Utilization of Retinoids in the Bullfrog Retina

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ABSTRACT The capacity to generate 11-cis retinal from retinoids arising naturally in the eye was examined in the retina of the bullfrog, Rana catesbeiana. Retinoids, co-suspended with phosphatidylcholine, were applied topically to the photoreceptor surface of the isolated retina after substantial bleaching of the native visual pigment. The increase in photoreceptor sensitivity associated with the formation of rhodopsin, used as an assay for the appearance of 11-cis retinal in the receptors, was analyzed by extracellular measurement of the photoreceptor potential; in separate experiments using the isolated retina or receptor outer segment preparations, the formation of rhodopsin was measured spectrophotometrically. Treatments with the 11-cis isomers of retinal and retinol induced significant increases in both the rhodopsin content and photic sensitivity of previously bleached receptors. The all-trans isomers of retinyl palmitate, retinol, and retinal, as well as the 11-cis isomer of retinyl palmitate, were inactive by both the electrophysiological and spectrophotometric criteria for the generation of rhodopsin. Treatment with any one of the "inactive" retinoids did not abolish the capacity of subsequently applied 11-cis retinal or 11-cis retinol to promote the formation of rhodopsin. The data are discussed in relation to the interconversions of retinoids ("visual cycle of vitamin A") thought to mediate the regeneration of rhodopsin in vivo after extensive bleaching.

INTRODUCTION

For the regeneration of visual pigment to occur in the vertebrate retina, there must be provision for the supply of the retinoid chromophore 11-cis retinal (or 11-cis-3-dehydroretinal) to the opsin contained within the photoreceptor outer segments (Wald, 1968). Numerous studies have indicated that a sequence of reactions, occurring in darkness within the eye, mediates the formation of this chromophore from all-trans retinal (or all-trans-3-dehydroretinal) produced on the bleaching of visual pigment. Through these reactions, ocular retinoid is recycled for incorporation into the visual pigment formed during dark adaptation (Wald and Hubbard, 1949; Futterman, 1965, 1974; Bridges, 1976a).

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The mechanisms underlying the interconversion of retinoids within the eye are largely unknown and probably differ among vertebrate species. However, there is reason to believe that the process responsible for the supply of chromophore to opsin in dark-adapting rod photoreceptors depends centrally on the association of the retina with the retinal pigment epithelium (RPE). Much of the evidence supporting this notion has come from studies of the isolated retina preparation, obtained by separation of the neural retina from the RPE. In the isolated retina, rod photoreceptors ordinarily exhibit little or no regeneration of their visual pigment after a bleaching exposure (Weinstein et al., 1967; Baumann, 1970; Frank, 1971; Hood et al., 1973; Azuma et al., 1977; Pepperberg et al., 1978; but cf. Cone and Brown, 1969; Goldstein and Wolf, 1973). However, placement of the previously bleached, isolated retina in contact with the RPE stimulates considerable regeneration in the rods (Kühne, 1878; Reuter et al., 1971; Bridges, 1973). Furthermore, both the substantial regeneration of rod visual pigment and the slow, "photochemical" phase of dark adaptation that has been linked with the regeneration process (Dowling, 1960, 1963; Rushton, 1961; Pepperberg et al., 1978) are exhibited in the eyecup preparation, where the association between the retina and the RPE is preserved (Donner and Reuter, 1965, 1968; Dowling and Ripps, 1970).

Additional evidence that the resynthesis of chromophore depends on an interaction of the photoreceptors and the RPE has come from studies analyzing the distribution of retinoids within the ocular tissues during light and dark adaptation (Hubbard and Colman, 1959; Dowling, 1960; Hubbard and Dowling, 1962; Zimmerman, 1974; Bridges, 1975, 1976a). On the bleaching of rhodopsin in the rods, the quantity of retinoid contained within the RPE increases; most of this retinoid, when extracted from the RPE, is found to be in the form of retinyl ester. During a subsequent period of darkness, the level of retinoid in the RPE slowly decreases, and the level of (regenerated) rhodopsin in the photoreceptors increases. Throughout this process, the total amount of retinoid contained in the retina and RPE remains approximately constant.

The data just reviewed have been interpreted to suggest that the RPE itself, or the extracellular matrix that exists in vivo between the retina and the RPE, is the locus of at least one reaction that is crucial for the resynthesis of chromophore from the retinoid product of bleaching. As yet, however, information regarding the participation of ocular retinoids in the pathway of resynthesis is limited. Of particular interest are the site and immediate substrate of a reaction clearly involved in this pathway: the isomerization of retinoid from the all-trans to the 11-cis form. Evidence that such an isomerization reaction can occur (in darkness) within the retina has been reported (Hubbard, 1956; Cone and Brown, 1969), but it remains unclear whether this represents a major pathway supporting the regeneration of visual pigment (cf. Futterman and Rollins, 1973a, b; Rotmans et al., 1973; Futterman, 1974; Zimmerman et al., 1974; Groenendijk et al., 1980). (Under appropriate conditions, the photosomization of retinal may support a significant degree of regeneration [cf., for example, Reuter, 1966, 1976; Shichi and Somers, 1974; Azuma and Azuma, 1980].)
The experiments reported here were undertaken to gain further information on the processes which, in the intact eye, mediate the formation of 11-cis retinal and its appearance in the rod photoreceptors during the period following a bleaching irradiation. Our approach has been to examine which of several naturally occurring retinoids, when applied topically to the isolated retina, or added to preparations of photoreceptor outer segments, can promote the regeneration of rhodopsin (through conversion of the test substance to 11-cis retinal). Recent studies of several isolated retina preparations suggested the workability of an electrophysiological assay for this process. These studies indicated that the formation of rhodopsin, induced by incubation of the previously bleached retina with 11-cis retinal, promotes a substantial increase in the photic sensitivity of the rods (Pepperberg et al., 1976; Pepperberg and Masland, 1978; Albani et al., 1980). We reasoned that the measurement of receptor sensitivity could similarly afford the analysis, within demonstrably functional photoreceptors, of the activities of putative precursors of 11-cis retinal. For these experiments, we have used the retina of the bullfrog, *Rana catesbeiana.* The bullfrog and other ranid species have been used in previous studies of the interconversion of retinoids in the ocular tissues (Bridges, 1973, 1975, 1976a, b); of particular relevance to our study were biochemical data reported by Bridges (1977) and by Yoshikami and Nöll (1978), which indicated the capacity of externally applied 11-cis retinol to stimulate the formation of rhodopsin in the bleached retina of the frog. Furthermore, electrophysiological and absorbance properties of the rod photoreceptors in the isolated frog retina have been studied closely (Frank, 1971; Baumann, 1972; Donner and Hemilä, 1975, 1979; Hemilä, 1977; Bäckström and Hemilä, 1979). Some of the results presented here were reported at recent meetings of the Association for Research in Vision and Ophthalmology (Perlman and Pepperberg, 1981; Perlman et al., 1982).

