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Histochemical localization of retinochrome and rhodopsin studied by fluorescence microscopy*

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Summary. Retinochrome is readily reduced by sodium borohydride into an N-retinyl protein that emits visible fluorescence upon irradiation with near-ultraviolet light. Rhodopsin is also converted to a similar fluorescent product, but only when denatured with formaldehyde before reduction. Based upon this difference, retinochrome was discriminated from rhodopsin on frozen sections. The distribution of these two photopigments in various photosensitive tissues was examined by means of epifluorescence microscopy.

In the octopus retina (*Octopus vulgaris*), the yellow-green fluorescence of reduced retinochrome was observed in both the basal regions of the outer segments and throughout the inner segments of the visual cells, while the fluorescence of reduced rhodopsin was restricted to within the rhabdomal layer of the outer segments. In the squid parolfactory vesicles (*Todarodes pacificus*), rhodopsin was present in the central lumen, which contains the distal processes of the photoreceptor cells, while retinochrome was detected in the myeloid bodies scattered within the vesicular wall. In the slug retina (*Limax flavus*), rhodopsin was found in the microvilli, and retinochrome appeared to be concentrated in the photic vesicles of the visual cells.

Key words: Retinochrome – Rhodopsin – Vitamin A – Fluorescence microscopy – Photoreceptor cell

Retinochrome is a photosensitive pigment which is found together with rhodopsin in the visual cells of various cephalopods (Hara and Hara 1965; Hara et al. 1967). The chemical and photochemical properties of retino-

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chrome have been reviewed by Hara and Hara (1972, 1973a, 1982). When retinochrome absorbs light, its retinaldehyde chromophore is isomerized from the all-*trans* to the 11-cis form. As this photoisomerization is the reverse of that which occurs upon irradiation of rhodopsin, we have suggested that the 11-*cis* photoproduct of retinochrome (metaretinochrome) might act as a direct supplier of active retinaldehyde required for rhodopsin formation (Hara and Hara 1967, 1968). Based on this, squid rhodopsin has recently been reconstituted by the use of membrane preparations (Seki et al. 1980a, b, 1982). It has also been revealed, however, that the light and dark reactions of retinochrome and metaretinochrome serve to restrict the various isomers of retinaldehyde into a simple system consisting of the two isomers, 11-*cis* and all-*trans*, only; i.e., in the dark, retinaldehyde is reserved as retinochrome as a "stock form", and in the light, it is converted into metaretinochrome as an "active form" available for rhodopsin formation (Hara and Hara 1973b; Ozaki et al. 1983).

Rhodopsin is believed to be localized mainly in the microvillar membranes of the rhabdoms. Since retinochrome was first found in the inner segments of cephalopod visual cells (Hara and Hara 1965), it was thought to be present in the myeloid bodies observed so abundantly there (Hara and Hara 1967). Subsequently, retinochrome was extracted from the outer segments, indicating an additional location in the basal regions of the outer segments. Myeloid bodies are also distributed in these regions (Hara and Hara 1976).

Eakin and Brandenburger (1978) demonstrated the fluorescence of the N-retinyl protein obtained by reduction of a photopigment. No reference has been made, however, to differences between retinochrome and rhodopsin. In the present study, by observing their reactions with the reducing agent sodium borohydride ($NaBH_4$), we were able to distinguish them by histochemical examination; retinochrome is reduced at once to an N-retinyl protein (Hara and Hara 1973a), whereas rhodopsin is reduced to it only when denatured before the addition of $NaBH_4$ (Bownds and Wald 1965). Although these two reduced photopigments emit the same yellow-green fluorescence upon exposure to near-ultraviolet light, they are identified separately by observing their fluorescence before and after denaturation. Preliminary accounts of the technique have been presented elsewhere (Ozaki et al. 1981, 1982a).

Materials and methods

Octopus (*Octopus vulgaris*), slug (*Limax flavus*), crayfish (*Procambarus clarkii*), and planarian (*Dugesia japonica*) were adapted to darkness for approximately 5 h. The retina or the eye was isolated under dim red light, transferred into a Bright Cryo-M-Bed embedding compound (Bright Instrument Co. Ltd., Cambs., England), and immediately frozen at −30° C. The ventral parolfactory vesicles were excised from dark-adapted squid (*Todarodes pacificus*) under dim white light (cf. Hara and Hara 1980), and similarly embedded at liquid-nitrogen temperature. All the following operations were carried out in a darkroom under a red safe-light.

