Some Properties of Old and New Rhodopsin in Single *Bufo* Rods

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ABSTRACT Rod photoreceptors renew the membranous disks of the outer segments (ROS). New disks are assembled at the proximal base and old disks are shed at the distal tip. Rhodopsin, the major protein of the disk, remains with the disk into which it was inserted. Thus, it is true that the oldest rhodopsin is at the tip and the newest at the base. A microspectrophotometer is used to examine the properties of rhodopsin in the two ends of the toad ROS. No differences between the two are found in absorption spectrum, concentration, dichroism, photoconversion rates, or lateral diffusion rates. Regeneration of rhodopsin from the bleached state is also studied but cannot be used to discriminate old from new rhodopsin because the point of entry of regeneration retinoids and/or their concentrations cannot be controlled. However, a new insight into pigment regeneration in the living toad eye is gained: regeneration is faster in the basal disks than in the distal.

INTRODUCTION

The vertebrate rod photoreceptor is a highly specialized cell: the outer segment (ROS) is a modified ciliary structure that is packed with double membranous disks separated from each other and from the surrounding plasma membrane. Residing within these disks and probably serving a structural function as well is the visual pigment of night vision, rhodopsin. The chromophore of the pigment, 11-*cis* retinal, is covalently bound to the pigment protein, opsin. When rhodopsin absorbs light, it bleaches, light is transduced to an electrical response, and eventually retinal dissociates from opsin (cf. Wald, 1968).

For reasons as yet unknown, the cell rapidly renews the ROS disk membranes. For example, amino acids incorporated into rod protein are used to a large extent in the synthesis of new opsin, and it has been shown by autoradiographic means that these newly synthesized opsin molecules stay together on the same disk and, moving as discrete packets from the outer segment bases to the distal tips, they are shed and phagocytized by the epithelial cells (Young, 1967). The ROS light-catching apparatus is regulated by a balancing of these renewal and shedding processes. In mammals the entire outer segment is turned over approximately every 9–11 d and in amphibia about every 30 d (Young, 1967). Such a rapid turnover implies a great metabolic demand for energy and material.

It is always true that the oldest opsin resides in the distal-most tip and the

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youngest at the proximal base. Therefore, the lifetime of the opsin is laid out in nearly linear chronology along the outer segment length (Kaplan, 1982). This is not true for phospholipids, the other major constituents of the disk, because they may be replaced during the lifetime of the disk (Bibb and Young, 1974). Therefore, a priori, it seems possible that disks are turned over because of some deficit or malfunction that may arise in the rhodopsin.

This hypothesis seems reasonable, is testable, and even derives some tentative support from electrophysiological experiments (Baylor et al., 1979; *vide infra*). However, it can be countered by reference to cone shedding.

Cone pigment proteins, once synthesized *de novo*, are reportedly able to diffuse along the entire outer segment length (Liebman, 1975). Thus, when cone tips are shed, both old and new pigment proteins are removed. This, in turn, implies that the age of cone pigment proteins has nothing to do with their disposal and renewal.

Why, then, are rod pigments segregated according to age along the ROS? Is it just an epiphenomenon—a simple consequence of the fact that rod disks are not connected together as are cone lamellae? Perhaps rod opsin, like cone pigment proteins, is shed for some reason other than its age. This would be a difficult question to resolve at the present time because there are so many possibilities that could be considered. I have chosen instead to test a simple hypothesis: rods shed their tips because the aged pigment in these tips does not have the properties of newly synthesized rhodopsin. If this hypothesis is sustained, it will indicate (but not prove) that rod and cone renewal processes are carried out for different reasons. If it is not sustained, a new hypothesis can be developed that states that both rods and cones renew their pigments for reasons other than the fact that they are aged.

With microspectrophotometers it is possible to study small parts of outer segments, especially those obtained from amphibian retinas. The toad retina, for example, typically has ROS that are ~50 μm long and 6–8 μm in diameter. These large cellular dimensions make it possible to impose a microbeam of light on a single ROS and to study no more than ~10% of its length.

