Squid Retinochrome

LINDA SPERLING and RUTH HUBBARD

From the Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138 and the Marine Biological Laboratory, Woods Hole, Massachusetts 02543. Ms. Sperling's present address is M. R. C. Laboratory of Molecular Biology, Cambridge, England.

ABSTRACT Retinochrome is a photosensitive pigment located primarily in the inner portions of the visual cells of cephalopods. Its absorption spectrum resembles that of rhodopsin, but its chromophore is all-trans retinal, which light isomerizes to 11-cis, the reverse of the situation in rhodopsin. The 11-cis photoproduct of retinochrome slowly reverts to retinochrome in the dark.

The chromophoric site of retinochrome is more reactive than that of most visual pigments: (a) Hydroxylamine converts retinochrome in the dark to all-trans retinal oxime + retinochrome opsin. (b) Sodium borohydride reduces it to N-retinyl opsin. (c) λmax of retinochrome shifts from 500 to 515 nm as the pH is raised from 6 to 10, with a loss of absorption above pH 8; meanwhile above this pH a second band appears at shorter wavelengths with λmax 375 nm. These changes are reversible. (d) If retinochrome is incubated with all-trans 3-dehydroretinal (retinal3) in the dark, some 3-dehydroretinochrome (retinochrome3, λmax about 515 nm) is formed. Conversely, when retinochrome3, made by adding all-trans retinal3 to bleached retinochrome or retinochrome opsin, is incubated in the dark with all-trans retinal some of it is converted to retinochrome. Retinal and 3-dehydroretinal therefore can replace each other as chromophores in the dark.

INTRODUCTION

Retinochrome is a photosensitive pigment found in the visual cells of cephalopods. In contrast to cephalopod rhodopsin, which is located in the rhabdomal membranes of the outer segments, retinochrome is localized primarily in the inner portions of the cells. It was first identified in the retinas of the Japanese squid, Todarodes pacificus, by Hara and Hara (1965) who subsequently found it in all species of squid, cuttlefish, and octopus that live in Japanese waters (cf. Hara and Hara, 1972). We have extracted it from the retinas of the Atlantic squid, Loligo pealei (Sperling and Hubbard, 1971). Dark-adapted retinas of Todarodes pacificus contain approximately the same amounts of retinochrome and rhodopsin, whereas those of Loligo pealei have approximately three to four times more rhodopsin than retinochrome (Hara and Hara, personal communication). This difference may in part reflect the very different conditions under which the animals are collected. Todarodes...
in Japan are collected from deep waters at night and are never exposed to daylight after capture, whereas at Woods Hole, *Loligo* are collected from shallower waters in the daytime, often in bright sunlight, and dark adapted several hours later in laboratory tanks in which they usually do not survive more than a day.

The absorption spectrum of retinochrome is similar to that of rhodopsin, with a main (α) band with $\lambda_{\text{max}}$ near 500 nm and a subsidiary β band in the near ultraviolet, both owing to absorption by the retinylede chromophore and a narrow γ band with $\lambda_{\text{max}}$ near 280 nm owing primarily to absorption by the aromatic amino acid residues of the protein. In all the species of cephalopods that have been examined, $\lambda_{\text{max}}$ of the main band of retinochrome lies at a somewhat longer wavelength than that of rhodopsin, but the precise position of $\lambda_{\text{max}}$ differs (cf. Hara and Hara, 1972). An important difference between retinochrome and rhodopsin lies in the geometrical configuration of their chromophores: the chromophore of retinochrome is in the all-trans configuration and is isomerized by light to 11-cis (Hara and Hara, 1967, 1968), whereas rhodopsin has an 11-cis chromophore that is photoisomerized to all-trans. Preliminary accounts of the properties of *Loligo* retinochrome have appeared elsewhere (Sperling and Hubbard, 1971, 1973; Hubbard and Sperling, 1973 a).

**METHODS**

Our method of extracting retinochrome is similar to that of Hara and Hara (1972). 25–50 retinas are used for each preparation and all operations are carried out at about 4°C in dim red light. Squids are dark adapted in running seawater at least 6 h in dim room light, followed by 2 h in total darkness. The eyes are excised and stored about 10 h at 4°C, since chilling the retinas splits them between the inner and outer segments of the visual cells. The chilled eyes are hemisected, the lens and anterior chamber removed, and the eyecups shaken in M/15 phosphate buffer, pH 6.3, to float off the outer segments and screening pigments. The remaining retina, which contains the inner segments and nervous tissue, is peeled out of the eyecup with fine forceps, homogenized by hand in a glass-Teflon homogenizer with a small amount of the same phosphate buffer, and collected by centrifugation at 15,000 rpm in a Spinco-Beckmann preparative centrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). All subsequent washings are carried out by stirring with about 12 ml solvent and centrifuging at this speed.

