Visual Pigment and Photoreceptor Sensitivity in the Isolated Skate Retina

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ABSTRACT Photoreceptor potentials were recorded extracellularly from the aspartate-treated, isolated retina of the skate (*Raja oscellata* and *R. erinacea*), and the effects of externally applied retinal were studied both electrophysiologically and spectrophotometrically. In the absence of applied retinal, strong light adaptation leads to an irreversible depletion of rhodopsin and a sustained elevation of receptor threshold. For example, after the bleaching of 60% of the rhodopsin initially present in dark-adapted receptors, the threshold of the receptor response stabilizes at a level about 3 log units above the dark-adapted value. The application of 11-*cis* retinal to strongly light-adapted photoreceptors induces both a rapid, substantial lowering of receptor threshold and a shift of the entire intensity-response curve toward greater sensitivity. Exogenous 11-*cis* retinal also promotes the formation of rhodopsin in bleached photoreceptors with a time-course similar to that of the sensitization measured electrophysiologically. All-*trans* and 13-*cis* retinal, when applied to strongly light-adapted receptors, fail to promote either an increase in receptor sensitivity or the formation of significant amounts of light-sensitive pigment within the receptors. However, 9-*cis* retinal induces a substantial increase in receptor sensitivity and promotes the formation of isorhodopsin. These findings provide strong evidence that the regeneration of visual pigment in the photoreceptors directly regulates the process of photochemical dark adaptation.

INTRODUCTION

The notion that visual pigment in the photoreceptors regulates visual sensitivity during light and dark adaptation was proposed by Hecht nearly 60 years ago (Hecht, 1920). However, only within the past two decades has evidence been provided for a link between the receptoral content of visual pigment and the sensitivity of the vertebrate visual system. Dowling (1960, 1963) and Rushton (1961) were the first to show that the concentration of rhodopsin and visual sensitivity are quantitatively related under particular conditions: namely, those which prevail long after the offset of an intense adapting light. These investigators found that during the slow phase of dark adaptation, the log sensitivity of the electroretinogram (ERG) *b*-wave or psychophysical discrimination varies in roughly linear fashion with the amount of rhodopsin regenerated in the rods (also cf. Wald et al., 1955). Further evidence linking the content of visual

pigment and the level of visual sensitivity has come from studies of the isolated retina, a preparation in which the regeneration of rhodopsin does not ordinarily occur to a significant extent. After the bleaching of visual pigment in the isolated retina, there occurs a rapid, "neural" component of dark adaptation; however, the final thresholds attained by both the ganglion cells and the ERG *b*-wave lie significantly above their initial, dark-adapted values (Weinstein et al., 1967; Baumann and Scheibner, 1968; Weinstein, 1969; Weinstein and Hobson, 1970). The final thresholds depend on the amount of pigment bleached; the relationship is again roughly linear between log threshold and the concentration of rhodopsin.

Together these data clearly imply that the content of rhodopsin somehow influences the mechanism which establishes the level of visual sensitivity during dark adaptation. Nevertheless, all of the evidence in support of this idea has been indirect; for in each preparation that has been examined (the intact eye, the eyecup, and the isolated retina), the slow phase of dark adaptation either coincides with pigment regeneration, or both processes are absent. It has therefore remained unclear whether the regeneration of rhodopsin directly affects visual sensitivity, or whether the observed correlation of pigment content and visual sensitivity results merely as a secondary (or fortuitous) consequence of adaptational processes. This latter possibility must be seriously considered; for, as described above, the approximately linear relationship linking log sensitivity and rhodopsin concentration appears valid only under certain conditions of adaptation (Wald et al., 1963). Specifically, this relationship does not at all describe the depression of visual sensitivity that occurs under sustained illumination (Rushton, 1959; Dowling, 1963; Dowling and Ripps, 1970); nor does it apply during the "neural" phase of dark adaptation which immediately follows the offset of a background light (Dowling, 1963; Frank, 1971; Green et al., 1975).

The present study was undertaken to examine the basis for the observed link between the content of visual pigment and the degree of visual sensitivity. In the first part of this paper, we address the following question: does the depression of photoreceptor sensitivity brought about by the bleaching of visual pigment match changes in the thresholds of visual responses arising proximally in the retina? It is generally agreed that intense illumination of the isolated retina permanently depresses photoreceptor sensitivity (Frank, 1971; Witkovsky et al., 1973; Grabowski and Pak, 1975); however, several studies have suggested that the effect of previous bleaching on receptor threshold is significantly less than that exerted on the threshold of the ERG *b*-wave (Frank, 1971; Ernst and Kemp, 1972; Hood et al., 1973). We report here that in the skate, both the photoreceptors and the *b*-wave appear to be similarly desensitized after intense light adaptation. Under such conditions, the level of visual sensitivity in the proximal skate retina therefore seems to be strongly influenced by the level of receptor sensitivity.

In the second part of this paper, we study the relationship between visual pigment and photoreceptor sensitivity in a new and more direct way. It is well established that upon illumination, the chromophore of rhodopsin, 11-*cis* retinal, is isomerized to the all-*trans* configuration (Hubbard and Kropf, 1958;

Wald, 1968). Ultimately, the bleached pigment molecule dissociates to yield opsin, and free vitamin A in the form of all-*trans* retinal or all-*trans* retinol (Matthews et al., 1963; Brin and Ripps, 1977). The inability of rod photoreceptors endogenously to convert these vitamin A compounds to 11-*cis* retinal normally prohibits the significant regeneration of rhodopsin in the isolated retina. In the present experiments, we utilize this fact and ask whether 11-*cis* retinal, exogenously supplied to a previously bleached isolated retina, directly promotes both the formation of rhodopsin in the receptors and an increase in receptor sensitivity. We find that this is indeed the case. Furthermore, with the similar use of 9-*cis* retinal, we find that a marked increase of receptor sensitivity accompanies the intracellular formation of isorhodopsin, an artificial visual pigment (Hubbard and Wald, 1952).

For these experiments, we have employed the isolated retina of the skate. This preparation is well suited for the study of photoreceptor adaptation, inasmuch as considerable electrophysiological and anatomical evidence has indicated that the skate possesses a single, rod-like class of photoreceptor; in addition, the adaptive properties of responses arising at several levels in the skate retina have been described (Dowling and Ripps, 1970, 1971, 1972, 1977; Green et al., 1975; Green and Siegel, 1975). Some of our results have been described in a brief report (Pepperberg et al., 1976).

MATERIALS AND METHODS

The dissections and electrophysiological experiments were carried out at a room temperature maintained at 15–19°C. Eyes from skates (*Raja oscellata* and *R. erinacea*) which had been dark-adapted for 12–18 h were removed under dim red light, hemisected, and drained of most of the vitreous. A piece of eyecup measuring ≅0.5 × 1.2 cm was trimmed from the tapetal region and immersed in a Ringer's solution which contained: Na-L-aspartate, 75 mM; NaCl, 215 mM; KCl, 6.0 mM; $CaCl_2$, 2.5 mM; $MgCl_2$, 1.8 mM; glucose, 3.0 mM; urea, 325 mM; and HEPES (*N*-2-hydroxyethyl-piperazine-*N*′-2-ethane-sulfonic acid), 5.0 mM, adjusted with NaOH to a pH (at 22°C.) of 7.6 (cf. Clusin and Bennett, 1977). After incubation in the Ringer's solution for a period of ≅15 min, the eyecup was removed and drained of additional vitreous with several wicks of facial tissue. The eyecup was further trimmed to ≅0.3 × 0.5 cm, and immersed again in the Ringer's solution for a period of 15–25 min.

After the second period of immersion, the retina was gently peeled away from the back of the eye onto a piece of Ringer-moistened filter paper (Whatman, qualitative; H. Reeve Angel & Co., Inc., Clifton, N. J.). This isolation of the retina placed the ganglion cell layer of the retina in contact with the filter paper, and left the photoreceptor layer exposed. The filter paper was placed in a 35-mm-wide plastic dish (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.; type 1008) above a grounded, chlorided silver wire which served as a reference electrode. The dish was mounted in a shielded cage, where moistened oxygen was gently blown over the preparation.

The massed photoreceptor potential was recorded extracellularly across the retina; the recording electrode was a glass pipette which contained a wick of glass fibers, and which was filled with Ringer's solution. The recording electrode was connected by a chlorided silver wire to either an AC-coupled amplifier (Grass Instrument Co., Quincy, Mass.; model P-15, band pass of 0.1 to 1,000 Hz) or a DC amplifier (MetaMetrics, Inc., Carlisle, Mass.). Amplified signals were displayed on an oscilloscope (Tektronix, Inc., Beaverton, Ore.; model 502 or model D12) and recorded on a Brush 280 pen writer

(Gould Inc., Instrument Systems Div., Cleveland, Ohio). For measurements of threshold and intensity-response behavior, the preparation was illuminated with test flashes of 0.2-s duration, controlled by a Uniblitz electronic timer and shutter assembly (Vincent Associates, Rochester, N. Y.). A photoreceptor response of 3 μV amplitude was usually used as a criterion for determining threshold intensities of the test flash; receptor sensitivity was defined as the inverse of the measured threshold. The interval between test flashes (at least 30 s) was sufficiently large that the flashes themselves had negligible effect on the sensitivity of the photoreceptors.

Photic stimulation of the isolated retina was carried out with a tungsten-halogen lamp mounted in a photostimulator previously described (Dowling and Ripps, 1971); the stimulating lamp was powered by a regulated supply and was operated at a fixed current within each experiment. The light was attenuated by a set of neutral density filters and spectrally shaped by passage through a Wratten 58 filter (Eastman Kodak Co., Rochester, N. Y.) and through Schott KG-1 and KG-3 heat filters (Schott Optical Glass, Inc., Duryea, Pa.). The resulting green light, focused onto the plane of the preparation, provided a field of illumination substantially larger than the isolated retina, and was used for both the adapting (bleaching) irradiations and the 0.2-s test flashes. For measurements of the quantal irradiance of the stimulating light, a 500 nm interference filter was substituted for the Wratten 58 filter, and the irradiance at 500 nm was determined with the use of a calibrated photodiode (PIN-5; United Detector Technology, Inc., Santa Monica, Calif.). During the course of this study, the operating current of the stimulating lamp was set at different values which yielded unattenuated quantal irradiances at 500 nm (I_{500}) in the range of 0.36×10^5–1.63×10^5 quanta (500 nm) per s/μm^2 at the plane of the preparation. The physiologically effective quantal irradiance transmitted to the retina was computed by comparing the attenuations at which 500 nm light (transmitted by the interference filter) and Wratten 58-filtered light evoked threshold responses from a dark-adapted preparation. The physiologically effective transmittances of the two Wratten 58 filters used in the present study were greater than that of the 500 nm interference filter by factors of 8.0 and 8.9, respectively. During the adapting irradiations (T seconds in duration) with unattenuated Wratten 58-filtered light, the effective number (N_{eff}) of 500 nm photons incident per μm^2 on the retina was therefore taken as $N_{eff} = f\, I_{500}\, T$, where f was appropriately set equal to 8.0 or 8.9.