This article reports results of experiments done with *Bufo marinus* outer segments. Six properties of old and new rhodopsin were examined in single, isolated ROS: absorption spectrum, concentration, dichroism, photoconversion rates, lateral diffusion rates, and the rate of dark regeneration from the bleached state.

MATERIALS AND METHODS

The microspectrophotometer (MSP) used in this study is a copy of the one designed by E. F. MacNichol, Jr., at the Marine Biological Laboratory, Woods Hole, MA. This is a single-photon counting apparatus that combines infrared full-field viewing of the ROS (by means of a television system) with rapid (0.75-s) scanning of the entire visible spectrum.

Unless otherwise specified, a 5-μm × 2-μm measuring beam spot was used in all of these experiments. This beam of light was polarized with a Nicol prism in such a way that the electric vector of the beam lay parallel to the transition moment of the retinal chromophore when the long axes of that ROS and spot were coincident. In this configuration, strong (~10%) absorption then occurred. In a few experiments, when dichroism was being

measured, a 3-μm-long spot was placed across the diameter of the ROS. In this condition the electric vector was mainly perpendicular to the transition moment of the retinal. (Note: the use of a high-numerical-aperture [1.3] condenser effectively depolarized the measuring beam by ~30%.)

Bufo marinus were obtained from Amphibians of North America (Nashville, TN) and fed with crickets. They were held in tanks with dripping water, and the ambient light (~500 lux) was cycled 12/12 L/D. After an animal had been dark-adapted overnight, it was decapitated in dim red light, the eyes were enucleated, and eyecups were prepared that were soaked in calcium-free Ringer's for ~5 min. A square patch of retina, ~3 mm on a side, was removed from the eyecup in a region that contained the optic nerve head. ROS from this region were deposited onto a coverslip by dabbing the piece of retina onto it. These coverslips had previously been coated with polylysine, which, as a monolayer on glass, acted to "glue" cells to the surface of the glass because of the charged nature of the glass, the cell surface, and the polylysine (Mazia et al., 1975). It was sometimes desirable to do the experiments in the presence of hydroxylamine, in which case the pieces of retina were soaked for ~4 min in 0.025 M hydroxylamine Ringer's before they were dabbed onto the coverslip. A thin line of silicone oil was spread around the perimeter of this coverslip and another, identical coverslip was applied to it. Surface tension between the two coverslips pulled them together and created an airtight seal that prevented evaporation of the medium in which the ROS resided. This preparation was then put onto the stage of the MSP. Focusing the ROS and the measuring and bleaching beams was accomplished in infrared light by means of an infrared-sensitive television system. In all the experiments described below, it was necessary to determine the baseline transmission of the medium between the coverslips. This was true whether the spectrum was to be scanned or whether a single wavelength was to be used in the examination of the pigment. Typically, 30 scans between 350 and 700 nm were averaged together to set a baseline. Then the measuring spot was imposed upon an ROS and a measurement on the old or new rhodopsin was made. Again, averages of 10–30 scans were carried out. The computer, which assisted the above procedures, automatically calculated the absorbance at each wavelength and displayed spectra on a video screen or on an *X–Y* recorder.

The best criterion for judging which end was proximal and which was distal was the appearance of a small bit of inner segment still attached to the ROS. In a typical preparation, ~15–20% of the ROS were in this condition and only these were used in the experiments.

Properties of Rhodopsin Examined

ABSORPTION SPECTRUM For these experiments a measuring beam was focused first at one end of the ROS and the spectrum was recorded and stored on a magnetic disk. Then the process was repeated using the other end of the ROS. Care was taken not to put the beam too close to either extremum in order to avoid problems of stray light and to avoid measuring open disks when the proximal end was studied. The MSP was run in the full-spectrum scan mode.

CONCENTRATION Observations on the rhodopsin concentration in either end of the ROS were a normal consequence of measuring the absorption spectra in those regions. Results pertaining to this will be reported along with the absorption spectrum results. No actual concentrations will be calculated because of uncertainties in the degree of polarization of the measuring beam and pathlength (pyramidal vs. rectangular geometry). It was sufficient to compare the uncorrected absorbance in the distal and proximal ends. The MSP was run in the full-spectrum scan mode.

