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Localization of Retinal Proteins in the Stalk and Dorsal Eyes of the Marine Gastropod, *Onchidium*

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ABSTRACT—*Onchidium* possesses stalk eye (SE) and dorsal eye (DE) which comprise part of a unique multiple photoreceptive system. The retina of SE consists of rhabdomeric-type visual cells, whereas the DE contains two types of photoreceptor cells; ciliary-type cells in the retina and rhabdomeric-type cells in the lens. High-performance liquid chromatography (HPLC) analyses revealed the presence of 11-*cis*-retinal as well as all-*trans*-retinal in both eyes. The amount of retinal of one DE (0.17 pmol) is far less than that in one SE (0.41 pmol) in the dark-adapted *Onchidium*. In the dark-adapted SE, the amount of all-*trans*-retinal was higher than that of 11-*cis*-retinal. This finding is consistent with the presence of photic vesicles, including retinochrome, in rhabdomeric-type visual cells. In contrast, a higher amount of 11-*cis*-retinal than all-*trans*-retinal was present in dark-adapted DE, although this was decreased in light-adapted DE. Upon UV irradiation following treatment with sodium borohydride ($NaBH_4$), the fluorescence (derived from retinochrome) was observed in the somatic layer of SE. Additional fluorescence (due to rhodopsin) was observed in the villous layer upon treatment with $NaBH_4$ after denaturation. However, only weak, obscure fluorescence of retinyl proteins was observed in the DE, not in a specific but an indefinite area on treatment with $NaBH_4$ with or without denaturation. With fluorescence histochemistry, the localization of rhodopsin and retinochrome was confirmed at specific regions in the retina of the SE, whereas no distinct localization of these photopigments in DE was demonstrated. The amount of retinal to detect the fluorescence may be too low in the DE, or photopigments of DE may differ in chemical nature from those of SE.

Key words: stalk eye, dorsal eye, HPLC, retinal protein, *Onchidium.*

INTRODUCTION

Rhodopsin localizes to the photoreceptive sites of photoreceptor cells in both vertebrate and invertebrate eyes. Visual cells in Cephalopods comprise two kinds of photopigments. Rhodopsin is localized in the microvilli of the outer segment, while retinochrome is present in myeloid bodies in the inner segment of visual cells (Robles *et al.*, 1987, 1995; Hara and Hara, 1991; Hara *et al*., 1992, 1995). Rhodopsin and retinochrome function cooperatively to mutually regenerate photopigments (Terakita *et al*., 1989). Upon illumination, rhodopsin is converted to metarhodopsin, and retinochrome to metaretinochrome. The 11-*cis*-retinal in rhodopsin is photoisomerized to the all-*trans* configuration. Conversely, all-*trans*-retinal in retinochrome is photoisomerized to its 11-*cis*-isomer. The 11-*cis*-retinal is required for rhodopsin formation. In the dark, rhodopsin and retinochrome are regenerated from these two isomers by chromophore exchange. Terakita *et al*. (1989) demonstrated that the turnover of 11-*cis*- and all-*trans*-retinal chromophores is mediated by retinal-binding protein, and proposed a conjugate rhodopsin-retinochrome system in visual cells of the squid eye. Using antibodies against squid retinal proteins, Katagiri *et al*. (2001) demonstrated the localization of three retinal proteins (rhodopsin, retinochrome and retinal-binding protein), and consequently the presence of a rhodopsin-retinochrome system in the stalk eye (SE) of the gastropod *Onchidium*. Retinochrome is readily reduced by sodium borohydride ($NaBH_4$) into N-retinyl protein that emits visible fluorescence upon UV irradiation. Rhodopsin is similarly converted to a fluorescent product, but only upon reduction following denaturation (Bownds and

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Wald, 1965; Hara and Hara, 1973). Based on these different fluorescent products, Ozaki *et al*. (1983) successfully identified rhodopsin in the microvilli and retinochrome in the cytoplasm of visual cells in *Octopus* and *Limax* eyes. Katagiri *et al*. (1995) further identified rhodopsin and retinochrome in the villous and somatic layers of *Onchidium* SE, using the method described by Ozaki *et al*. (1983) and reported that fluorescence from reduced retinal proteins disappears rapidly.