To ensure uniform illumination of the retina during exposures to the intense adapting light, the recording electrode was temporarily withdrawn from the field of illumination with the use of a hydraulic microdrive (David Kopf Instruments, Tujunga, Calif.). Control experiments were performed in which the electrode was retracted from the surface of the retina and re-advanced to the initial position several minutes later; these movements of the recording electrode were found not to affect the measured level of receptor threshold. The electrode was also retracted for a 5-min period after each application of retinal to the isolated retina.

Absorption spectra of intact retinas were obtained in a microspectrophotometer previously described (Brown, 1961, 1972). A scanning beam 300 μm in diameter was used for the recording of these spectra. For the spectrophotometric analysis of preparations treated with retinal, dark-adapted isolated retinas were mounted (with the layer of photoreceptors up) on Ringer-moistened filter paper, so as to span a rectangular hole previously cut in the paper. The retina and its base of filter paper were placed on a supporting metallic mesh (Vitallium, no. 6510; Howmedica, Inc., Rutherford, N. J.) in a 35-mm-wide plastic dish. To achieve optical clarity, sufficient Ringer's solution was applied to each preparation to fill the interstices of the mesh beneath the retina.

Skate rhodopsin was prepared by a single extraction into a solution containing digitonin (Merck Chemical Div., Merck & Co., Inc., Rahway, N. J.); the procedure employed was modified from that of Hubbard et al. (1971). Absorption spectra of

extracted rhodopsin and regenerated pigments were obtained in a Cary 14 spectrophotometer (Varian Associates, Instrument Division, Palo Alto, Calif.).

All-*trans* retinal (Distillation Products, Inc., Eastman Kodak Co., Rochester, N. Y.) and 11-*cis* retinal (generously provided by Hoffmann-LaRoche, Inc., Basel, Switzerland) were each recrystallized and stored at −20° C. under nitrogen; 9-*cis* and 13-*cis* retinal were products of Sigma Chemical Co. (St. Louis, Mo.). Ethanolic stock solutions of retinal (90-365 mM) were prepared under dim red light and stored in the dark at −20°C.; the solutions were typically used within 1 wk of the date of preparation. Aliquots of freshly prepared stock solutions were analyzed by absorption spectrophotometry, and concentrations of retinal were determined from published extinction coefficients (Brown and Wald, 1956; Hubbard et al., 1971).

RESULTS

Responses Recorded from the Dark-Adapted Retina

Fig. 1 A, B, and C illustrate responses recorded extracellularly across the dark-adapted skate eyecup or isolated retina, upon the presentation of a 0.2-s test

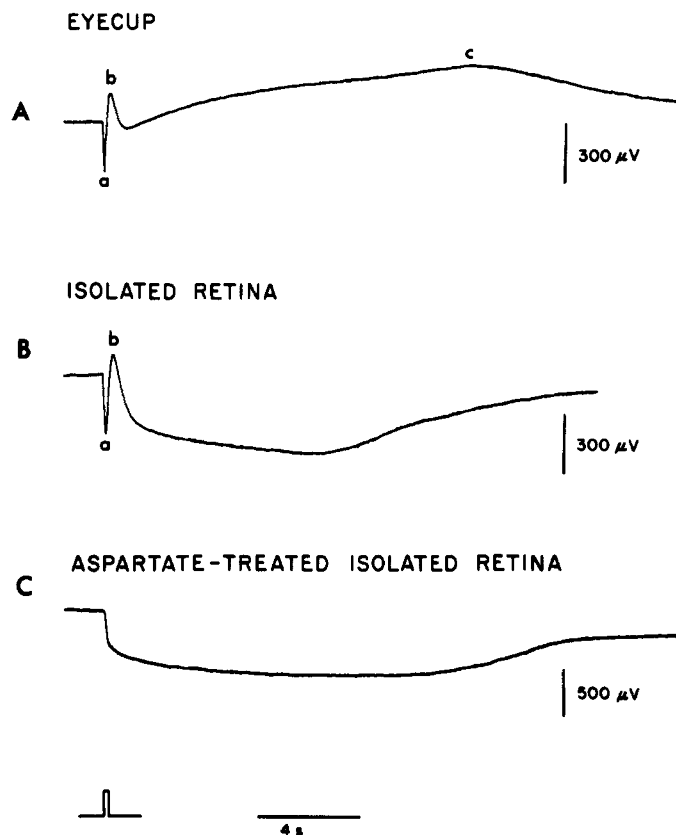

FIGURE 1. Responses of the dark-adapted skate eyecup and isolated retina to a 0.2-s stimulus (N_{eff} = 2 × 10^3 photons [500 nm] per μm^2); DC recordings from three separate preparations. For comparison with the recordings obtained from the isolated retina, the response recorded from the eyecup has been inverted.

flash. The preparations described in Fig. 1 A and B were treated during dissection with normal skate Ringer's solution, in which NaCl was substituted for the Na-L-aspartate. In the presence of the normal Ringer's solution, the response of the skate eyecup exhibits the *a*-, *b*-, and *c*-wave components commonly observed in the electroretinogram (Granit, 1955); however, the isolation of the retina from the back of the eye eliminates the *c*-wave (Noell, 1954; Ames and Gurian, 1963; Steinberg et al., 1970). As Fig. 1 C indicates, treatment of the isolated retina with sodium aspartate suppresses the *b*-wave and leaves only a negative potential, designated PIII in Granit's analysis (Sillman et al., 1969). The initial phase of this potential ("fast PIII"), which accounts for the greater part of the response, reflects the activity of the photoreceptors (Faber, 1969; Witkovsky et al., 1975). A later negative component is also evident in Fig. 1 C; this potential, designated "slow PIII," is believed to arise from the activity of the Müller cells of the retina (Faber, 1969; Witkovsky et al., 1975; Arden, 1976), and becomes predominant in the PIII response of the skate retina at very high intensities of the test flash (not illustrated).

The responses of the aspartate-treated isolated retina are graded with intensity of the test flash, as shown by the DC recordings in Fig. 2. For routine measurements of threshold and amplitude of the photoreceptor potential, responses were recorded with the use of an AC-coupled amplifier (band pass of 0.1–1,000 Hz). Receptor thresholds determined in this way were found to be virtually identical to those obtained by measurement of the DC-recorded PIII; furthermore, maximal response amplitudes obtained by the AC amplification closely matched the maximal amplitude of the fast phase of the DC-recorded response. Fig. 3 illustrates the receptoral intensity-response curve of a typical dark-adapted isolated retina, determined with the use of AC amplification. Near threshold (usually 3 μV), the amplitude of the recorded response is linearly graded with intensity of the test flash; at high intensities of the flash, the response approaches a limiting value (Naka and Rushton, 1966; Fain and Dowling, 1973). The incident energy of a flash required to elicit a maximal response bleaches an insignificant fraction of the rhodopsin present in dark-adapted photoreceptors (compare the data of Fig. 3 with those of the inset of Fig. 5, below).

Electrophysiological and Spectrophotometric Changes Resulting from Intense Illumination

Fig. 4 shows the effect of intense illumination on photoreceptor sensitivity. After the determination of dark-adapted threshold, an isolated retina was progressively light-adapted by brief exposures to the unattenuated light of the photostimulator. After each period of intense illumination, values of threshold for the photoreceptor response were monitored until they showed no further decrease. As the data of Fig. 4 indicate, these adapting irradiations substantially reduced the sensitivity of the photoreceptors, i.e., led to a permanent elevation of receptor threshold.

In the absence of further treatment, receptor thresholds in some light-adapted preparations slowly increased, reflecting an apparent deterioration of the retina. Usually, this slow desensitization could be prevented by the periodic

application of several drops of Ringer's solution to the upper (photoreceptor) surface of the retina. This procedure probably served to regulate the moisture or the ionic composition of the extracellular environment, and was adopted for

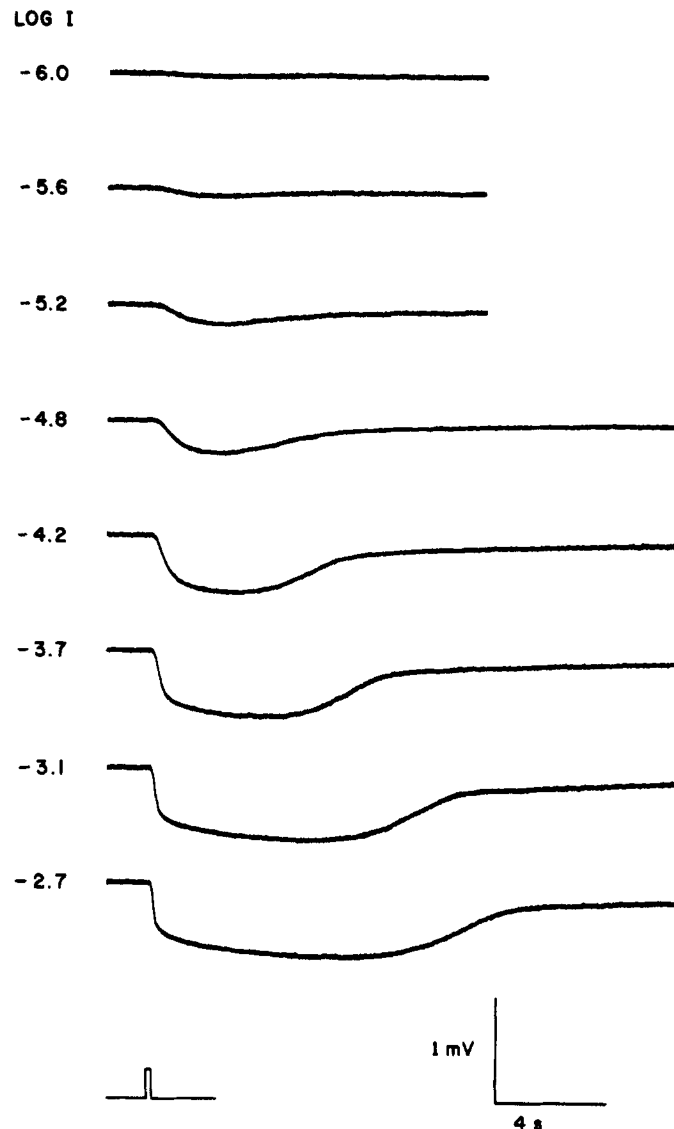

FIGURE 2. Responses recorded from the dark-adapted isolated retina after stimulation with 0.2-s test flashes of graded intensity.

a number of the experiments; for example, in the experiment shown in Fig. 4, Ringer's solution was applied to the retina at the times indicated by asterisks. The application of the Ringer's solution was, however, never observed to promote a significant increase in the receptor sensitivity of either dark-adapted or light-adapted retinas.

