

# Visual Pigment Bleaching in Isolated Salamander Retinal Cones

## *Microspectrophotometry and Light Adaptation*

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**ABSTRACT** Visual pigment bleaching desensitizes rod photoreceptors greatly in excess of that due to loss of quantum catch. Whether this phenomenon also occurs in cone photoreceptors was investigated for isolated salamander red-sensitive cones. In parallel experiments, (a) visual pigment depletion by steps of bleaching light was measured by microspectrophotometry, and (b) flash sensitivity was measured by recording light-sensitive membrane current. In isolated cones, visual pigment bleaching permanently reduced flash sensitivity significantly below that due to the reduction in quantum catch, and there was little spontaneous recovery of visual pigment. The "extra" desensitization due to bleaching was most prominent up to bleaches of ~80% visual pigment and reached a level ~1 log unit beyond that due to loss of quantum catch. At higher bleaches, the effect of loss of quantum catch became more important. Bleaching did not greatly reduce the maximum light-suppressible membrane current. A 99% reduction of the visual pigment permanently reduced the circulating current by only 30%. Visual pigment bleaching speeded up the kinetics of dim flash responses. All electrical effects of bleaching were reversed on exposure to 11-*cis* retinal, which probably caused visual pigment regeneration. Light adaptation in photopic vision is known to involve significant visual pigment depletion. The present results indicate that cones operate with a maintained circulating current even after a large pigment depletion. It is shown how Weber/Fechner behavior may still be observed in photopic vision when the contributions of bleaching to adaptation are included.

### INTRODUCTION

Rod photoreceptors in the isolated retina, i.e., after separation from the pigment epithelium, are not able to regenerate more than a few percent of their visual pigment after bleaching (Weinstein, Hobson, and Dowling, 1967; Coccozza and Ostroy, 1987). A permanent loss in visual pigment is associated with a permanent loss

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in sensitivity to light that is much greater than can be accounted for by the decrease in quantum catch of the cell (see summaries in Grabowski and Pak, 1975, and Leibovic, Dowling, and Kim, 1987), implying some mechanism by which a product, or products, of photoisomerization are able to desensitize the rod cell.

Recording from single cones of the turtle, Baylor and Hodgkin (1974) noted a long-lasting desensitization after bright light steps and suggested that this was associated with visual pigment bleaching. More recently, during a study of background adaptation in isolated cones of the salamander, Matthews, Fain, Murphy, and Lamb (1990) found permanent reductions in flash sensitivity and changes in response kinetics after the termination of bright background lights, which they attributed to effects of pigment bleaching, above that due to loss of quantum catch. Normann and Perlman (1990), recording from horizontal cells in the isolated turtle retina, inferred that bleaching desensitizes cones by a mechanism independent of the loss in quantum catch, though, for extensive bleaches, quantum catch appeared to be the more important factor. Schnapf, Nunn, Meister, and Baylor (1990), recording from single primate cones, report that bleaching produces a permanent desensitization, but the desensitization appeared to be due only to the loss in quantum catch.

In the human visual system, an indication that bleaching lowers sensitivity in cone photoreceptors below that due to the loss in quantum catch comes from studies of visual pigment and visual sensitivity during dark adaptation. Here there is an elevation of threshold for cone-mediated photopic vision substantially above that due to the limitation of quantum catch (Baker and Rushton, 1965; Hollins and Alpern, 1973). The elevation is less extensive than that seen for rod-mediated vision during dark adaptation (Rushton, 1961).

This article presents a direct study of the effects of visual pigment bleaching in isolated cone photoreceptor cells. Previous work indicating that there is little visual pigment regeneration in the isolated cone cell (Marks, 1965; Liebman, 1972) is confirmed by our microspectrophotometry, and also inferred from measurements of membrane current responses to light flashes. In isolated salamander cones, bleaching produces a permanent desensitization that is only partly accounted for by the loss in quantum catch. A simple model is presented indicating how, after bleaching, both the effects of quantum catch and an additional mechanism independent of quantum catch can be included in discussion of the process of light adaptation in the photopic visual system.

#### METHODS

Isolated photoreceptor cells were obtained from dark-adapted retinas of the larval tiger salamander, *Ambystoma tigrinum*, using mechanical dissociation. Under infrared illumination, the retinas were removed into a small volume (~1 ml) of physiological salt solution, chopped into small pieces using fine iris scissors, and triturated by passage six or seven times through the tip of a truncated, fire-polished Pasteur pipette. An additional step was added in preparing cells for microspectrophotometry. Before dissociation, the retinas were thoroughly washed with six or seven changes of solution to remove any elements of the interphotoreceptor matrix and any fragments of pigment epithelial cells remaining after separation from the pigment epithelium. This was because preliminary microspectrophotometry indicated some very low values of photosensitivity on bleaching (and even no bleaching for one cone), which may have

been due to transfer of retinal from other cells or fragments of cells within the small sealed chamber used in the microspectrophotometer (cf. Cone and Brown, 1969).

The physiological salt solution contained (mM): 108 NaCl, 2.4 KCl, 1.2 MgCl<sub>2</sub>, 0.75 NaH<sub>2</sub>PO<sub>4</sub>, 1.6 CaCl<sub>2</sub>, 0.5 NaHCO<sub>3</sub>, and 15 glucose, and was buffered at pH 7.8 with 10 mM HEPES.

Isolated red-sensitive cone cells were identified by their round cell bodies and tapering inner and outer segments. The cone cells used probably correspond to the large single cones in Mariani's (1986) classification of the photoreceptor cells of the larval tiger salamander retina. The absorbance spectrum of the visual pigment of these cells peaks at between 600 and 620 nm (see Fig. 1). In electrophysiological experiments, these cone cells were identified as those having high sensitivity to brief flashes of light at 600 or 620 nm (Nakatani and Yau, 1989; Matthews et al., 1990). All experiments were at room temperature, maintained at ~20°C.

#### *Microspectrophotometry*

The single beam photon-counting microspectrophotometer is a modified version of that described by MacNichol (1978) and Levine and MacNichol (1985). Measurements were made by scanning repetitively through the spectrum at 1-s intervals. The rapid, repetitive scanning reduces distortion of the spectrum due to bleaching by the measuring beam. A group of scans (blank) was first measured in a cell-free area of the preparation, and was followed by a second group of scans (sample) measured through a cone outer segment. For the experiments described here, the crossed-slit field diaphragm defining the area of measurement was demagnified in two stages, first by a Zeiss Ultrafluar projection eyepiece, then by a ×40, 0.85 NA dry Nikon FLUOR objective. A Nikon UVF ×100, 1.3 NA glycerine immersion lens was used as objective. A 2-mm-diam aperture in front of the projection eyepiece limited the angle of the cone of light falling on the specimen.