Onchidium has a unique multiple photoreceptive system comprising SE, dorsal eyes (DE), extraocular dermal photoreceptors and photosensitive ganglion cells in the CNS (Hisano *et al*., 1972, Katagiri *et al*., 1985, 1990). This multiple photoreceptive system contains one ciliary-type and three kinds of rhabdomeric-type photoreceptor cells. The retina of the SE contains rhabdomeric-type visual cells characterized by microvilli and photic vesicles in the cytoplasm. DE is designated 'vertebrate-type eye', due to similar morphology to the vertebrate eye (Stantschinsky, 1908). However, previous studies by Katagiri *et al*. (1983, 1985) demonstrate that the DE exhibits some unique structures and responses to light. The DE contains two different types of photoreceptor cells, specifically, ciliary-type photoreceptor cells in the retina and rhabdomeric-type lens cells in the lens. The ciliary-type photoreceptor cell is believed to be the intrinsic photoreceptor since the retina contains no other cell types. The lens cell may dually function as the dioptric apparatus and photoreceptor (Katagiri *et al*., 1983, 1985, 1998).

To examine the function of SE and DE and their relationship in the *Onchidium* multiple photoreceptive system, it is of considerable importance to compare the photopigments of SE and DE in addition to elucidating the structure and photoresponse. As both eyes of *Onchidium* are too minute for biochemical study, it is difficult to generate antibodies against *Onchidium* photopigments. Immunohistochemical analyses using anti-squid retinal protein antibodies were therefore employed to determine the localization of rhodopsin and retinochrome in the SE (Katagiri *et al*., 2001). In the present investigation, photopigments in both SE and DE of *Onchidium* were examined by HPLC and fluorescence histochemistry. The localization of rhodopsin and retinochrome in the SE is in concurrence with earlier immunohistochemical results (Katagiri *et al*., 2001) but photopigments of DE did not show the specific localization.

MATERIALS AND METHODS

Animals

Onchidium sp. (Gastropoda, Mollusca, 3–5 g body weight) were collected on the seashore of Tateyama (Chiba Prefecture) between June to August and maintained in a tank of aerated artificial seawater (Tetra Marine) under natural light/dark and an additional 12hr-light/12hr-dark cycle by a submergible NS-fluorescent lamp (Nissei, 15W) at 20–23°C. The intensity of illumination on the water surface at the center of the tank was about 600–1000 lux but varied depending on the weather. Animals inhabit under the stones in the artificial seawater or clung to the corners of the tank except for feeding time.

HPLC analyses

Onchidium were dark-adapted overnight before use. SEs and DEs were excised under deep red light with the scissors under a Wild stereoscopic microscope and frozen immediately. Samples were maintained at –80°C in the dark until use. Light-adapted animals were picked up from the tank at noon, then, DEs were excised from the dorsal mantle under a ring-type fluorescent lamp (SZ-FLR, 10W, about 1500–2000 lux) of a binocular microscope (Olympus SZ 4045) and frozen, similar to the dark-adapted samples. Retinals were extracted and analyzed according to the method of Makino-Tasaka and Suzuki (1986). Seventeen dark-adapted SEs, 50 dark-adapted DEs and 50 light-adapted DEs were homogenized in 0.2 ml phosphate buffer (0.06 M, pH 6.8) and 0.1 ml 1M hydroxylamine (neutralized with NaOH). Methanol (1ml) was added to the homogenate to convert retinals to retinaloximes. Oximes were extracted three times with 1 ml dichloromethane and 2 ml n-hexane. Extracts were evaporated under reduced pressure, dissolved in 100 μl elution solvent and subjected to HPLC, using the JASCO system equipped with a Zorbax SIL column (2.1×250mm, Shimadzu). The elution solvent used was 7% ether in n-hexane containing 0.075% ethanol, at a flow rate of 0.6 ml/min. Absorbance at 360 nm was monitored with a UV monitor (JASCO UVIDEC 100-III). Peak areas were determined by integrating the absorbance at 360 nm with a Chromatopac E-1A integrator (Shimadzu). The 11-*cis* and all-*trans*-retinal isomers were quantified from peak areas and standard curves determined with authentic compounds.