To determine the extent of rhodopsin bleaching which results from intense light adaptation, we spectrophotometrically analyzed several preparations before and after exposures to the calibrated adapting light of the photostimulator. In each of these experiments, a dark-adapted isolated retina was mounted in a sealed microcell and positioned in the microspectrophotometer (sample chamber thermostatted at about 16°C.). After an absorption spectrum had been recorded, the microcell was transferred in the dark to the focal plane of the

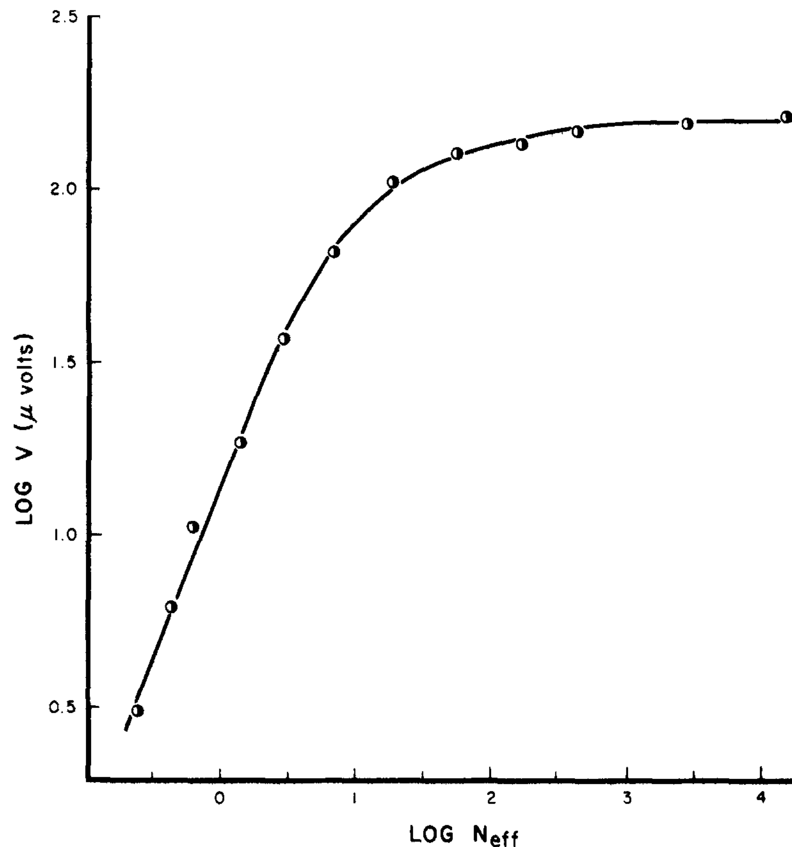

FIGURE 3. Intensity-response characteristics of the AC-coupled receptor potential recorded from a dark-adapted isolated retina.

photostimulator, and the retina was light-adapted as in the electrophysiological experiment of Fig. 4. During the exposure to the intense light, a piece of moist filter paper was placed beneath the microcell to simulate the reflectance of the filter paper present in the electrophysiological experiments. Immediately after this irradiation, the retina was returned in the dark to exactly the same position in the microspectrophotometer. Then, over a period of at least 15 min from the time of offset of the adapting light, several absorption spectra were recorded. During this period the decay of metarhodopsin III proceeded virtually to completion (Brin and Ripps, 1977), and the absorbance of the retina at 530 nm became stable; in no instance was there observed any significant increase in

absorbance at 530 nm. The retina was progressively light-adapted by the repetition of this procedure, and the loss of rhodopsin resulting from each intense irradiation was measured by the decrease in absorbance at 530 nm. At the conclusion of each experiment, an exhaustive bleaching (5 min in duration) of the remaining rhodopsin was carried out in the microspectrophotometer, to provide a baseline value of absorbance at 530 nm.

The data from six such experiments are shown in the inset of Fig. 5; these data describe the bleaching of skate rhodopsin under conditions almost identical to those present in the electrophysiological experiment of Fig. 4. The bleaching curve derived from these data clearly shows that exposures to light which

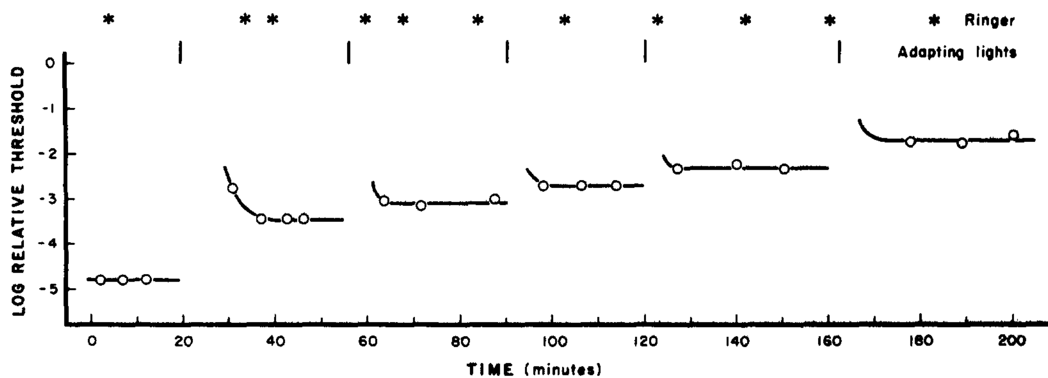

FIGURE 4. Desensitization of photoreceptors in the isolated retina by brief irradiations (log intensity = 0.0). During the five periods of light adaptation (vertical bars), the intensity of light incident on the retina was 1.26×10^6 photons (500 nm) per $\mu m^2/s$; the durations of the five irradiations were, sequentially: 2.1, 2.1, 4.2, 6.0, and 23 s. The rapid, "neural" phase of dark adaptation was usually complete before the determination of receptor threshold. Asterisks denote the times of application of Ringer's solution to the upper surface of the retina.

permanently desensitize the photoreceptors also result in the elimination of significant amounts of rhodopsin. In calculating the fraction of rhodopsin bleached, we have assumed the absorbance of rhodopsin (at 530 nm) to be proportional to the density of rhodopsin in the photoreceptors. (At 530 nm, the absorbance of rhodopsin in the dark-adapted retinas varied between 0.10 and 0.28.) This assumption, used in earlier investigations (Dowling and Ripps, 1970; Witkovsky et al., 1973), neglects a small contribution to the transmittance of the retina due to the passage of light through spaces outside the receptor outer segments (Rushton, 1965; Grabowski and Pak, 1975).

An objective of the present study was to examine closely the variation of photoreceptor threshold with rhodopsin content in the strongly light-adapted skate retina, and to compare the properties of the receptoral response with those of visual responses arising proximally in the skate eye. Shown in Fig. 5 are the results of 15 electrophysiological experiments in which an isolated retina was progressively light-adapted, as in the experiment of Fig. 4. Each data point in Fig. 5 represents the stable value of relative threshold attained after a given stage of light adaptation, plotted against the percent of rhodopsin permanently

eliminated by bleaching. In each case, the percent of rhodopsin bleached was determined through use of the bleaching curve shown in the inset. These data show that, in the isolated skate retina, the bleaching of visual pigment is associated with a marked reduction of photoreceptor sensitivity. This loss of

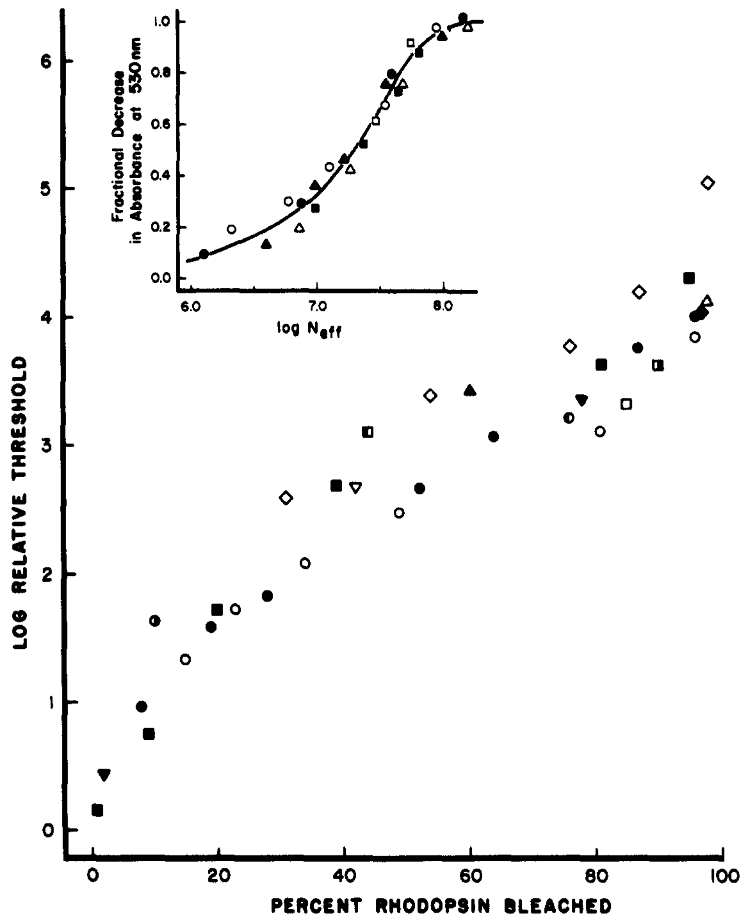

FIGURE 5. Sustained elevations of receptor threshold exhibited after the bleaching of rhodopsin. Thresholds measured in the same experiment are represented by identical symbols. The percent of rhodopsin bleached at each stage of light adaptation was determined from the bleaching curve shown in the inset; the bleaching efficiencies of the two weakest adapting irradiations ($N_{eff} < 10^6$ photons [500 nm] per μm^2) were calculated by extrapolation of the bleaching curve.

sensitivity amounts, for example, to 3 log units when ≅60% of the rhodopsin has been bleached, greatly exceeding the desensitization expected merely from the reduced quantal absorptivity of bleached receptors (Weale, 1964).