The frozen samples were sectioned at 10 μm with a Bright model 5030/WDE rotary microtome set up in a Bright model OT/FASM cryostat adjusted to −25° C (Bright Instrument Co. Ltd., Cambs., England). Each section was mounted on a glass slide and air-dried for

approximately 4 h at about 20° C. Prior to histochemical treatment, light and fluorescent images of the specimen were observed and photographed using an Olympus model BHA-RF-A epifluorescence microscope (Olympus Co. Ltd., Tokyo, Japan). If the specimen contained retinol and retinyl ester, a yellow-green fluorescence would appear at this stage. The specimen was immersed in a 0.2% aqueous solution of $NaBH_4$ for 1 s at 4° C, rinsed gently with water, and photographed to examine the fluorescence of reduced retinochrome. In order to reduce rhodopsin with $NaBH_4$, it was first denatured by immersing the specimen in a 20% aqueous solution of formaldehyde (HCHO) for 3 min at about 20° C, and washed with water. The denatured rhodopsin was then reduced by a second treatment of 0.2% $NaBH_4$ for 5 s at 4° C. The fluorescence was observed microscopically after rinsing the specimen in water.

To excite the specimen, 334- and 365-nm beams from a 100 W high-pressure mercury lamp were isolated by combining a UG-1 filter and a DM-400 dichroic mirror. The specimen was observed through an L-420 filter to exclude reflected light upon excitation, and photographed at 20–60 s exposures with Kodak Tri-X film (ASA-400). It was sensitized to an equivalent of about ASA-2400 by using a Fuji Pandol high-energy developer.

Results

Five sets of micrographs, each derived from different animals, are presented in Figs. 1 to 5. Each set contains four micrographs, A, B, C and D, prepared from a single specimen and shown in the order of the steps of observation. Micrographs A and B are light and fluorescent images of the frozen section, respectively, while C and D are fluorescent images taken after the first and second treatment with $NaBH_4$, respectively; C allows the detection of retinochrome and D of rhodopsin.

Both reduced retinochrome and reduced rhodopsin, like retinol, emit a characteristic yellow-green fluorescence only when irradiated with near-ultraviolet light, but do not emit with either violet or blue light. Under near-ultraviolet light, the fluorescence fades rapidly and disappears after about 1 min. In the text such an emission is termed "yellow-green fluorescence".

Octopus (Octopus vulgaris) retina

The octopus retina is composed mainly of a large number of visual cells, each of which is divided into an inner and outer segment by a constriction that corresponds to the so-called basement membrane observed in histological sections. The inner segments contain many lamellated bundles of retinochrome-bearing membranes (myeloid bodies) and nuclei, while the outer segments contain rhabdomeres in the anterior parts and black pigment immediately adjoining the basal regions, forming the rhabdomal layer and the black-pigment layer, respectively (Fig. 1A; cf. Yamamoto et al. 1965). Frozen sections showed weak, bluish fluorescence over the entire area (Fig. 1B). However, this fluorescence could be completely eliminated by washing the specimen with water, suggesting the absence of retinol and retinyl ester. When the nonfluorescent specimen thus obtained was treated with $NaBH_4$, a distinct band of yellow-green fluorescence due to reduced retinochrome appeared on both sides of the basement membrane, indicating the presence of retinochrome in the basal regions of the outer segments

