the tip exceeded 1.0, suggesting an underestimation of \( c_0 \) (see Methods). Thus, overly steep gradients may be due to errors in estimating \( c_0 \).

**Figure 2.** Absorbance spectra from a ROS exposed to 500-nm light in situ. Curves a-h were obtained at increasing distances from the base in a single ROS, respectively. The maximum amplitude of each curve is proportional to the concentration of rhodopsin remaining at that location. All curves approach a baseline at wavelengths >650 nm.

**Figure 3.** The axial profile of bleaching. Each filled symbol corresponds to a curve shown in Fig. 2. The line represents the Simplex fit to Eq. 1 for a photon dosage of 7.29 x 10^7 photons \( \mu \text{m}^{-2} \). The region above the points represents the pattern of bleaching; the region below represents the distribution of rhodopsin remaining. A ROS is shown below the graph for reference; the darkened regions indicate the positions where measurements were made.
Bleaching with Regeneration Inhibited: Nonmonochromatic Light

Replacing the 500-nm monochromatic light with a yellow nonmonochromatic light had an effect qualitatively similar to that of moving the wavelength of the monochromatic light away from the rhodopsin lambda max; the bleaching gradients diminished for any given percent of bleach (Fig. 5 A). The observed gradients were fit by numerical analysis to a version of Eq. 1 generalized for nonmonochromatic illuminants (see Appendix):

\[
\frac{c(\xi, \tau)}{c_0} = \frac{F[G^{-1}[G(\xi) + \tau]]}{F(\xi)}
\]

where \(F, G,\) and \(G^{-1}\) (the inverse function of \(G\)) are functions that can be computed numerically from the spectral density \(i_0(\lambda)\) of the input light and the absorbance coefficient \(\alpha(\lambda)\) of rhodopsin (as explained in the Appendix), and \(c(\xi, \tau)\) is the
rhodopsin concentration at position $\xi$ after exposure to standardized photon dosage $\tau$. Similar results were obtained when the yellow light was presented either as a brief flash or as a longer duration exposure, consistent with the Bunsen–Roscoe law (Fig. 5A).

Bleaching with a flash of white light also conformed to Eq. 2 as long as the intensity of the flash was low (Fig. 5B). After a very bright flash the rhodopsin was more evenly distributed than Eq. 2 predicted. This was particularly true near the base of the ROS, but in the extreme cases the rhodopsin content was axially uniform throughout (Fig. 5B). Furthermore, the maximal extent of bleach observed in any single ROS from the central retina was only 67%, even though the photon dosage was sufficient to bleach all of the rhodopsin in that part of the retina (from Eq. 2).

**Figure 5.** Bleaching patterns in ROSs exposed to nonmonochromatic light. (A) The upper set of symbols shows the bleaching pattern in a ROS exposed to yellow nonmonochromatic light for 3 min, where the ROS received $2.20 \times 10^8$ photons $\cdot \mu m^{-2}$. The lower set of symbols shows the pattern in a ROS exposed to higher intensity light for several milliseconds, where the ROS received $5.92 \times 10^8$ photons $\cdot \mu m^{-2}$. The continuous lines were derived from Eq. 2, using numerical analysis. (B) The filled symbols show the rhodopsin distributions in ROSs exposed to a “dim” flash of white light; the open symbols show the distribution in a ROS exposed to a bright flash of white light. The continuous curves show the fits to Eq. 2 using numerical analysis with photon dosages of $3.37 \times 10^7$, $7.36 \times 10^7$, $1.35 \times 10^8$, and $2.86 \times 10^8$ photons $\cdot \mu m^{-2}$ (from top to bottom, respectively). Note the pronounced deviation of the open symbols from the fitted curve. The photon dosage given for this curve represents a conservative lower limit.

**Intraretinal Variation in the Extent of Bleach**

In spite of attempts to make the lighting uniform at the cornea, ROSs from different regions of the retina bleached to different extents. ROSs from the peripheral retina bleached less than did ROSs located more centrally. This was consistent with observations in rabbit made by Young (1981). Since the amount of rhodopsin remaining depends in part on the length of the outer segment, which varies across the retina, it was more meaningful to compare the estimated photon dosages
received by the ROSs (Fig. 6 A). There was some variability in the amount of light received by ROSs from within a retinal region (Fig. 6 B), but it was much smaller than the variability across retinal regions.