The data of Fig. 5 may be directly compared with those of Dowling and Ripps (1970), who examined the effects of flash bleaching on ERG *b*-wave and ganglion cell thresholds in the skate eyecup preparation. These investigators found that throughout the slow phase of dark adaptation, the log relative sensitivities of

both the *b*-wave and the ganglion cells grow linearly with increases in the receptoral content of (regenerated) rhodopsin; the regeneration of ≅80% of the full (dark-adapted) complement of rhodopsin is found to be associated with an increase of 4 log units in the *b*-wave and ganglion cell sensitivities. Together, the present and earlier data show that under conditions of equal rhodopsin content, the sustained desensitization of the receptors in the isolated retina roughly approximates the transient depression of *b*-wave and ganglion cell sensitivities in the dark-adapting eyecup. Thus, adaptation of the skate visual system to bleaching irradiation is already expressed to a significant extent at the most distal level of the retina.

Interestingly, the present data indicate relatively small but systematic differences between the behavior of receptor threshold and the (logarithmic-linear) relationship previously found to describe *b*-wave and ganglion cell thresholds. This disparity is such as to suggest that the bleaching of an initial fraction of the rhodopsin exerts a disproportionately large effect upon the log threshold of the photoreceptor response. Previous results obtained in the isolated retinas of the frog, axolotl, and skate have suggested a similar tendency in the threshold properties of bleached photoreceptors (Matsuura, 1975; Grabowski and Pak, 1975; Brin, 1975; Witkovsky et al., 1976). Although this feature of receptor adaptation cannot yet be explained, it may reflect an intrinsic property of the cellular mechanism which regulates photoreceptor sensitivity.

Treatment of Strongly Light-Adapted Photoreceptors with 11-cis Retinal

Fig. 6 illustrates the primary observation of the present study: the sensitizing influence of externally applied 11-*cis* retinal on strongly light-adapted skate photoreceptors. After determination of the dark-adapted threshold, an isolated retina was briefly exposed to the intense adapting light. From the bleaching

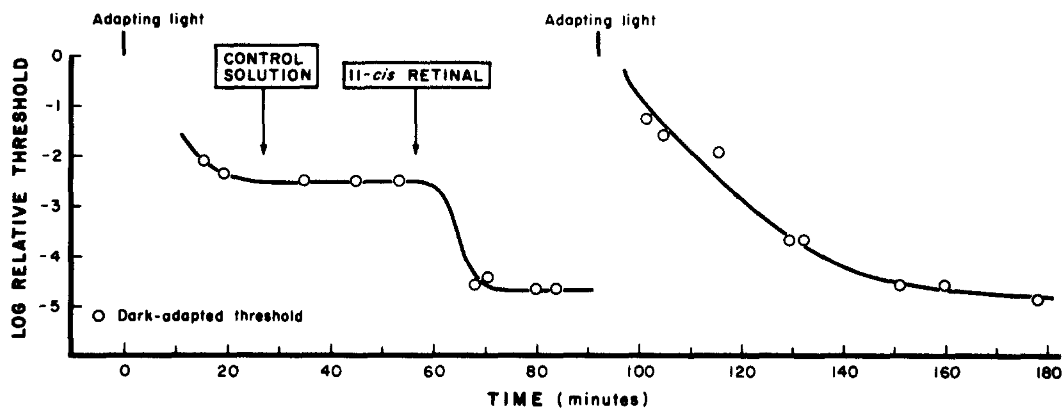

FIGURE 6. The sensitization of photoreceptors in a strongly light-adapted retina induced by treatment with 11-*cis* retinal. The first adapting irradiation lasted 12 s and was terminated at time zero. At the times shown by the arrows, the retina was treated sequentially with: 0.05 ml of ethanol-Ringer's solution, 1:100 by volume ("control solution"); and 0.04 ml of 11-*cis* retinal suspended in the ethanol-Ringer's solution at a concentration of 2.5 μmol/ml. The second adapting irradiation was of the same intensity as the first, but lasted three times as long.

curve of Fig. 5, we determined that this irradiation eliminated ≅42% of the rhodopsin initially present. As the receptor threshold stabilized at a new, elevated value, a control solution (consisting of ethanol-Ringer's solution, 1:100 by volume) was gently applied dropwise to the retina from a 1-ml disposable syringe fitted with a 26-gauge hypodermic needle. This treatment, carried out at the time indicated by the first arrow, had no effect on receptor sensitivity; for subsequent to the application of this solution, receptor threshold remained constant at a value about 2.7 log units above the initial, dark-adapted value, consistent with the behavior of untreated preparations (cf. Fig. 5).

At the time indicated by the second arrow, a suspension containing 11-*cis* retinal in ethanol-Ringer's solution was similarly applied to the upper surface of the retina; this suspension, prepared from an ethanolic stock solution of 11-*cis* retinal during the course of the experiment, had been thoroughly mixed just before its application by repeated passage through a hypodermic syringe. As the suspension was applied to the surface of the retina, it appeared temporarily to bathe the preparation in 11-*cis* retinal; it then appeared to disperse over the surface of the retina and to be absorbed by the surrounding (and underlying) filter paper. Presumably, a portion of the applied 11-*cis* retinal adhered to the plasma membranes of the photoreceptors. As Fig. 6 indicates, this treatment promoted a rapid and substantial increase in the sensitivity of the receptors; over a period of ≅20 min, receptor threshold fell to a new value almost as low as the original, dark-adapted value.

The virtually complete recovery of sensitivity brought about by this treatment suggested that the presence of the applied retinal caused no significant attenuation of the (Wratten 58-filtered) light incident on the photoreceptors. However, a subsequent adapting irradiation of the treated retina led to a threshold behavior which differed strikingly from that of untreated controls (cf. Figs. 4 and 5). The second irradiation shown in Fig. 6 would have bleached ≅78% of the rhodopsin in an untreated, dark-adapted retina; but after the offset of this light, and in the absence of further treatment with retinal, receptor threshold decreased by a least 3.5 log units to return again almost to the original, dark-adapted value. Thus, intense light still served to desensitize the receptors, but the subsequent recovery of sensitivity in the dark proceeded far more extensively than in untreated preparations.

Fig. 7 illustrates amplitudes of the photoreceptor potential recorded before and after treatment with 11-*cis* retinal. Strong light adaptation of the isolated retina both decreased the maximal response that could be elicited by the test flash, and shifted the intensity-response curve in the direction of decreased sensitivity (Grabowski et al., 1972; see also Alpern et al., 1970). On treatment with 11-*cis* retinal, there occurred a progressive shift of the entire response curve in the direction of increased sensitivity; concomitantly, there appeared an increase in the maximal amplitude of the photoreceptor response. Thus, the treatment of strongly light-adapted receptors with 11-*cis* retinal not only leads to the lowering of receptor threshold, but also serves to augment the photoreceptor response to suprathreshold intensities of a test flash.

Fig. 8 illustrates the spectrophotometric effects of exogenous 11-*cis* retinal on a strongly light-adapted retina. In this experiment a dark-adapted isolated

retina, mounted on a supporting wire mesh, was positioned in the microspectrophotometer, analyzed for absorbance, and then exposed for 5 min to intense, Wratten 58-filtered light. This irradiation bleached at least 96% of the rhodopsin initially present. A suspension of 11-*cis* retinal was then applied to the upper

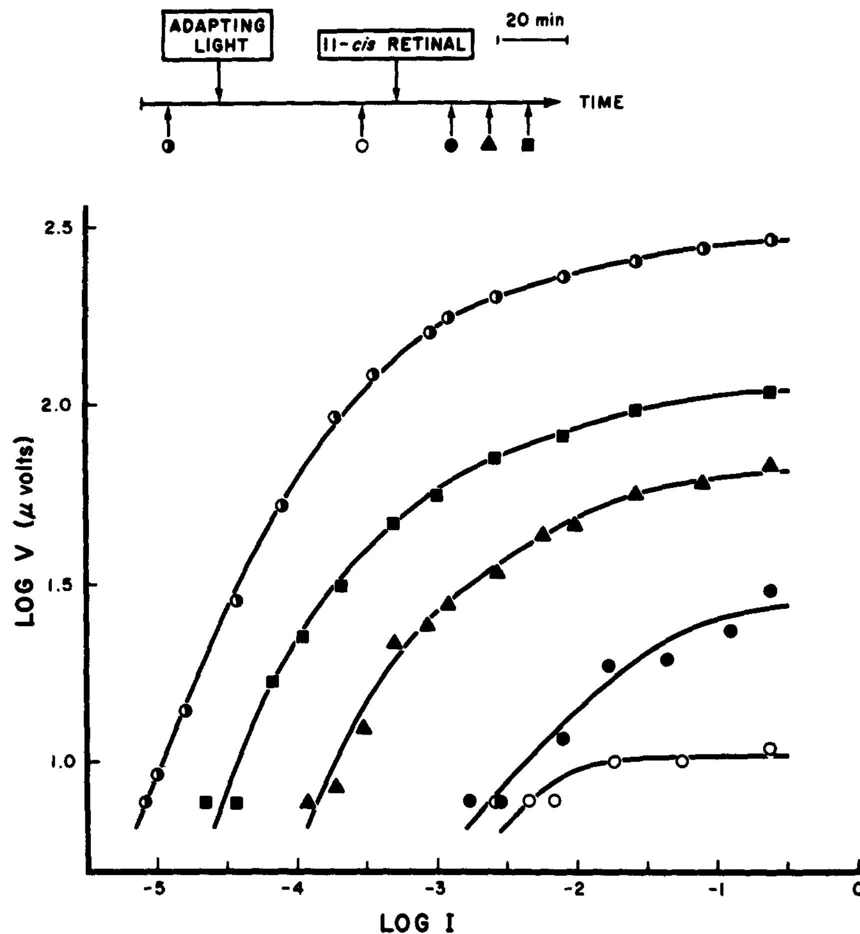

FIGURE 7. The effects of 11-*cis* retinal on the amplitude of the photoreceptor potential. The key at the top of the figure indicates the times at which the data were obtained. The adapting irradiation, 30 s in duration, was of sufficient intensity to bleach ≅37% of the rhodopsin initially present. At the time shown, 0.07 ml of a suspension containing 11-*cis* retinal (3.6 μmol/ml in ethanol-Ringer's solution, 1:100 by volume) was applied to the retina.