DICHROISM The dichroic ratio was measured on each of the two ends of ROS as

described above. The dichroic ratio was defined as the ratio of absorbance with the E-vector parallel to the plane of the disks divided by the absorbance when the E-vector was perpendicular to them. Full spectra were determined with the MSP in the scan mode.

PHOTOCONVERSION RATE This was measured at a single wavelength, 550 nm, and it was determined in conjunction with lateral diffusion rates as described immediately below.

LATERAL DIFFUSION RATES OF RHODOPSIN The methods used to study lateral diffusion and the photoconversion rate require some elucidation. Fig. 1 shows the beam configuration and expected absorbance changes. This method will be referred to as "single beam, high intensity." In this case, the MSP did not scan the spectrum. Rather, the wavelength of the measuring beam was set at 550 nm—a wavelength chosen because it is strongly absorbed by rhodopsin and is capable of causing rapid bleaching and yet is

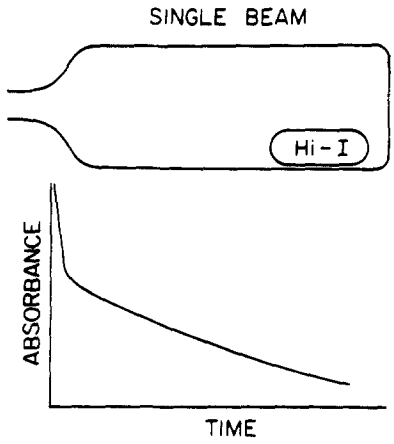

FIGURE 1. Geometry and expected results of a single-beam high-intensity experiment. The measuring-beam intensity is increased so that it causes rapid bleaching of the edges of the disks it subtends. The beam intensity is reduced at the photomultiplier face by neutral filters so as to permit single-photon counting. Under these conditions the absorbance change with time is double-branched: the first, fast change is related to the photosensitivity of the rhodopsin and the second is slower and is controlled by the lateral diffusion of rhodopsin into and out of the beam volume.

sufficiently remote from the absorption spectra of metarhodopsin I and III to permit rhodopsin measurements uncontaminated by absorbance changes of these products. The intensity of this measuring beam was made great enough to cause bleaching intentionally as it traversed the ROS, but its intensity was subsequently reduced at the photon-counting tube (to avoid coincident photons) by means of a neutral filter. Absorbance changes under these conditions of beam geometry, intensity, placement on the cell, and wavelength were as follows. A rapid drop of absorbance occurred as the measuring-bleaching beam began to bleach a path through one edge of the subtended disks. For this method to work, the bleaching rate had to be slightly greater than the lateral diffusion rate (approximately four times worked well). Then, as the rate of bleaching fell and became commensurate with the lateral diffusion rate, further absorbance loss was controlled by the diffusion of unbleached molecules into and bleached molecules out of the region subtended by the

measuring beam. Thus, two branches of absorbance loss were obtained: a fast one, controlled by the rate of photoconversion, and a second, controlled by the rate of lateral diffusion.

REGENERATION Experiments on the rate of regeneration from the bleached state were carried out in three ways: in vivo, in vitro, and in eyecups. The in vivo studies were done by bleaching living eyes in intact toads, dark-adapting the animals to regenerate some pigment, and subsequently isolating and examining ROS for the sites of pigment regeneration. The bleaching was carried out in the following way. A dark-adapted (overnight) toad was put into a special cubicle that provided uniform illuminance, 80 ± 20 lx, at all visual angles. The lights were turned on for 1 h and this exposure was sufficient to bleach at least 95% of the rhodopsin in the ROS of the posterior pole of the toad eye. Dark adaptation was done in a water bath at 30 ± 1°C. In vitro experiments were performed by isolating dark-adapted ROS as usual, and then bleaching them to completion on the coverslip either in ambient light or with an orange bleaching beam from the MSP. The ROS were then perfused with 0.025 M NH_2OH for ~3 min to