Bleaching with Regeneration Allowed: Low Intensity, 500 nm

The red ROSs of toads exposed to diffuse 2–3 lx, 500-nm light for 1.5 h displayed nonuniform axial distributions of rhodopsin (Fig. 7 A) that were similar in form to those obtained with regeneration inhibited. From Eq. 1 it was estimated that a ROS from the central retina received \((4.38 \pm 0.87) \times 10^8\) photons \(\mu\text{m}^{-2}\) (mean \(\pm\) SD, \(n = 6\)).

A similar result was obtained after a 4-h light exposure, except that the amount of rhodopsin remaining was reduced further (Fig. 7 B). The application of Eq. 1 yielded an estimate of \((1.36 \pm 0.21) \times 10^8\) photons \(\mu\text{m}^{-2}\) (mean \(\pm\) SD, \(n = 9\)) for the number of photons received by centrally located ROSs. This value is approximately three times higher than the photon dosage received by ROSs after 1.5 h in the light, suggesting that little regeneration occurred throughout the 4-h exposure to dim monochromatic light.
Bleaching with Regeneration Allowed: High Intensity, 500 nm

A 10-fold increase in the intensity of the light (to 25–35 lx, 500 nm) bleached nearly all of the rhodopsin in the perioptic disk region of the retina in 1 h (Fig. 8 A). Eq. 1 provided a good description of the intracellular axial rhodopsin distributions. The ROSs from this region of the retina received \((2.21 \pm 0.41) \times 10^8\) photons \(\mu m^{-2}\) (mean \(\pm\) SD, \(n = 15\)), as estimated from Eq. 1. The fraction of rhodopsin remaining over the entire outer segment measured microspectrophotometrically \((R_m)\) was \(0.14 \pm 0.10\) (mean \(\pm\) SD, \(n = 11\)), similar to the value obtained from the rhodopsin extraction technique \((R_e)\), 0.12. In the peripheral retina, where single ROSs were found to contain higher levels of rhodopsin, \(R_e = 0.38\). \(R_m\) was not calculated for peripherally located ROSs because the individual variation was high. Over the entire retina, \(R_e\) was 0.35.

After 2 h in high intensity, 500-nm light the whole retina \(R_e\) decreased to 0.28. Little change was observed in the rhodopsin measurement of the central retina, where \(R_e = 0.11\). However, the MSP analysis of the central retina indicated otherwise; \(R_m\) reduced to \(0.03 \pm 0.03\) (mean \(\pm\) SD, \(n = 15\)). In many ROSs the rhodopsin level dropped below the level of detection by the MSP. The slight discrepancy between \(R_e\) and \(R_m\) was attributed to the larger retinal sampling area of the extraction method.

For those ROSs containing measurable levels of rhodopsin, Eq. 1 provided a reasonable description of the axial rhodopsin distributions. In a few ROSs the
FIGURE 8. Monochromatic 500-nm light-exposed rods: 25–35 lx. The intracellular distributions of rhodopsin in perioptic disk ROSs from toads after a 1-h (1.68 x 10⁸ photons · μm⁻²) (A), 2-h (3.54 x 10⁸ photons · μm⁻²) (B), and 4-h light exposure (C). Because of the poor fit of the 4-h light-exposed ROS to Eq. 1, the photon dosage was not computed. (D) The rhodopsin in a peripheral ROS after 4 h in the light (8.91 x 10⁷ photons · μm⁻²). The continuous lines show the Simplex fits to Eq. 1.
rhodopsin content at the base appeared to be slightly higher than at the tip, although the low level of rhodopsin present made it difficult to be certain of this (Fig. 8 B).

After a total of 4 h in high intensity, 500-nm light the whole-retina rhodopsin exhibited little further change; $R_e = 0.23$. Similarly, the rhodopsin level in the central retina appeared to have stabilized; $R_c = 0.09$. $R_m$ increased slightly to $0.07 \pm 0.04$ (mean $\pm$ SD, $n = 20$), but since every ROS from the central retina examined microspectrophotometrically was now found to contain rhodopsin, it was clear that some rhodopsin recovery had occurred. The rhodopsin was localized predominantly at the bases of the ROSs (Fig. 8 C). In many ROSs the tips contained little or no measurable rhodopsin. Thus it would appear that the odd distributions observed in a few of the 2-h light-exposed ROSs had anticipated the 4-h light exposure result. This reversal in the axial gradient of rhodopsin with time in the light is not explicable in terms of the "bleaching only" model. Rhodopsin gradient reversals were observed less consistently in ROSs in the far periphery; sometimes more rhodopsin was

![Figure 9](Image)
present at the tip than at the base throughout the 4-h light exposure period (Fig. 8 D).