**METHODS**

**Electrophysiology**

Bullfrogs (*Rana catesbeiana*), obtained from commercial suppliers, were maintained on a constant cycle of 8 h light (white light from a 5-W tungsten lamp positioned in the aquarium), 16 h darkness. Animals were killed by pithing after 8–12 h of dark adaptation. Dissections and all subsequent procedures were carried out under dim red light, at a room temperature of 21–23°C. After removal and hemisection of an eye, the eyecup was transferred to a 35-× 10-mm plastic dish (Falcon 3001; Becton, Dickinson & Co., Oxnard, CA), which contained ~6 ml of Ringer's solution. The standard, bicarbonate Ringer's solution, modified from that used by Miller and Steinberg (1977), contained: 82.5 mM NaCl; 2.0 mM KCl; 1.8 mM CaCl₂; 1.0 mM MgCl₂; 10 mM glucose; 10 mM l-aspartate; 27.5 mM NaHCO₃, bubbled with 95% O₂:5% CO₂, pH ≈ 7.2. While the eyecup was immersed in the Ringer's solution, the retina was gently teased away from the pigment epithelium and then fully isolated by cutting of the optic nerve.

The section of isolated retina (typically 1 cm in diameter) was placed photoreceptor side up on a 15-× 15-mm piece of filter paper (ashless; Whatman, Ltd., Maidstone, Kent, England), and excess Ringer's solution was drained away. The retina and its
base of filter paper were mounted on a second piece of filter paper (6 × 3 cm) which covered the sloping floor of a Lucite recording chamber; the design of this chamber, illustrated in Fig. 1, afforded both continuous replacement of the solution surrounding the retina and access to the retina from above (for the application of retinoid). Throughout each experiment, except during brief periods immediately preceding and following the application of retinoid, the retina was moistened by freshly bubbled Ringer's solution that flowed through the underlying filter paper. A peristaltic pump (Gilson Minipuls 2; Gilson Medical Electronics, Middleton, WI) maintained the flow of Ringer's solution at 1–2 ml/min. (On passage through the recording chamber, the bicarbonate-containing medium underwent an increase in pH. The pH of the solution, measured after movement through the filter paper to the approximate position of the retina, was 7.4.) The sides and base of the recording chamber were in contact with a jacket of brass plate; positioned beneath this jacket was a Cambion thermoelectric stage (model 806-1036-01; Cambridge Thermionic Corp., Cambridge, MA; the controlling unit was designed and constructed by P. Hamilton of Purdue University), which maintained the temperature of the chamber at 19–20°C.

Full-field test flashes, 0.2 s in duration, were delivered from above the retina; the duration of the stimulus was regulated by an interval generator (model 830; WPI Instruments, Inc., New Haven, CT) and an electronic shutter (Vincent Associates, Rochester, NY). The light source used for the test flashes was a tungsten-halogen lamp powered by a regulated supply (model SP20-10; Deltron, Inc., North Wales, PA; operating current = 6 A). Light from this source passed through a series of focusing lenses, Schott heat filters (KG-1, KG-3, and θ; Schott Optical Glass, Inc., Duryea, PA), and a Wratten 65A filter (maximum transmittance at 497 nm; Eastman Kodak Co., Rochester, NY), and was suitably attenuated with the use of Schott neutral density filters. For the dark-adapted retina, the physiologically effective irradiance of the unattenuated, Wratten 65A-filtered light was \(-4.3 \times 10^8\) quanta \((500 \text{ nm}) \cdot \mu\text{m}^{-2} \cdot \text{s}^{-1}\) (cf. Pepperberg et al., 1978). Adapting irradiations, 2–20 min in

![Diagram](image_url)
duration, used either unattenuated white light from the photostimulator or full-field orange light from a microscope illuminator (model 651; American Optical Corp., Buffalo, NY; light was filtered by passage through Schott KG-1 and KG-3 heat filters, and through a Wratten 22 [orange] cutoff filter). Spectrophotometric data (see below) indicated that these irradiations bleached ≥67% of the rhodopsin/porphyrnosin initially present in the dark-adapted retina.

The photoreceptor potential (rapid component of the PIII response to a test flash), isolated by the presence of L-aspartate in the Ringer's solution, was recorded transretinally (Sillman et al., 1969). A pipette filled with Ringer's solution, mounted in a Stoelting MH-1 microelectrode holder (Stoelting Co., Chicago, IL) and placed in contact with the upper, photoreceptor surface of the retina, served as the recording electrode. The reference electrode was a loop of platinum or chlorided silver wire positioned beneath the filter paper supporting the retina. Responses were amplified (P-16 amplifier; Grass Instrument Co., Quincy, MA), displayed on an oscilloscope (model 5113; Tektronix Inc., Beaverton, OR), and recorded on a pen writer (Brush model 220 or 2400; Gould, Inc., Cleveland, OH). Responses were measured with the use of AC amplification (band pass of 0.1–1,000 Hz) or DC amplification (0–1,000 Hz). An amplitude of 10 μV of the AC-amplified response routinely was used as the criterion for photoreceptor threshold; throughout this paper, photoreceptor sensitivity is defined as the inverse of the measured threshold. When DC amplification was used, the amplitude of the photoreceptor response to relatively weak test flashes (intensities within 1.5 log units of the threshold value) was defined as the peak voltage of the PIII response; the amplitude of the photoreceptor response to more intense flashes (intensities >1.5 log units above threshold, which typically elicited a discernible slow component of the PIII waveform; cf. Fig. 2) was defined as the peak voltage developing within 1.0 s after onset of the stimulus.

Spectrophotometry of the Isolated Retina

Absorption spectra were obtained with the use of a Cary 118 (Varian Instruments, Inc., Palo Alto, CA) or a Perkin-Elmer 320 (Perkin-Elmer Corp., Oakbrook, IL) spectrophotometer. An optical assembly positioned in the sample chamber of the spectrophotometer passed the scanning beam vertically through the retina. The solution used for preparation and incubation of the retina was either the standard, bicarbonate Ringer's (see above), or a modified solution (pH = 7.4) containing 10 mM Tris (Tris(hydroxymethyl)aminomethane; Sigma Chemical Co., St. Louis, MO) and no bicarbonate. L-Aspartate was omitted from the Ringer's solution used in several experiments; similar results were obtained in the presence and absence of L-aspartate. The dark-adapted retina was placed photoreceptor side up on a piece of Ringer-moistened filter paper so as to span a hole (3–8 mm in diameter) previously cut in the paper. The preparation was placed in a 35 × 10-mm Falcon dish and positioned on the horizontal stage of the optical assembly; the spectrophotometric experiments described in this paper did not use superfusion of the retina. The temperature of the preparation was maintained at 19–20°C by a metallic plate (thermoelectric or water-cooled stage) located immediately beneath the dish. Spectra were recorded over the range of 350–650 or 350–700 nm (absorbance at 650 or 700 nm was defined as reference) at a scanning rate of ≥200 nm/min; the scanning beam induced negligible bleaching of the retina. Bleaching irradiations used either orange (Wratten 22-filtered) light from a microscope illuminator, or white light from a General Electric Quartzline lamp (model EJY; General Electric Co., Cleveland, OH), which passed through a fiber optic guide. The standard application of hydroxylamine
was 100–150 μl of a 0.6-M solution of hydroxylamine, pH = 6.5 (cf. Pepperberg, 1982).

Relative absorbance at 537 nm was used as a measure of the prevailing level of visual pigment; long-lived photoproducts (e.g., metarhodopsin III) and added retinoids contribute relatively little absorbance at this wavelength. The formation of rhodopsin in treated retinas was judged to be insignificant if either (a) on incubation for >2 h with a test retinoid, the net increase in transretinal absorbance at 537 nm (referred to the value measured shortly after application of the retinoid) amounted to a recovery of ≤10% of the loss of absorbance at 537 nm resulting on the bleaching of the native visual pigment; or (b) application of hydroxylamine to the retina, after incubation with the retinoid, decreased the absorbance at 537 nm to a value below that corresponding to a 10% recovery.