(photoreceptor) surface of the retina in a manner identical to that used in the electrophysiological experiments. The treated retina was incubated for a period of ≅26 min, during which time changes in absorbance at 530 nm were repeatedly measured (Fig. 8, inset). A solution of hydroxylamine was then applied to the retina, and an absorption spectrum (spectrum 1) was recorded shortly thereaf-

ter. The preparation was then again exposed to the bleaching light for 5 min; immediately after the offset of this light, spectrum 2 was obtained.

The data of Fig. 8 indicate that the treatment with 11-*cis* retinal had promoted

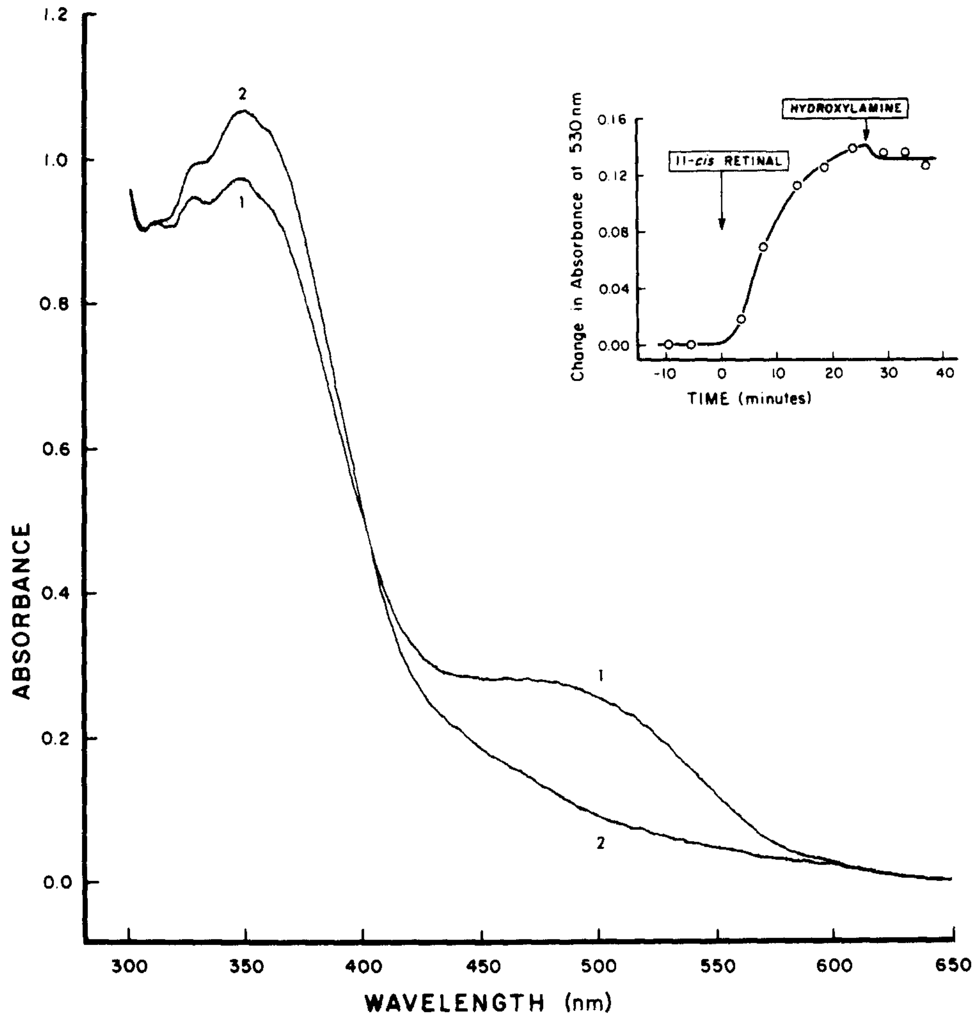

FIGURE 8. Absorption spectra recorded from an isolated retina that had undergone (*a*) exhaustive bleaching; (*b*) treatment with 11-*cis* retinal (1.7 μmol suspended in 0.11 ml of ethanol-Ringer's solution, 1:5 by volume); and (*c*) subsequent treatment with hydroxylamine (0.10 ml of a 0.70 M solution, pH 6). Spectrum 1 was obtained just before and spectrum 2 just after a second exposure to the bleaching light. The inset shows changes in absorbance at 530 nm which followed the applications of 11-*cis* retinal and hydroxylamine.

the formation of a light-sensitive pigment which, like rhodopsin, was unreactive with hydroxylamine (Wald and Brown, 1950; Hubbard and Wald, 1952); the absorbance of this pigment at 500 nm was 56% of that due to the rhodopsin initially present in this preparation. The inset of Fig. 8 shows that the application of hydroxylamine caused relatively little change in the measured absorbance at

530 nm; thus, the kinetic data obtained before the treatment with hydroxylamine appeared to represent closely the time-course of formation of the pigment itself. Spectra 1 and 2 further show that irradiation of this pigment led to increases in absorbance at wavelengths in the near ultra-violet range. The differences in absorbance between these spectra are plotted in Fig. 9, and indicate that this increase is maximal at wavelengths near 367 nm, the absorption maximum of retinyl oxime. The open circles in Fig. 10 show, in normalized form, the difference spectrum obtained from spectra 1 and 2 of Fig. 8. For comparison with this result, a separate experiment was performed in which a dark-adapted

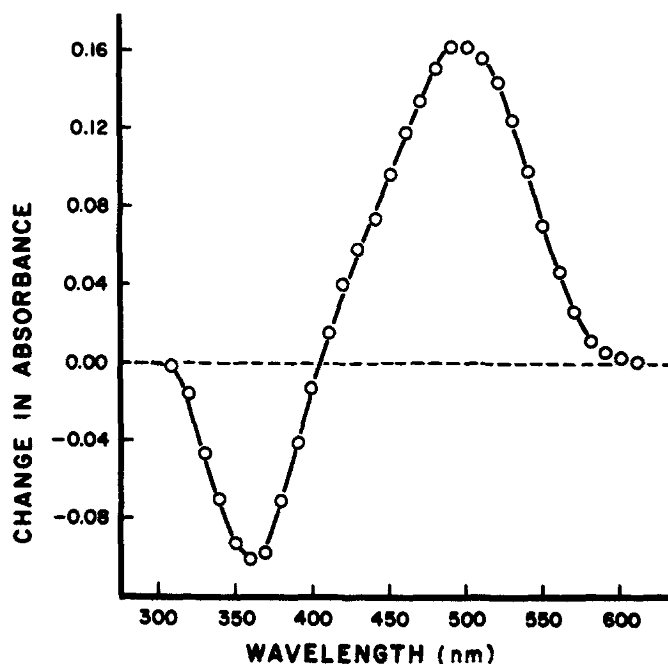

FIGURE 9. Difference spectrum obtained from spectra 1 and 2 of Fig. 8.

isolated retina was first treated with hydroxylamine, and then exhaustively bleached. The resulting normalized difference spectrum of native skate rhodopsin, shown by the filled squares in Fig. 10, agrees closely with the spectrum obtained from the retina treated with 11-*cis* retinal. Together, these findings indicate that 11-*cis* retinal, when applied to the strongly light-adapted retina under conditions similar to those of the electrophysiological experiments, promotes the formation of rhodopsin in the photoreceptors. Furthermore, the time-course of formation of the rhodopsin corresponds closely with the time course of the receptor sensitization described above.