The homogenate is washed once with 1% Na$_2$CO$_3$ to remove a soluble pink pigment, several times with distilled water, then hardened for a few minutes in M/5 KH$_2$PO$_4$ and washed again with distilled water. It is soaked 1 h in Weber-Edsall solution (0.6 M KCl, 0.04 M NaHCO$_3$, 0.01 M Na$_2$CO$_3$) and washed successively in distilled water, M/5 KH$_2$PO$_4$ and more distilled water. It is finally extracted 1 h with 1–2 ml 2% digitonin (Merck & Co., Inc., Rahway, N. J.) in M/15 phosphate buffer, pH 6.3. Unless otherwise specified our observations are made with 0.5-ml samples at this pH. The pH of individual samples is changed by addition of small
amounts of solid Na₂CO₃ or KH₂PO₄. At room temperature, the solutions are stable for many hours between pH 6 and 7 and for shorter times at higher pH's, depending on the age and purity of the extracts. When frozen and stored at −15°C, they change slowly as described below. Emulphogene, a relatively mild detergent that is sometimes used to extract rhodopsin, destroys the spectral integrity of retinochrome.

The chromophore-free protein portion of retinochrome, which we call retinochrome ops or opsin, is prepared by following the same procedure in diffuse room light, with one extra step. Before the tissue is extracted with digitonin, it is soaked 5 min in several milliliters M/5 hydroxylamine (NH₂OH), pH 6.5, and washed with four 12-ml portions of distilled water to remove the excess NH₂OH. The digitonin extracts contain small amounts of retinaldehyde oxime, which cannot be removed by extracting the lyophilized tissue with petroleum ether, as is usual in the preparation of cattle opsin (cf. Hubbard et al., 1971), since we find that petroleum ether destroys retinochrome opsin. The contaminating retinaldehyde oxime is inert, but since it absorbs in the near ultraviolet, the absorption spectrum of the opsin preparation must be used to correct the spectra we measure in experiments in which we use opsin (cf. Figs. 8 and 9). Retinal and 3-dehydroretinal are dissolved in 2% digitonin in M/15 phosphate buffer, pH 6.3, as described by Hubbard et al. (1971).

Absorption spectra are measured with a Perkin-Elmer 402 (Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.) or a Cary model 14 (Cary Instruments, Monrovia, Calif.) recording spectrophotometer against a distilled water blank and subsequently corrected for absorption by 2% digitonin. This is necessary because digitonin precipitates from water or buffer solutions much more rapidly than from tissue extracts. The absorption spectrum of 2% digitonin departs from the base line below about 520 nm and slowly rises to an absorbance of about 0.3 at 320 nm. Unless otherwise stated, retinochrome is bleached by irradiation with orange light from a tungsten filament microscope lamp shielded by a Wratten 12 filter.

RESULTS

Reactions of Retinochrome with Hydroxylamine and with Sodium Borohydride

Fig. 1 shows the α and β bands and the toe of the γ band of Loligo pealei retinochrome. In fresh solutions at pH 6.3, λmax of the α band is at 500 nm. Addition of NH₂OH in the dark converts retinochrome to opsin and all-trans retinaldehyde oxime with λmax 367 nm, as shown in the figure. The isomeric identity of the product has been established by Hara and Hara (1967, 1968), whose findings we confirm. The rate of the reaction depends on the concentration of NH₂OH; in 0.2 M NH₂OH the reaction goes to completion in a few minutes at room temperature. The experiment in Fig. 1 can be used to calculate the molar absorption coefficient (εmax) of retinochrome (cf. Wald and Brown, 1953). We find that it is approximately 40,000

1 Hara and Hara (personal communication) prefer the name apo-retinochrome for the protein portion of retinochrome.
FIGURE 1. Bleaching of retinochrome by hydroxylamine in the dark. To 0.5 ml of a fresh solution of retinochrome at pH 6.3 ($\lambda_{\text{max}}$ 500 nm), 0.01 ml 1 M NH$_2$OH was added in dim red light and the spectrum was measured after 5 min at 25°C. The $\alpha$ and $\beta$ bands of retinochrome are replaced by the absorption band of all-trans retinaldehyde oxime ($\lambda_{\text{max}}$ 367 nm).