Fluorescence histochemistry

SEs and DEs were isolated from animals dark-adapted overnight, embedded in Tissue Tek OCT compound (Sakura Finetechnical Co.) and frozen immediately by immersion in liquid nitrogen under red light. Cryosections of both eyes (10 μm thickness) were mounted onto the same 3-aminopropyltriethoxy-silan-coated glass slides and simultaneously treated. Sections were air-dried for 3 hr at room temperature. Some specimens were immersed in 0.2% $NaBH_4$ for 1 sec at 4°C to induce fluorescence by conversion of retinochrome to N-retinyl protein, and rinsed gently with water. Other specimens were treated with a 20% formaldehyde aqueous solution for 3 min at room temperature to induce rhodopsin fluorescence, immersed in methanol for 2 min and 0.2% $NaBH_4$ for 5 sec at 4°C, and rinsed with water after each step. Fluorescence was observed with an epifluorescence microscope (Nikon Optiphoto EFD) through a L-420 filter to exclude reflected light upon excitation. To excite samples, UV light (334-365 nm) from a 100W high-pressure mercury lamp was isolated by combining a UG-1 filter and DM-400 dichromatic mirror. Since the fluorescence from reduced retinal proteins faded very quickly, it was difficult to record data from *Onchidium* samples with a conventional camera attached to an epifluorescent microscope. For recording a drastic change in faint fluorescence, the image observed with an epifluorescent microscope was amplified using a highly sensitive image intensifier (Hamamatsu Photonics, V1366P) and recorded with a high-speed VTR system (nac, MHS-200). Still pictures were subsequently photographed from VTR. Each fluorescent micrograph (Figs. 4, 5, 7 and 8) was printed from the same film under similar conditions to compare fluorescence

RESULTS

I. HPLC analyses

Stalk eye: Chromophores of photopigments in the 17 dark-adapted SEs were extracted as retinaloximes. HPLC analyses revealed the presence of both 11-*cis*-retinal and all-*trans*-retinal, but no 3-dehydroretinal in SE (Fig. 1). The total amount of retinal was calculated as 6.90 pmol (average

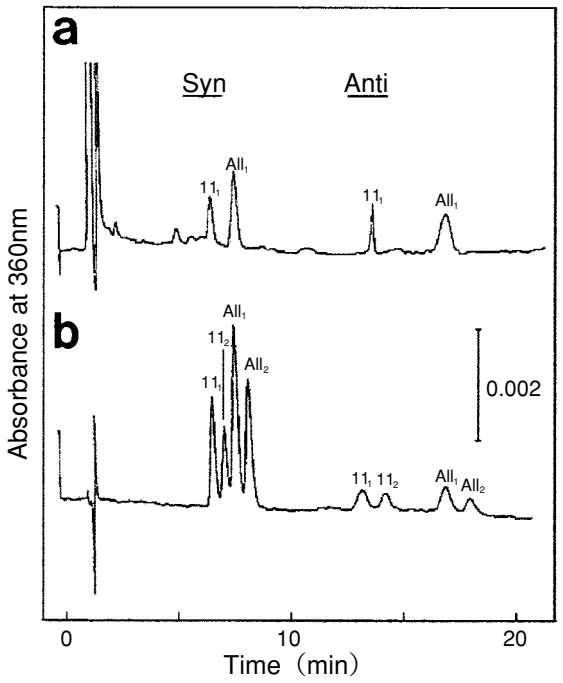


Fig. 1. High–performance liquid chromatogram of stalk eye extract (**a**) and standard oximes (**b**). (**a**) Seventeen dark-adapted stalk eyes (SE) contained 6.90 pmol retinal. The proportions of 11-*cis*- and all-*trans*-retinal were 41.3% and 58.7%, respectively. The average amount of 11-*cis*-retinal was 0.17 pmol, while that of all-*trans*-retinal was 0.24 pmol per SE. (**b**) Standard oximes (5 pmol each). 11_1, 11-*cis*-retinaloxime; 11_2, 11-*cis*-3-dehydroretinaloxime; All_1, all-*trans*-retinaloxime; All_2, all-*trans*-3-dehydroretinaloxime.