**Bleaching with Regeneration Allowed: “White” Light**

ROSs exposed to 20–30-lx white light for 1.5 h exhibited rhodopsin distributions describable by Eq. 2 (Fig. 9 A). After 4 h nearly all ROSs, even those from the peripheral retina, possessed more rhodopsin at their bases than at their tips (Fig. 9, B and C). The axial rhodopsin distributions in peripheral ROSs were nonhomogeneous; however, the rhodopsin level in some ROSs was actually lowest in the middle. In comparison to 25–35-lx, 500-nm light, 20–30-lx white light produced more pronounced gradient reversals at higher overall ROS rhodopsin levels.

**DISCUSSION**

**Applicability of the “Bleaching Only” Model**

In the absence of regeneration, bleaching of rhodopsin in toad red rods exposed to light through the physiological optics of the intact eye conformed to the model of light absorption by an unstirred, photosensitive solution (Eq. 2). The model accurately described the systematic changes in the bleaching patterns that occurred as a function of the photon dosage, as well as the changes brought about by varying the wavelength of the bleaching light.

Eqs. 1 and 2 ignore waveguide effects in the ROS and absorption by the photoproducts of bleaching. Frog rods have been shown to act as waveguides when light passes through them longitudinally (Enoch and Tobey, 1973). However, it would appear that waveguide effects on the topography of bleaching were minimal in toad rods. It should be noted that since waveguide effects increase as the radius of the guide decreases (cf. Rohler and Fischer, 1971), the axial pattern of bleaching in a mammalian rod could differ from that predicted by Eq. 2.

The photointermediates MI, MII, and MIII could act as screening pigments (cf. Goldstein and Williams, 1966). Screening provides a likely explanation for the observations on rods exposed to the brief, high intensity flash of white light. The gradients of bleach were much shallower than predicted by Eq. 2, particularly toward the basal regions. Furthermore, the extents of bleach were lower than predicted. Such anomalies did not occur when the light was filtered through a yellow cutoff filter (50% transmission at 550 nm) that removed the medium and short wavelength components. The latter two observations were reminiscent of a study (Williams, 1974) in which the same type of flash gun, used to bleach rhodopsin in solution, photoreversed MI and MII to rhodopsin. The extremely large photon flux delivered over a very short period of time produced a photoequilibrium, where the rate of rhodopsin conversion equaled the rate of photoreversal.

The photoequilibrium occurred at the base of the ROS and then spread axially as the intensity of the flash was increased. Thus ROSs from the peripheral retina, which received the lowest photon fluxes in an eye exposed to diffuse light, showed more uniform bleaching patterns than predicted from Eq. 2 at their bases only. Proceeding to the central retina, the extent of ROS bleach increased and the axial uniformity of bleach extended further and further toward the tip. In some centrally located
ROSs, which were exposed to the highest photon fluxes, the rhodopsin content was uniform throughout the entire length of the outer segment. In these ROSs the "upper limit of bleaching" was ~65-70%, similar to the value observed for rhodopsin in solution at room temperature (Williams, 1974). Photoreversal has been demonstrated in the living eyes of rabbits (Hagins, 1955), rats (Dowling and Hubbard, 1963), and man (Rushton, 1963; Alpern, 1971; Pugh, 1975).

Screening effects attributable to MIII were conspicuously absent. Since the absorbance spectrum of MIII is blue-shifted from that of rhodopsin (c.f. Bowmaker, 1973), screening effects should have been most pronounced when toads were exposed to the shorter wavelength illuminants (e.g., 435 and 487 nm). However, no such wavelength dependence was observed; with 546-, 500-, 487-, and 435-nm light exposures, the great majority of the axial patterns of bleaching adhered to Eq. 1. It has been reported that the decay of MIII is more rapid after a bleach of <15% (Donner and Hemila, 1975) and that the rate of decay increases with temperature (Williams, T.P., and E.F. MacNichol, unpublished observations). Yet in this study the axial patterns of bleaching followed Eq. 1 regardless of temperature (6°C vs. 17-21°C) and/or extent of bleach. The simplest interpretation is that under these experimental conditions MIII was not formed to any significant extent during the period of illumination.