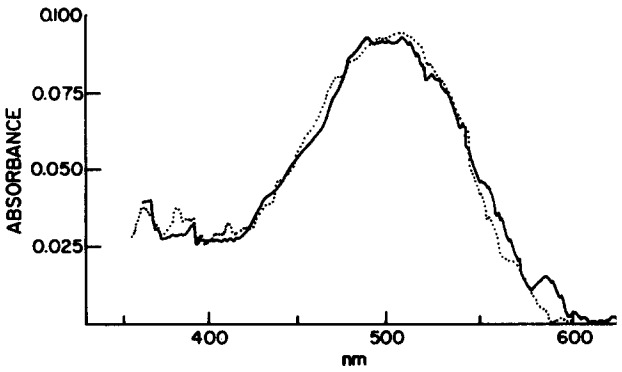

FIGURE 2. Comparison of rhodopsin absorption spectra from distal (solid) and proximal (dotted) ends of a single ROS.

remove endogenous retinal and excess NH_2OH was washed out with two to three flushes of Ringer's solution. Finally, the ROS were perfused with 9-*cis* retinal (10 mg in 0.2 ml ethanol, diluted with 10 ml Ringer's solution) and the rate of regeneration of isorhodopsin was followed over the next 3–4 h. The eyecup experiments were carried out as follows. Eyecups were prepared from dark-adapted toads and then bleached in ambient light (150 lx) to virtual completion. These were then put into a dark chamber (at 30 ± 1°C) through which H_2O-saturated O_2/CO_2 (95%/5%) flowed. At various times in the dark, an eyecup was removed and the ROS were studied for local regeneration of pigment.

In all of the above-mentioned regeneration experiments, care was taken to examine only the ROS from the posterior pole and to ensure that the bleaching of these ROS was >95% complete. This assured that patterns of regeneration were not seriously distorted by patterns of unbleached pigment.

RESULTS

Fig. 2 shows absorption spectra obtained from proximal and distal rhodopsin within the same ROS. This figure discloses two repeatable observations. First,

the total absorbance of rhodopsin in these two regions is identical within experimental error (±5%). This indicates that there is overt loss of neither opsin nor of its competence to dark-regenerate pigment as the protein ages. Second, the absorption spectrum of the rhodopsin in the two ROS regions is identical.

The dichroic ratio, which is a measure of the interaction of rhodopsin with polarized light, is always ~3.7 in the present experiments. This is shown in Fig. 3, *A* and *B*. Theoretically, this ratio should be >>3.7, but with high-power, high-numerical-aperture lenses such as those used here, the measuring beam is greatly depolarized as it traverses the thickness of the ROS. This results in considerable

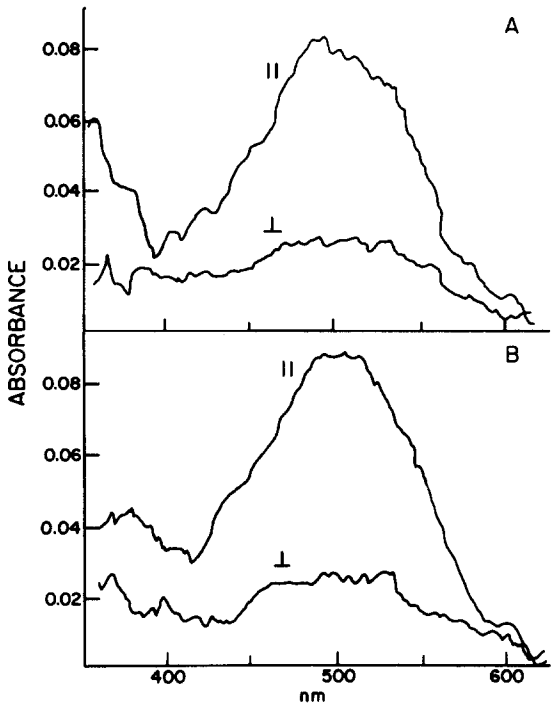

FIGURE 3. Comparison of dichroic ratio of rhodopsin in distal (*A*) and proximal (*B*) ends of a single ROS. Perpendicular and parallel symbols refer to the relative orientation of the plane of polarized measuring light and the plane of the disks. No significant differences are seen here or in the other ROS examined.

absorption, which occurs even when the electric vector is (supposedly) perpendicular to the transition moment of the retinal. Liebman (1975) reported a dichroic ratio of ~5 under more optimal conditions. Nevertheless, the concern here is whether old and new rhodopsins are different in this regard; they are not. Measurements at both ends of six ROS resulted in similar calculated dichroic ratios of 3.7 ± 0.2.