Treatment of Skate Photoreceptors with All-trans, 13-cis, and 9-cis Retinal

In the following set of experiments, we asked whether the sensitizing activity of 11-*cis* retinal represents a unique property of this molecule, or whether other isomers of retinal possess similar activity. We were particularly interested to learn the degree to which the sensitization of strongly light-adapted receptors is

correlated with the induced formation of visual pigment. Figs. 11 and 12 show that sensitizing activity depends markedly on the form of retinal applied externally to the receptors. In each of the experiments indicated, multiple applications of either all-*trans* or 13-*cis* retinal failed to promote any significant

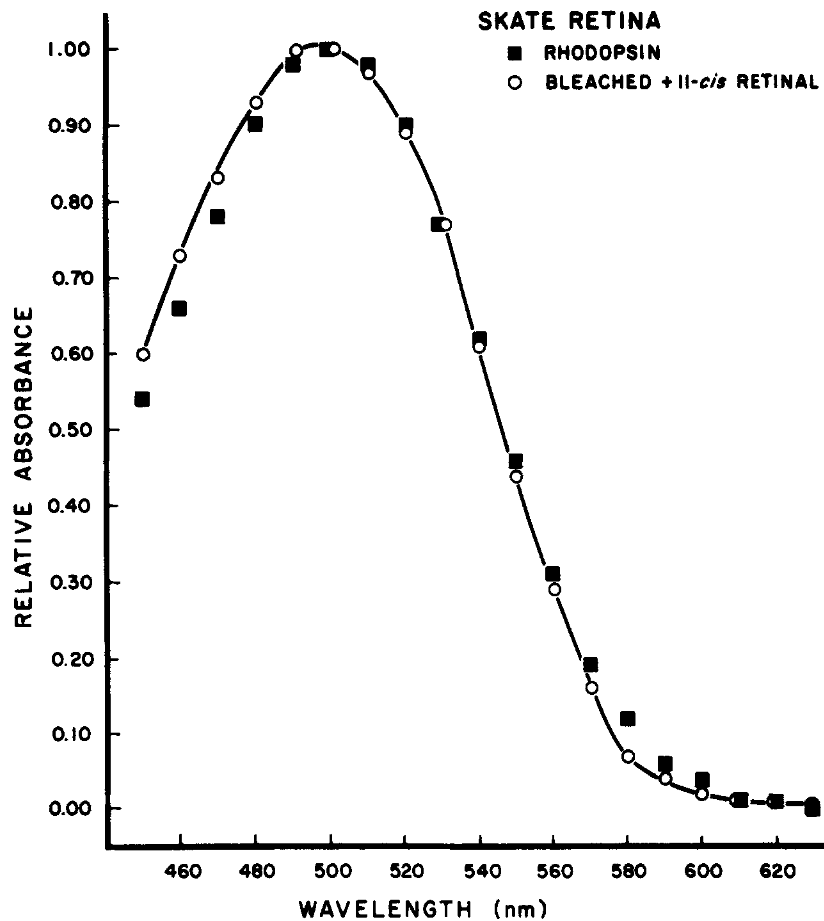

FIGURE 10. (■) Normalized difference spectrum resulting from the exhaustive bleaching of a dark-adapted isolated retina in the presence of hydroxylamine. (○) Normalized difference spectrum of pigment regenerated in a bleached isolated retina to which 11-*cis* retinal had been applied (obtained from spectra 1 and 2 of Fig. 8).

change in the threshold of previously light-adapted receptors. The failure of these compounds to induce a sensitization could not have resulted from a deterioration of the preparations, because in each case, subsequent treatments with an equal concentration of 11-*cis* retinal promoted substantial increases in receptor sensitivity. Similar results (not illustrated) were obtained when the concentration of applied all-*trans* retinal exceeded by 40% the concentration of subsequently applied 11-*cis* retinal. The spectrophotometric effects of treatment

with all-*trans* and 13-*cis* retinal were examined in a separate set of experiments, under conditions identical to those used with 11-*cis* retinal (cf. legend of Fig. 8 and accompanying text). In each case, only insignificant amounts of light-sensitive pigment were formed on incubation of the previously bleached retina

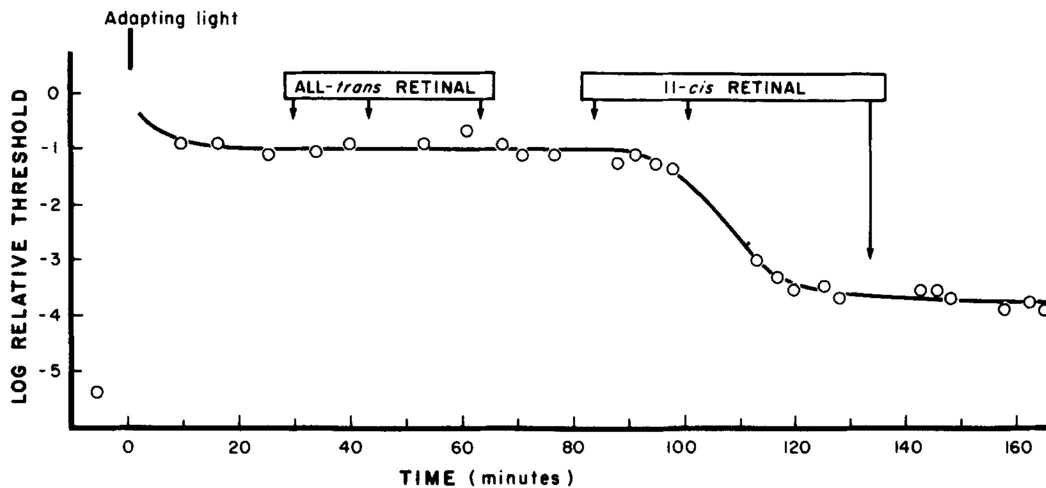

FIGURE 11. Treatment of a strongly light-adapted isolated retina with all-*trans* and 11-*cis* retinal. The adapting irradiation bleached ≅97% of the rhodopsin initially present. At the times indicated by arrows, all-*trans* or 11-*cis* retinal, suspended at a concentration of 1.5 μmol/ml in ethanol-Ringer's solution (1:100 by volume) was applied to the retina. The volumes applied were (sequentially): 0.12, 0.15, and 0.09 ml of all-*trans* retinal; and 0.11, 0.15, and 0.11 ml of 11-*cis* retinal.

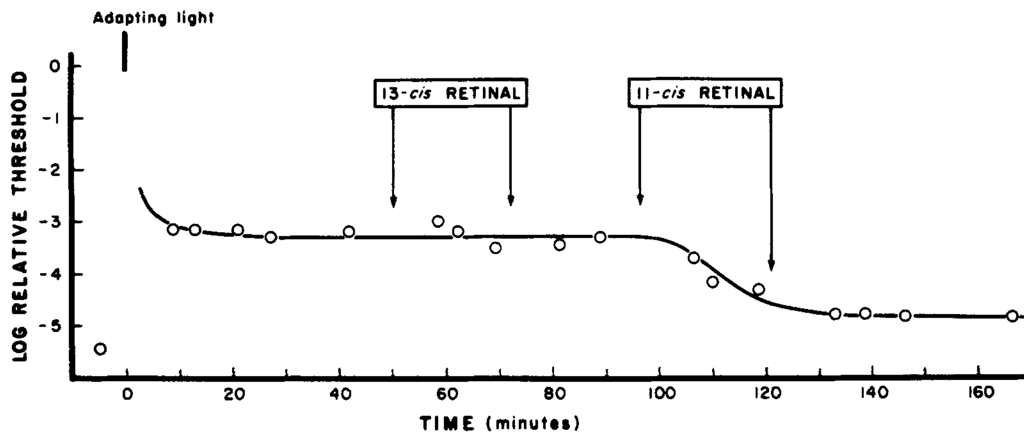

FIGURE 12. Treatment of a strongly light-adapted isolated retina with 13-*cis* and 11-*cis* retinal. After irradiation (which bleached ≅31% of the rhodopsin) and the stabilization of receptor threshold, the retina was treated with 13-*cis* or 11-*cis* retinal, suspended in ethanol-Ringer's solution (1:100 by volume) at a concentration of 2.6 μmol/ml. At the times shown by the arrows, 0.07 ml of the indicated suspension was applied.

with all-*trans* or 13-*cis* retinal; in both experiments, for example, the absorbance at 500 nm of light-sensitive pigment present after the treatment with hydroxylamine (as determined by the difference spectrum resulting from a subsequent irradiation) represented 8% or less of the absorbance due to the rhodopsin initially present in the receptors.

All-*trans* retinal is an ultimate product of the bleaching of rhodopsin, and the formation of the all-*trans* isomer therefore accompanies the depression of receptor sensitivity brought about by strong light adaptation. In order to learn if all-*trans* retinal itself promotes a substantial decrease in sensitivity when applied to receptors containing a full complement of rhodopsin, we treated a *dark-adapted* retina with this compound. Fig. 13 shows that the application of all-

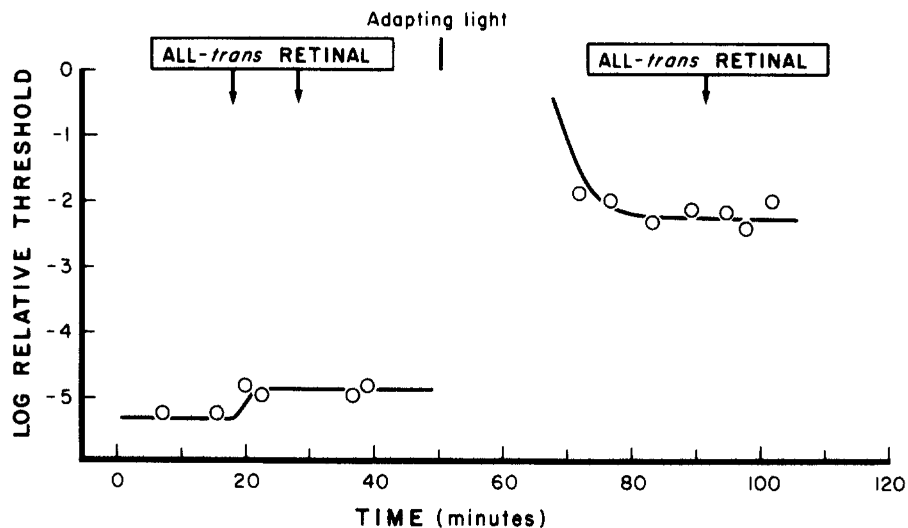

FIGURE 13. Treatment of a dark-adapted isolated retina with all-*trans* retinal. All-*trans* retinal was suspended at a concentration of 2.6 μmol/ml in ethanol-Ringer's solution (1:100 by volume). At the times indicated by arrows, the retina was treated with this suspension, twice before intense light adaptation (applications of 0.08 and 0.09 ml), and once after the light adaptation (0.10 ml). The adapting irradiation, 30 s in duration, was of sufficient intensity to bleach $\cong$41% of the rhodopsin in an untreated preparation.

trans retinal results in only a slight desensitization of the receptors. A small decrease in sensitivity was also observed when a dark-adapted retina was similarly treated with 11-*cis* retinal. Together, these results indicate that under our experimental conditions, external treatment with retinal somewhat diminishes the sensitivity of fully dark-adapted photoreceptors; however, all-*trans* retinal appears to exert no specific effect on the level of receptor sensitivity.