**Preparation and Spectrophotometry of Photoreceptor Outer Segments**

The standard preparation, modified from that used by Wheeler and Bitensky (1977), was carried out under dim red light and used two retinas obtained from a single dark-adapted animal. Retinas were isolated in the standard manner (see above) after immersion of the eyecups in buffer A at room temperature. The composition of buffer A was: 117 mM NaCl; 2.0 mM KCl; 1.0 mM MgCl₂; 1.8 mM CaCl₂; 10 mM glucose; and 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), pH = 7.4. The retinas were transferred to a vial containing 2 ml of buffer B (24 mM HEPES and 0.54 g/ml sucrose, pH = 7.4). The capped vial was shaken manually for ~90 s. The contents of the vial then were transferred to a 3.7 ml polycarbonate centrifuge tube; the tube was filled by the addition of buffer B, and the preparation was centrifuged at 50,000 g at 4°C for 1 h (Beckman SW 50.1 rotor; Beckman Instruments, Inc., Palo Alto, CA).

After this initial centrifugation, during which the receptor outer segments accumulated near the air/fluid interface, an aliquot of fluid containing the receptor outer segments (volume ~1 ml) was gently withdrawn with the use of a Pasteur pipette and dispersed into ~1.5 ml of buffer A. This suspension was centrifuged at 20,000 g at 4°C for 15 min (Dupont/Sorvall SE-12 or SS-34 rotor; Dupont Instruments, Newton, CT). After this centrifugation, the supernatant was removed and discarded, and 0.6 ml of buffer A was added to the pellet. Material in the pellet was resuspended by repeated passage through a 25-guage, stainless steel hypodermic needle. The resulting suspension of receptor outer segments, henceforth referred to as the ROS preparation, typically contained ~0.8 mg of protein (Bradford, 1976; Markwell et al., 1978).

The experiments employed aliquots of 0.5 ml of the ROS preparation and used either a Cary 14 (Varian Instruments, Inc.) or a Perkin-Elmer 320 recording spectrophotometer. Experiments were performed at ambient temperatures of ~25 or ~34°C; qualitatively similar results were obtained under these two conditions. Absorption spectra were obtained over the range of wavelengths from 350 to 650 nm, at a scanning speed of >120 nm/min; the measured absorbance at 650 nm was defined as reference. For bleaching of the visual pigment, the cuvette containing the ROS preparation was removed from the sample chamber of the spectrophotometer and positioned 6 cm in front of a microscope illuminator. Light from this source passed through Schott KG-1 and KG-3 heat filters and a Wratten 22 cutoff filter, and then passed horizontally through the cuvette.

Suspensions containing retinoid were prepared in the standard manner (see below) and then supplemented with NADP⁺ (Sigma Chemical Co.), at a concentration of 1.5 mM, before addition to the ROS. Immediately after the addition of retinoid (25–30 μl of a suspension) or of hydroxylamine (100 μl of a 0.7-M solution, pH ~6.0),
the cuvette was capped and momentarily inverted for mixing of the contents. In addition, just before the recording of each spectrum (intervals of ~30 min), the contents of the cuvette were mixed by brief, gentle stirring with a thin Teflon rod.

**Retinoids**

Crystalline 11-cis retinal was the generous gift of Paul K. Brown of Harvard University. The 11-cis isomer of retinyl palmitate used in early experiments was generously provided by C. David Bridges (Baylor College of Medicine); this material was synthesized and purified through high-performance liquid chromatography (HPLC) by Dr. Bridges and by Dr. Richard A. Alvarez (Alvarez et al., 1981). Later experiments used 11-cis retinyl palmitate prepared in our laboratory; the procedures used were based on those described by Bridges and Alvarez (1982). The all-trans isomers of retinal, retinol, and retinyl palmitate were obtained from Sigma Chemical Co. Absorption spectra of retinoids were obtained in a Spectronic 505 spectrophotometer (Bausch & Lomb, Inc., Rochester, NY). Concentrations of retinoids were calculated with the use of published extinction coefficients (Brown and Wald, 1956; Hubbard et al., 1971).

The 11-cis isomer of retinol was prepared from 11-cis retinal by reduction with borohydride. The procedure, based on that described by Hubbard et al. (1971), was as follows. To a 13- × 100-mm test tube containing 2 ml of ethanol-water (9:1 by volume), 1-5 μmol of 11-cis retinal was added (typically, <100 μl of an ethanolic or hexane solution). The reduction was initiated by the addition of solid potassium borohydride (at least ~250 mg, representing at least ~900 times the molar quantity of 11-cis retinal present). After incubation at room temperature for ~5 min, the reaction mixture was supplemented with hexane (~1 ml) and thoroughly mixed; the resulting hexane phase, containing 11-cis retinol, was subjected to repeated extraction with water (total volume = 10–20 ml). In several control experiments, 11-cis retinal was omitted from the reaction mixture containing borohydride; the final hexane extract obtained from this mixture, to be referred to as “control extract,” was then supplemented with 11-cis retinal and used for preparation of a (control) suspension (see Fig. 6 and accompanying text).

The purity of the 11-cis retinol obtained by borohydride reduction was examined by HPLC (Bridges et al., 1980). The analyses used a Waters 6000A solvent delivery system, a U6K injection system, an RCM-100 radial compression module containing a 10-μm silica column, and a model 440 absorbance detector (Waters, Inc., Milford, MA). Elutions were carried out with 10% ether in hexane; absorbance was measured at 365 nm. By comparing the chromatographic profiles of 11-cis retinol preparations with those exhibited by known molar quantities of authentic 11-cis retinal (analyzed under identical conditions), we obtained values for the degree of contamination by 11-cis retinal. The level of contamination was found, on a molar basis (that is, [concentration of 11-cis retinal] + [concentration of 11-cis retinol]) to be 0.12 ± 0.21% (mean ± 1 SD for five preparations).

**Suspensions Containing Retinoid**

**Preparation** For addition to both isolated retina and ROS preparations, all retinoids were co-suspended with phosphatidylcholine (type VII-E chloroform solution, 100 mg/ml; Sigma Chemical Co.). The procedure routinely used was as follows. To a 1-dram vial were added an ethanolic or hexane solution of retinoid (typically 10–100 μl) and 150–200 μl of the commercially obtained solution of phosphatidylcholine. The mixture was dried under a gentle stream of nitrogen. To the residue was added 0.5 ml of the aqueous solution in use for incubation of the (retina or ROS)
preparation; the mixture was sonicated for 10 min (model W-375 sonicator, equipped with a tapered microtip, model 420; Heat Systems-Ultrasonics, Inc., Plainview, NY). During sonication, the vial containing the suspension was partially immersed in a water bath at room temperature. The suspensions were applied in dropwise fashion to the isolated retina (Pepperberg et al., 1976, 1978), or added in small volumes to the ROS preparation (see above).