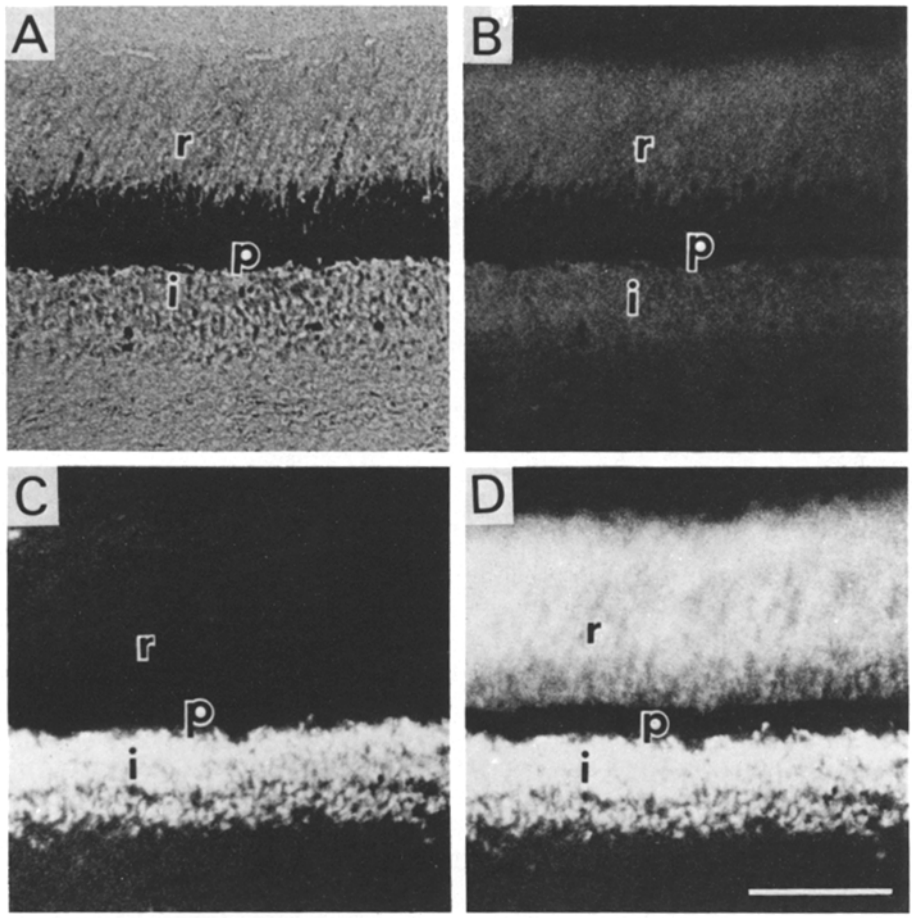

Fig. 1 A–D. Octopus retina. **A** Light micrograph of a 10-μm frozen section. **B** Micrograph of the same specimen under near-ultraviolet illumination showing a faint bluish fluorescence that can be readily removed by rinsing the section with water. **C** Yellow-green fluorescence of retinochrome reduced by $NaBH_4$. **D** Additional yellow-green fluorescence of rhodopsin treated with HCHO and $NaBH_4$; *i* area consisting of the inner segments and basal regions of the outer segments (retinochrome); *p* black pigment layer; *r* rhabdomal layer (rhodopsin). × 190. Bar = 100 μm

and throughout the entire inner segments (Fig. 1 C). This bright band consisted of many fluorescent spots, each of which seemed to correspond to the retinochrome-bearing myeloid bodies. Upon subsequent treatment with HCHO and $NaBH_4$, an additional band of yellow-green fluorescence due to reduced rhodopsin appeared in the rhabdomal layer of the outer segments (Fig. 1 D).

The retinas of the paper nautilus (*Argonauta argo*) and the squid (*Todarodes pacificus*) were also examined using this technique. The results were essentially similar to the pictures shown in Fig. 1 for the *Octopus* retina. In general, the histochemical localization of retinochrome and rhodopsin