Sodium borohydride (NaBH$_4$) also attacks retinochrome in the dark and reduces it to N-retinyl opsin, with $\lambda_{\text{max}}$ near 330 nm. Addition of a small grain of powdered borohydride to 0.5 ml retinochrome effects the reduction as quickly as we can measure the spectrum at room temperature (cf. also Hara and Hara, 1973 a).

**pH Dependence of the Absorption Spectrum**

The absorption spectrum of retinochrome changes with pH as shown in Fig. 2. In this experiment the pH of a retinochrome solution is raised in steps from 6.3 to 9.7. The solution had been prepared at pH 6.3 and stored 3 wk at $-15^\circ$C, during which time $\lambda_{\text{max}}$ began to shift to shorter wavelengths as described in the next section. The initial spectrum at pH 6.3 therefore has $\lambda_{\text{max}}$ 495 nm and the subsequent spectra also are displaced by about 5 nm from their positions in fresh solutions. As the pH is raised to 7.8 (Fig. 2 a), $\lambda_{\text{max}}$ moves to longer wavelengths and the absorption at $\lambda_{\text{max}}$ increases slightly. There are isosbestic points at 412 and 487 nm. As the pH is increased above 7.8 (Fig. 2 b), $\lambda_{\text{max}}$ of the long wavelength band continues to move toward the red, but its absorption decreases and a new band appears at shorter wavelengths, with $\lambda_{\text{max}}$ 375 nm. All these changes are reversible as long as the pH remains below 10. The shift in $\lambda_{\text{max}}$ of the main absorption band...
follows a straight line when the ratio of absorptions at 530 and 470 nm ($A_{530}/A_{470}$) is plotted as a function of pH (Fig. 2 c).

**Effects of Aging and of Cysteine**

When excised eyes or retinochrome solutions are stored at $-15^\circ C$, $\lambda_{max}$ begins to shift to shorter wavelengths, although the other properties of the pigment remain unchanged.\(^2\) The shift is larger in more acid solutions. The most shifted spectrum we have seen is shown in curve 1 of Fig. 3. The solution used in this experiment was prepared at pH 6 and stored 7 mo at $-15^\circ C$. During this time, $\lambda_{max}$ moved from 500 to 475 nm, the position shown in

\(^2\) This type of spectroscopic change does not happen with *Todarodes* retinochrome (Hara and Hara, personal communication).
Figure 3. Effects of aging and of cysteine. A solution of retinochrome at pH 6 was stored 7 mon at -15°C, at which time $\lambda_{\text{max}}$ had shifted from its initial position at 500 nm to 475 nm (curve 1). Within 2 min after addition of cysteine to a final concentration of 0.02 M, $\lambda_{\text{max}}$ had shifted to 491 nm (curve 2) and after 30 min to 496 nm (curve 3). Addition of cysteine to a final concentration of about 0.02 M, shifts $\lambda_{\text{max}}$ to 491 nm within 2 min (curve 2) and to 496 nm after 1/2 h (curve 3). Similar effects are observed when cysteine is added to preparations in which $\lambda_{\text{max}}$ has shifted less. We have not been able to mimic the effect of aging by bubbling oxygen through the solution or by adding copper sulfate or ferricyanide.

Bleaching of retinochrome, the 11-cis photoprodut

The bleaching of retinochrome by orange light is shown in curves 1 and 2 of Fig. 4 (see also Fig. 7). Hara and Hara (1967) have shown that this involves the photoisomerization of the all-trans chromophore to the 11-cis configuration. We have confirmed this for Loligo retinochrome by incubating the products of its thermal denaturation and its photoprodut with excess cattle opsin. Like the Haras we find that the former does not react with opsin, but the latter combines with it to form rhodopsin.

The photoprodut (curve 2) has a broad absorption band maximal in the neighborhood of 440 nm and a secondary hump near 370 nm. The absorbance of the photoprodut is much lower than that of retinochrome and the ratio of the areas under the two absorption spectra is approximately 1:2. The secondary hump may in part be due to absorption by 11-cis retinal that has been hydrolyzed off the chromophoric site, since the 11-cis photoprodut is less stable than retinochrome. However this is unlikely, because synthesis of the 11-cis photoprodut from 11-cis retinal and retinochrome yields a compound with two absorption maxima like those shown in Figs. 4 and 7 (cf. Hara and Hara 1973 a and b).

Like retinochrome, the 11-cis photoprodut is converted to opsin + retinaldehyde oxime upon addition of NH$_2$OH, though the oxime is of course 11-cis. It is also reduced by NaBH$_4$. However, Hara and Hara (1973 a) have
shown that the product formed by NaBH₄ is at least in part retinal. At alkaline pH, the photoproduct is converted to a compound with λ_max about 370 (see Fig. 5, curve 2; also Hara and Hara, 1973 a), which does not change back to the original spectrum of the 11-cis photoproduct upon lowering the pH to 6.3. The 370-nm compound is a mixture of Schiff bases of retinal with amino groups on opsin of the sort that is usually called alkaline indicator yellow (cf. Hara and Hara, 1973 a).