A small aliquot (~10 μl) of physiological salt solution containing photoreceptor cells (from a washed retina; see above) was sealed between two #1 coverslips. An isolated cone was localized under infrared illumination, and oriented so that the measuring beam was in the center of the outer segment and parallel to its long axis, with the light polarized at right angles to the long axis. After obtaining an absorbance spectrum, a fraction of the visual pigment was bleached using a uniform, focused field, larger than the outer segment, of 600-nm light from the auxiliary beam of the microspectrophotometer with its infrared filter replaced by an interference filter (FWHM, 10 nm). The cell was repositioned and refocused and an absorbance spectrum was again obtained. This was repeated until visual pigment absorbance could no longer be detected. To estimate the extent of bleaching by the measurement scans, a second isolated cone was positioned and focused in the measuring beam and the procedure was repeated, but without bleaching light steps; i.e., the visual pigment was progressively bleached by repeated groups of scans.

#### *Electrical Recording*

Light-induced changes in membrane current were measured using suction microelectrodes, with either the outer segment or the inner segment and cell body drawn into the microelectrode (Baylor, Lamb, and Yau, 1979; Yau, McNaughton, and Hodgkin, 1981). Microelectrodes were fire-polished to internal tip diameters of ~15 μm (for recording from cone inner segments) or ~8 μm (for recording from cone outer segments). The suction microelectrodes were silanized and filled with physiological salt solution. The suction microelectrodes and the reference electrode (an agar bridge filled with physiological salt solution) were connected via Ag/AgCl pellets to a conventional current-to-voltage converter.

The experimental chamber was formed by the space between a coverslip and the glass

surface of a cap fitted onto an achromat objective lens ( $\times 10$ , 0.25 NA) used as condenser on an inverted microscope. The volume, 1.5 ml, between the two glass surfaces was perfused at a rate of  $\sim 0.5$  ml  $\text{min}^{-1}$  with physiological salt solution. Light stimuli were from a photostimulator with a tungsten-halogen 150-W projector lamp as light source, operated at 6 A from a voltage-regulated power supply. The final stimulus was a focused circular spot of diameter 50–100  $\mu\text{m}$ . The suction electrodes and the cone outer segments were oriented at an angle of  $\sim 10^\circ$  to the horizontal.

Light stimuli were flashes of monochromatic or white light (obtained by removing the interference filter from the light beam), of the duration given in the figure legends. Flash sensitivities were measured as peak response amplitude divided by flash photon density for responses in the range that scaled linearly with light intensity.

#### *Light Calibration*

Neutral density filters were calibrated in situ using a silicon photodiode placed on the stage of the microscope at the same level as the preparation, or placed at the exit of the photostimulator. Current from the photodiode was measured using an operational amplifier in virtual ground configuration.

The absolute intensities of the unattenuated light beams used for bleaching in the microspectrophotometer or stimulation and bleaching during electrical recording were measured at regular intervals using a calibrated photometer (model 80 $\times$ ; UDT Instruments, Orlando, FL).

#### *Recovery from Bleaching*

Bleached cone cells were exposed to 11-*cis* retinal using either ethanolic physiological salt solution or physiological salt solution containing liposomes (Pepperberg, Brown, Lurie, and Dowling, 1978; Yoshikami and Nöll, 1978; Jones, Crouch, Wiggert, Cornwall, and Chader, 1989). 11-*cis* Retinal was dissolved in ethanol at 10 mg/ml (35 mM) and diluted 100-fold with physiological salt solution. Liposomes were prepared by sonication and loaded with 11-*cis* retinal as described (Jones et al., 1989). Usually, aliquots of solution containing 11-*cis* retinal were added to the bath (final retinal concentrations are given in the figure legends) after halting superfusion of the bleached cell, and the superfusion remained halted until the sensitivity recovered to, or close to, its original dark-adapted level. In some experiments a high concentration of 11-*cis* retinal was obtained by flooding the bath with the retinal solution. In this study, only cells oriented in the suction microelectrode so that the outer segment was exposed were treated with 11-*cis* retinoid.

## RESULTS

#### *Microspectrophotometry*

A necessary first step in this study of visual pigment bleaching in cone photoreceptors was to determine the extent of depletion of visual pigment by bleaching light. This was done by measuring the in situ photosensitivity of the visual pigment by microspectrophotometry. The absorbance of the outer segment of a red-sensitive salamander cone was obtained initially for the dark-adapted cell and after several steps of bleaching light (Fig. 1 A). The squares in Fig. 1 B show the fall in peak visual pigment absorbance as a function of the cumulative bleaching time (i.e., the amount of bleaching light). But part of the fall in absorbance is due to bleaching by the measuring beam, since for the second cone of this experiment (Fig. 1 C) the absorbance of the outer segment decreased during repeated measurements without

bleaching light steps. To take account of bleaching by the measuring beam, it was assumed that each measurement bleached the same fraction of visual pigment. This is likely since each set of measuring scans used the same amount of light and 2–3 min elapsed between each measurement, sufficient time for equilibration by diffusion across bleached and nonbleached regions of the cone outer segment (see Gupta and Williams, 1990). If  $f$  is the fraction of visual pigment remaining after a single measurement (a fraction  $1 - f$  is bleached by each measurement) and there are no

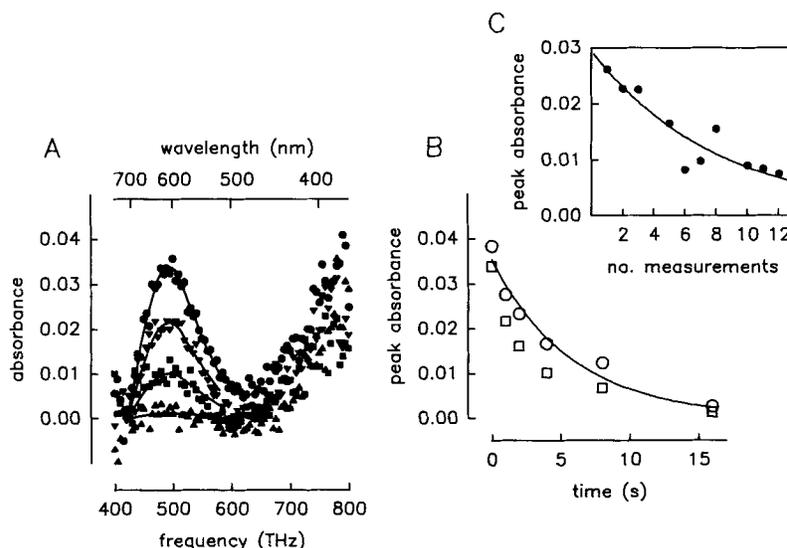


FIGURE 1. Microspectrophotometry of bleaching of the visual pigment of an isolated salamander red cone. (A) Absorbance spectra (*symbols*) from the outer segment, dark-adapted and after steps of bleaching light. Curves are the dark-adapted absorbance spectrum after digital filtering and truncation. Fitting of a normalized version of this curve was used to obtain the amplitudes and adjust the baselines of the measured absorbance spectra. Two spectra are omitted for clarity. A high, photostable ultraviolet absorbance was usually present in the outer segments of these cone cells. (B) Peak absorbances from A plotted against the cumulative bleaching time before ( $\square$ ) and after ( $\circ$ ) correction for bleaching by the measuring beam. The data were corrected according to Eq. 1 with  $f$  equal to 0.88. The curve is a single exponential fitted to the circles. (C) Peak absorbances from the outer segment of a second isolated cone cell during repeated measurements without bleaching light steps. The curve is Eq. 1, with  $I$  set to zero, fitted to the data points. Two missing points were due to artifacts in the recordings producing spurious absorbance spectra. Experiment 2 of Table I.

