

# Squid Retinochrome

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**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)  $\lambda_{\text{max}}$  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  $\lambda_{\text{max}}$  375 nm. These changes are reversible. (d) If retinochrome is incubated with *all-trans* 3-dehydroretinal (retinal<sub>2</sub>) in the dark, some 3-dehydroretinochrome (retinochrome<sub>2</sub>,  $\lambda_{\text{max}}$  about 515 nm) is formed. Conversely, when retinochrome<sub>2</sub>, made by adding *all-trans* retinal<sub>2</sub> 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 ( $\alpha$ ) band with  $\lambda_{\text{max}}$  near 500 nm and a subsidiary  $\beta$  band in the near ultraviolet, both owing to absorption by the retinylidene chromophore and a narrow  $\gamma$  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<sub>2</sub>CO<sub>3</sub> to remove a soluble pink pigment, several times with distilled water, then hardened for a few minutes in M/5 KH<sub>2</sub>PO<sub>4</sub> and washed again with distilled water. It is soaked 1 h in Weber-Edsall solution (0.6 M KCl, 0.04 M NaHCO<sub>3</sub>, 0.01 M Na<sub>2</sub>CO<sub>3</sub>) and washed successively in distilled water, M/5 KH<sub>2</sub>PO<sub>4</sub> 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  $\text{Na}_2\text{CO}_3$  or  $\text{KH}_2\text{PO}_4$ . 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  $-15^\circ\text{C}$ , 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 opsin* or *opsin*,<sup>1</sup> 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 ( $\text{NH}_2\text{OH}$ ), pH 6.5, and washed with four 12-ml portions of distilled water to remove the excess  $\text{NH}_2\text{OH}$ . 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  $\alpha$  and  $\beta$  bands and the toe of the  $\gamma$  band of *Loligo pealei* retinochrome. In fresh solutions at pH 6.3,  $\lambda_{\text{max}}$  of the  $\alpha$  band is at 500 nm. Addition of  $\text{NH}_2\text{OH}$  in the dark converts retinochrome to opsin and all-*trans* retinaldehyde oxime with  $\lambda_{\text{max}}$  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  $\text{NH}_2\text{OH}$ ; in 0.2 M  $\text{NH}_2\text{OH}$  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 ( $\epsilon_{\text{max}}$ ) of retinochrome (cf. Wald and Brown, 1953). We find that it is approximately 40,000

<sup>1</sup> Hara and Hara (personal communication) prefer the name apo-retinochrome for the protein portion of retinochrome.

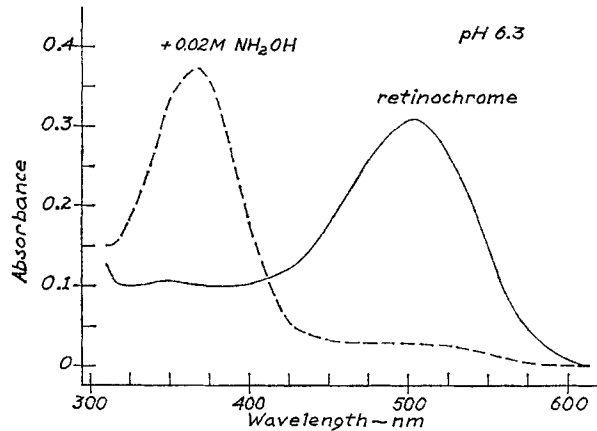


FIGURE 1. Bleaching of retinochrome by hydroxylamine in the dark. To 0.5 ml of a fresh solution of retinochrome at pH 6.3 ( $\lambda_{\text{max}}$  500 nm), 0.01 ml 1 M  $\text{NH}_2\text{OH}$  was added in dim red light and the spectrum was measured after 5 min at 25°C. The  $\alpha$  and  $\beta$ 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

$\text{cm}^2\text{mol}^{-1}$ , similar to that of rhodopsin. Hara and Hara (personal communication) find that  $\epsilon_{\text{max}}$  of *Todarodes* retinochrome is about 1.5 times that of rhodopsin.