**Regeneration of Retinochrome from the 11-cis Photoproduct**

The 11-cis photoproduct slowly reverts to retinochrome in the dark. At pH 6.3 (Fig. 4), the absorption spectra measured during the first few hours of the regeneration have isosbestic points at 407 and 490 nm, which suggests that the photoproduct initially gives rise to at least two compounds one of which absorbs at shorter wavelengths with an apparent λ_max at about 375 nm, the other at longer wavelengths (apparent λ_max above 490 nm). The absorption spectrum measured after 19 h of regeneration (curve 6) resembles the initial retinochrome spectrum shown in curve 1, with λ_max 500 nm. Almost all the retinochrome present at the beginning of the experiment is regenerated in 3 days at 25°C.

The regeneration of retinochrome at pH 9.5 is shown in Fig. 5. The product of bleaching (curve 2; λ_max 370 nm) disappears as retinochrome regenerates, with an isosbestic point near 450 nm.

---

**Figure 4.** Thermal regeneration of retinochrome from its photoproduct at pH 6.3. A fresh solution of retinochrome (curve 1) was irradiated 5 min with orange light. The photoproduct, shown in curve 2, was left in the dark at 28°C and spectra were recorded 28 min (3), 45 min (4), 2.5 h (5), and 19 h (6) after the bleaching light was turned off. Curves 2-5 cross at 407 and 490 nm.
FIGURE 5. Thermal regeneration of retinochrome from its photoproduct at pH 9.5. Retinochrome (curve 1) was irradiated 20 min with orange light. The photoproduct, shown in curve 2, was incubated in the dark at 15-18°C. Absorption spectra were recorded 3 h (3), 4.45 h (4), and 17 h (5) after the bleaching light was turned off. Curves 2–5 cross at approximately 450 nm.

The rates of regeneration at pH 6.4 and 9.5 are shown in Fig. 6 for two experiments at 23.5°C. Along the ordinate is plotted log, of the concentration of a hypothetical precursor of retinochrome on the assumption that at the moment the light is turned off, its concentration is the same as that of photoisomerized retinochrome. At later times, t, the concentration of the precursor therefore is the difference between its initial concentration and the concentration of regenerated retinochrome. Concentration is measured in terms of absorbance. The absorbance at 0 time, A0, is expressed as the difference at 530 nm between the absorbances of the original sample of retinochrome and of its photoproduct at the moment the light is turned off. A0 is the absorbance at 530 nm at time, t, minus the same correction. Since the photoproducts absorb 530 nm light (cf. Figs. 4 and 5) this procedure introduces errors of about 10–20%.

Fig. 6 presents the data in terms of the kinetic equation for the first-order reaction, precursor → retinochrome, although the reaction clearly is not homogeneous in acid solution (Fig. 4), nor probably at pH 9.5 where the starting material is probably a mixture of Schiff bases with several different amino groups on opsin. The fact that at both pH's the points fall on straight lines after the first few minutes, suggests that whatever the mechanisms, the rates of the regenerations are limited by one reaction, which need not be the same at the two pH's.
Fig. 6 shows that the rate is about nine times faster at pH 9.5 than at pH 6.4; the rate constants, \( k \), are, respectively, \( 2.0 \times 10^{-6} \) and \( 2.4 \times 10^{-4} \) s\(^{-1}\). Experiments at two temperatures show marked increases in the rates of regeneration with temperature.

**Retinochrome**

Hara and Hara (1968, 1973b) have shown that incubation of irradiated *Todarodes* retinochrome or of retinochrome opsin with all-trans retinal yields retinochrome with a half-time of a half minute or less at room temperature. The comparable synthesis of the *Loligo* pigment is also rapid. By analogy, it is possible to synthesize the artificial retinal\(_2\) (3-dehydroretinal) analog of retinochrome by adding all-trans 3-dehydroretinal as shown in Fig. 7. Hara and Hara (personal communication) have prepared a similar pigment from *Todarodes* retinochrome.