complications (such as screening by photoproducts), the fraction of visual pigment remaining after  $n$  measurements will be related to the intensity of the bleaching light,  $I$ , by (cf. Dartnall, 1972),

$$F_n = f^n \exp\left(-PI \sum_1^n t_{n-1}\right) \quad (1)$$

where  $t_n$  is the duration of the  $n$ th bleaching light step (there was no bleaching light before the first measurement,  $t_0 = 0$ ) and  $P$  is the in situ photosensitivity, in this case for light incident transversely to the long axis of the cone outer segment. For the data of Fig. 1 C, fitting of Eq. 1 to the peak absorbances, with  $I$  set to zero, gave a value for  $f$  of 0.88; i.e., each measurement bleached 12% visual pigment. This was used to correct the peak absorbances in the bleaching experiment (Fig. 1 B) by dividing by  $f^n$  (Eq. 1). The curve in Fig. 1 B is a single exponential fitted to the corrected data points. The rate constant of this exponential is the product of the in situ photosensitivity and the intensity of the bleaching beam (Eq. 1).

Measurement of the in situ photosensitivity in this way is very sensitive to the correction for bleaching by the measuring beam: a 1–2% change in the estimate of the fraction of pigment bleached by each measurement translates into a 5–10% change in the calculated photosensitivity (see Table I). The measurements were

TABLE I  
Photosensitivity of the Visual Pigment for Transverse Illumination of Salamander Red Cones

Cone	$f$	$\alpha_1$ $s^{-1}$	$\alpha_2$ $s^{-1}$	$I$ $photons \mu m^{-2} s^{-1}$	$P_1$ $\mu m^2$	$P_2$ $\mu m^2$
1	0.93	0.148	0.134	$2.5 \times 10^7$	$5.9 \times 10^{-9}$	$5.3 \times 10^{-9}$
2*	0.88	0.171	0.197	$2.5 \times 10^7$	$6.8 \times 10^{-9}$	$7.8 \times 10^{-9}$
3	0.94	0.131	0.115	$2.1 \times 10^7$	$6.2 \times 10^{-9}$	$5.5 \times 10^{-9}$
4	0.91‡	0.111	0.114	$2.1 \times 10^7$	$5.3 \times 10^{-9}$	$5.4 \times 10^{-9}$
5	0.90	0.091	0.101	$2.1 \times 10^7$	$4.3 \times 10^{-9}$	$4.8 \times 10^{-9}$
6	0.92	0.269	0.264	$3.6 \times 10^7$	$7.5 \times 10^{-9}$	$7.4 \times 10^{-9}$
Mean	0.91				$6.0 \times 10^{-9}$	$6.0 \times 10^{-9}$
SD					$1.1 \times 10^{-9}$	$1.3 \times 10^{-9}$

$f$  is the fraction of pigment remaining after each measurement from the control experiment using repeated measurements without bleaching light.  $\alpha_1$  is the rate constant at which pigment was bleached from the experiment with bleaching light, after correction for bleaching by the measuring beam from the corresponding control experiment.  $\alpha_2$  is the rate constant for bleaching after correction for bleaching by the measuring beam using the mean value for  $f$  from all experiments.  $I$  is the bleaching light intensity, wavelength 600 nm.  $P_1$  and  $P_2$  are the photosensitivities corresponding to  $\alpha_1$  and  $\alpha_2$ .

\*Illustrated in Fig. 1.

‡Mean of two values, 0.906 and 0.911.

corrected for bleaching by the measuring beam using either the estimate of the fraction of pigment bleached by each measurement from the associated control experiment or a mean value from all control experiments. The two sets of calculations gave the same average photosensitivity,  $6.0 \times 10^{-9} \mu m^2$  (Table I). This result confirms measurements of others (Gupta and Williams, 1990; Makino, Taylor, and Baylor, 1991) and is close to that expected for vitamin A<sub>2</sub>-based visual pigments (Dartnall, 1972; see Discussion). Taken together with the simple exponential time course of bleaching, it appears that there can be little regeneration of visual pigment in the isolated cone cell, and that there is little screening by photoproducts during bleaching. Moreover, there can be little or no photoreversal of visual pigment bleaching, though photoreversal was not expected at the light intensities used in this study.

### Membrane Current Responses after Bleaching

Fig. 2 shows the membrane current responses of a red-sensitive salamander cone to brief flashes, recorded first from a dark-adapted cell and then from the same cell after steps of light that progressively bleached away visual pigment. Bleaching produced large rightward shifts in the response amplitude versus flash intensity

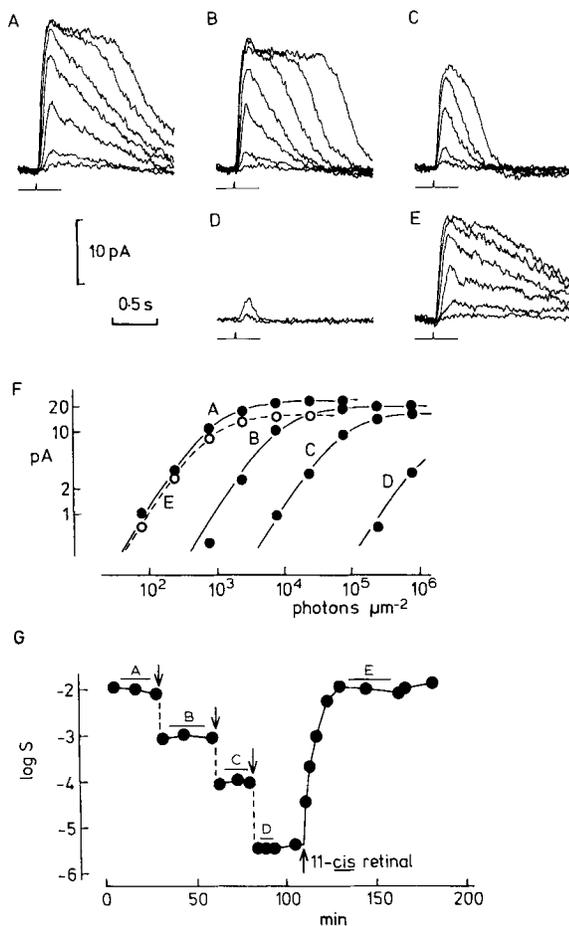


FIGURE 2. Membrane current responses to light flashes of an isolated salamander red cone before and after visual pigment bleaching, and after recovery from bleaching. (A) Superimposed responses of the dark-adapted cell. (B-D) Responses after three bleaching light steps. (E) Responses after subsequent exposure to 11-*cis* retinal. Flash duration, 10 ms; wavelength, 600 nm. Recording bandwidth, 0–30 Hz; each trace is the average response to 10 flashes. (F) Peak amplitudes of responses in A–E plotted against flash photon density. (G) Complete time course of changes in flash sensitivity ( $S$ , pA/(photons  $\mu\text{m}^{-2}$ )) during this experiment. Horizontal bars indicate time periods when responses in A–E were recorded. Bleaching light steps are indicated by downward arrows: light intensity,  $8.0 \times 10^6$  photons  $\mu\text{m}^{-2} \text{ s}^{-1}$ ; durations, 15, 41, and 98 s from left to right. Superfusion was halted between 105 and 120 min to prevent washout of 11-*cis* retinal. 11-*cis* Retinal concentration, 23  $\mu\text{M}$ ; in physiological salt solution containing 0.1% ethanol.