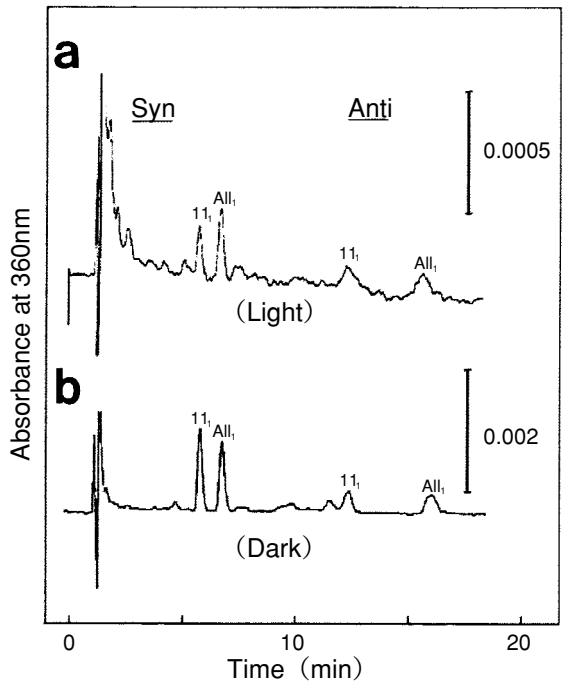


Fig. 2. High–performance liquid chromatogram of light-adapted (**a**) and dark-adapted (**b**) dorsal eye extracts. (**a**) Fifty light-adapted dorsal eyes (DE) contained 2.05 pmol retinal. The proportions of 11-*cis*- and all-*trans*-retinal were 53.7% and 46.3%, respectively. The average amount of 11-*cis*-retinal was 0.022 pmol, while that of all-*trans*-retinal was 0.019 pmol per DE. (**b**) Fifty dark-adapted DEs contained 8.64 pmol retinal. The proportions of 11-*cis*- and all-*trans*-retinal were 59.9% and 40.1%, respectively. The average amount of 11-*cis*-retinal was 0.10 pmol, while that of all-*trans*-retinal was 0.07 pmol per DE. 11_1, 11-*cis*-retinaloxime; All_1, all-*trans*-retinaloxime.

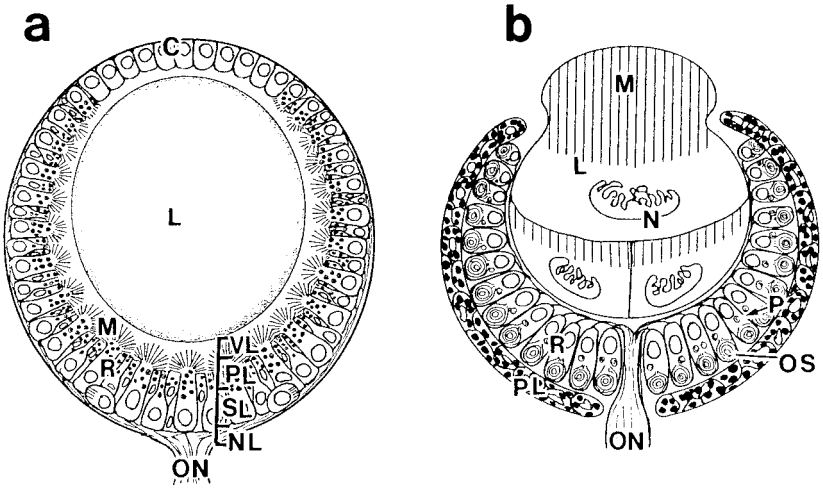
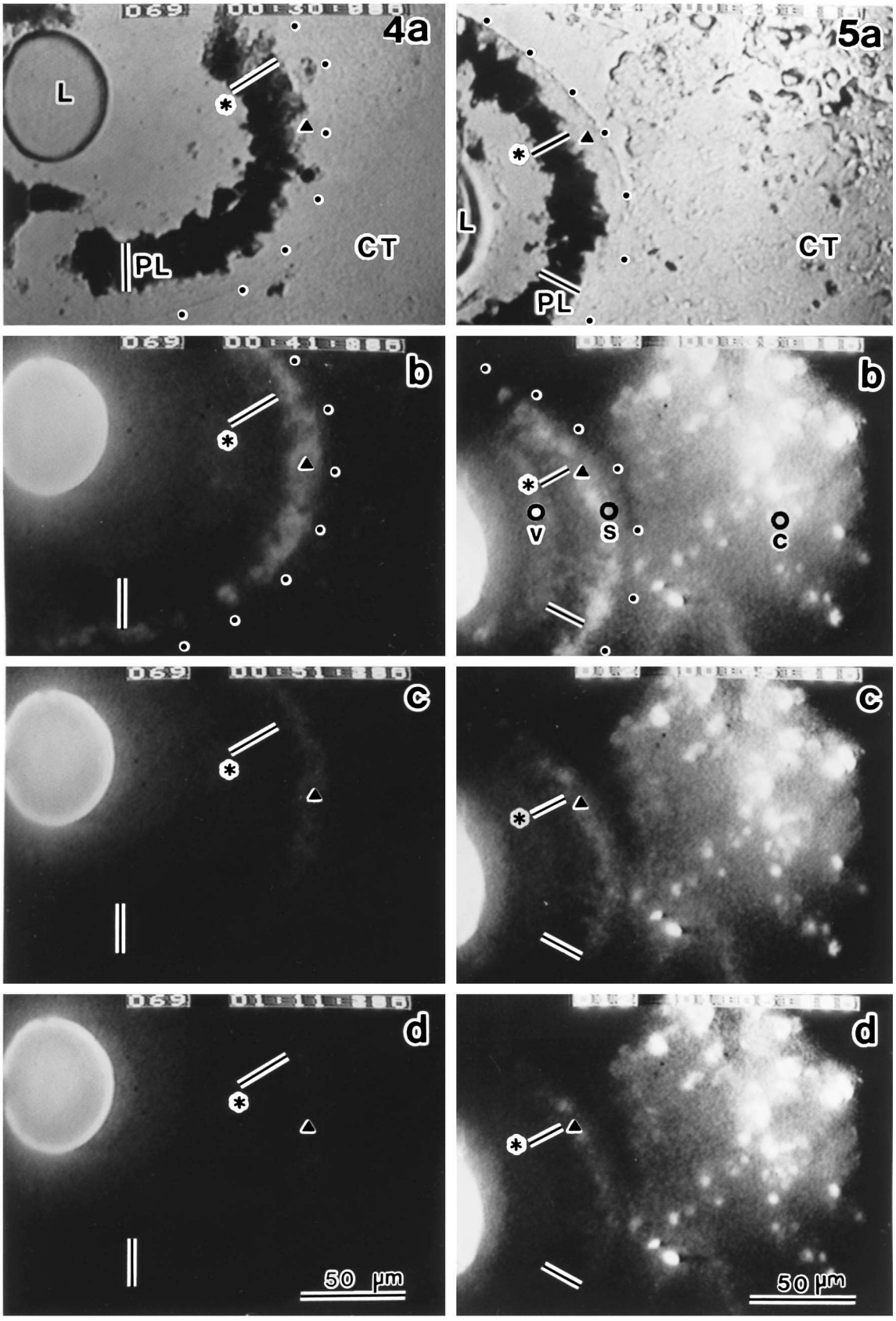