Fig. 7 shows an experiment in which retinochrome at pH 6.4 (curve I,
FIGURE 7. Synthesis of retinochrome from bleached retinochrome and all-trans retinal. A fresh solution of retinochrome at pH 6.4 (curve 1, \( \lambda_{\text{max}} = 500 \) nm) was irradiated 5 min with orange light. The photoproduct shown in curve 2 was incubated in the dark with the approximate molar equivalent of all-trans retinal. Spectrum 3 was recorded after 5 min at 23°C. It has a long wavelength band with \( \lambda_{\text{max}} = 515 \) nm owing to absorption by retinochrome and a short wavelength band maximal near 380 nm owing to absorption by 11-cis retinal that has been displaced from the photoproduct with perhaps a small admixture of excess all-trans 3-dehydroretinal.

with \( \lambda_{\text{max}} = 500 \) nm is bleached 5 min with orange light. The photoproduct shown in curve 2 is incubated in the dark with an approximate molar equivalent of all-trans retinal and spectrum 3 is recorded 5 min later. It exhibits a long wavelength band with \( \lambda_{\text{max}} \) about 515 nm due to absorption by retinochrome (3-dehydroretinochrome) and a band in the near ultraviolet due to the mixture of excess all-trans retinal and of the 11-cis retinal it has displaced from the photoproduct.

Replacement of the Chromophores of Retinochrome and Retinochrome by all-trans Retinal and Retinal in the Dark

Fig. 7 shows that all-trans retinal in the dark replaces the 11-cis chromophore of bleached retinochrome. Fig. 8 shows that it also replaces the all-trans chromophore of retinochrome. Curve 1 is the absorption spectrum of a sample of retinochrome prepared by mixing a solution of retinochrome opsin at pH 6.3 with a slight molar excess of all-trans retinal in order to make sure that all the chromophoric sites are occupied by retinal. A 1.6-fold excess of all-trans retinal is then added in the dark and curve 2 is recorded as quickly as possible. Curves 3, 4, and 5 are measured at stated times during the subsequent 2 h in the dark at 23°C, after which we observe no further changes.
Conversion of retinochrome to retinochrome<sub>2</sub> by addition of all-trans retinal<sub>2</sub> in the dark. Retinochrome opsin was incubated 2 h with a small excess of all-trans retinal at pH 6.3 and room temperature to convert all the opsin to retinochrome. It was then stored 2 days at −15°C, thawed, and the absorption spectrum measured. The absorption spectrum of the opsin used to synthesize the retinochrome was subtracted from this and all subsequent spectra. Curve 1, the corrected spectrum of the thawed sample, therefore, is the absorption spectrum of the retinochrome chromophore and of any excess retinal that was left over after all the opsin had been converted to retinochrome. A 1.6-fold excess of all-trans retinal<sub>2</sub> was added in dim red light and spectra were recorded after 1.8 min (2), 7.5 min (3), 30.7 min (4), and 2 h (5) in the dark at 23°C. The long wavelength limb of the absorption band moves to longer wavelengths; there is an isosbestic point at about 505 nm.

As can be seen from the figure, the absorbance rises at long wavelengths and falls at shorter wavelengths with an isosbestic point near 505 nm as a pigment is formed that absorbs at longer wavelengths than retinochrome. If all-trans retinal is added at this time, the long wavelength limb of the absorption band shifts back toward shorter wavelengths.

The reverse experiment is shown in Fig. 9. In this experiment an aliquot of the opsin used in Fig. 8 is incubated at pH 6.3 with excess all-trans retinal<sub>2</sub> to form the mixture of retinochrome<sub>2</sub> and excess retinal<sub>2</sub> shown in curve 1. An approximate molar equivalent of all-trans retinal is now added in the dark and the calculated spectrum of the mixture at the instant of mixing is plotted in curve 2. Curves 3–5 are recorded at stated intervals during the next few hours in the dark. The spectra are isosbestic at 520 nm as the long wavelength absorption band shifts to shorter wavelengths. This is the opposite of the changes seen in Fig. 8 and suggests that a pigment is formed that absorbs at shorter wavelengths than does retinochrome<sub>2</sub>. If all-trans retinal<sub>2</sub> is added to the mixture shown in curve 5, the long wavelength absorption band shifts back to longer wavelengths.
FIGURE 9. Conversion of retinochrome2 to retinochrome by addition of all-trans retinal in the dark. An aliquot of the opsin solution used in Fig. 8 was incubated with an excess of all-trans retinal1 h in the dark at room temperature and its spectrum measured and corrected as described in Fig. 8. Curve 1 is the corrected spectrum of the chromophore of retinochrome, plus the excess all-trans retinal that remains after all the opsin has been converted to retinochrome. An approximately equimolar amount of all-trans retinal was added to the sample in dim red light and the calculated spectrum of the mixture plotted in curve 2. It represents the absorption spectrum of the experimental sample at 0 time. The mixture was incubated in the dark at 23°C and spectra were recorded after 1.6 min (3), 3.8 min (4), and 200 min (5). The long wavelength limb of the absorption band moves to shorter wavelengths; there is an isosbestic point at 520 nm.