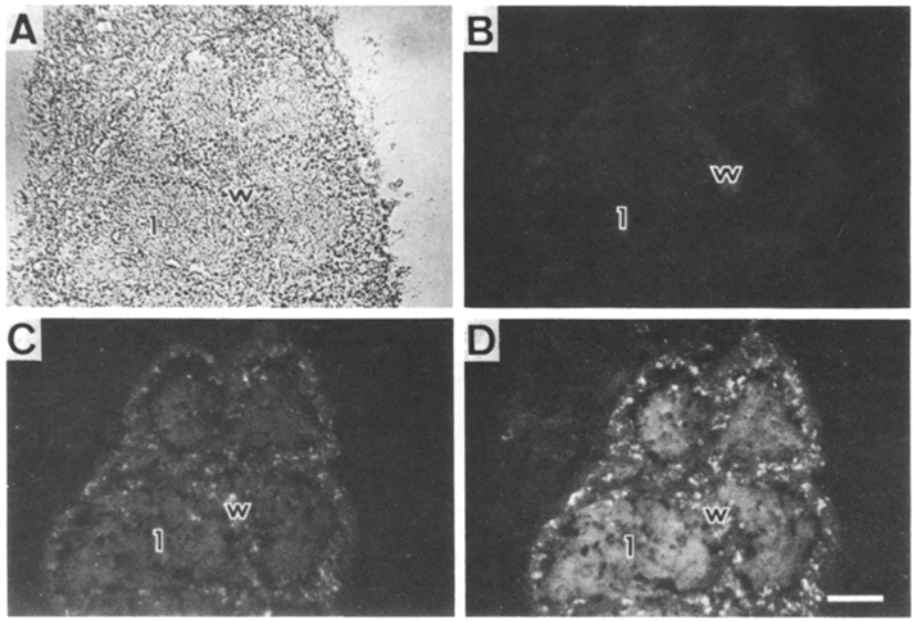

Fig. 2A–D. Ventral parolfactory vesicles of the squid. **A** Light micrograph of a 10-μm frozen section including four vesicles. **B** Fluorescence micrograph of the same specimen showing a very weak fluorescence, which can be rinsed out with water. **C** Yellow-green fluorescence of reduced retinochrome in the wall and lumen of the vesicle. **D** Additional yellow-green fluorescence of reduced rhodopsin; *l* central lumen of the vesicle; *w* wall of the vesicle. ×80. Bar = 100 μm

in cephalopod retinas was consistent with results obtained from other experiments performed with the *Todarodes* retina, i.e., the extraction of retinochrome (Hara and Hara 1976), isolation of the myeloid bodies (Seki et al. 1980a, b; Ozaki et al. 1982b) and immunocytochemical examination (Fukushima et al. 1980).

Squid (Todarodes pacificus) parolfactory vesicles

The parolfactory vesicles are extraocular photoreceptors that are well developed in the deep-sea squid. They are attached to the surface of the optic tract immediately behind the optic lobe. The ventral set of the vesicles forms a strand-shaped organ, 2–3 mm long and approximately 0.5 mm wide. A vesicle consists of many photoreceptor cells, each of which roughly resembles the visual cell in structure, and contains rhodopsin and retinochrome (Hara and Hara 1980). The wall of the vesicle is composed mainly of nucleated cell bodies of photoreceptor cells, and the central lumen is filled with photoreceptor cell processes surrounded entirely by microvilli (Fig. 2A; cf. Hara and Hara 1980). Frozen sections of the vesicle showed a weak, blue fluorescence (Fig. 2B), which was easily rinsed out with water. Upon treatment with $NaBH_4$, the yellow-green fluorescence of reduced retino-