Fig. 3. Schematic drawings of stalk eye (**a**) and dorsal eye (**b**) of *Onchidium*. (**a**) Stalk eye is a closed vesicle, consisting of a cornea (C), lens (L) and retina (R). The retina is conveniently divided into villous (VL), pigmented (PL) somatic (SL) and neural (NL) layer. The PL corresponds to the distal cytoplasm of visual cells and pigmented supportive cells which contain abundant dark pigment granules. Incident light enters from the top, corneal side. M, microvilli of type 1 visual cell; ON, optic nerve. (**b**) Dorsal eye is an open vesicle. It comprises a pigment layer (PL), an inverted retina (R) and a lens (L) but lacks cornea. The retina is a single layer of ciliary-type photoreceptor cells of which the distal outer segments (OS) show a concentric lamellar structure and face the PL. Paranucleus (P) is located near the base of the OS. Axons extending from ciliary-type photoreceptor cells converge to the optic nerve (ON). The pear- shaped lens is located in the central portion of the eye. It is mostly composed of several large lens cells, which are rhabdomeric-type photoreceptors. The distal portion of the lens cell is composed of massive microvilli (M). Incident light enters from the microvilli of the lens. N, nucleus of lens cell.



of 0.41 pmol/ SE), of which 11-*cis*- retinal comprised 2.85 pmol (average of 0.17 pmol/ SE) and all-*trans*-retinal, 4.05 pmol (average of 0.24 pmol/ SE). The proportion of all-*trans*-retinal was greater (58.7%) than that of 11-*cis*-retinal (41.3%). **Dorsal eye:** Chromophores of photopigments were extracted as retinaloximes from the 50 dark-adapted and 50 light-adapted DEs. HPLC analyses similarly revealed 11-*cis*-retinal and all-*trans*-retinal, but no 3-dehydroretinal in DE (Fig. 2). In light-adapted DE, the total amount of retinal was calculated as 2.05 pmol (average of 0.04 pmol/ DE), of which 11-*cis*-retinal was 1.10 pmol (average of 0.022 pmol/ DE) and all-*trans*-retinal was 0.95 pmol (average of 0.019 pmol/DE). The relative proportions of 11-*cis*-retinal and all-*trans*-retinal were 53.7% and 46.3%, respectively (Fig. 2a). In the dark-adapted DE, the total amount of retinal was 8.64 pmol (average of 0.17 pmol/ DE), of which 11-*cis*-retinal comprised 5.18 pmol (average of 0.10 pmol/ DE) and all-*trans*-retinal, 3.46 pmol (average of 0.07 pmol/DE**).** In the dark-adapted DE, the proportions of 11-*cis*-retinal was greater (59.9%) than that of all-*trans*-retinal (40.1%) (Fig. 2b).