In conclusion, the bleaching model of Eqs. 1 and 2 was shown to be valid under a variety of illumination conditions. A flash of high intensity white light proved to be an exception. In this case multiple photon absorptions caused a photoreversal phenomenon that significantly affected the axial pattern of bleaching. However, a flash of this strength probably lies outside the bounds of the physiological range. Eqs. 1 and 2, therefore, provide a working model for bleaching within single rods in an intact toad eye.

Intracellular Rhodopsin Distributions after Prolonged Bleaching with Regeneration Allowed

The axial distributions of rhodopsin remaining in ROSs after bleaching and regeneration in vivo for 1 h also conformed to the "bleaching only" model for all three lighting conditions tested. This was unexpected, because when placed in total darkness, fully bleached toad ROSs regenerate rhodopsin in vivo at the base first (Williams and Penn, 1985). While the extent of regeneration in toads is low after 1 h in darkness at temperatures <30°C (Williams, T.P., unpublished observations), if it had occurred during the light exposure period (Kuhne, 1879; Zewi, 1939; Hall and Bok, 1974) it should have perturbed the axial patterns of bleaching observed here. The results of this study indicate that regeneration was minimal during the first hour in the light. Furthermore, the rods exposed to low intensity, 500-nm light showed no evidence of rhodopsin regeneration throughout the entire 4-h light exposure period. However, during other lighting conditions the rate of regeneration increased and significant amounts of regeneration did occur.

Rods from the central retina exposed to a 10-fold increase in the light intensity regenerated significant amounts of rhodopsin during the latter half of the 4-h light exposure. The rhodopsin at the base first decreased to a minimum after 2 h then returned to surpass the level at the tip after 4 h, suggesting that the regeneration
rate rapidly increased during this interval. A similar change in the rate of regeneration was reported in frog (Zewi, 1939).

Although the ROSs exposed to 500-nm light bleached to completion before exhibiting rhodopsin gradient reversals, this was not a prerequisite. Gradient reversals were present in peripheral ROSs exposed to white light. The high rhodopsin content after 1.5 and 4 h in the light made it unlikely that these ROSs bleached to completion between the two measurement intervals. It is not clear why the peripheral rods exposed to high intensity, 500-nm light did not exhibit gradient reversals. Possibly, the rate of regeneration was lower in the periphery (Kemp et al., 1988).

**Single-Cell Rhodopsin vs. Whole-Retina Rhodopsin**

Traditional methods of extracting and measuring whole-retina rhodopsin as a function of time in the light obscure two important aspects of light adaptation: changes occurring within single cells and regional differences across the retina. This was illustrated by the long duration exposures of toads to 25–35-Lx, 500-nm light with regeneration allowed. The whole-retina rhodopsin content stabilized in ~2 h. Spatially restricted sampling of the retina indicated that the central retina achieved a steady-state condition of bleaching and regeneration during the first hour in the light. The peripheral retina stabilized more slowly. This was not surprising, since the central retina received more light than the peripheral retina during diffuse illumination of the intact eye.

Examination of individual ROSs from the central retina revealed that they did not simply stabilize at a particular rhodopsin level during the 4-h light exposure. Instead, they bleached to near completion and then began to recover some rhodopsin. There was no evidence that these rods ever attained a steady state of bleaching and regeneration. The steady state apparent in rhodopsin extracts of the central retina probably arose from different rods bleaching and regenerating at slightly different rates. Thus the conclusions about the kinetics of bleaching were dependent on the sampling method.

**Rod Signaling and Adaptation**

The proposed bleaching model may be important to rod physiology as it pertains to vision. It has been shown that a photoexcited rhodopsin activates the transduction machinery in a spatially localized region of the ROS (Hagins et al., 1970; Jagger, 1979; Lamb et al., 1981). A single photoexcitation is thought to result in the closure of the light-regulated channels in a circumferential ring of ROS plasmalemma surrounding the site of photon absorption. Higher intensity lights recruit more rings of closed channels, causing a larger hyperpolarization. The spatial pattern of recruitment will be related to Eq. 2, with adjustments made for the spread of excitation.