curves (Fig. 2 F), reflecting a fall in sensitivity of the cell. This was associated with a small reduction in the maximum response amplitude. In the experiment illustrated, only small responses were recorded after the third bleach due to insufficient light intensity. Other experiments (see Fig. 4) in which more intense flashes were used

showed saturating response amplitudes to flashes after bleaches producing sensitivity losses of up to 3 log units.

After bleaching, the flash sensitivity of the cell of Fig. 2 remained reduced and steady for periods of 20–30 min. The sensitivity returned to close to its dark-adapted level, however, on exposure to 11-*cis* retinal, although in this experiment the maximum response amplitude did not recover completely. Recovery of the sensitivity to flashes with failure of the maximum light-suppressible membrane current to recover to its dark-adapted level occurred for many, though not all, cells in this study, and is believed to be due simply to deterioration of the cells.

#### Flash Sensitivity after Bleaching

For the cell of Fig. 2, the fractions of visual pigment remaining after the bleaching light steps were 50, 7.0, and 0.063%, based on the known amounts of bleaching light and the microspectrophotometric measurements described above. These reductions

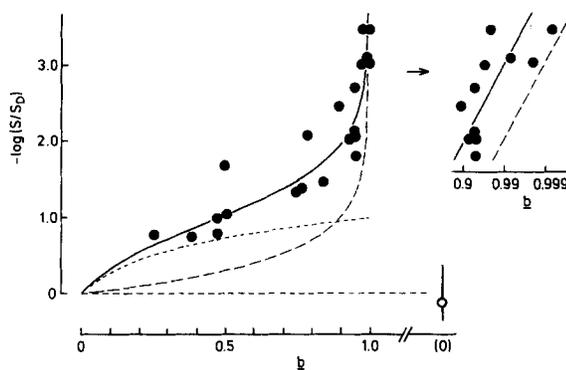


FIGURE 3. Dependence of flash sensitivity of salamander red cones ( $S$ , relative to sensitivity before bleaching,  $S_D$ ) on the fraction of visual pigment bleached ( $b$ ) after one or more steps of bleaching light. Filled circles are results from 16 separate experiments. Sensitivity was tested with 10-ms, 600-nm flashes or 50-ms, 620-nm flashes (three experiments). Open circle and vertical line

show the mean sensitivity ( $\pm 2$  SD) for nine experiments where sensitivity was recovered after bleaching upon exposure to 11-*cis* retinal (six cells) or 11-*cis* retinol (three cells). Solid line is a fit of Eq. 2 to the data points. Long-dashed curve indicates a decrease in sensitivity expected from loss in quantum catch. Short-dashed curve shows the extra decrease in sensitivity beyond that due to loss in quantum catch. (*Inset*) The results after extensive bleaches on an expanded (logarithmic) abscissa. The mean dark-adapted sensitivity was  $7.6 (\pm 5.7 \text{ SD}) \times 10^{-3} \text{ pA/} (\text{photons } \mu\text{m}^{-2})$ .

in the amount of visual pigment would reduce flash sensitivity by 0.31, 1.2, and 3.2 log units simply because of a reduced quantum catch. The corresponding reductions in flash sensitivity were 1.0, 2.0, and 3.5 log units, however, in each case greater than expected if quantum catch were the only mechanism reducing sensitivity after bleaching.

Collected results showing the relationship between fall in flash sensitivity and the reduction in visual pigment after bleaching are shown in Fig. 3. (Note that the inverse of the fall in relative sensitivity is plotted against the fraction of visual pigment, equivalent to plotting threshold against visual pigment bleached; cf. Baylor and Hodgkin, 1974). The criterion for inclusion of an experiment in this figure was either that sensitivity was reduced to a stable level after bleaching, or that sensitivities were

measured at short times after bleaching, before any spontaneous recovery occurred (see below for a description of spontaneous recovery). For a number of experiments, recovery from bleaching was obtained after exposure to 11-*cis* retinal (see Fig. 2) or 11-*cis* retinol (see Jones et al., 1989). On average, the recovery was to the same level as the initial dark-adapted sensitivity (Fig. 3, open circle).

For all measurements shown in Fig. 3, the flash sensitivity fell below the level expected from the reduction in quantum catch. The solid line fitted to the data points represents the following equation:

$$-\log (S/S_D) = -\log (1 - b) + \log (1 + kb) \quad (2)$$

where  $S$  is the flash sensitivity after bleaching,  $S_D$  is the initial dark-adapted sensitivity,  $b$  is the fraction of pigment bleached, and  $k$  is a constant. The best-fit value of  $k$  was 8.6. Writing  $F$  (equal to  $1 - b$ ) as the fraction of pigment remaining after each bleach, Eq. 2 is formally equivalent to

$$(S/S_D)^{-1} = (1 + kb)/F \quad (3)$$

In Eq. 3 the term in parenthesis on the right represents the extra factor by which the relative sensitivity falls below that due to loss of quantum catch; division by  $F$  then includes the loss in quantum catch. Both terms are plotted separately as dashed curves in Fig. 3. Eqs. 2 and 3 were written with the assumption that the inverse of the extra fall in relative sensitivity rises linearly with the fraction of pigment bleached (see Discussion). This provided a simple and adequate description of the data, though scatter in the data points is such that other formulations cannot be excluded. The main conclusion to be drawn from the data of Fig. 3 is that bleaching desensitizes cone cells by two mechanisms. For bleaches up to 90% or so, both mechanisms are about equally effective. For bleaches greater than this, quantum catch becomes more important (Fig. 3, inset).

#### *Reduction in Dark Current*

Bleaching produces both a rightward shift in the response-intensity curve, reflecting a decrease in sensitivity as described above, and a small downward shift, indicating a reduction in the maximum light-suppressible current (see Fig. 2  $F$ ). Is this reduction due to a reduction in the circulating dark current, or to some other change in the transduction mechanism of the cone cell? The current recording of Fig. 4  $A$  shows that, when the circulating dark current returned to a steady level after bleaching, the absolute level of this current was clearly different from the level before bleaching. In contrast, the absolute peak level of the saturating response to flashes was close to that before bleaching. This suggests that the reduction in the maximum light-suppressible current after bleaching corresponds to a reduction in the circulating dark current, and that saturation of the response to flashes represents closure of all outer segment light-sensitive channels both before and after bleaching. The lower maximal response to flashes after bleaching would then be due to a lower level of circulating dark current.