As indicated in Materials and Methods, photoconversion rates and lateral diffusion rates of rhodopsin are measured in the same experiments. In order to calibrate the bleaching effectiveness of the measuring beam at 550 nm, it was

necessary to prevent lateral diffusion of the rhodopsin. This is because absorbance changes caused by the lateral diffusion process would otherwise be convoluted with the loss of absorbance caused by photoconversion. Rods were treated with 4% glutaraldehyde for 30 min. This rigidly fixes and cross-links the membrane components of the disks. Under these conditions, no lateral diffusion of rhodopsin occurs (Leibman and Entine, 1974; Poo and Cone, 1974) and absorbance changes seen in such a system are due solely to the photoconversion of rhodopsin. The single high-intensity beam was imposed midway between the ends of the ROS; thus, rhodopsin of intermediate age was being examined. Results of an individual run are shown in Fig. 4. The fact that this first-order rate plot is linear over more than eight half-lives indicates that a single process, viz., the photoconversion of rhodopsin, is indeed being measured under these

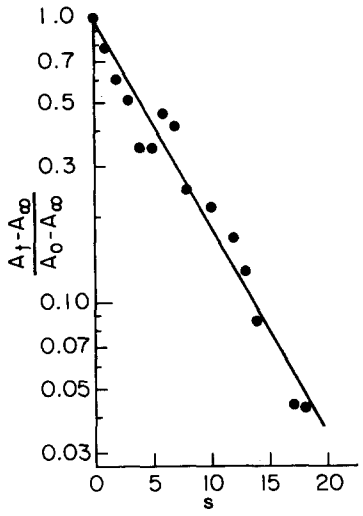

FIGURE 4. Calibration of photoconversion of rhodopsin in glutaraldehyde-treated ROS. Since lateral diffusion was prevented, the absorbance loss with time is a simple exponential function.

circumstances. This experiment was done on six ROS with virtually identical results. For example, the slope of this line is 0.18/s and the average slope for all the ROS was 0.21/s. Fig. 5 shows that absorbance is lost in two stages with fresh rods in the single-beam high-intensity experiment. The first stage occurs immediately upon exposure of the ROS and at a rate that is exactly equal to that given in the previous figure. Thus, the slope of the first branch of the function is the rate of photoconversion of rhodopsin; furthermore, these rates are, within experimental error, identical to each other and to the rate of photoconversion of rhodopsin of intermediate age. This agrees with the results of Baylor et al. (1979). The second branch is that caused by lateral diffusion. The open circles are measurements made at the distal tips of the rods and the filled circles indicate measurements made at the bases. Despite the considerable scatter, it appears

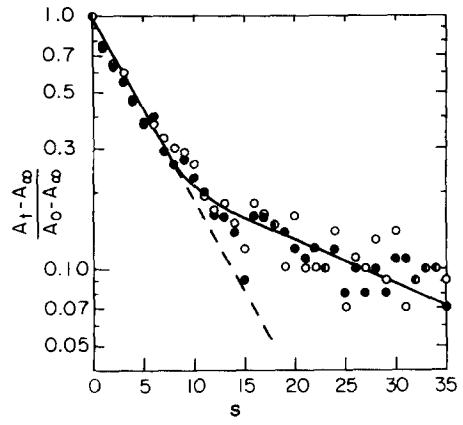

FIGURE 5. Results of a single-beam high-intensity experiment on fresh ROS. Double-branched loss of absorbance is evident and conforms to expectations. The first branch is photoconversion of rhodopsin (the dashed line is a redrawing of the line in Fig. 4) and the second is caused by lateral diffusion control (see Materials and Methods). Each point is the average of seven individual runs. Open circles are rhodopsin in distal end, and filled circles in the proximal end of ROS.

that there is no major difference between old and new rhodopsin in lateral diffusion rates. Each datum in this figure is the average of seven individual points.