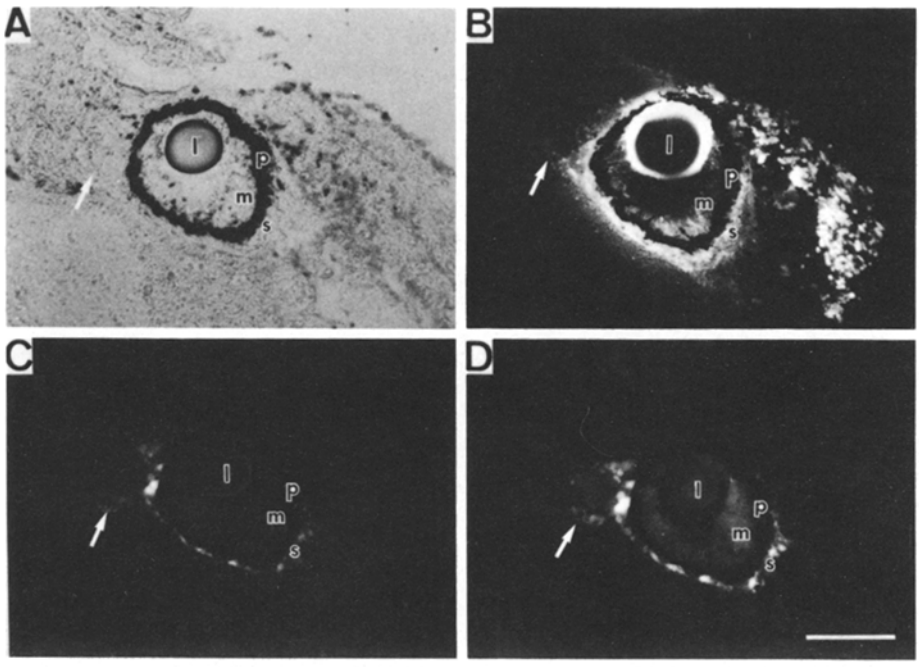

Fig. 3A–D. Main and accessory eye of the slug. **A** Light micrograph of a 10-μm section of the eye. **B** Micrograph of the above section showing a strong red fluorescence inside and outside the eye which disappears completely when the tissues are treated with $NaBH_4$. **C** After treatment with $NaBH_4$, yellow-green fluorescence, produced by reduced retinochrome, is seen. **D** Additional yellow-green fluorescence due to reduced rhodopsin. The *arrows* indicate the accessory eye which also bears retinochrome and rhodopsin; *l* lens of the main eye; *m* microvillar regions of the visual cells; *p* the layer of black pigment; *s* somata of the visual cells. × 120. Bar = 100 μm

chrome appeared in the central lumen and in the wall. While the fluorescence observed was faint but uniform in the lumen, many bright spots were found in the wall (Fig. 2C). They were due to retinochrome located on the myeloid bodies, similar to those found in the inner segments of visual cells. The dim fluorescence in the lumen, however, may be associated with retinochrome carried by much smaller structures scattered uniformly in the processes of the photoreceptor cells. Following treatment with HCHO and $NaBH_4$, the yellow-green fluorescence increased, most markedly in the central lumen (Fig. 2D). This indicated that rhodopsin was concentrated on the microvillar membranes in the photoreceptor cells.

Slug (Limax flavus) eye

The slug has a very small camera-like eye, and a histological section shows layers of microvilli, black pigment and somata (Fig. 3A; cf. Kataoka 1975). Frozen sections emitted a marked red fluorescence from the tissues present within and around the eye, as seen in Fig. 3B. This fluorescence could not

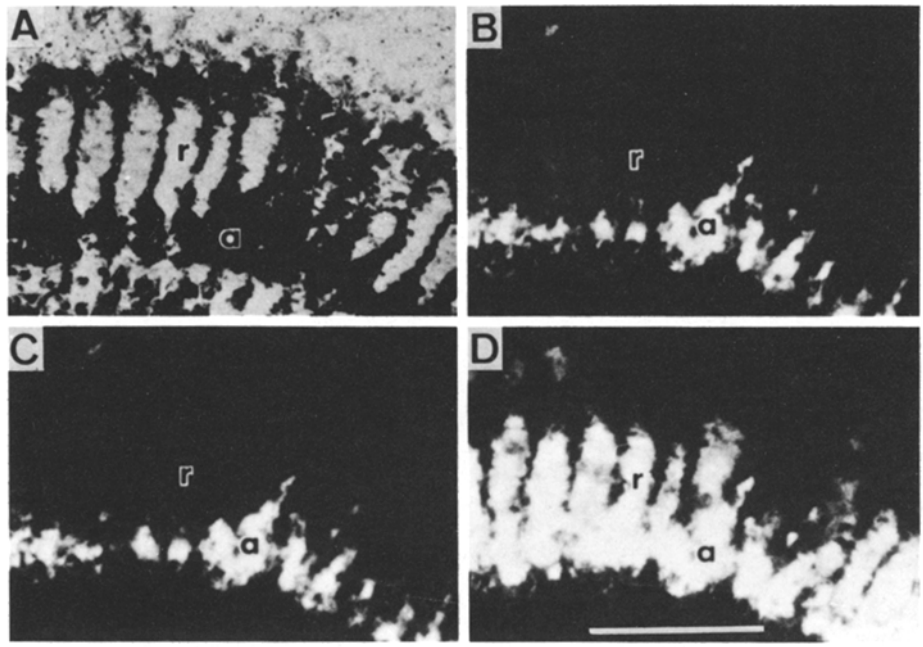

Fig. 4A–D. Retina of the crayfish. **A** Light micrograph of a 10-μm section of the compound eye. **B** Micrograph of the same section showing a strong whitish-blue fluorescence in the accessory pigment cells. **C** Fluorescence after treatment with $NaBH_4$; no change from B. **D** Yellow-green fluorescence of reduced rhodopsin after treatment with HCHO and $NaBH_4$; *a* accessory pigment cells; *r* rhabdomal regions of the visual cells. × 230. Bar = 100 μm

be washed out with water but was completely extinguished by treatment with $NaBH_4$ (cf. Fig. 3C). According to the fluorescence spectroscopy of the tissue extract, the red fluorescence seemed to be due to protoporphyrin. After the specimen was treated with $NaBH_4$, a spotted band of yellow-green fluorescence appeared, indicating the presence of retinochrome along the external side of the layer of black pigment (Fig. 3C). Since this area corresponds to the somata of visual cells that contain masses of photic vesicles in addition to the nuclei (Kataoka 1975), it seems likely that retinochrome is retained in the photic vesicles. Upon subsequent treatment with HCHO and $NaBH_4$, the yellow-green fluorescence of reduced rhodopsin was observed in the microvillar regions inside the layer of black pigment (Fig. 3D).