**ANALYSIS** After sonication, an aliquot of the suspension (typically 20 μl) was added to 2 ml of hexane and the mixture was vigorously agitated (vortexing for 5-10 min). The concentration of retinoid in the hexane then was analyzed spectrophotometrically; the value obtained was used to calculate the concentration of (hexane-extractable) retinoid in the sonicated suspension. In later experiments, results obtained by this procedure were compared with those obtained when retinoid (and phosphatidylcholine) contained in a small aliquot of the suspension were solubilized by the addition of ethanol. We found that the routinely used (hexane) method underestimated the quantity of retinoid contained in the suspension; among 14 such comparisons, the concentration of retinoid determined by the method of ethanol solubilization was 3.4 ± 2.6 (± 1 SD) times as great as that determined by extraction with hexane. Thus, the concentration of suspended retinoid calculated on the basis of extraction with hexane should be viewed as a lower limit for the actual concentration of suspended retinoid. In this paper, $c_H$ and $c_E$ identify, respectively, concentrations determined by the procedures of hexane extraction and ethanol solubilization; $q_H$ and $q_E$ identify the molar quantity of retinoid delivered in each application ($q = [\text{calculated concentration}] \times [\text{volume applied}]$). In all experiments using the isolated retina, values of $q_H$ and $q_E$ were ≥24 nmol. We routinely obtained a complete absorbance spectrum of the retinoid which was extractable by hexane from the freshly prepared suspension. In several experiments, an extract of the suspension was similarly prepared and analyzed after completion of the experiment (i.e., several hours after preparation of the suspension). In all cases, the absorbance spectrum of the extracted material corresponded with that expected for the retinoid under study.

**RESULTS**

11-cis Retinal and 11-cis Retinol

Evidence that the appearance of 11-cis retinal in bullfrog photoreceptors can be measured electrophysiologically came from experiments involving the application of 11-cis retinal to previously bleached retinas. Fig. 2, which shows DC responses recorded from a single aspartate-treated retina, illustrates both the general protocol of our experiments and the sensitizing effect of treatment with 11-cis retinal. The data in column 1, obtained early in the experiment from the dark-adapted retina, illustrate the growth and ultimate saturation, with increasing intensity of the stimulating flash, of the rapidly developing, receptoral component of the PIII response. (Responses to relatively intense stimuli also exhibit a well-defined, more slowly developing component, believed to be due to the activity of the Müller cells; cf. Witkovsky et al., 1975.) Shortly after the recording of the responses in column 1, the retina was exposed for 20 min to intense orange light that bleached ~88% of the visual pigment initially present in the principal rods (i.e., those containing rhodopsin [$\lambda_{max} = 502$ nm] or porphyropsin [$\lambda_{max} = 522$ nm]; cf. Reuter et al., 1971; Witkovsky et al., 1981). Immediately after the extinction of this adapting light
(time zero in the experiment), the retina exhibited a partial recovery of responsiveness to the test flashes, presumably through the action of receptoral processes not dependent on the regeneration of visual pigment (Frank, 1971; Hood et al., 1973; Brin and Ripps, 1977; Donner and Hemilä, 1979). This

Figure 2. Effects of light adaptation and treatment with 11-cis retinal on PIII responses of the isolated retina. The DC responses shown in each column were obtained during periods of \( \leq 8 \) min. Numbers shown in parentheses above columns 2–4 identify the approximate times at which the data were obtained, relative to the time of extinction of the adapting light \( (t = 0 \text{ min}) \). Asterisks identify responses recorded at reduced sensitivity. At \( t = 86 \) min, 200 \( \mu l \) of a suspension containing 30–40 mg/ml of phosphatidylcholine (PC) was applied to the retina. At each of two later times \( (t \approx 169 \text{ and } 226 \text{ min}) \), 200 \( \mu l \) of a suspension containing 11-cis retinal \( (c_H = 1.4 \mu \text{mol/ml}) \) and phosphatidylcholine \( (30–40 \text{ mg/ml}) \) was similarly applied.

partial recovery, analyzed by periodic measurements of photoreceptor thresholds, was complete by \( t = 42 \) min (data not illustrated; for examples of the time course of this recovery, cf. Figs. 3 and 9). The responses of column 2, recorded over a brief period centered at \( t = 66 \text{ min} \), describe the stabilized condition attained after the bleaching irradiation. In this light-adapted state, the photoreceptors displayed a relatively high value of threshold, and the
saturating responses to intense stimuli were relatively small in amplitude (Hood et al., 1973; Donner and Hemilä, 1979; Bäckström and Hemilä, 1979). (Also evident in columns 2 and 3 is a notch separating the rapid and slow components of the responses to intense stimuli; previous studies have suggested that this feature of the PIII response is due in significant part to the relatively rapid decay of a cone-generated component of the photoreceptor potential [Whitten and Brown, 1973; Hemilä, 1977]. The data in columns 1 and 4 of

![Graph](image.png)

**Figure 3.** Treatment of a light-adapted retina with 11-cis retinol. The adapting irradiation (16 min in duration, using unattenuated white light from the photostimulator) was terminated at time zero; the filled circle at the left identifies the initial, dark-adapted value of photoreceptor threshold. Arrows indicate the times of application of 200 μl of a suspension containing 11-cis retinol (c 낱자 = 0.64 μmol/ml). During brief (<9 min) periods centered about the times shown by the upward-pointing arrows, complete response functions for the photoreceptor potential were obtained (cf. Fig. 4). The inset defines the parameters τ₁ and τ₂ used in Table I; the open circle in the illustrated hypothetical experiment represents the first data point obtained after application of the retinoid and the resumption of superfusion.

the figure also suggest the contribution of a cone component to the responses elicited by the more intense stimuli. It is unlikely, however, that the responses in columns 1 and 4 obtained on the presentation of relatively weak stimuli contain a significant contribution from the cones [cf. Discussion].) At t = 83 min, superfusion of the retina was stopped, and a suspension containing phosphatidylcholine, but no retinoid, was applied (over a period of <2 min) to the receptoral surface of the retina; superfusion then was resumed (t = 93
min). This treatment had little effect on the responsiveness of the preparation (column 3). At $t = 169$ min and $t = 226$ min, aliquots of a suspension containing 11-cis retinal and phosphatidylcholine were applied to the retina (during similar, brief interruptions of superfusion). In response to these treatments, there gradually developed large increases in the responses elicited by each of the test flashes; the responses in column 4 show the ultimate extent of these induced changes.

When similarly applied to previously bleached retinas, 11-cis retinol induced a significant, overall increase in photoreceptor sensitivity, evident both as a

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4}
\caption{Voltage-intensity data obtained during the experiment described in Fig. 3. Data illustrated by filled squares were obtained shortly before the onset of the adapting light; the remaining data were obtained during the periods identified by the corresponding symbol in Fig. 3. Shown at the right are (AC-amplified) responses to test flashes of log intensity = -3.9 and -3.4; accompanying symbols identify the series of responses to which these data belong.}
\end{figure}

lowering in the value of the threshold for a criterion response (Fig. 3) and as a shift of the photoreceptor response function in the direction of increased sensitivity (Fig. 4). (For brevity, Fig. 3 and later figures omit reference to the presence of phosphatidylcholine in all suspensions applied to the isolated retina or ROS.) Typically, treatment with 11-cis retinol promoted relatively little change in the maximal amplitude ($V_{\text{max}}$) of the photoreceptor potential, and the value of $V_{\text{max}}$ ultimately exhibited after treatment usually was significantly less than that exhibited before bleaching.