After excitation, activated regions desensitize or adapt to subsequent illumination. Like excitation, adaptation is restricted to a region surrounding the site of photon absorption (Jagger, 1979; Lamb et al., 1981; Baylor and Lamb, 1982; Cornwall et al., 1983). Recovery from desensitization follows a spatiotemporal pattern; after equal bleaching of base and tip, the base recovers faster than the tip. Baylor and
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Lamb (1982) showed that after bleaching with a light attenuated exponentially along a ROS in accord with the Beer–Lambert law, the entire ROS recovered uniformly. Recovery was studied after light exposures that bleached <10% of the pigment. Recovery is likely to be nonuniform after more extensive bleaches. In these rods the Dowling–Rushton relation applies (Dowling, 1960, 1963; Rushton, 1961; Cornwall et al., 1983); the log sensitivity of an ROS region is proportional to the fraction of the rhodopsin bleached there. Bleaching at the distal tip may cause greater desensitization than bleaching at the base (Hemila and Reuter, 1981).

Since the spectral composition of the illuminant, the extent of bleach, and the rate of regeneration determine the axial distribution of rhodopsin in a ROS, they will also determine its adaptational state (Hemila and Reuter, 1981). For example, a ROS from the central retina of a toad will be more sensitive after a 4-h exposure to 2–3-lx, 500-nm light than after an exposure to 25–35-lx, 500-nm light because its rhodopsin content will be higher. But after being exposed to 25–35 lx, the rod should dark-adapt faster due to its faster rate of regeneration. In addition, the adaptational states of rods will vary across the retina, because even with diffuse illumination of the cornea ROSs in different parts of the retina bleach and regenerate at different rates.

In the rat regeneration in fully bleached rods occurs at the tip first (Williams and Penn, 1985), while in the catfish regeneration is virtually uniform along the ROS (Ingui, S., and T.P. Williams, unpublished observations). There are also differences in the rate of regeneration between species. Therefore, even if the patterns of bleaching are similar, differences in the light- and dark-adaptational processes could exist across species.

APPENDIX

Bleaching of pigment in toad rod photoreceptors exposed to monochromatic light proceeds according to Eq. 1. This is a special case of the more general description provided by Eq. 2:

\[
\frac{c(\xi, \tau)}{c_0} = \frac{F^{-1}[G(\xi) + \tau]}{F(\xi)}
\]

where the illuminant can be nonmonochromatic light. In the derivation of Eq. 2 that follows, these symbols will be used:

- \(c_0\): the initial dark-adapted concentration of rhodopsin, assumed to be uniform throughout the rod
- \(a(\lambda)\): \(\alpha(\lambda)/\alpha_{\text{max}}\), where \(\alpha(\lambda)\) is the Napierian molar absorbance coefficient of rhodopsin for unpolarized light incident normal to a planar layer of rhodopsin molecules randomized in two dimensions
- \(\xi\): \(\alpha_{\text{max}}c_l\), a dimensionless measure of distance \(l\) from the base
- \(i(\lambda,\tau)\): the spectral density of light of wavelength \(\lambda\) at the base (photon flux per unit wavelength interval)
- \(I_0\): \(\int i(\lambda,\tau)\,d\lambda\), integrated over all wavelengths
- \(j(\lambda)\): \(i(\lambda)/I_0\)
- \(\tau\): \(\alpha_{\text{max}}\gamma l\), a dimensionless measure of time \(t\); this can also be interpreted as a standardized photon dosage
The spectral density of light of wavelength \( \lambda \) present at position \( \xi \) in a ROS exposed to standardized photon dosage \( \tau \), divided by \( I_\lambda \) is:

\[
j(\lambda, \xi, \tau) = \frac{j(\lambda, \xi)}{I_\lambda} \]

The concentration of rhodopsin at position \( \xi \) after being exposed to standardized photon dosage \( \tau \) is:

\[
c(\xi, \tau) = c(\xi) \exp[-a(\xi)\tau] \]

The derivation of Eq. 2 is based on the mathematical description of the axial attenuation of the light within the ROS given (in dimensionless form) by the equation:

\[
\frac{dj}{d\xi} = -a(\lambda)j(\lambda, \xi, \tau)c(\xi, \tau)/\xi_0 \]

and that of the resultant change in rhodopsin content given by:

\[
\frac{dc}{d\tau}c(\xi, \tau)/\xi_0 = -c(\xi, \tau)/\xi_0 \int a(\lambda)j(\lambda, \xi, \tau) d\lambda \]

Let

\[
F(z) = 1 - \int f(\lambda) \exp[-a(\lambda)z] d\lambda = 1 - \int f(\lambda) d\lambda = 1 \]

where \( u(\xi, \tau) = \int c(\xi', \tau)/\xi_0 d\xi' \)

where \( u \) is a dimensionless measure of the total amount of rhodopsin located between the base and position \( \xi \) at time \( \tau \). Integration of Eq. 5 from 0 to \( \xi \) thus gives:

\[
\frac{du}{d\tau} = \int j(\lambda, \xi, \tau) d\lambda - 1 \]