Sodium borohydride ( $\text{NaBH}_4$ ) 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  $-15^\circ\text{C}$ , 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

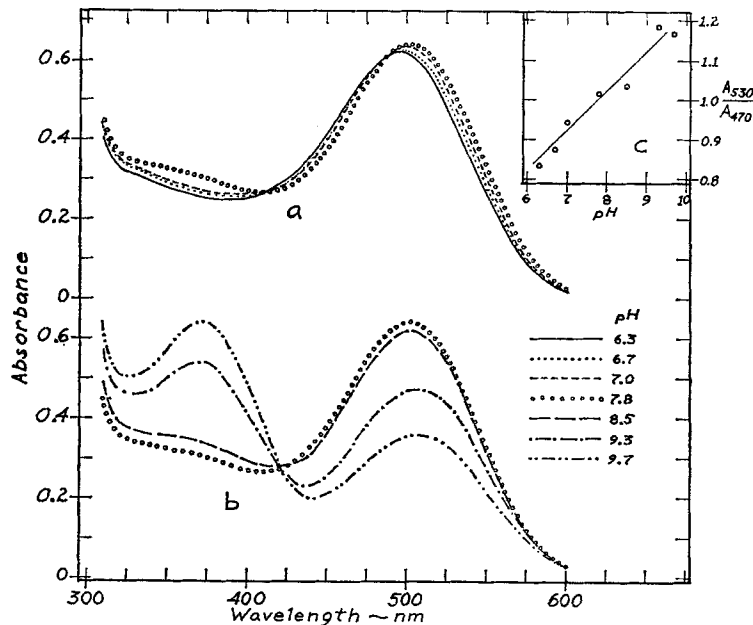


FIGURE 2. Changes in the absorption spectrum of retinochrome with pH. The pH of a retinochrome solution was raised in steps from 6.3 to 9.7 by adding small amounts of solid  $\text{Na}_2\text{CO}_3$ . (a) Between pH 6.3 and 7.8,  $\lambda_{\text{max}}$  shifted from 495 to 503 nm and the absorption at  $\lambda_{\text{max}}$  increased slightly. The curves cross at 412 and 487 nm. (b) Above pH 7.8,  $\lambda_{\text{max}}$  of this band continued to move to longer wavelengths but its absorption decreased and a second band appeared at shorter wavelengths with  $\lambda_{\text{max}}$  375 nm. The curves cross at 421 nm. (c) The ratio of absorbances at 530 and 470 nm ( $A_{530}/A_{470}$ ) is plotted as a function of pH to show that the shift in the wavelength position of the long wavelength band is continuous. Throughout this experiment,  $\lambda_{\text{max}}$  is shifted about 5 nm to shorter wavelengths than where it is in fresh solutions, because this preparation had been stored 3 wk at  $-15^\circ\text{C}$ , but the phenomena are the same as are observed with fresh solutions.

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  $-15^\circ\text{C}$ ,  $\lambda_{\text{max}}$  begins to shift to shorter wavelengths, although the other properties of the pigment remain unchanged.<sup>2</sup> 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  $-15^\circ\text{C}$ . During this time,  $\lambda_{\text{max}}$  moved from 500 to 475 nm, the position shown in

<sup>2</sup> This type of spectroscopic change does not happen with *Todarodes* retinochrome (Hara and Hara, personal communication).