The effects of treatment with 11-cis retinol were relatively complex in that they, unlike changes observed with 11-cis retinal, typically included an initial, transient increase in the value of log threshold. Data relating to this phenomenon are contained in Table I, which summarizes results obtained in similar
**TABLE I**

**TREATMENT OF LIGHT-ADAPTED RETINAS WITH 11-cis RETINOL OR 11-cis RETINAL**

| Experiment | Retinoid applied (nmol)* | Plus retinoid§ | Dark-adapted | Light-adapted | \( \tau_1 \) \( \tau_2 \) |
|------------|--------------------------|----------------|--------------|---------------|------------------|
| 11-cis retinol |                         |                |              |               |                  |
| 1          | 440**                    | -5.7           | -4.2         | -3.7          | -5.3            | 60 27            |
| 2¶         | 130                      | -4.9           | -3.7         | -3.6          | -3.9            | >34 >25          |
| 2¶¶        | 130                      | -2            | -3.7         | -3.8          | -4.7            | 36 15            |
| 3          | 190                      | -5.8           | -3.9         | -3.7          | -4.7            | 121 77           |
| 4          | 140                      | -5.3           | -3.9         | -3.5          | -3.9            | >98 >88          |
| 4¶¶        | 70                       | -3.6           | -4.3         | -3.6          | -4.3            | >206 67          |
| 5          | 1,100**                  | -5.8           | -4.4         | -3.9          | -4.7            | >108 34          |
| 6          | 400                      | -5.1           | -3.9         | -2.9          | -4.4            | 142 17           |

| 11-cis retinal |                         |                |              |               |                  |
| 7            | 680                      | -5.5           | -3.9         | -4.1          | -5.3            | 21 19            |
| 8            | 290                      | -5.7           | -3.8         | -3.7          | -4.1            | >44 >35          |
| 8¶¶         | 530                      | -3.3           | -4.3         | -3.6          | -4.6            | 24 >24           |
| 9            | 810                      | -5.2           | -3.3         | -3.6          | -4.7            | 6 2              |
| 10           | 200                      | -4.9           | -2.7         | -3.7          | -4.1            | <6 >43           |
| 10¶¶        | 200                      | -3.9           | -4.6         | -3.9          | -4.6            | 68 11            |
| 11¶¶        | 440                      | -5.7           | -3.9         | -4.6          | -5.5            | 10 36            |
| 12¶¶        | 220                      | -5.5           | -3.9         | -4.3          | -5.3            | 10 12            |
| 13¶¶        | 240                      | -5.2           | -3.8         | -3.8          | -4.3            | 14 19            |
| 13¶¶¶        | 240                      | -5.2           | -3.8         | -4.4          | -5.0            | 26 23            |

* The quantity of retinoid applied was, in most cases, determined by the method of hexane extraction (qH); in experiments 1 and 5, applied 11-cis retinol was quantitated by the method of ethanol solubilization (qE; values are accompanied by a double asterisk).

† Stabilized value of threshold attained after the exposure to intense light.

§§ A is the value of log threshold obtained on the first measurement after application of the retinoid and the resumption of superfusion (typically, =12 min after the application). B is the value of log threshold ultimately attained, in the absence of (or immediately before) further treatment.

|| For definition of the parameters \( \tau_1 \) and \( \tau_2 \), see inset of Fig. 3. In some cases, the experiment was terminated, or the retina was further treated with retinoid, before a decrease of 0.5 log unit (measured from the appropriate starting level; cf. Fig. 3) was attained. In these cases, the appropriate period of measurement was taken as a lower limit for \( \tau_1 \) or \( \tau_2 \).

†† This experiment is illustrated in Figs. 3 and 4.

††† The indicated data describe the effects of a second application of the retinoid. The value of log threshold prevailing immediately before this second treatment was used as a reference for determination of \( \tau_1 \).

§§ Before the application of 11-cis retinal, the retinas of experiments 11 and 12 were treated with all-trans retinyl palmitate. In each experiment, the value of log threshold measured immediately before initial application of the 11-cis retinal was =-3.9. Experiment 11 is illustrated in Fig. 9.

|| Before the application of 11-cis retinal, the retina was treated with all-trans retinol. The value of log threshold measured immediately before the initial application of 11-cis retinal was =-3.2. This experiment is illustrated in Fig. 11.
experiments using 11-cis retinol or 11-cis retinal. To describe the time course of changes in log threshold observed in the experiments of Table I, we have employed two time parameters, $\tau_1$ and $\tau_2$ (cf. inset of Fig. 3). These parameters characterize, respectively, the period after each treatment which was required for a net decrease of 0.5 log unit ($\tau_1$), and the period required for a decrease of 0.5 log unit from the value of the first data point obtained after application of the retinoid ($\tau_2$). As the data of Table I show, the effects of treatment with each retinoid varied with respect to time course and overall extent; however,

![Figure 5](image)

**Figure 5.** Treatment of the isolated retina with 11-cis retinol. An initial bleach of the retina (white light, 30 s in duration) was terminated at time zero. At $t \approx 93$ and 306 min, respectively, the preparation received 150 µl of a suspension containing 11-cis retinol ($c_R = 0.16 \mu$mol/ml), and 150 µl of 0.6 M hydroxylamine. After these treatments, the retina again was exposed to the bleaching light ($t = 341-343$ min). At each wavelength, data illustrated in the figure represent values of absorbance referred to that measured immediately after the second bleach. The key identifies the prevailing conditions and the approximate times at which the data were obtained.

the sensitizing change induced by 11-cis retinol appeared to be somewhat slower in its development than that occurring on treatment with 11-cis retinal. For example, in experiment 1 of Table I, the value of threshold measured immediately after treatment was 0.5 log unit higher than that measured immediately before treatment; the development of a net decrease in threshold of 0.5 log unit (i.e., attainment of a logarithmic value of $-4.7$) required a period of 60 min from the time of treatment. The value of $\tau_1$ measured in this experiment was among the smaller of those observed with 11-cis retinol, but
it was larger than the values of $\tau_1$ observed in the majority of experiments involving 11-cis retinal.

Fig. 5 illustrates absorbance data obtained from a retina which, after bleaching of the native visual pigment, was treated with 11-cis retinol; this

![Graph showing absorbance data](image)

**Figure 6.** Formation of rhodopsin in bleached retinas. In each of the seven experiments described, 11-cis retinal or 11-cis retinol, suspended in the Ringer's solution used for incubation of the retina, was delivered in a single application after bleaching of the native visual pigment (\(\geq 74\%\)) and the stabilization of absorbance at 537 nm. The time of an absorbance measurement carried out \(\leq 4\) min after application of the retinoid defined time zero; the value of absorbance at this time was taken as the reference value used for calculation of $\Delta A$. Data for each experiment are plotted as the net change in absorbance at 537 nm ($\Delta A$), relative to $(\Delta A)_0$, the overall loss of absorbance induced by the prior bleach. The key identifies the retinoid applied and the type of Ringer's solution used in each experiment. Quantities of retinoid applied were (in nanomoles): $\Delta$, $q_H = 68$; $\triangledown$, $q_R = 49$; $\triangle$, $q_H = q_E = 910$; $\bullet$, $q_E = 510$; $\diamond$, $q_H = 24$; $\square$, $q_H = 130$; and $\blacksquare$, $q_H = 150$. For the experiment described by $\bullet$, an aliquot of "control extract" was used for preparation of the suspension containing 11-cis retinal.

experiment employed incubation of the retina with the bicarbonate Ringer's solution used in all of the electrophysiological experiments described in Table I. Treatment with 11-cis retinol led to the formation in the preparation of a substance which, on the basis of its absorption spectrum in the presence of hydroxylamine and its sensitivity to light, was identified as rhodopsin.