Eight cells provided reliable measurements of the absolute values of the dark current before and after bleaching. In Fig. 4  $B$  (triangles) the relative reduction in the circulating dark current from these experiments is shown as a function of the fraction

of pigment bleached. Also plotted are results from nine other experiments, showing the reduction in the maximum light-suppressible current. Only results from these nine cells are shown, since a strict criterion for inclusion was that a clear increase in the maximum light-suppressible current was obtained during recovery from bleaching on exposure to 11-*cis* retinal or retinol (Jones et al., 1989). Many cells did not satisfy this criterion; as mentioned above, during the (fairly) extended periods of the experiments described here there was usually considerable rundown in the size of the maximum light-suppressible current. Fig. 4 *B* shows that after bleaching 99% of the visual pigment, the circulating dark current (or the maximum light-suppressible current) was  $\sim 70\%$  of its dark-adapted level.

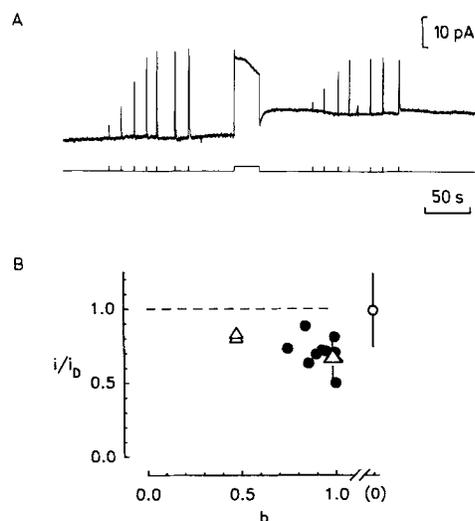
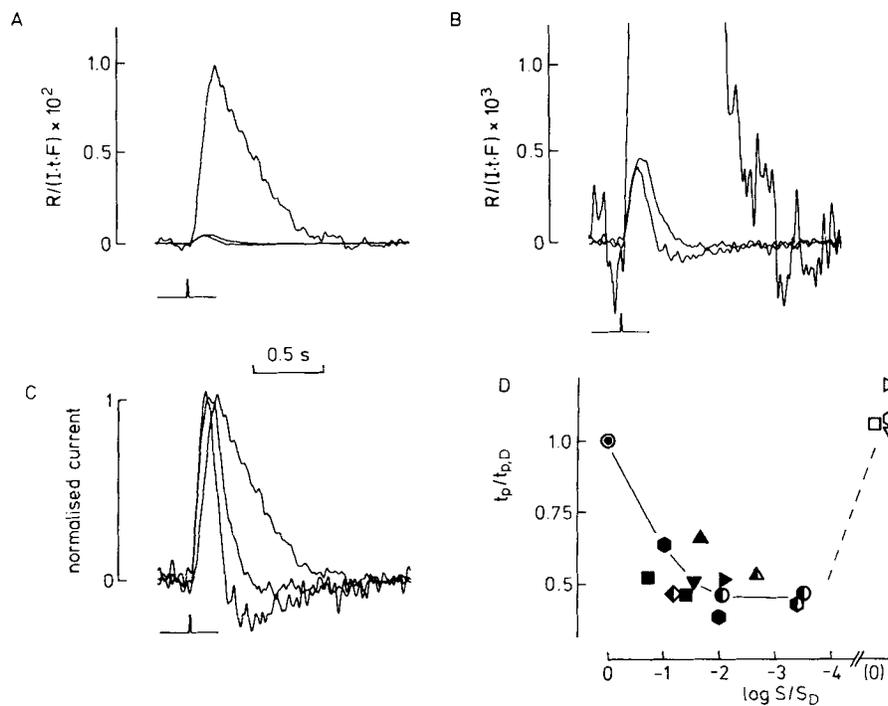


FIGURE 4. Reduction in circulating dark current after bleaching. (A) Membrane current recording showing responses to light flashes of increasing intensity (brief deflections on the lower, light monitor trace) before and after a light step bleaching 99% visual pigment. Sensitivity was reduced by 2.5 log units on bleaching; flash light intensities on the right are 2.5 log units brighter than on the left. White light flashes; duration, 200 ms. Bleaching light was  $2.5 \times 10^7$  photons  $\mu\text{m}^{-2} \text{s}^{-1}$ ; wavelength, 600 nm; duration, 28 s. (B) Dependence of the circulating dark current and the maximum light-suppressible membrane current ( $i$ , relative to the initial, dark-

adapted level,  $i_D$ ) on the fraction of visual pigment bleached ( $b$ ). Open triangles show reduction in circulating dark current. The point with a vertical line indicates the mean  $\pm 2$  SD for six cells after 99% bleaches; one of these is illustrated in *A*. Filled circles show reduction in maximum light-suppressible current from nine separate experiments. The open circle and vertical line indicate the mean and  $\pm 2$  SD of the maximum light-suppressible current for these nine cells after recovery from bleaching on exposure to 11-*cis* retinal or 11-*cis* retinol (two cells).

#### *Kinetics of Linear Range Responses*

Visual pigment bleaching not only reduces the sensitivity of cone photoreceptors but also produces a speeding up of the response to flashes. This was examined more closely for small amplitude responses in the range that scaled linearly with flash intensity. Fig. 5, *A* and *B*, shows responses in this range plotted as sensitivity; i.e., the responses before and after bleaching have been divided by the flash intensity and by the fraction of pigment remaining (to account for the loss in quantum catch). For this cell, 50% visual pigment was initially bleached, and the cumulative bleach after the second bleaching light step was 95%, producing reductions in flash sensitivity of 1.7 and 2.7 log units, respectively. The predominant effect of desensitization by bleach-



**FIGURE 5.** Kinetics of linear range flash responses before and after visual pigment bleaching. (A) From a dark-adapted cone and from the same cell after two bleaches. Traces ( $\text{pA}/(\text{photons } \mu\text{m}^{-2})$ ) are current responses ( $R$ ) divided by the product of light intensity ( $I$ ) and flash duration ( $t$ ), and corrected for quantum catch by dividing by the fraction of pigment remaining ( $F$ ). (B) Same responses as in A, but with the ordinate scaled up by a factor of 10. Traces are averages of 50, 20, and 40 responses. Mean amplitudes and flash intensities were: dark-adapted, 2.5 pA, 260 photons  $\mu\text{m}^{-2}$ ; after first bleach, 3.9 pA, 16,800 photons  $\mu\text{m}^{-2}$ ; after second bleach, 0.96 pA, 48,300 photons  $\mu\text{m}^{-2}$ . Flash duration 10 ms, wavelength 600 nm. Bleaching light steps were  $6.4 \times 10^6$  photons  $\mu\text{m}^{-2} \text{ s}^{-1}$ , duration 18 s, and  $8.0 \times 10^6$  photons  $\mu\text{m}^{-2} \text{ s}^{-1}$ , duration 48 s ( $F = 0.50$  and  $0.05$ ). Recording bandwidth 0–30 Hz, followed by digital filtering at 0–20 Hz. The time course of changes in sensitivity of this cell are shown as open circles in Fig. 6 B. (C) Same current responses as in A and B, but after normalization at the peak amplitude. (D) Dependence of time-to-peak ( $t_p$ ) for linear range responses relative to the initial dark-adapted time-to-peak ( $t_{p,D}$ ) on the relative desensitization due to bleaching. Each symbol represents a different cell. Half-filled symbols indicate responses showing an overshoot of the falling phase (as in C). Open symbols show relative times-to-peak after recovery to 11-*cis* retinal or 11-*cis* retinol. The mean dark-adapted time-to-peak was 0.25 s ( $\pm 0.07$  s, SD,  $n = 7$ ).