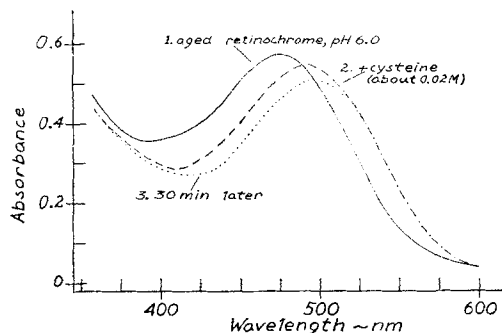


FIGURE 3. Effects of aging and of cysteine. A solution of retinochrome at pH 6 was stored 7 mon at  $-15^{\circ}\text{C}$ , at which time  $\lambda_{\text{max}}$  had shifted from its initial position at 500 nm to 475 nm (curve 1). Within 2 min after addition of cysteine to a final concentration of 0.02 M,  $\lambda_{\text{max}}$  had shifted to 491 nm (curve 2) and after 30 min to 496 nm (curve 3).

curve 1. 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  $\frac{1}{2}$  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 photoproduct*

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 photoproduct 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 photoproduct (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 photoproduct 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* photoproduct is less stable than retinochrome. However this is unlikely, because synthesis of the 11-*cis* photoproduct 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* photoproduct is converted to opsin + retinaldehyde oxime upon addition of  $\text{NH}_2\text{OH}$ , though the oxime is of course 11-*cis*. It is also reduced by  $\text{NaBH}_4$ . However, Hara and Hara (1973 *a*) have

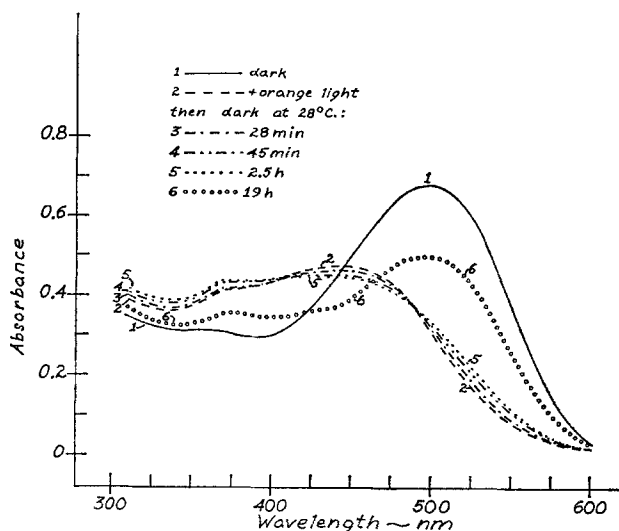


FIGURE 4. Thermal regeneration of retinochrome from its photoproduct at pH 6.3. A fresh solution of retinochrome (curve 1) was irradiated 5 min with orange light. The photoproduct, shown in curve 2, was left in the dark at 28°C and spectra were recorded 28 min (3), 45 min (4), 2.5 h (5), and 19 h (6)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

shown that the product formed by  $\text{NaBH}_4$  is at least in part retinal. At alkaline pH, the photoproduct is converted to a compound with  $\lambda_{\text{max}}$  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  $\lambda_{\text{max}}$  at about 375 nm, the other at longer wavelengths (apparent  $\lambda_{\text{max}}$  above 490 nm). The absorption spectrum measured after 19 h of regeneration (curve 6) resembles the initial retinochrome spectrum shown in curve 1, with  $\lambda_{\text{max}}$  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;  $\lambda_{\text{max}}$  370 nm) disappears as retinochrome regenerates, with an isosbestic point near 450 nm.