DISCUSSION AND CONCLUSIONS

Color of the Retinochrome Chromophore

The chromophores of the visual pigments are protonated Schiff bases of 11-cis retinal with aliphatic amino groups on opsin. In cattle and squid rhodopsin the anchoring amino group has been identified as the e-amino group of an internal lysine residue (Bownds, 1967; Hagins, 1973). After the photoisomerization of the rhodopsin chromophore, the intermediates of bleaching continue to be protonated until the transition from metarhodopsin I (acid metarhodopsin in cephalopods) to metarhodopsin II (alkaline metarhodopsin in cephalopods) (for a recent discussion of the color of these chromophores, see Hubbard and Sperling, 1973 6).

The retinochrome chromophore is analogous to the protonated chromophores of the visual pigments and participates in an acid-base equilibrium
like that of the cephalopod metarhodopsins (cf. Hubbard and St. George, 1958; Hara and Hara, 1972). The reaction is illustrated in Fig. 2 b and can be described by the equation,

\[
\text{retinochrome} \longrightarrow \text{retinochrome-375} = \text{retinochrome-500} \quad \text{[1]}
\]

In addition, in retinochrome \( \lambda_{\text{max}} \) of the protonated form itself is pH variant as shown in Fig. 2. The only other instance of this has recently been observed in crayfish rhabdomes, in which \( \lambda_{\text{max}} \) of what is assumed to be a visual pigment changes reversibly with pH (Goldsmith, 1973). We have elsewhere discussed possible mechanisms of this color change (Hubbard and Sperling, 1973 b).

**Accessibility of the Chromophoric Site**

The fact that the Schiff base linkage in retinochrome loses and adds protons depending upon the pH of the solution shows that it must be accessible to the solvent. By contrast we must assume that the Schiff base linkage in the pH invariant pigments is shielded from the solvent because it is buried inside the hydrophobic portions of opsin or perhaps because it is surrounded by phospholipid molecules that are known to be associated with the visual pigments in the photoreceptor membranes (cf. Chen and Hubbell, 1973).

The retinochrome chromophore is accessible also to other hydrophylic molecules such as \( \text{NH}_3\text{OH} \) and \( \text{NaBH}_4 \) (cf. Fig. 1). The reactions are summarized in Eqs. 2 and 3,

\[
\begin{align*}
\text{retinochrome} + \text{NH}_3\text{OH} & \rightarrow \text{retinal oxime} + \text{opsin} \\
\text{retinochrome} + \text{NaBH}_4 & \rightarrow \text{N-retinyl opsln}
\end{align*}
\]

No known rhodopsin reacts with \( \text{NH}_3\text{OH} \) or \( \text{NaBH}_4 \) before it is photoisomerized and transformed to metarhodopsin, but the cone pigment, chicken iodopsin, reacts in the dark with low concentrations of \( \text{NH}_3\text{OH} \) (Wald et al., 1954-55) and with \( \text{NaBH}_4 \) (Matsumoto et al., in press).

Another indication that the chromophoric site of retinochrome is accessible

---

3 Though Eqs. 2 and 3 are written with the imino nitrogens in their protonated (quaternary) form, we do not wish to imply that \( \text{NH}_3\text{OH} \) and \( \text{NaBH}_4 \) react with the protonated chromophores. The equations merely are meant to show that at the pH at which the reactions have been carried out, the bulk of the starting material and of the product is protonated.
to the surrounding solvent is the capacity of all-trans retinal and 3-dehydroretinal to replace each other in the dark as shown in Figs. 8 and 9. It is usual for a more firmly bound chromophore to replace the more loosely bound product of its photoisomerization. For example, once the 11-cis chromophore of rhodopsin is photoisomerized to trans, it is released from opsin more rapidly in the presence of added 11-cis retinal (Hubbard, unpublished experiments).

In the same way, the 11-cis photoprodct of retinochrome is attacked by all-trans retinal and converted to retinochrome with the release of 11-cis retinal (Hara and Hara, 1968; Fig. 7 above). What is unusual in the present case is that two molecules which are bound equally well replace each other. This reaction is in some ways analogous to reaction 2 in which the amino group of NH₂OH displaces the amino group on opsin. In the exchange reaction summarized in Eq. 4, the retinyl and 3-dehydroretinyl radicals displace each other, so forming equilibrium mixtures of retinochrome and 3-dehydroretinochrome:

\[
\begin{align*}
+ & \quad C_{19}H_{27} \cdot C(H) = N(H) \cdot R + C_{19}H_{27} \cdot C(H) = O \\
\text{retinochrome} & \quad \text{retinal} \\
\text{retinochrome} & \quad \text{retinal}
\end{align*}
\]

This type of reaction has not been observed before. However recently Matsumoto et al. (in press) have shown that incubation of chicken iodopsin with 9-cis retinal in the dark yields iodopsin, perhaps by a similar mechanism.