ing was a shortening of the time course of the linear range responses. Most of this effect occurred already after the first bleach; after the second bleach there was little further desensitization (after correction for quantum catch), but the linear range responses now showed an overshoot in the falling phase beyond the level of the dark current. These changes in response kinetics are very reminiscent of those reported by Matthews et al. (1990) to occur during similar desensitizations by background light

and to remain after extended exposure to bright backgrounds. In Fig. 5 C the same responses are replotted after normalization to the peak amplitude, showing more clearly the presence of the overshoot and a reduction in time-to-peak after bleaching. A pattern of changes very similar to those of Fig. 5, A–C was found for seven cells, and collected results from these experiments are shown in Fig. 5 D. Desensitization by up to  $\sim 1.5$  log units by bleaching produced a fall in the time-to-peak of the linear responses. Further desensitization had little effect on the time-to-peak but was usually accompanied by the appearance of an overshoot. On recovery from bleaching, the time-to-peak returned to close to its dark-adapted level.

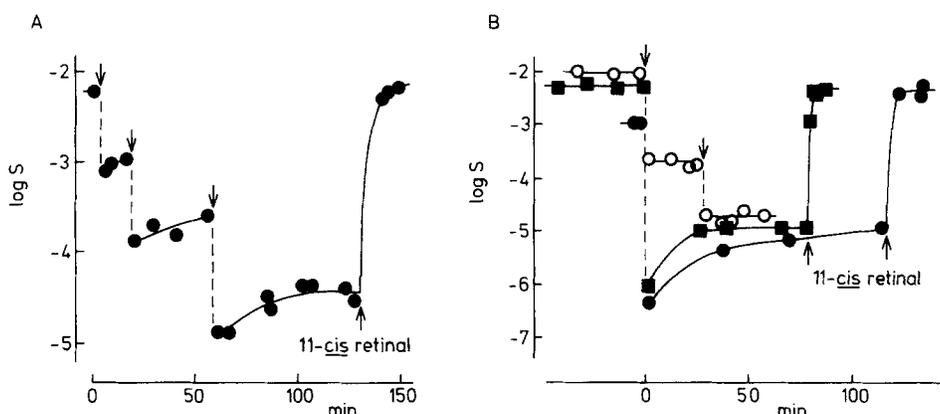


FIGURE 6. Spontaneous recovery of sensitivity after visual pigment bleaching. (A) Changes in flash sensitivity ( $S$ , pA/(photons  $\mu\text{m}^{-2}$ )) for a cone that showed recovery back toward the dark-adapted level after each of three bleaches (downward arrows). Bleaching light steps were  $6.4 \times 10^6$  photons  $\mu\text{m}^{-2} \text{s}^{-1}$ ; wavelength, 600 nm; durations, 20, 40, and 62.5 s, from left to right. Sensitivity was tested using 200-ms flashes; wavelength, 600 nm. 11-*cis* Retinal was added in liposomes; concentration, 0.6 mM. (B) Recovery of sensitivity toward dark-adapted levels after an intense bleach (left downward arrow) for two cones (filled symbols). Bleaching light steps were  $3.1 \times 10^8$  photons  $\mu\text{m}^{-2} \text{s}^{-1}$ ; wavelength, 620 nm; duration, 10 s (circles) or 5 s (squares). Sensitivity was tested using 50-ms flashes; wavelength, 620 nm. Open circles show changes in sensitivity for a third cone after two bleaches (downward arrows). Bleaching light steps were  $6.4 \times 10^6$  photons  $\mu\text{m}^{-2} \text{s}^{-1}$ ; duration, 18 s; and  $8.0 \times 10^6$  photons  $\mu\text{m}^{-2} \text{s}^{-1}$ ; duration, 48 s; calculated to bleach 50% and, cumulatively, 95% visual pigment. Recovery with 11-*cis* retinal was attempted, but this cell was lost during addition of the retinoid.

#### *Spontaneous Recovery of Sensitivity after Bleaching*

Bleaching by moderately intense light steps, either singly or repeated, usually produced a desensitization that remained steady with time (Fig. 2 G). This was generally not so after bleaching with steps of more intense light, when a subsequent slow spontaneous recovery of sensitivity was often observed.

Of the  $> 60$  cells tested in this study, about one-third (20) showed some spontaneous recovery of sensitivity after bleaching and about the same number (18) clearly showed no spontaneous recovery. Results for the other cells were equivocal. Two cells

showed spontaneous recovery after each step of relatively weak bleaching light, and the results from one of these are shown in Fig. 6A. The spontaneous recovery was most evident after the third step of bleaching light, though far from being as extensive as the final recovery on exposure to 11-*cis* retinal. The spontaneous recovery seen after single steps of intense bleaching light is illustrated by the sensitivity changes for two cells shown as filled symbols in Fig. 6B. The initial desensitization of these cells was by 3.5–4 log units, recovering spontaneously by ~1 log unit.

The spontaneous recovery in sensitivity could represent a phase of recovery of sensitivity of the transduction mechanism in the cone cell. However, it is a very slow process and was not always present, even after extensive brief bleaches. It seems more likely that it represents a slow regeneration of a small fraction of the visual pigment of the cell. After very extensive desensitization, the relationship between sensitivity and visual pigment bleached is very steep (Fig. 3, inset), and a recovery in sensitivity of 1 log unit, such as those of Fig. 6B, would correspond to a recovery of visual pigment of only a few percent. The open circles of Fig. 6B show the changes in sensitivity of a third cell which fell almost to the same level as that of the two strongly bleached cells, but in this cell there was no apparent spontaneous recovery and the calculated final fraction of visual pigment remaining was 5%. Careful comparison with the results of Fig. 3 indicate, moreover, that the spontaneous recovery in sensitivity seen in the cell of Fig. 6A would also represent only small amounts of visual pigment regeneration. Thus, the slow recovery of sensitivities by 0.1, 0.2, and 0.4 log units after each bleach correspond, at equivalent levels of desensitization, to visual pigment recoveries of ~6, 8, and 3%, respectively.