The rate of regeneration of rhodopsin from the bleached state was studied in vivo, in eyecups, and in vitro. Fig. 6 shows typical results of in vivo experiments. After 2 h in the dark, the proximal end of the ROS had regenerated about twice as much as had the distal. Usable spectra were obtained from 21 ROS from four

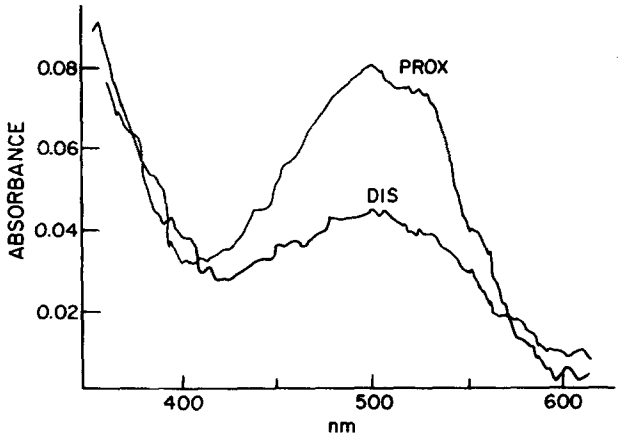

FIGURE 6. In vivo regeneration of rhodopsin in proximal and distal ends after a complete bleach (≤5% rhodopsin remaining). This particular ROS was obtained from a toad that had been dark-adapted for 2 h and is typical of most ROS after such a dark period: the proximal end regenerated about twice as much pigment as did the distal.

different toads, and it was found in 19 of these ROS that the proximal regeneration was faster than the distal. The other two ROS showed nearly equal regeneration in tip and base. The most typical result was that the base had regenerated about twice as much as the tip in 2 h of dark adaptation. The ROS with the largest difference between tip and base was obtained from a toad dark-adapted for 160 min; the rhodopsin in the base was 4.5 times that in the tip.

A major problem in trying to measure a detailed time course of regeneration is the inter-ROS variability. Attempts to reduce the variability have not been successful. It could simply be that individual toad cells vary considerably in the regeneration rates. Despite these cellular idiosyncrasies, the tip was never seen to regenerate faster than the base in vivo. However, all possible results were found in eyecups and in vitro preparations: distal regeneration was sometimes faster than proximal; at other times the reverse was true, and one time the rates were equal within experimental error.

DISCUSSION

Before beginning a detailed discussion of these results, it may be well to indicate the overall conclusion suggested by them. Proximal and distal rhodopsin in toad ROS are not different from each other in five of the six parameters studied; the sixth (regeneration) is inconclusive as regards differences in the rhodopsin. The latter, however, does provide a new insight into the mechanism of in vivo regeneration of red rod pigment in the toad retina.

A close and highly specific association of retinal with opsin is necessary to produce the rhodopsin absorption spectrum (cf. Harosi et al., 1978). If aging had disrupted this association, one would have expected to see spectral differences between distal and proximal rhodopsin. Since such differences were absent, it was possible to conclude that disruption had not occurred. This does not mean, however, that opsin was completely spared from all aging effects; rather, it indicates only that such effects were not present in the region of the chromophore.

The concentration of distal rhodopsin in the toad rod is the same as proximal. This agrees with the results of Kaplan et al. (1978) for the frog. Thus, it appears that if disk membranes become peroxidized with age and if aldehydic products of this peroxidation attack opsin (Dillard and Tappel, 1973), either such products cannot react with the chromophoric sites, or, if they do, 11-*cis* retinal can successfully displace them during dark regeneration.

The absence of reduction in the dichroic ratio of rhodopsin, as it ages, means that the chromophoric site has not been tilted appreciably out of the plane of the disk. Again, however, nothing can be said about regions of opsin that are remote from retinal.

The results here indicate that photoconvertibility is unaltered as rhodopsin ages in situ. This agrees with the work of Baylor et al. (1979), who arrived at this conclusion from electrophysiological measurements on single toad rods.