The slug possesses an accessory eye (lens-less) in contact with the main eye (arrows in Fig. 3). The accessory eye lacks black pigment, as does the parolfactory vesicle of the squid. Even in this small eye, both retinochrome and rhodopsin are distributed in the same way as in the main eye (cf. Fig. 3C, D).

Crayfish (Procambarus clarkii) compound eye

When a frozen section of the compound eye (Fig. 4A) was placed under near-ultraviolet light, a whitish-blue fluorescence was strongly emitted from

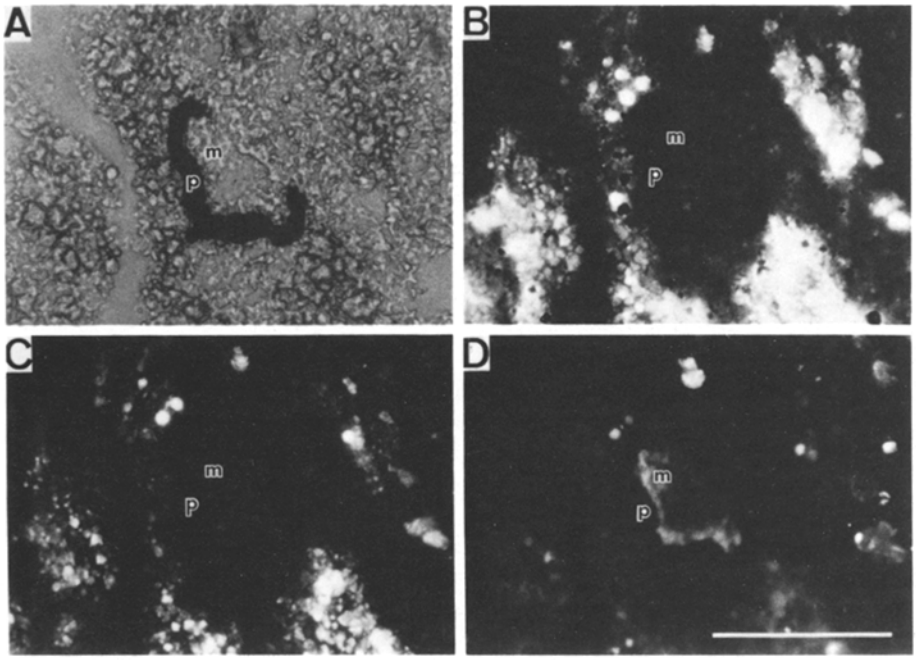

Fig. 5A–D. Eye of the planarian, *Dugesia japonica*. **A** Light micrograph of a 10-μm section of the cup-shaped eye. **B** Fluorescence micrograph of the above specimen. **C** Fluorescence micrograph of the specimen treated with $NaBH_4$; the eye emits no fluorescence, indicating the absence of retinochrome. **D** Yellow-green fluorescence of reduced rhodopsin observed inside the cup-shaped layer of black pigment; *m* microvillar regions of the visual cells; *p* layer of black pigment. × 240. Bar = 100 μm

the regions that surround the proximal half of the rhabdoms and contain the accessory pigment cells (Fig. 4B; cf. Kong and Goldsmith 1977). This fluorescence could not be removed by washing with water, by steady irradiation with near-ultraviolet light or by treatment with HCHO and $NaBH_4$. Because the fluorescent image did not change upon treatment with $NaBH_4$, retinochrome appeared to be absent in the crayfish retina (Fig. 4C). In contrast, the yellow-green fluorescence from reduced rhodopsin appeared in the rhabdomal regions of the visual cells after treatment with HCHO and $NaBH_4$ (Fig. 4D).