#### DISCUSSION

The values for the *in situ* photosensitivity of the visual pigment of salamander red-absorbing cones presented in Table I depend somewhat critically on the correction for bleaching by the measuring beam in the microspectrophotometer. A significant amount of bleaching (~10%) by each measurement was necessary to obtain a reasonable signal-to-noise ratio (cf. Marks, 1965; Liebman, 1972), even though the outer segments of these cones are fairly large compared with cones of many other species. Recent reports of very similar values for the photosensitivity of cone pigments suggest that the correction was satisfactory. Gupta and Williams (1990), also using microspectrophotometry, measured the photosensitivity of the visual pigment of red-sensitive cones of the catfish. They obtained  $8.9 (\pm 2.2) \times 10^{-9} \mu\text{m}^2$  for the equivalent solution photosensitivity (i.e., after correction for the dichroism of light absorption by the visual pigment of the cone outer segment), not significantly different from the present results. Their work relied on measuring bleaching by the measuring beam alone at a fixed wavelength. This has the advantage of not requiring correction, but suffers from other difficulties (as noted by these authors), principally that of measuring the absolute intensity of the measuring beam, which was very weak compared with the bleaching beam used in this study. Makino et al. (1991) have obtained the photosensitivity of cone pigments *in situ* by a completely different technique: from whole-cell recordings of the early receptor current after strobe stimulation. In salamander red-sensitive cones, the *in situ*

photosensitivity measured in this way was  $5.4 (\pm 0.7) \times 10^{-9} \mu\text{m}^2$ , again not significantly different from the present measurements.

The equivalent solution photosensitivity corresponding to the mean values of Table I is  $6.7 (\pm 1.3) \times 10^{-9} \mu\text{m}^2$  if the dichroic ratio for absorbance is taken to be 2.0 (measured values of dichroic ratio for cones are between 1.7 and 3; Hárosi and MacNichol, 1974; Hárosi, 1976; Gupta and Williams, 1990; Hárosi, F. I., personal communication). This is close to that measured for vitamin A<sub>2</sub>-based rod pigments in solution ( $7.4 \times 10^{-9} \mu\text{m}^2$ ; Dartnall, 1972). This is presumably because the photoproducts of bleaching of cone visual pigments, especially red-absorbing pigments, are expected to absorb at wavelengths well displaced from the absorbance peak of the intact pigment (Liebman, 1972; Hárosi and MacNichol, 1974). Photoproducts are also very short-lived (Hárosi and MacNichol, 1974) and were not detected in this study (Fig. 1). This is not the case for rod pigments where, even in solution, the presence of relatively long-lived photoproducts with overlapping absorbance spectra means that measurements of photosensitivity are consistent only if bleaching is done in the presence of hydroxylamine (which removes absorbance in the visible due to photoproducts), or if bleaching is done in brief steps between which sufficient time is allowed for decay of the photoproducts (Dartnall, 1972). This study suggests very little or no contribution by photoproduct absorption during bleaching of cone visual pigments.

Measurements of the sensitivity to light flashes of salamander retinal cones after bleaching often show a spontaneous recovery toward dark-adapted levels, which we interpret as indicating that the isolated cone photoreceptor can regenerate a small amount of its visual pigment. The effect is seen especially after brief, intense bleaches when the relationship between visual pigment and sensitivity becomes very steep (Fig. 3). A similar amount of visual pigment regeneration has been measured directly in rods of the isolated amphibian retina (Cocozza and Ostroy, 1987). Isolated cone photoreceptors are probably able to regenerate a few percent of their visual pigment because they have a small store of 11-*cis* retinal or retinol. Under our conditions, about half of the cells appeared to have this capability; occasionally, cells could regenerate larger amounts.

Exposure of bleached salamander cones to exogenous 11-*cis* retinal (or retinol) returns their flash sensitivity, linear-range response kinetics, and dark current to their initial dark-adapted values. Measurements by microspectrophotometry under similar experimental conditions have not yet been made (in particular, information concerning the important question of the relative time courses of recovery of sensitivity and regeneration of visual pigment is not yet available), but other work indicates that this recovery is due to formation of new visual pigment. Thus, Hárosi (1984) reports that new visual pigment is found in bleached cone outer segments after exposure to 11-*cis* retinal and that cone outer segments previously loaded with 11-*cis* retinal regenerate visual pigment spontaneously after bleaching. Spectral sensitivity measurements of red-sensitive salamander cones, obtained under similar experimental conditions to ours, show a large blue shift consistent with replacement of a dehydroretinal-based visual pigment with a retinal-based visual pigment (Makino, Kraft, Mathies, Lugtenburg, Miley, van der Steen, and Baylor, 1990). At the same time, this replacement

produces an increase in the molar extinction coefficient (Dartnall, 1972; Makino et al., 1991). Combination of these two effects is sufficient to explain why flash sensitivity returns to the same level at the wavelengths, 600 or 620 nm, used in this study. Indeed, the choice of these wavelengths was based on an estimate that the isosbestic wavelength for the replacement lies between 600 and 620 nm, using templates (Jones, G. J., and E. F. MacNichol, manuscript in preparation) derived from the shape of other visual pigment absorption spectra.

The dependence of the fall in relative flash sensitivity in salamander cones on visual pigment bleaching (Fig. 3) is similar to that inferred for cones in the turtle retina from horizontal cell recordings (Normann and Perlman, 1990). A significant proportion of the fall in sensitivity cannot be explained by visual pigment depletion, i.e., by a loss in quantum catch. This conclusion is not very sensitive to error in the calculations based on the *in situ* photosensitivity. For there to be no component in the desensitization above that due to loss in quantum catch, the photosensitivity would have to be about twice that measured in this and other studies. Other evidence that bleaching of visual pigment does light-adapt cone photoreceptors comes from the observation of a speeding up of the membrane current responses to dim flashes. Similar changes in response kinetics to those described here, and the development of a response overshoot, have been reported for both voltage and current responses of cones to dim flashes during adaptation to background light (Baylor and Hodgkin, 1974; Matthews et al., 1990). The changes in response kinetics occurring during background adaptation were interpreted to indicate that the underlying mechanism of desensitization is the development of a feedback acting on the recovery phase of the photoresponse (Baylor and Hodgkin, 1974; Baylor, Hodgkin, and Lamb, 1974). The changes seen after desensitization by bleaching, involving predominantly the falling phase of the response and including a shortening of the time to peak (Fig. 5), strongly suggest that the same or a similar mechanism is involved.

If only bleaches up to ~90% are considered, the data points in Fig. 3 could be taken to indicate a straight line with a slope of ~2 (i.e., corresponding to a desensitization of ~2 log units for a full bleach). A linear relationship between the fall in log sensitivity and the fraction of pigment bleached, with a similar slope, has been reported for turtle cones (O'Bryan and Schmidt, 1980; Norman and Perlman, 1990) and might correspond to the relationship found for human cones during dark adaptation (where the slope is closer to 3 than to 2; Baker and Rushton, 1965; Hollins and Alpern, 1973). A simple linear relationship between log sensitivity and the fraction of pigment bleached (the Dowling/Rushton relationship) cannot provide a complete description of desensitization due to bleaching, however, because it predicts a finite desensitization at infinite pigment bleaches (Barlow, 1972). A single straight line cannot provide a full description of the present data because it cannot include the limitations of quantum catch at high pigment bleaches (those > 90%; Fig. 3).