In dark-adapted eyes, the amount of retinal of one DE was far less (two-fifth) than that of one SE. Furthermore, in light-adapted DE, the total amount of retinal was far less (one-fourth), with a slightly lower proportion of 11-*cis* to all-*trans*-retinal, than in dark-adapted DE (Figs. 2a, 2b).

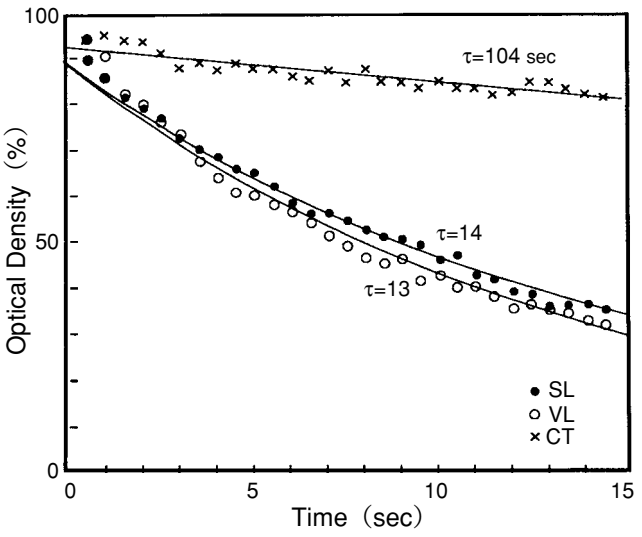


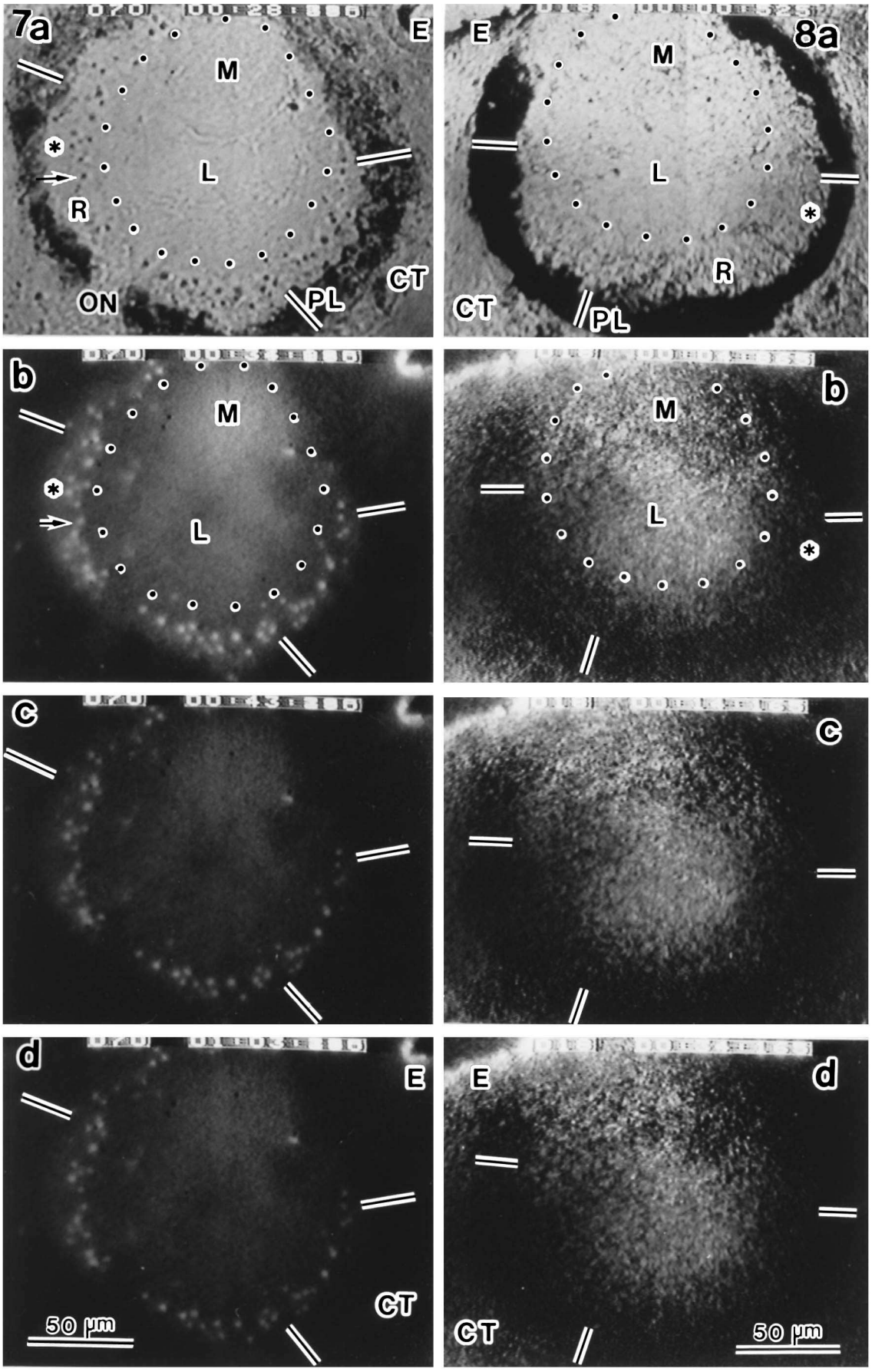
Fig. 6. Time-course of the optical density that corresponds to fluorescent intensity in two portions of the stalk eye (SE) and connective tissue (CT). Three circled portions were examined; the villous (v) and somatic (s) layers, and the CT (c) in Fig. 5b. The lifetime of decay of fluorescence in the SE differs from that of CT. Fluorescence from the villous (○) and somatic (●) layers declined within 15 sec to about 35%, but that from CT (x) did not change considerably during irradiation. The time constants (τ) of the villous and somatic layers were 13 and 14 sec respectively, while that of CT was 104 sec.