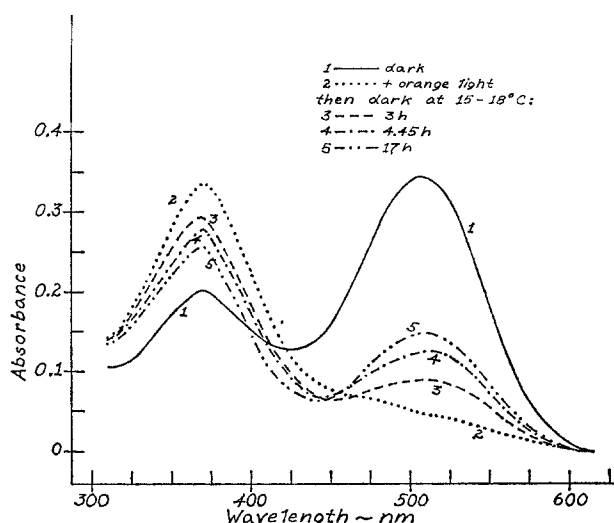


FIGURE 5. Thermal regeneration of retinochrome from its photoproduct at pH 9.5. Retinochrome (curve 1) was irradiated 20 min with orange light. The photoproduct, shown in curve 2, was incubated in the dark at 15–18°C. Absorption spectra were recorded 3 h (3), 4.45 h (4), and 17 h (5) after the bleaching 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  $\log_e$  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,  $A_i$ , 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.  $A_t$  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  $\rightarrow$  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,  $2.0 \times 10^{-5}$  and  $2.4 \times 10^{-6} \text{ s}^{-1}$ . Experiments at two temperatures show marked increases in the rates of regeneration with temperature.

### *Retinochrome<sub>2</sub>*

Hara and Hara (1968, 1973 *b*) 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<sub>2</sub> (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,

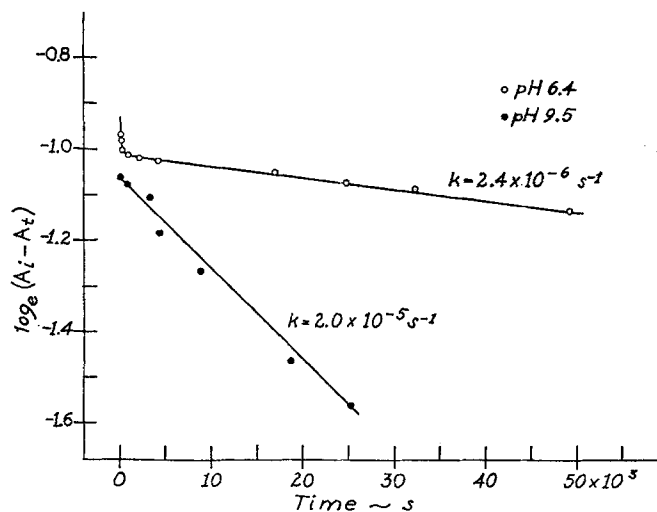


FIGURE 6. Rates of regeneration of retinochrome from its photoproducts at pH 6.4 and 9.5. Solutions of retinochrome at the two pH's were bleached 5 min with orange light and incubated in the dark at 23.5°C. Spectra were recorded at the times after the irradiation that are shown along the abscissa. The ordinate is  $\log_e$  of the absorbance at 530 nm of the original retinochrome solution less that of the photoproduct immediately after the bleaching light is turned off ( $A_i$ ) minus the absorbance at 530 nm after  $t$  s in the dark less the same base-line correction ( $A_t$ ). Since the photoproducts at the two pH's have about  $\frac{1}{10}$ – $\frac{1}{5}$  the absorption of retinochrome at 530 nm, the values of  $A_i$  and  $A_t$  are in error by about that amount. ( $A_i - A_t$ ) is equivalent to the concentration of a hypothetical precursor of retinochrome, which is converted to retinochrome by a first-order or pseudo first-order process. The slopes,  $k$ , of the lines are the first-order rate constants for the reaction, precursor  $\rightarrow$  retinochrome.