The examination of 9-*cis* retinal held particular interest for the present study. It is well established that in digitonin and in isolated rod outer segments, 9-*cis* retinal can combine with opsin to form isorhodopsin, a photosensitive pigment (Hubbard and Wald, 1952; Shichi and Somers, 1974); before our work, however, it was not known if the 9-*cis* isomer possessed any physiological activity. Fig. 14 shows that 9-*cis* retinal possesses substantial activity. In each of the experiments

illustrated, the treatment of strongly light-adapted photoreceptors with this compound brought about a large increase in receptor sensitivity. The experiment described by the open circles further shows that, once treated with 9-*cis* retinal, the photoreceptors display an augmented recovery of sensitivity following intense irradiation. Finally, the sensitization induced by treatment with 9-*cis* retinal was found to be accompanied by a shift of the entire intensity-response curve in the direction of increased sensitivity (not illustrated).

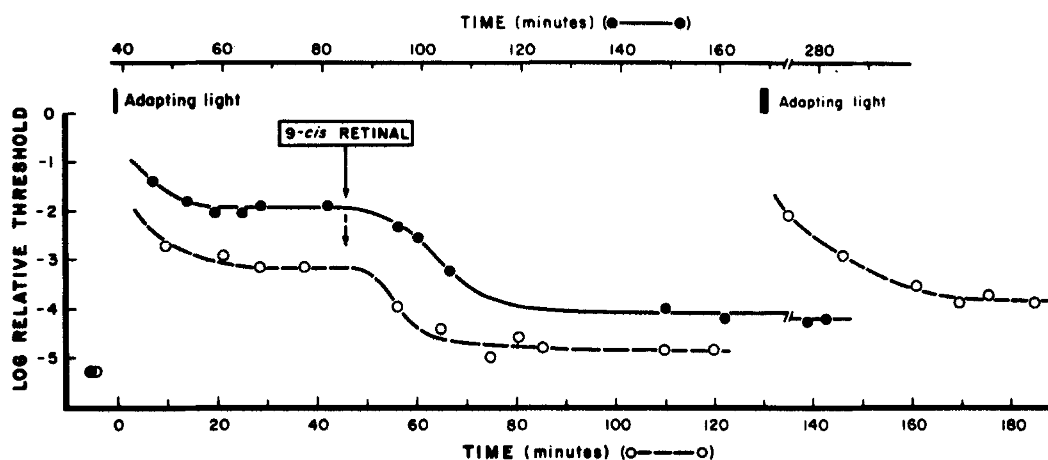

FIGURE 14. The effects of exogenous 9-*cis* retinal on photoreceptor sensitivity. In the experiment illustrated by (O), 0.09 ml of a suspension containing 9-*cis* retinal (3.1 μmol/ml in ethanol-Ringer's solution, 3:100 by volume) was applied at the indicated time to an isolated retina that had previously been strongly light-adapted. This initial period of light adaptation (first vertical bar), 30 s in duration, bleached ≅36% of the rhodopsin initially present. After the treatment with 9-*cis* retinal, this preparation was again strongly light-adapted (second vertical bar); the second intense irradiation was of the same intensity as the first, but lasted three times as long. In the experiment illustrated by (●), 9-*cis* retinal (suspended at a concentration of 3.7 μmol/ml in ethanol-Ringer's solution, 3:100 by volume; volume delivered = 0.08 ml) was applied to a strongly light-adapted retina. The single adapting irradiation used in this experiment bleached ≅85% of the rhodopsin initially present, and was terminated at time zero. The data from this experiment have been shifted vertically to allow matching of the initial, dark-adapted thresholds measured in the two experiments shown.

Although the electrophysiologically measured activity of 9-*cis* retinal appears to duplicate that of the 11-*cis* isomer, treatment with these compounds yields quite different spectrophotometric results. Fig. 15 indicates the results of an experiment similar to that described by Fig. 8, except that the bleached isolated retina was incubated with 9-*cis* retinal. The data of Fig. 15 show that the light-sensitive pigment formed in the presence of 9-*cis* retinal exhibits a difference spectrum with hydroxylamine whose peak wavelength (λ_{max} = 489 nm) is approximately 12 nm shorter than that of skate rhodopsin (dashed curve). To obtain further information bearing on this result, we spectrophotometrically analyzed rhodopsin and isorhodopsin prepared from extracted skate opsin.

The data of Fig. 16 show that in digitonin, both native and regenerated skate rhodopsin exhibit a λ_{max} near 497 nm, in agreement with the value found by Beatty (1969). (The absorption spectrum of skate rhodopsin in the extracts

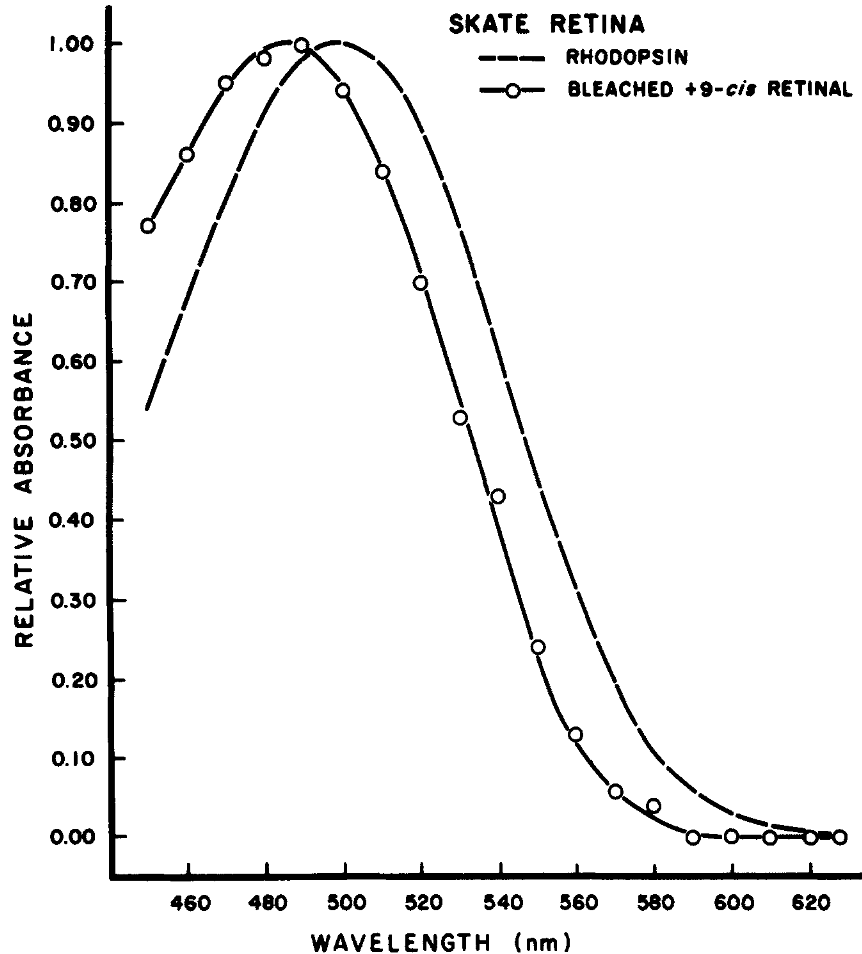

FIGURE 15. Light-sensitive pigment formed in the presence of 9-*cis* retinal; see text for details. (O) Normalized difference spectrum that resulted from intense irradiation of a preparation previously treated with 9-*cis* retinal and hydroxylamine. At 490 nm, the absorbance of this pigment (0.094) amounted to 72% of the absorbance at 500 nm contributed by the rhodopsin initially present in this retina. Dashed curve represents normalized difference spectrum of rhodopsin in the isolated retina, bleached in the presence of hydroxylamine (spectrum obtained from the data of Fig. 10).

appears slightly different from that measured in the intact retina. Similar variations in the absorption properties of rhodopsin have been reported by previous investigators; cf. Wald and Brown, 1958; Tokunaga et al., 1976.) The data also show that the absorption spectrum of skate isorhodopsin in digitonin

(λ_{max} = 485 nm) is significantly displaced from that of rhodopsin. Furthermore, the spectral relationship between rhodopsin and isorhodopsin in digitonin closely resembles that found in the intact retina between rhodopsin and the

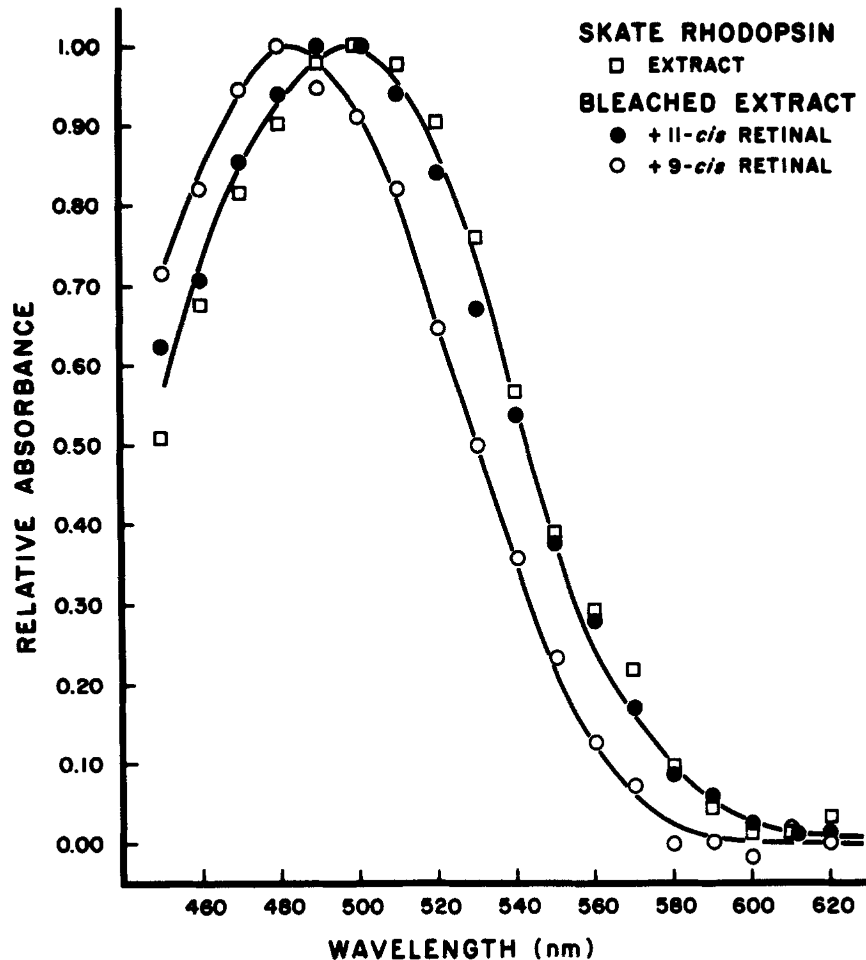

FIGURE 16. Absorption spectra of skate visual pigments in digitonin. Extracted skate rhodopsin was bleached with orange light (Corning no. 3484 filter, Corning Glass Works, Corning, N. Y.; and Schott KG-3 heat filter) in the presence of 30 mM hydroxylamine, yielding the difference spectrum shown by (□). For the regeneration of visual pigments, extracted rhodopsin was exhaustively bleached with orange light, and the resulting opsin was incubated for about 18 min in the presence of 11-*cis* or 9-*cis* retinal (5 nmol/ml). The reaction mixtures were then supplemented with hydroxylamine and bleached as just described, yielding difference spectra for skate isorhodopsin (O) and regenerated rhodopsin (●).

pigment formed in the presence of 9-*cis* retinal (compare Figs. 15 and 16). These results lead to the conclusion that the receptor sensitization induced by exogenous 9-*cis* retinal is accompanied by the intracellular formation of isorhodopsin.