**Isomerase Activity and Stereospecificity of Retinochrome**

Hara and Hara (1968) have shown that catalytic amounts of retinochrome in orange light (that is, light not absorbed by retinal) isomerize all-trans retinal to 11-cis. More recently they have shown that retinochrome opsin accepts also the 13- and 9-cis isomers of retinal as chromophores and converts them to 11-cis in orange light (Hara and Hara, 1973 b). Retinochrome opsin therefore is a photoisomerase with a low stereospecificity for its substrate that converts all the substrates to 11-cis retinal. In the dark, retinochrome opsin isomerizes 11-cis retinal to trans and regenerates retinochrome as shown in Figs. 4 and 5. The opsin-activated isomerization of 11-cis retinal proceeds at much lower temperatures and exhibits a much higher activation energy \(E_a\) than the thermal isomerization of 11-cis retinal in aqueous digitonin (cf. Hubbard, 1966).

Like retinochrome opsin, the retinal isomerase that has been extracted from cattle retinas converts 11-cis retinal to all-trans in the dark and catalyzes
the reverse isomerization in orange light. However, it does not accept any
other isomer of retinal as substrate nor form stable complexes with either
isomer.

Physiological Function of Retinochrome

Hara and Hara (1972) have shown that most of the retinochrome is located
between the basement membrane and the nuclei of the inner segments of
the visual cells. This region contains bundles of membranes that have been
described by Zonana (1961), by Yamamoto et al. (1965), and by Cohen
(1973). The organelles stain like rhabdomal membranes and have been
called myeloid or somal bodies. It seems likely that retinochrome is located
in the somal bodies since the cellular elements from which it is extracted
by means of detergents float on 40% sucrose (Hara and Hara, 1968; Sperling
and Hubbard, 1971).

Electron micrographs of the rhabdomal outer segments show membranous
filaments within the cytoplasmic core of the rhabdomes (Zonana, 1961;
Cohen, 1973; Sperling et al., unpublished observations). Hara and Hara
(personal communication) have extracted small amounts of retinochrome
from outer segments, where it may be contained in these membranes.

It has been suggested that retinochrome functions in the resynthesis of
rhodopsin by exchanging the 11-cis chromophore of its photoproduct for
the all-trans chromophore of metarhodopsin. In this way, rhodopsin and
retinochrome would be regenerated simultaneously from their respective
photoproducts. There are several problems with this hypothesis. In the first
place, very little retinochrome occurs in the rhabdomes, which contain a
lot of rhodopsin (cf. Hubbard and St. George, 1957–58). Photons are there-
fore much more likely to be absorbed by rhodopsin than by the retinochrome
in the outer segments and considerably more rhodopsin will be isomerized
to trans than retinochrome to 11-cis. Secondly, cephalopod metarhodopsins
are stable at physiological temperatures in the presence of excess 11-cis retinal
(Hubbard, unpublished observations) or of photoisomerized retinochrome
(see Hara and Hara, 1968, 1972). It is therefore difficult to see how retino-
chrome in the outer segments can be a source of retinal for regenerating
rhodopsin by a light-dependent mechanism.

It is possible that retinochrome could be isomerized in the inner segments
and the 11-cis photoprodut migrate into the outer segment. However, pre-
liminary experiments suggest that little, if any, of the retinochrome in the
inner segments is photoisomerized in vivo, probably because it is shielded
from incoming light by dense layers of black screening pigment as well as
by rhodopsin in the rhabdomes (Sperling and Hubbard, 1971; Hara and
Hara, personal communication). It is possible that the all-trans chromophore
of retinochrome is isomerized to 11-cis by an unknown thermal reaction.
However, the stability of cephalopod metarhodopsin makes it difficult to see how providing it with 11-cis retinal can regenerate rhodopsin. Another possibility needs to be explored. In bright light the pupil of cephalopod eyes becomes very small and a dense layer of black screening pigment moves to the tips of the outer segments. Very little light therefore reaches the rhodopsin in the visual cells. Even in bright daylight only about 5% rhodopsin is isomerized to metarhodopsin in vivo (Sperling, unpublished experiments; Hara and Hara, personal communication). Perhaps the small bits of rhabdomal membrane that contain photoisomerized rhodopsin are extruded and replaced by somal membrane and during this exchange retinochrome is converted to rhodopsin.