II. Fluorescence histochemistry

Stalk eye: The SE is an ellipsoidal vesicle of about 200 μm in diameter, comprising a cornea, lens and retina (Figs. 3a, 4a, 5a). The fine structure of the SE has been described in detail elsewhere (Katagiri *et al.,* 1995). The rhabdomeric-type photoreceptor cell extending well-developed microvilli in the SE is designated the 'type 1 visual cell'. The retina is a pigmented columnar epithelium divided into villous, pigmented, somatic and neural layers (Figs. 3a, 4a, 5a). The villous layer consists of an enormous tuft of microvilli of type 1 visual cells. The dark pigmented layer corresponds to the distal heavily pigmented cytoplasm of visual cells and pigmented supportive cells, while the somatic layer contains the nuclei and cytoplasm of visual and pigmented supportive cells. In the neural layer, axons of visual cells converge to form the optic nerve.

Upon immersion in water or formaldehyde solution, autofluorescence was observed in the epidermis and its surface and in unknown cells in the connective tissue of the tentacle. The SE does not show autofluorescence except for the lens. The particularly strong autofluorescence of the lens interfered with the intrinsic fluorescence of the villous layer in the retina when it was treated with $NaBH_4$ with or without denaturation. When cryosections of the SE were treated with $NaBH_4$, yellow-green fluorescence was observed in the somatic layer, which faded rapidly and disappeared completely within 60 sec after UV irradiation (Figs. 4b–d). Upon $NaBH_4$ treatment after denaturation, additional fluorescence was observed in the villous layer (Fig. 5b). Fluorescence in