Fig. 6, which illustrates changes in absorbance at 537 nm exhibited by
bleached retinas after treatment with either 11-cis retinol or 11-cis retinal, compares the effects of these treatments on the rhodopsin content of the photoreceptors. For each experiment illustrated, values of absorbance are referred to the value measured shortly after application of the (suspended) retinoid. This was done to exclude from consideration the increase in absorbance at 537 nm that occurred on the introduction of the turbid suspension. The data of Fig. 6 indicate that, in retinas incubated in the bicarbonate Ringer's solution (open symbols), the formation of rhodopsin with 11-cis retinal proceeded more rapidly than with 11-cis retinol. For example, with retinal, a recovery amounting to 25% of the absorbance change induced by bleaching occurred within less than ~40 min after treatment of the retina; with retinol, a similar percentage recovery required ~100 min. Regenerations with both 11-cis retinol and 11-cis retinal also were examined in the presence of Tris-buffered Ringer's solution. The time courses and extents of regenerations in the Tris-containing medium (filled triangles and filled squares in Fig. 6) did not differ substantially from those exhibited in the presence of the bicarbonate Ringer's solution.

We considered the possibility that the relative slowness of regenerations occurring on treatment with 11-cis retinol was due to the presence in the applied suspension of some (unidentified) inhibitory substance, introduced during and carried through the preparation of 11-cis retinol. To test this possibility, we subjected several bleached retinas to treatment with a suspension prepared from a mixture of 11-cis retinal and "control extract" (cf. Methods); data obtained from a representative experiment are illustrated in Fig. 6 by the filled circles. The similarity between regenerations observed in these experiments and those observed under standard conditions with 11-cis retinal argues against the possibility just raised.

Evidence that the photoreceptors themselves are a principal site for the oxidation of 11-cis retinol came from experiments using ROS preparations. Under our experimental conditions, additions of 11-cis retinol and NADP⁺ (Bridges, 1977) to bleached ROS induced regenerations that, with respect to both rate and overall extent, typically exceeded those induced in the intact retina (Fig. 7). Accompanying the formation of rhodopsin in such preparations was the appearance of material that exhibited considerable absorbance at wavelengths near 420 nm, and that, unlike rhodopsin, was reactive with hydroxylamine. To elucidate the nature of this material, we analyzed the effects of addition of 11-cis retinol and NADP⁺ to unbleached ROS, where little or no formation of rhodopsin would be expected to occur. Results obtained in the presence and absence of hydroxylamine (squares and circles, respectively, in Fig. 8) showed that increases in absorbance near 420 nm develop in unbleached ROS on supplementation with 11-cis retinol and NADP⁺, and that the material formed is reactive with hydroxylamine. The data of Figs. 7 and 8 are consistent with the possibility that, in both bleached and unbleached ROS, the hydroxylamine-reactive material absorbing near 420 nm is free 11-cis retinal (generated from the added 11-cis retinol) and/or one or more Schiff-base derivatives of the retinal formed by interaction with
amine-containing substances in the ROS. It remains to be determined whether hydroxylamine, when present throughout the period of incubation with 11-cis retinol, merely scavenges the retinal product (forming retinyl oxime; cf. Hubbard et al., 1971) or also influences the rate of generation of 11-cis retinal. (The hydroxylamine-reactive material evident in Figs. 7 and 8 [also cf. Fig. 5] could consist, in part, of the blue-sensitive rod visual pigment [\(\lambda_{\text{max}} \approx 430-440\) nm] observed to constitute a minor fraction of the visual pigment in frog ROS preparations [Reuter, 1966; Dartnall, 1967; Makino et al., 1980; also cf. Witkovsky et al., 1981]. Unlike the rhodopsin of the principal rods, the blue-sensitive rod pigment is readily degraded by hydroxylamine [Reuter, 1966; Dartnall, 1967]. However, results obtained on the addition of hydroxylamine to unbleached ROS gave no indication of the presence of a rapidly degrading pigment [data not illustrated].)

**Retinyl Palmitate, All-trans Retinol, and All-trans Retinal**

Examination of the activity of retinyl palmitate was of central interest in this study; retinyl palmitate is among the most abundant of the esterified retinoids found in the pigment epithelium of the frog, and it has been hypothesized...
that all-trans retinyl ester is the substrate for a reaction, occurring within the photoreceptors, which mediates the all-trans to 11-cis isomerization crucial for the ultimate resynthesis of 11-cis retinal (Bridges, 1976a). Under our experimental conditions, both all-trans and 11-cis retinyl palmitate lacked sensitizing activity (Figs. 9 and 10); neither of these retinoids abolished the capacity of the receptors to respond to subsequent treatment with 11-cis retinal.

In spectrophotometric experiments, we observed no significant increase in the level of rhodopsin when either bleached retinas or bleached ROS were treated with 11-cis retinyl palmitate (data not illustrated). For example, in
one experiment, after incubation of an isolated retina for 126 min with 650 nmol (qe) of this ester, the prevailing value of \(\Delta A/(\Delta A)_o\) at 537 nm (cf. Fig. 6 and accompanying text) was only 0.07; subsequent treatment of the retina with 630 nmol (qe) of 11-cis retinol led, within 117 rain, to an (additional) increase of 0.23 in \(\Delta A/(\Delta A)_o\) at 537 nm. Another experiment involved successive treatment of a retina with 11-cis retinyl palmitate (qe = 880 nmol, applied at \(t = 0\) min) and hydroxylamine (applied at \(t = 255\) min). Again, the

![Figure 9](image-url)  
**Figure 9.** Electrophysiological effects of treatment with all-trans retinyl palmitate. The filled circle identifies the initial, dark-adapted value of photoreceptor threshold. At each of the two times indicated by arrows, the retina received 200 \(\mu\)l of a suspension containing all-trans retinyl palmitate (\(c_H = 1.2 \, \mu\text{mol/ml}\)); the later, single application of 11-cis retinal (\(c_H = 2.2 \, \mu\text{mol/ml}\)) was 200 \(\mu\)l. Shown in the inset are (AC-amplified) responses obtained during the experiment on presentation of a test flash of log intensity = -3.9. The times of recording of these responses were as follows: A, prior to light adaptation; B, at \(t = 48\) min; C, at \(t = 166\) min; and D, at \(t = 336\) min.

incubation with retinyl palmitate caused no significant change in \(\Delta A/(\Delta A)_o\) at 537 nm; values of this parameter prevailing at \(t = 252\) min and \(t = 286\) min were, respectively, 0.07 and -0.02.