Baylor et al. found, however, that distal responses of the toad rod had slower time courses than proximal ones. Since a recent theory of transduction in the rod (Liebman and Pugh, 1979) involves the lateral diffusion of rhodopsin, it seemed possible, a priori, that a lower diffusion rate might be observable in the

distal disks. The present results do not support this hypothesis and other reasons for the Baylor et al. result should be sought. For example, is lateral diffusion of rhodopsin not a requirement for transduction? If it is, perhaps it is not rate limiting. Another possibility is that the concentration of some critical ingredient (e.g., cGMP) might be lower in the distal region than in the base. This would affect the electrophysiology of the ROS but not necessarily the rhodopsin diffusion rate.

The regeneration experiments contribute nothing to a differentiation of old from new rhodopsin. This is because it is impossible to control the point of entry into the ROS of the retinal and/or its concentration at that point. These factors, no doubt, have contributed to the diverse results seen in the regeneration experiments. However, what has been disclosed by the in vivo experiments is a new finding: rhodopsin regeneration in the rod of the living toad is faster in the basal disks than in those at the tip. (Note: open disks were not being measured; cf. Methods.) Additional experiments are being done in order to explain this intracellular pattern of regeneration of the toad. Some of this work includes studies of rat rhodopsin regeneration in vivo and we find that the rat regenerates rod pigment much faster in the tip than in the base—that is, rat and toad are opposites in this regard (T. P. Williams and J. S. Penn, unpublished observations). This is true even though Zimmerman (1974) has shown with the rat that retinoids leave the RPE and begin to accumulate in the inner segment during dark adaptation. The answer to some of these questions about regeneration patterns would be clearer if the membrane binding site of the interstitial retinol-binding protein were known (cf. Liou et al., 1982).

The results reported here are a partial test of the hypothesis that old disks are shed because of a defect in aged rhodopsin; they do not support that hypothesis. However, at least two other possibilities should be examined before the hypothesis is completely rejected: (*a*) the toad may shed old disks before the deficit appears, and (*b*) a deficit in the rate (and/or extent) of the bleaching sequence reactions may exist. The former would be possible to test if the shedding of old disks could be inhibited, thus permitting the accumulation of still older disks. Unfortunately, ways to do this add other variables whose effects on rhodopsin function are not known. For example, constant light inhibits shedding (Currie et al., 1978) but can also cause retinal light damage (cf. Williams and Baker, 1980). The other possibility, bleaching sequence effects, requires modification of the MSP to accommodate an intense, short-duration bleaching flash and faster recording capabilities. These changes are presently being accomplished.

Thus, the work reported here is incomplete. However, the additional studies on bleaching sequence, etc., could also show that rhodopsin's age has nothing to do with its being shed from the ROS. If this is eventually demonstrated—this study representing a start in that direction—then a strong connection between rod renewal and cone renewal may have been made because it seems clear, as mentioned in the Introduction, that cones shed pigment molecules regardless of their ages. Extending this logic, the question obviously becomes: why, then, is there outer segment turnover? At least two possibilities suggest themselves. (*a*) The first idea retains the concept of aging. In this case, it could be that some

other intrinsic membrane protein that is segregated along both rod and cone outer segments according to its age becomes functionless or nearly so as it gets old. (*b*) The other possibility is that ROS turnover has nothing to do with aging of any molecules but, instead, is a mechanism that allows the animal to adapt to its ambient lighting. There is no evidence for the former at the present time and only scant evidence for the latter, which is implied by the experiments of Battelle and LaVail (1978) and Organisciak and Noell (1977). Battelle and LaVail showed that ROS lengths in rats depended upon the ambient lighting: rats raised in darkness had longer ROS than those raised in cyclic light. Furthermore, they showed that the dark-reared animals had more rhodopsin than could be accounted for by the additional ROS length. Organisciak and Noell did a similar experiment on light history and showed that the lipid/rhodopsin ratio was lower in dark-reared rats than in light-reared ones. Taken together, these two studies indicate that rats are able to modify the turnover mechanism in such a way as to adjust the rhodopsin per disk and ROS length in response to their lighting environment.

Thus, if membrane aging turns out to be irrelevant to outer segment turnover, should we next examine an hypothesis that considers ROS turnover an adaptation phenomenon?

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