Planarian (Dugesia japonica) head

When a frozen section of the head including the eye (Fig. 5A) was irradiated with near-ultraviolet light, many spots of orange and blue fluorescence were observed in the tissues surrounding the eye (Fig. 5B). The fluorescent image within the eye remained virtually unchanged after treatment with $NaBH_4$, indicating the absence of retinochrome (Fig. 5C). However, after treatment with HCHO and $NaBH_4$, a yellow-green fluorescence appeared in the area cupped by a layer of black pigment (Fig. 5D). This fluorescence is derived

from the reduction of rhodopsin localized in the microvillar membranes of the visual cells.

Discussion

In order to establish the identity of rhodopsin in the present study, rhodopsin was denatured by HCHO and then reduced by $NaBH_4$ to convert it into N-retinyl opsin (λ_{max}: approximately 330 nm), the yellow-green fluorescence of which was observed under near-ultraviolet light. As shown from the results with various species, rhodopsin was concentrated without exception in the microvillar regions of the photoreceptor cells. Regarding the planarian eye, the electrophysiological study suggested that the visual pigment might be a rhodopsin-like photopigment with a λ_{max} near 508 nm (Brown et al. 1968). In our histochemical experiments, the planarian photopigment behaved in the same way as rhodopsins found in Mollusca, Arthropoda and Vertebrata, and is probably therefore a chromoprotein (rhodopsin) which possesses retinaldehyde as the chromophore.

In a recent experiment, Yoshida and his group (1983) detected the yellow-green fluorescence indicating the presence of rhodopsin in the ocellar microvilli of the starfish. In this case, however, a slight modification in the histochemical procedures was required; prior to the reduction of photopigment with $NaBH_4$, it was denatured not only by 20% HCHO for 1 min but successively by 100% methanol for 2 min at approximately 20° C.

Rhodopsin is little affected by the addition of $NaBH_4$ only, whereas retinochrome readily reacts with this reagent to yield an N-retinyl protein with a λ_{max} of 340 nm (Hara and Hara 1973a). The yellow-green fluorescence of this reduced protein was used for the detection of retinochrome in this study. The location of retinochrome in the retinas and the parolfactory vesicles of cephalopods was associated primarily with the myeloid bodies, as had been expected from our earlier works (Hara and Hara 1976; Seki et al. 1980a; Fukushima 1980; Ozaki et al. 1982b). Retinochrome might be also contained in a compact structure consisting of small vesicles regularly arranged in the proximal segments of the sensory cells of the snail (Hara and Hara 1967). Correspondingly, the present experiment with the slug has suggested the presence of retinochrome in the photic vesicles of the gastropod retina.

When exposed to light, retinochrome changes to metaretinochrome in order to provide opsin with 11-*cis* retinal for rhodopsin formation. In the dark, metaretinochrome slowly changes back to retinochrome which retains all-*trans* retinaldehyde as a chromophore (cf. Hara and Hara 1982; Ozaki et al. 1983). In Mollusca, the myeloid bodies and the photic vesicles may be regarded as a store for retinaldehyde in the form of retinochrome-chromophore. In Vertebrata, the retinoid necessary for rhodopsin synthesis is stored in the form of retinyl ester in the pigment epithelium (cf. Bridges 1976). Frozen sections of the rat retina revealed that the pigment epithelium emitted a yellow-green fluorescence under near-ultraviolet light before any histochemical preparation, indicating the presence of retinoid (unpublished

observation). We could not, however, find any retinol, retinyl ester or retinochrome in the retinas of the crayfish and the planarian. In these species, the photoreceptor cells may be provided with a different retinoid metabolic system from that in molluscans and vertebrates.

In the present work, we have distinguished retinochrome from rhodopsin by using the difference in reactivity to $NaBH_4$. Although a higher reactivity to $NaBH_4$ is one of the characteristic properties of retinochrome, the most desirable method of distinguishing these two photopigments would be to examine them in extracts, with special reference to the essential difference in the configuration of the retinaldehyde chromophore (11-*cis* in rhodopsin, but all-*trans* in retinochrome). Recently we have observed the yellow-green fluorescence in the photic vesicles of the red mouthed conch (*Conomurex luhuanus* L.) similar to that found in the slug (unpublished observation). Because the eye of this animal is far larger than that of the slug, a biochemical investigation is to be performed on the photic vesicles of the conch. Nevertheless, the technique that is presented here is both convenient and useful in determining the presence of retinal photopigments in various species before extraction experiments are carried out.

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