This function may be regarded as a known, since it is determined by the spectral density of the incoming light at the base and the absorbance spectrum of rhodopsin; it can be computed numerically from these spectral data. It is obviously a monotonically increasing function of \( z \) with value 0 at \( z = 0 \), and its derivative is given by:

\[
F'(z) = \int a(\lambda)j_0(\lambda) \exp[-a(\lambda)z] d\lambda \]
After integrating over all wavelengths, Eq. 8 can be restated in terms of Eq. 9 as

\[ \int j(\lambda, \xi, \tau) \, d\lambda = 1 - F[u(\xi, \tau)] \]

Substituting this into Eq. 7:

\[ \frac{du}{d\tau} = -F[u(\xi, \tau)] \] 

From this equation and the initial condition \( u(\xi, 0) = \xi \) (which is obvious from Eq. 6), \( u \) can be implicitly determined as the solution of:

\[ \int_{\xi}^{u(\xi, \tau)} \frac{dz}{F(z)} = \tau \] 

Let \( G(z) \) be a function with \( G'(z) = -1/F(z) \)

\[ G(z) = \int_{z_0}^{z} \frac{dz'}{F(z')} \] 

where \( z_0 \) is some convenient constant. Like \( F \), \( G \) may be regarded as known; it can be computed from the spectral data. Then Eq. 12 may be expressed as:

\[ G[u(\xi, \tau)] = G(\xi) + \tau \] 

(Since \( F(0) = 0 \) and \( F'(0) > 0 \) (see Eq. 10), the integral in Eq. 13 diverges logarithmically as \( z \to 0 \); \( G \) is also clearly monotonically decreasing for \( z > 0 \). Therefore, Eq. 14 can always be solved uniquely for \( u \), for any \( \tau \geq 0 \). The inverse function \( G^{-1} \) of \( G \) thus exists and may be regarded as known).

Differentiating Eq. 14 with respect to \( \xi \) gives \( c(\xi, \tau) = c_0 \), and since \( du/d\xi = c(\xi, \tau)/c_0 \) and \( G' = -1/F \) this becomes \( c(\xi, \tau)/c_0 = F[u(\xi, \tau)]/F(\xi) \), or, solving Eq. 14,

\[ \frac{c(\xi, \tau)}{c_0} = \frac{F[G^{-1}[G(\xi) + \tau]]}{F(\xi)} \] 

This is Eq. 2; it determines \( c(\xi, \tau) \) for any specified \( \xi, \tau \) via the known functions \( F, G \), and \( G^{-1} \).

In the case of monochromatic light the spectral density \( j_0(\lambda) \) is localized at one wavelength \( \lambda_1 \) (a \( \delta \) function), so Eq. 9 becomes:

\[ F(z) = 1 - \exp[-a(\lambda_1)z] \]

To simplify the writing, suppose \( \lambda_1 = \lambda_{\text{max}} \), so \( F(z) = 1 - \exp(-z) \). In this case the indefinite integral \( G \) can be explicitly given by the formula:

\[ G(z) = -\ln[\exp(z) - 1] \]

Also, the equation \( G(z) = w \) can be solved explicitly by a formula:

\[ z = G^{-1}(w) = \ln[1 + \exp(-w)] \]

Consequently,

\[ F[G^{-1}(w)] = 1 - [1 + \exp(-w)]^{-1} = [\exp(w) + 1]^{-1} \]

Putting \( w = G(\xi) + \tau, \exp(\omega) = \exp(\tau)/[\exp(\xi) - 1] \), so

\[ F[G^{-1}[G(\xi) + \tau]] = [\exp(\xi) - 1]/[\exp(\xi) + \exp(\tau) - 1] \]
Since \( F(\xi) = 1 - \exp(-\xi) = \exp(-\xi)[\exp(\xi) - 1] \), Eq. 15 becomes
\[
c(\xi, \tau) / c_0 = \exp(\xi) / [\exp(\xi) + \exp(\tau) - 1]
\]
i.e., Eq. 1. (A similar result will obtain for any \( \lambda_i \)). In the nonmonochromatic case the functions \( F \) and \( G \) must be computed numerically.

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