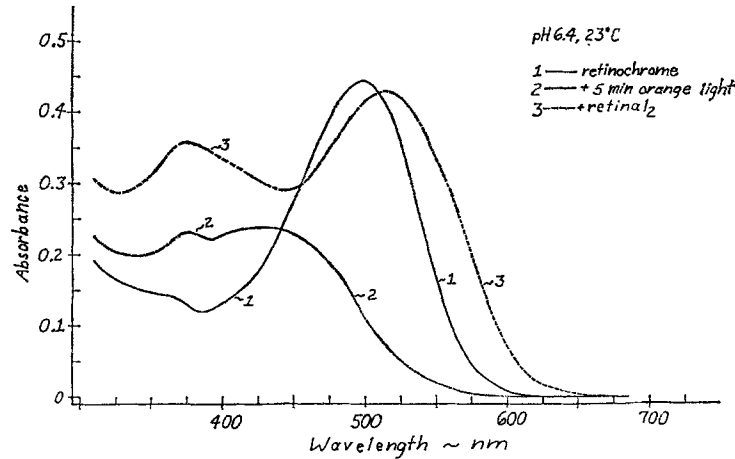


FIGURE 7. Synthesis of retinochrome<sub>2</sub> from bleached retinochrome and all-*trans* retinal<sub>2</sub>. A fresh solution of retinochrome at pH 6.4 (curve 1,  $\lambda_{\text{max}}$  500 nm) was irradiated 5 min with orange light. The photoproduct shown in curve 2 was incubated in the dark with the approximate molar equivalent of all-*trans* retinal<sub>2</sub>. 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> (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<sub>2</sub> by all-*trans* Retinal<sub>2</sub> and Retinal in the Dark*

Fig. 7 shows that all-*trans* retinal<sub>2</sub> 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<sub>2</sub> 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.

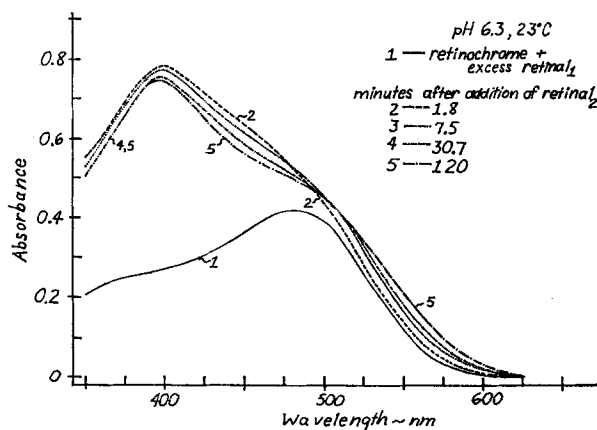


FIGURE 8. Conversion of retinochrome to retinochrome<sub>2</sub> by addition of all-*trans* retinal<sub>2</sub> in the dark. Retinochrome opsin was incubated 2 h with a small excess of all-*trans* retinal at pH 6.3 and room temperature to convert all the opsin to retinochrome. It was then stored 2 days at  $-15^{\circ}\text{C}$ , 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  $23^{\circ}\text{C}$ . The long wavelength limb of the absorption band moves to longer wavelengths; there is an isobestic 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 isobestic 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 isobestic 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.