Treatment of the bleached retina with all-trans retinol failed to induce a sensitization of the receptors (Fig. 11) or the formation of rhodopsin (data not illustrated). As the data of Fig. 12 illustrate, incubation of the bleached retina with all-trans retinal induced significant increases in transretinal absorbance at wavelengths near 470 nm (inset), but only relatively small changes in absorbance occurred at wavelengths (e.g., 537 nm) indicative of the formation
of rhodopsin. Data obtained on the subsequent application of hydroxylamine gave further indication that little or no rhodopsin had been formed during the incubation with all-trans retinal; in the presence of hydroxylamine, values of absorbance at wavelengths near 537 nm were similar to those exhibited immediately before treatment with the all-trans retinal. The hydroxylamine-dependent changes in absorbance evident in Fig. 12 presumably resulted from the conversion to retinyl oxime of either free all-trans retinal, or Schiff-base derivatives of this retinoid, formed by interaction of the applied retinal with amine-containing substances in (or on the surface of) the retina (Shichi and Somers, 1974; Groenendijk et al., 1980; also cf. text accompanying Figs. 7 and

![Graph showing electrophysiological effects of treatment with 11-cis retinyl palmitate.](image)
8, above). All-trans retinal had no sensitizing effect on bleached receptors; in both electrophysiological and spectrophotometric experiments, 11-cis retinal exhibited significant activity when applied after treatment of the retina with the all-trans isomer (data not illustrated).

**DISCUSSION**

**11-cis Retinal and 11-cis Retinol**

Our results indicate that the formation of visual pigment in the bleached, isolated retina of the bullfrog, induced by the topical application of 11-cis retinal, leads to a pronounced increase in photoreceptor sensitivity. The presence of several types of photoreceptors in the bullfrog retina tends to complicate the analysis of the extracellularly recorded photoreceptor potential. However, several lines of evidence suggest that the sensitizing activity of 11-cis retinal observed here arises largely, if not entirely, from the ability of this retinoid to promote the formation of rhodopsin in the principal rods. Goldstein and Wolf (1973) have reported that, in the bleached, isolated retina of the frog, the visual pigment contained in the blue-sensitive rods regenerates spontaneously. Electrophysiological evidence further suggests that, in this
preparation, considerable regeneration of the visual pigment of the principal cones occurs after a bleaching exposure (Goldstein, 1967, 1970; Hood and Mansfield, 1972; Hood and Hock, 1973). However, there is general agreement that principal rods in the isolated frog retina spontaneously regenerate at most only a small fraction of their visual pigment (Baumann, 1970; Azuma et al., 1977; Yoshikami and Nöll, 1978; and present data). These observations, and other studies cited earlier (cf. text accompanying Fig. 2), indicate that, in the present experiments, the sustained depression of sensitivity exhibited after bleaching resulted in significant part from the (permanent) depletion of visual pigment in the principal rods. Thus, our treatments with 11-cis retinal increased sensitivity (lowered threshold) from a level that probably was governed by the responsiveness of the principal rods.

Figure 12. Treatment of the isolated retina with all-trans retinal (Tris Ringer). Open symbols identify values of absorbance at 537 (O), 477 (□), and 430 nm (Δ); filled symbols at the left identify values of absorbance at the corresponding wavelength measured before an initial bleaching irradiation (orange light, 20 min in duration, terminated at time zero). Further treatment of the retina (times indicated by arrows) involved: the application of 150 μl of a suspension containing all-trans retinal (cR = 7.3 μmol/ml); the application of hydroxylamine; and a second exposure to the bleaching light (t = 472–474 min). For each wavelength, values of absorbance are referred to the value measured immediately after the second bleaching irradiation (time indicated by the "×"); cf. experiment of Fig. 5. The difference spectrum shown in the inset represents changes in absorbance occurring between t = 224 and t = 434 min.
Further indication that applied 11-cis retinal influences the sensitivity of principal rods comes from the consideration of thresholds attained by treated retinas. In several experiments, final values of receptor threshold exhibited after the application of 11-cis retinal (to the bleached, desensitized retina) differed by only 0.2 log unit from the value exhibited before bleaching. Under the conditions of stimulation and recording used in this study, cones and blue-sensitive rods would be expected to contribute negligibly to near-threshold responses recorded from the fully dark-adapted retina (Frank, 1970; Hood, 1972; Hood et al., 1973); thus, it is unlikely that recoveries to levels within several tenths of a log unit of this minimal, dark-adapted value could be mediated solely by sensitization of the cones or blue-sensitive rods.

For reasons not yet clear, the response of bleached photoreceptors to treatment with 11-cis retinol was more complex than that occurring on similar treatment with 11-cis retinal. (The transient elevation of threshold observed with 11-cis retinol, and the absence of a substantial recovery of $V_{\text{max}}$, could be due to a [nonspecific] deteriorative activity of retinol on the photoreceptors; cf. Fig. 11 and Bangham et al., 1964.) However, incubation with 11-cis retinol led ultimately to a net sensitization of the photoreceptors, an effect consistent with the ability of this retinoid to promote the regeneration of rhodopsin in situ. The present data obtained from both the intact retina and ROS preparations support the interpretation, arising from previous biochemical results (Bridges, 1977; Yoshikami and Nöll, 1978), that frog photoreceptors possess at least one oxidoreductase which under physiological conditions can catalyze the generation of 11-cis retinal from externally applied 11-cis retinol.

The existence of a receptoral mechanism for the oxidation of 11-cis retinol appears not to be a universal feature of the vertebrate eye. For example, in studies of the mammalian eye, the capacity for oxidizing 11-cis retinol has been detected in fractions obtained from the RPE, but not in those obtained from the retina (Daemen et al., 1974; Lion et al., 1975; Zimmerman et al., 1975; Zimmerman, 1976; however, cf. Sack, 1982). Further indication that the mammalian retina lacks the capacity to oxidize 11-cis retinol comes from the observation that treatment of the bleached, isolated retina of the rat with vesicles containing 11-cis retinol does not promote the regeneration of rhodopsin (Yoshikami and Nöll, 1978, footnote 9). We have found that 11-cis retinol, when applied to the bleached, isolated retina of the rabbit, fails to induce a sensitization of the photoreceptors (unpublished observations); under similar experimental conditions, 11-cis retinal exhibits considerable sensitizing activity (Pepperberg and Masland, 1978).

It is of interest to compare the spectrophotometric data of this study with those obtained from the isolated retina of *R. pipiens* by Yoshikami and Nöll (1978). Despite differences in the species investigated, conditions of incubation, and method of treatment with retinoid used in the two studies, there is a fairly close agreement in the maximal rate of rhodopsin formation ($\approx 0.3-0.4%/\text{min}$) exhibited by the bleached retina on incubation with 11-cis retinol. However, our results with 11-cis retinal differ considerably from theirs. We observed an apparently maximal rate of regeneration of $\approx 1.0-1.5%/\text{min}$
on the application of retinal in quantities \((q \text{m})\) of \(<1,000\ \text{nmol}\) (cf. Fig. 6); on
the conservative assumption that our sections of bleached, isolated retina
contained at least \(2\ \text{nmol}\) of opsin (cf. Bridges, 1977), such quantities of retinal
would correspond to \(<500\ \text{mol}\) applied \(11\text{-cis}\) retinal per mole of opsin.
Yoshikami and Nöll observed maximal rates of regeneration (sustained for
relatively longer periods) of \(~0.2\%/\text{min}\) on treatment, by superfusion of the
retina, with \(1,260\ \text{mol}\ 11\text{-cis}\) retinal per mole of opsin (Fig. 2 of their paper),
and of \(~0.5\%/\text{min}\) when the molar ratio of superfused \(11\text{-cis}\) retinal
to available opsin was \(6,900\) (their footnote 15). These considerations suggest
that, in the isolated retina preparation, quantities of \(11\text{-cis}\) retinal required for
near-maximal rates of induced regeneration may depend significantly on the
methodology used for application of the retinoid.