The data shown in Fig. 3 were fitted by a model in which desensitization after bleaching has a component due to loss in quantum catch and a second component for which sensitivity (rather than log sensitivity) falls linearly with the fraction of

pigment bleached. Several arguments suggest that a linear formulation for the extra desensitization is appropriate. Thus, Lamb (1981) has argued that thresholds during scotopic dark adaptation are related linearly to the concentration of some (so far unspecified) photoproducts of bleaching. Pepperberg (1984) has shown that steady-state desensitization in the all-rod retina of the skate after bleaching can be described by a model in which that component of the loss in sensitivity due directly to bleached pigment is linearly related to the fraction of visual pigment bleached. It is likely, judging from the data presented by Leibovic et al. (1987), that a model similar to that of Pepperberg (1984) would also account for steady-state bleaching desensitization in amphibian rods.

A linear relationship between steady-state desensitization and the fraction of visual pigment bleached is not necessarily contradictory to the exponential relationship (Baker and Rushton, 1965; Hollins and Alpern, 1973) seen during photopic dark adaptation, since the latter does not include the effects of loss of quantum catch (Hollins and Alpern, 1973). However, if the extra desensitization increases linearly with the fraction of pigment bleached, it can be shown that the effects of background light on cone cells (producing both cellular adaptation and pigment bleaching) will combine to give behavior that is in accord with the Weber/Fechner rule for light adaptation. This may explain why, for human photopic vision, there is no deviation from near-unity slope in the relationship between log increment threshold and log background light intensity (the Weber/Fechner rule) for backgrounds extending from  $\sim 2$  log trolands to well above 5 log trolands (see, for example, Giesler, 1978), backgrounds that include those for which significant visual pigment depletion occurs (from  $\sim 3.5$  log trolands; Rushton and Henry, 1968; Hollins and Alpern, 1973) and where the extra desensitization due to bleaching would appear.

Studies of cone cells of several different species have shown that the predominant effect of background adaptation is to shift the response amplitude/flash intensity curve toward higher intensities without a change in shape and with little decrease in the maximal response (e.g., Normann and Perlman, 1979; Valetton and van Norren, 1983); the same effect is found after visual pigment depletion by bleaching (see Fig. 2). The result of this shift is that thresholds for incremental light flashes ( $\Delta I$ ) increase in accord with the Weber/Fechner rule,

$$\Delta I = C \cdot (I_0 + I) \quad (4)$$

where  $C$  is a constant and  $I_0$  is a "dark light" (Barlow, 1972) that fixes the dark-adapted threshold. In the presence of pigment depletion, however, this formulation must be modified so that light intensities become quantum catches. Moreover, pigment depletion itself produces a shift in the response curve above and beyond the reduction in quantum catch. Assuming that this adds to the effect of the background, we replace Eq. 4 by

$$\Delta I \cdot F = C \cdot [I_0 + I \cdot F + k \cdot (1 - F)] \quad (5)$$

where  $F$  is the fraction of pigment remaining and  $k$  is a constant.

During steady illumination of human cones the fraction of pigment remaining depends on background intensity  $I$  as  $I_b / (I_b + I)$ , where  $I_b$  is a light intensity of

4.3–4.5 log trolands (Rushton and Henry, 1968; Hollins and Alpern, 1973). Substituting for  $F$  in Eq. 5,

$$\Delta I \cdot \left( \frac{I_b}{I_b + I} \right) = C \cdot \left[ I_o + I \cdot \left( \frac{I_b}{I_b + I} \right) + k \cdot \left( \frac{I}{I_b + I} \right) \right] \quad (6)$$

representing the way in which threshold rises with background light intensity when the effects of pigment depletion and bleaching desensitization are included. When  $I$  is zero or very low, threshold will be constant. As  $I$  rises, threshold will rise with a constant Weber fraction. However, at levels where visual pigment bleaching becomes important, the second and third terms in the expression in square brackets will tend to constant values: any effect of background light on adaptation must, as it were, run out of steam as pigment is bleached away because its quantum catch will fall. We have found in this study that there is very little compression in the responses to flashes after bleaching, implying that in this region the cone photoreceptors can operate with a maintained circulating current. At high backgrounds, when both terms in parenthesis on the right of Eq. 6 tend to constant values, thresholds will be determined solely by the left-hand side of this equation (i.e., solely by quantum catch), and one arrives at what is essentially Hecht's (1935) original photochemical theory of sensitivity in photoreceptors.

The above analysis makes an initial attempt to account for the effects of visual pigment bleaching during background adaptation of the photopic visual system. Matthews et al. (1990) have reported studies of background adaptation in isolated salamander cones under conditions where we find very little visual pigment regeneration. During background adaptation, the isolated salamander cone cell showed behavior in good agreement with the Weber/Fechner rule despite the observation that, on termination of a (strong) background light, the cell remained permanently desensitized. A scheme along the lines described above for photopic light adaptation will probably be able to explain why bleaching desensitization did not result in a deviation from the Weber/Fechner rule while visual pigment was being depleted as the isolated cone cell adapted to background light.

This report shows directly that bleaching produces a desensitization in cones that is in excess of that due to loss of quantum catch. The extra desensitization in cones is similar to, but smaller than, the same effect in rods. It is not associated with a dramatic fall in the circulating light-sensitive current, as in rods (compare our Figs. 2 and 4 with Figs. 2 and 4 of Leibovic et al., 1987). The present results differ from those of Schnapf et al. (1990), who, working with primate cones, did not find an excess desensitization above that due to loss in quantum catch. However, in the experiments of Schnapf et al. (1990), values for the visual pigment photosensitivity were obtained only indirectly and were all very low, probably indicating that these cells were regenerating visual pigment. If so, the deduced fractional bleaches might have been seriously in error. Alternatively, it may be that any extra desensitization is a small effect in the primate cone cell.

The mechanism by which bleaching produces an excess desensitization remains obscure at present. It is, presumably, related to the presence of opsin in the bleached cell, for which only a very small residual transduction activation, relative to that of photoactivated rhodopsin, would be sufficient to explain the desensitization (Corn-

wall and Fain, 1992). Bleaching desensitization can be reversed by forming new visual pigment. However, formation of new pigment is not a requisite for reversal of bleaching desensitization. In cones, combination of opsin with retinal analogues that simply occupy the opsin binding site is sufficient to reverse bleaching desensitization (Jin, Cornwall, Corson, Katz, and Crouch, 1992), in the same way that treatment of bleached rods with 11-*cis* locked analogues of retinal, which form pigments but are not photoactivatable, also reverses bleaching desensitization (Corson, Cornwall, MacNichol, Jin, Johnson, Derguini, Crouch, and Nakanishi, 1990).

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