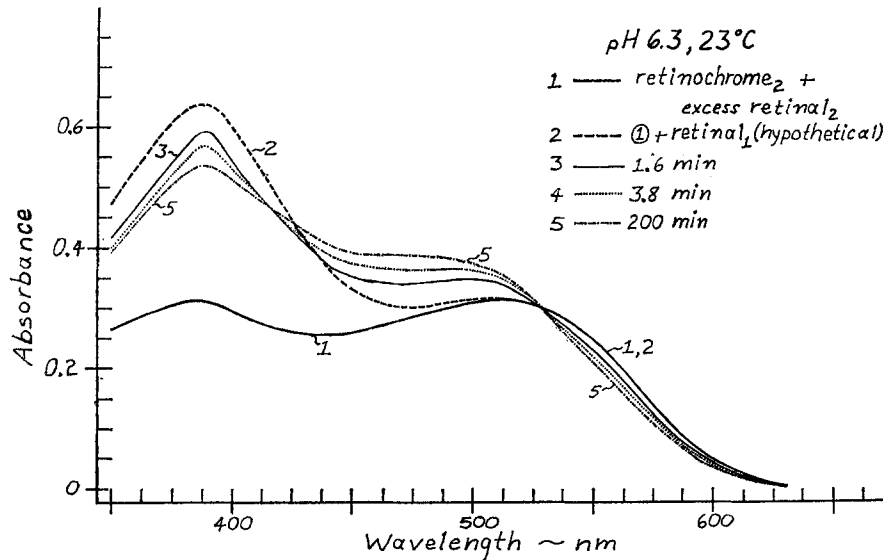


FIGURE 9. Conversion of retinochrome<sub>2</sub> to retinochrome by addition of all-*trans* retinal in the dark. An aliquot of the opsin solution used in Fig. 8 was incubated with an excess of all-*trans* retinal<sub>2</sub> 1 h in the dark at room temperature and its spectrum measured and corrected as described in Fig. 8. Curve 1 is the corrected spectrum of the chromophore of retinochrome<sub>2</sub> plus the excess all-*trans* retinal<sub>2</sub> that remains after all the opsin has been converted to retinochrome<sub>2</sub>. 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.

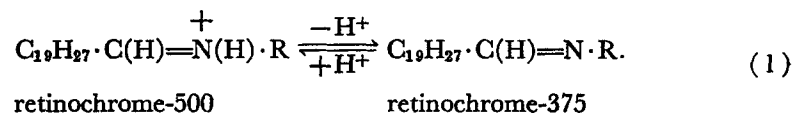
## DISCUSSION AND CONCLUSIONS

### *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  $\epsilon$ -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 *b*).

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,

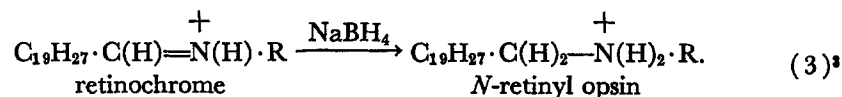
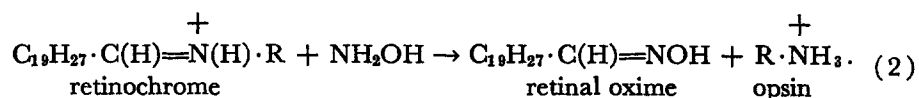


In addition, in retinochrome  $\lambda_{\text{max}}$  of the protonated form itself is pH variant as shown in Fig. 2. The only other instance of this has recently been observed in crayfish rhabdomes, in which  $\lambda_{\text{max}}$  of what is assumed to be a visual pigment changes reversibly with pH (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  $\text{NH}_2\text{OH}$  and  $\text{NaBH}_4$  (cf. Fig. 1). The reactions are summarized in Eqs. 2 and 3,

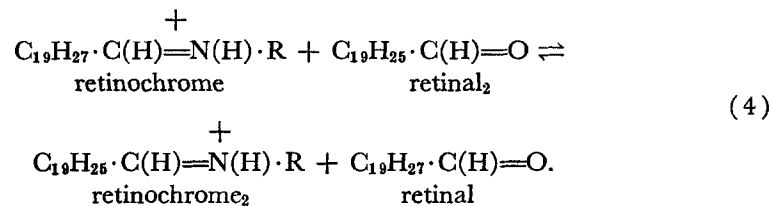


No known rhodopsin reacts with  $\text{NH}_2\text{OH}$  or  $\text{NaBH}_4$  before it is photoisomerized and transformed to metarhodopsin, but the cone pigment, chicken iodopsin, reacts in the dark with low concentrations of  $\text{NH}_2\text{OH}$  (Wald et al., 1954–55) and with  $\text{NaBH}_4$  (Matsumoto et al., in press).

Another indication that the chromophoric site of retinochrome is accessible

\* Though Eqs. 2 and 3 are written with the imino nitrogens in their protonated (quaternary) form, we do not wish to imply that  $\text{NH}_2\text{OH}$  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* photoproduct 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 NH<sub>2</sub>OH 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:



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 therefore 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 retinochrome 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* photoproduct migrate into the outer segment. However, preliminary 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;<sup>4</sup> 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.

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