All-trans Retinal and All-trans Retinol

There is ample evidence that all-trans retinal is a direct product of the
bleaching event. However, much if not all of the all-trans retinal so generated
is thought to undergo conversion, within the photoreceptors, to all-trans retinol;
furthermore, at least in the intact eye, most of the all-trans retinoid generated
on bleaching is transported (or diffuses) out of the retina (Wald and Hubbard,
1949; Hubbard and Dowling, 1962; Futterman, 1963; Zimmerman, 1974;
Bridges, 1976a; Brin and Ripps, 1977). The departure from the retina of
retinol and/or retinal in the all-trans form suggests the absence of a receptoral
process capable of (rapidly) isomerizing either substance to the \(11\text{-cis}\) config-
uration. However, data available prior to this study did not directly address
the possibility that isomerization of (one or both of) these retinoids in frog
photoreceptors occurs gradually during the 2–3 h period within which rod
visual pigments in the frog eye fully regenerate after virtually complete
bleaching (Zewi, 1941; Peskin, 1942; Donner and Reuter, 1965, 1968; Reuter,
1966; Bridges, 1976a). Under this possibility, a steady supply of all-trans retinal
or all-trans retinol from a source outside the retina (e.g., the RPE) would
support the slow but ultimately significant accumulation of \(11\text{-cis}\) retinal (and
therefore, rhodopsin) in the photoreceptors. The present experiments, involv-
ing prolonged incubation of the bleached retina with large quantities of these
all-trans retinoids, provided a relatively direct test of this possibility. Our
results argue against a role for either substance as an immediate substrate for
dark isomerization in the receptors.

Retinyl Palmitate

Retinyl esters constitute a major fraction of retinoid found in the RPE.
However, the significance of the esterifying reaction, and the involvement of
retinyl esters in the resynthesis of \(11\text{-cis}\) retinal, have remained unclear. A
principal aim of this study was to test an interesting hypothesis, directly
relevant to these questions, which was developed by Bridges several years ago
(1976a). On the basis of several lines of evidence, Bridges postulated a direct
link between the esterification of retinol, known to occur predominantly in
the RPE, and the dependence of visual pigment regeneration on the associa-
tion of the photoreceptors with the RPE; he hypothesized that all-trans retinyl ester, once formed in the RPE, returns to the retina, where it serves as the substrate of an isomerase located in the photoreceptors. Our results with retinyl palmitate, a likely candidate for this substrate in view of its abundance in the pigment epithelium, do not disprove this hypothesis in its most general form; it is conceivable, for example, that 11-cis retinyl palmitate is generated (from the all-trans isomer) solely in a receptoral reaction, but must return to the RPE for hydrolysis to 11-cis retinol. However, our data suggest the absence in the receptors of either (a) an efficient mechanism for the hydrolysis of 11-cis retinyl palmitate, or (b) a single, complex reaction generating 11-cis retinol from all-trans retinyl palmitate. Thus, our results argue against the operation of certain, relatively simple pathways based on Bridges' hypothesis, i.e., pathways involving a single passage of retinoid to the RPE for each round of synthesis of 11-cis retinal.

\( \text{Scheme for the Visual Cycle of Vitamin A} \)

The preceding considerations lead naturally to a reexamination of two questions bearing on the pathway of resynthesis of 11-cis retinal in the bullfrog eye; Fig. 13, which illustrates postulated steps in this pathway, is intended as a focus for the following discussion. (The interpretations that follow concern the interconversion of A1 retinoids. It is likely, however, that analogous reactions mediate the formation of 11-cis-3-dehydroretinal from all-trans A2 retinoids arising on the bleaching of porphyropsin; cf. Reuter et al., 1971; Bridges, 1973; Tsin and Beatty, 1980.) First, what is the identity of the retinoid which, during dark adaptation, is returned to the retina to support the regeneration of rhodopsin? Available data suggest that this substance is either 11-cis retinol or 11-cis retinal. The ability of externally applied 11-cis retinol to promote regeneration in electrophysiologically active photoreceptors of the isolated retina clearly emphasizes the possibility that, in vivo, the principal substance returned is 11-cis retinol. Interestingly, 11-cis retinol is thought to be less susceptible than 11-cis retinal to thermal or catalytic isomerization in vivo (Futterman and Rollins, 1973a, b; Sack and Seltzer, 1978; Groenendijk et al., 1980). As yet, however, there is no firm basis for
excluding 11-cis retinal alone, or a mixture of the two retinoids, as the substance(s) delivered to the retina. The incorporation in Fig. 13 of dual pathways linking 11-cis retinol outside the photoreceptors with 11-cis retinal inside the receptors is intended to reflect the uncertainty on this point.

Second, what is the identity of the all-trans retinoid which, in vivo, functions as the substrate for isomerization (in darkness) to the 11-cis form? The nature of this isomerization mechanism remains unclear, and it is likely that conclusive identification of the substrate(s) involved will come only on successful isolation of the substance(s) mediating this reaction. However, we believe it useful to comment here on several notions regarding the isomerization step. Central to the scheme shown in Fig. 13 is a proposition that previous investigators have discussed (cf., for example, Futterman, 1965): namely, that all-trans retinol functions as the substrate for isomerization in a reaction occurring outside the retina (i.e., in the RPE, or in the extracellular space separating the retina and the RPE). This possibility is consistent with the observations of the present study. It supposes that isolation of the retina, which involves the removal of the RPE and (presumably) the dispersal of the native fluid residing between the retina and RPE, results in the loss of isomerase activity, and thus eliminates the capacity for conversion of any all-trans retinoid to 11-cis retinol.

Clearly, however, the possibility of an all-trans to 11-cis isomerization of retinyl ester, occurring in concert with the extraretinal hydrolysis of 11-cis retinyl ester, presents no inconsistency with the observations reported here. Why, then, do we suppose that all-trans retinol is the principal substrate for isomerization? This view is based on the implications of previous data, obtained by Hubbard and Dowling (1962), and by Bridges (1976a), regarding the depletion and replenishment of the ocular store of 11-cis retinyl ester during and after bleaching in the intact eye of the frog (also cf. Alvarez and Bridges, 1981; Tsin and Bridges, 1982). These studies showed that, during a period of irradiation that bleaches most of the rhodopsin in the photoreceptors, the ocular level of 11-cis retinyl ester decreases significantly; however, the quantity of rhodopsin regenerated during a subsequent period of dark adaptation substantially exceeds, on a molar basis, the overall decrease in the level of 11-cis retinyl ester (i.e., the decrease cumulatively developing over the periods of bleaching and regeneration). Furthermore, on completion of the regeneration of rhodopsin (attained at ~2–3 h of dark adaptation), the level of 11-cis retinyl ester remains well below the maximal level prevailing in animals kept in darkness for much longer periods (≥20 h). A possibility noted by Hubbard and Dowling that could account for these observations, and which is incorporated within the scheme of Fig. 13, is that 11-cis retinyl ester is not an obligatory intermediate in the principal pathway mediating the resynthesis of 11-cis retinal. The 11-cis retinyl ester may function only as a storage form of 11-cis retinoid which, after bleaching, reaccumulates (through esterification of 11-cis retinol, its presumed immediate precursor) over a period greatly exceeding that required for the regeneration of rhodopsin (Hubbard and Dowling, 1962; Futterman, 1965).
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