We should like to thank Drs. Reiko and Tomiyuki Hara for many helpful discussions and Dr. Toru Yoshizawa for showing us results of unpublished experiments performed in his laboratory. This work was supported in part by grants from the National Eye Institute of the National Institutes of Health to Ruth Hubbard and to George Wald and by a grant to G. W. from the National Science Foundation.

References

BOWNDS, D. 1967. Site of attachment of retinal in rhodopsin. Nature (Lond.). 216:1178.

CHEN, Y. S., and W. L. HUBBELL. 1973. Temperature- and light-dependent structural changes in rhodopsin-lipid membranes. Exp. Eye Res. 17:517.

COHEN, A. I. 1973. An ultrastructural analysis of the photoreceptors of the squid and their synaptic connections. I. Photoreceptive and non-synaptic regions of the retina. J. Comp. Neurol. 147:351.

Daw, N. W., and A. L. Pearlman. 1974. Pigment migration and adaptation in the eye of the squid, Loligo pealei. J. Gen. Physiol. 63:22.

GOLDSMITH, T. H. 1973. Dependence of absorption on pH in rhabdoms of crayfish (Orconectes, Procambarus). Biol. Bull. 145:1436.

HAGINS, F. M. 1973. Purification and partial characterization of the protein component of squid rhodopsin. J. Biol. Chem. 248:3298.

HARA, T., and R. HARA. 1965. New photosensitive pigment found in the retina of the squid Ommastrephes. Nature (Lond.). 206:1331.

HARA, T., and R. HARA. 1967. Rhodopsin and retinochrome in the squid retina. Nature (Lond.). 214:573.

HARA, T., and R. HARA. 1968. Regeneration of squid retinochrome. Nature (Lond.). 219:450.

HARA, T., and R. HARA. 1972. Cephalopod retinochrome. In Handbook of Sensory Physiology H. Autrum, R. Jung, W. R. Loewenstein, D. M. MacKay, and H. L. Teuber, editors. Vol. VII/1: Photochemistry of Vision H. J. A. Dartnell, editor. Springer-Verlag, Berlin. 729-746.

HARA, T., and R. HARA. 1973 a. Biochemical properties of retinochrome. In Biochemistry and Physiology of Visual Pigments H. Langer, editor. 181-191. Springer-Verlag, Berlin.

Hara, T., and R. Hara. 1973 b. Isomerization of retinal catalysed by retinochrome in the light. Nature (Lond.). 242:39.

Hubbard, R. 1966. The stereo-isomerization of 11-cis-retinal. J. Biol. Chem. 241:1814.

Hubbard, R., P. K. Brown, and D. Bownds. 1971. Methodology of vitamin A and visual pig-

4 Cited in Daw and Pearlman (1974).
ments. In Methods in Enzymol. V. 18: Vitamins and Coenzymes, Part C. D. B. McCormick and L. D. Wright, editors. Academic Press, Inc., New York. 615-653.

HUBBARD, R., and R. C. C. ST. GEORGE. 1958. The rhodopsin system of the squid. J. Gen. Physiol. 41:501.

HUBBARD, R., and L. SPERLING. 1973 a. Accessibility of the chromophoric site of squid retinochrome. Biol. Bull. (Woods Hole). 145:440.

HUBBARD, R., and L. SPERLING. 1973 b. The colors of the visual pigment chromophores. Exp. Eye Res. 17:581.

MATSUMOTO, H., F. TOKUNAGA, and T. YOSHIZAWA. Accessibility of the iodopsin chromophore. In press.

SPERLING, L., and R. HUBBARD. 1971. The identification of retinochrome in Loligo pealei. Biol. Bull. (Woods Hole). 141:402.

SPERLING, L., and R. HUBBARD. 1973. Squid retinochrome. Biophys. Soc. Annu. Meet. Abstr. 13:232 a.

WALD, G., and P. K. BROWN. 1953. The molar extinction of rhodopsin. J. Gen. Physiol. 37:189.

WALD, G., P. K. BROWN, and P. H. SMITH. 1955. Iodopsin. J. Gen. Physiol. 38:623.

YAMAMOTO, T., K. TABASI, Y. SUGAWARA, and A. TONOSAKI. 1965. Fine structure of the octopus retina. J. Cell Biol. 25:345.

ZONANA, H. V. 1961. Fine structure of the squid retina. Bull. Johns Hopkins Hosp. 109:185.