DISCUSSION

The results presented here show that strong light adaptation of the isolated skate retina ordinarily leads to a large and sustained reduction of photoreceptor sensitivity. The extent of this desensitization appears to depend predominantly on a single parameter, the fractional depletion of rhodopsin in the receptors. For example, adapting irradiation which bleaches 80% of the rhodopsin, whether delivered in a single brief exposure or in steps over a period of several hours, ultimately reduces the initial, dark-adapted level of receptor sensitivity by ≅3.3 log units (cf. Fig. 5). Moreover, the relationship observed here between pigment content and receptor sensitivity is roughly similar to that describing *b*-wave and ganglion cell thresholds in the eyecup under equivalent conditions of previous bleaching irradiation (Dowling and Ripps, 1970; Green et al., 1975). Simply interpreted, the present and earlier data together imply that, during the course of slow dark adaptation, the photoreceptors themselves largely determine the relative degree of sensitivity expressed by more proximal neurons in the skate retina.

Earlier studies performed on the isolated rat and frog retinas, in which receptoral responses were compared with those arising proximally, suggested that strong light adaptation exerts only a relatively small change in the sensitivity of the photoreceptors (Frank, 1971; Ernst and Kemp, 1972; Hood et al., 1973). The variance of these findings from our results in the all-rod skate retina is difficult to interpret. However, the rat and frog retinas are known to contain multiple classes of photoreceptor which subserve scotopic and photopic vision (Frank, 1971; Green, 1973; Ernst and Kemp, 1975; Cicerone, 1976), and it is reasonable to believe that the scotopic and photopic receptors adapt differently to intense (full-field) illumination. Conceivably, the large disparity of receptoral and *b*-wave thresholds exhibited by these preparations reflects a varying mixture of contributions from the scotopic and photopic systems to visual responses developed at various levels in the retina. A second possibility is that *b*-wave thresholds in the strongly light-adapted frog and rat retinas are specifically elevated by an adaptive mechanism operating in the proximal retina. Such a mechanism of "network adaptation" has been described (Green et al., 1975); but in the skate eyecup, the influence of this mechanism on visual thresholds seems to decay shortly after background illumination has been extinguished.

In the present study it has been possible to examine the effects of externally applied retinal on the electrophysiological properties of partially bleached photoreceptors. Most importantly, this approach has revealed that incubation with 11-*cis* retinal, the naturally occurring chromophore of rhodopsin, specifically promotes a sensitization of strongly light-adapted skate photoreceptors. Accompanying this sensitization is the formation of a pigment whose properties closely match those of native skate rhodopsin. At the present time, we do not know if the applied 11-*cis* retinal merely diffuses into the photoreceptors, or if its entry may be facilitated by a system designed for the transport of vitamin A compounds (Wiggert and Chader, 1975; Heller, 1976; Bridges, 1977). In either case, the entry of 11-*cis* retinal under our experimental conditions appears to occur with low efficiency; for on a molar basis, the applications of retinal typically employed here (which we have found to be optimal) exceeded by up to

several 1,000-fold the total amount of opsin in our preparations. Once applied to the retina, the large quantity of 11-*cis* retinal may have served as an extracellular reservoir for the indefinite supply of fresh chromophore to the photoreceptors. The results of Figs. 6 and 14 support this view, for they show that the presence of excess 11-*cis* (or 9-*cis*) retinal continues to affect receptor threshold after a subsequent period of bleaching irradiation.

The comparative examination of several isomers of retinal has shown that the sensitizing activities of these compounds are closely correlated with their known abilities to combine with opsin (Hubbard and Wald, 1952). We have shown here that all-*trans* and 13-*cis* retinal, when applied to the strongly light-adapted isolated skate retina, fail to induce either the significant formation of visual pigment or an increase in receptor sensitivity. However, externally applied 9-*cis* retinal, which combines with opsin *in situ* to form isorhodopsin, exerts a sensitizing effect similar to that of the 11-*cis* isomer. Considered together, these results provide clear evidence that photoreceptor sensitivity in vivo is directly and strongly regulated by the level of visual pigment; for it appears that the formation of visual pigment in previously bleached receptors is itself sufficient to bring about a marked increase in receptor sensitivity. This pigment-linked recovery of receptor sensitivity appears, in turn, largely to influence the progress of slow ("photochemical") dark adaptation throughout the entire skate retina.

A question immediately raised by the present results is how the regeneration of visual pigment may bring about the increase of receptor sensitivity. It is now well established that the bleaching of rhodopsin involves a series of configurational changes in both opsin and retinal (Wald and Brown, 1952; Hubbard et al., 1965; Cone and Cobbs, 1969; Ostroy, 1974), although the mechanisms which may link these changes to the generation of the photoreceptor potential are not fully understood. Recently, several investigators have suggested that some of these light-induced changes may be specifically involved in the process of light adaptation (Bownds and Brodie, 1975; Keirns et al., 1975; Brodie and Bownds, 1976); an obvious extension of this idea is that during dark adaptation, chemical or conformational changes in opsin may activate cellular processes which resensitize the photoreceptor. The binding of chromophore by opsin, a highly stereospecific interaction, may itself be the chemical event which initiates such changes in opsin (cf. Koshland and Neet, 1968). Thus, we suggest that 11-*cis* retinal, in addition to serving as the chromophore for the photoexcitation of rhodopsin, may also directly regulate a chemical activity of opsin, by inducing (or stabilizing) a "dark-adapted" configuration of the pigment molecule. Our results with 9-*cis* retinal are particularly interesting in this regard; for they suggest that isorhodopsin, though structurally distinct from rhodopsin, mimics the sensitizing activity of the naturally occurring visual pigment.

Another interesting question raised by the present study is whether isorhodopsin *in situ* can function in the process of visual excitation. That is, isorhodopsin may lack the molecular configuration required for activity in the transduction of photic stimuli, even though it is able to function in the sensitization of strongly light-adapted receptors. On the other hand, if isorhodopsin can mediate visual excitation, its activity in this process would be expected to match

its absorbance spectrum in the photoreceptor (Wald and Brown, 1958). For example, the complete substitution of isorhodopsin for the rhodopsin initially contained in dark-adapted skate receptors should shift the action spectrum of the photoreceptor potential by $\cong$12 nm to the blue of its initial position (cf. Fig. 15).

By applying 9-*cis* retinal to strongly light-adapted photoreceptors, we have attempted to learn whether the intracellular formation of isorhodopsin is accompanied by a shift in the spectral sensitivity of the receptoral response. Our data, although suggesting a slight blue shift in spectral sensitivity on treatment with 9-*cis* retinal, are as yet inconclusive. One difficulty with such measurements is that, except after the most extreme conditions of previous bleaching, sufficient rhodopsin remains in the photoreceptors to contribute significantly to the spectral sensitivity, particularly at long wavelengths. To this problem must be added the possibly selective screening of short wavelength incident light by the excess 9-*cis* retinal applied to the retina. Recently, Huddleston and Williams (1977) have used an entirely different technique to obtain evidence on this question. By photoisomerizing retinal contained within rat photoreceptors, these investigators have induced the intracellular formation of isorhodopsin, and found that the appearance of this pigment is accompanied by a blue-shift in the sensitivity of the extracellularly recorded PIII response.

A final note concerns the implications of our findings for the role of the pigment epithelium. As Kühne first demonstrated a century ago, the regeneration of visual pigment within vertebrate rods ordinarily requires the association of the receptors with the pigment epithelium (Kühne, 1877). Furthermore, the interaction of the retina with this tissue seems to be necessary for the complete recovery of visual sensitivity after strong light adaptation (cf., for example, Weinstein et al., 1967). Under certain nonphysiological conditions a significant degree of (light-independent) pigment regeneration can occur in the rods even in the absence of the pigment epithelium (Cone and Brown, 1969; Rotmans et al., 1973). On the other hand, our experiments on the isolated skate retina have shown no evidence for an endogenous regeneration of rhodopsin in the dark; thus, in vivo, skate photoreceptors appear to rely on the pigment epithelium for the supply of vitamin A that can ultimately be utilized as the chromophore of rhodopsin (Dowling, 1960; Zimmerman, 1974; Bridges, 1976). As the present results indicate, the exogenous supply of 11-*cis* retinal to skate photoreceptors mimics the effects of association with the pigment epithelium; for, in the presence of 11-*cis* retinal, strongly light-adapted receptors display an extensive (pigment-linked) recovery of sensitivity in the dark, as they do in the eyecup preparation (Dowling and Ripps, 1972; Normann and Werblin, 1974; Kleinschmidt and Dowling, 1975). As yet, no one has identified the form of vitamin A that is supplied in vivo by the pigment epithelium to bleached photoreceptors. However, our results suggest that the provision of this material may be the major role of the pigment epithelium specific to the slow phase of dark adaptation.

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