Fig. 4. (**a**) Differential interference light micrograph of the stalk eye (SE) was taken from the same section as that in Fig. 4b–d after VTR recording. The pigmented layer (PL, lines) is located between the inner villous (⋆) and outer somatic layer (SL, ▲). To identify the location of fluorescence in the SE, the height of the PL is drawn with the lines and the outline of the SE is depicted with dots. CT, connective tissue. (**b**)–(**d**) Fluorescent micrographs of the stalk eye (SE) treated with sodium borohydride. (**b**) Immediately after irradiation, a yellow-green fluorescent band was observed in the SL (▲) under the PL. The lens (L) displayed strong autofluorescence. (**c**) The fluorescence image of the same specimen (Fig. 4b) at 10 sec after UV irradiation. Fluorescence in SL (▲) diminished rapidly. (**d**) After 30 sec irradiation, fluorescence derived from retinochrome in the SL (▲) disappeared. **Fig. 5.** (**a**) Differential interference light micrograph of the stalk eye was taken from the same section as that in Fig. 5b-d after VTR recording. See abbreviations of Fig. 4a. (**b**)–(**d**) Fluorescence micrographs of the stalk eye (SE) treated with sodium borohydride after denaturation with formaldehyde and methanol. (**b**) Upon UV irradiation, yellow-green fluorescence of the retinochrome was visualized in the SL (▲) under the PL. Additional fluorescence was observed in the VL (⋆) along the internal side of PL. Fluorescence in the SL was stronger than that in VL. The optic density of three circled areas, the villous layer (v), somatic layer (s) and connective tissue (c), were examined. The lens displayed intense autofluorescence. (**c**) At 10 seconds after irradiation, fluorescence of the retinochrome considerably faded in the SL (▲), but that of rhodopsin in the VL (⋆) disappeared. Autofluorescence of the lens was observed throughout. (**d**) At 30 seconds after irradiation, the fluorescence of the SL (▲) remained faintly, although endogeneous fluorescence of the lens and connective tissue remained.



the villous layer was weaker than that in the somatic layer. At 10 sec after irradiation, the yellow-green fluorescence in the villous layer had almost disappeared, but remained weakly in the somatic layer (Fig. 5c). Fluorescence in both layers faded considerably at 30 sec (Fig. 5d), disappearing completely within 60 sec after UV irradiation, although the endogeneous fluorescence of the lens and connective tissue was observed throughout.

A typical case of quick decay of fluorescence derived from photopigments is shown in Fig. 6. Time courses of optical density corresponding to a change in fluorescence were analyzed at three spots in a sample of Fig. 5, the villous and somatic layers in SE and connective tissue, that was treated with $NaBH_4$ treatment after denaturation with formaldehyde and methanol. The lifetime of decay of fluorescence intensity in both villous and somatic layers differed from that in connective tissue. The optical density of fluorescence from the villous and somatic layers decreased to 30% (τ-13) and 35% (τ-14) after 15 sec, while that from connective tissue was maintained at 85% (τ-104) during exposure to light (Fig. 6).

Dorsal eye: The DE is spherical in shape (not a closed vesicle) and about 200 μm in diameter. The DE comprises a cup-shaped pigment layer, inverted retina and lens, but lacks cornea (Figs. 3b, 7a, 8a). The retina is composed of a single columnar epithelium of only ciliary-type photoreceptor cells. The distal portion (outer segment) of the ciliary-type photoreceptor cell shows a concentric lamellar structure and faces the pigmented cells of the pigment layer. The supranuclear region contains rough and smooth endoplasmic reticulum, several paranuclei (dense inclusions) and other organelles. A biconvex pear-shaped transparent lens is situated in the center of DE. It consists of several large lens cells. Each lens cell contains massive microvilli in the distal portion, numerous lamellar bodies and a network of endoplasmic reticulum in the proximal cytoplasm. Based on fine structure and photoresponse data, the lens cell is identified as a rhabdomeric-type photoreceptor cell (Katagiri *et al*., 1983, 1985, 1998).

In control sections that were immersed in water or formaldehyde solution, autofluorescence was observed in the epidermis, its surface and in cuboidal cells in the connective tissue of the dorsal mantle, which did not disappear with reducing treatment. When non-specific fluorescence was evident in the surrounding connective tissue, weak, obscure fluorescence overspread diffusely the DE (not shown). The paranuclei of the ciliary-type photoreceptor cell in the retina emitted strong autofluorescence that faded slightly in the control sections and rapidly after $NaBH_4$ treatment (Fig. 7b–7d) and was not observed after methanol treatment (Fig. 8b) upon UV irradiation.

Fluorescence in DE after treatment with $NaBH_4$ with or without denaturation was observed in samples that showed less autofluorescence in connective tissue. Upon $NaBH_4$ treatment, unclear fluorescence was observed in indefinite and vague areas in individual DE at various intensities and different fading times. Faint fluorescence observed in the retina and the distal microvilli of the lens faded within 30 sec (Figs. 7b–7d). Upon $NaBH_4$ treatment after denaturation with formaldehyde and methanol, vague fluorescence was observed (Fig. 8b). However, additional fluorescence was either not present or very faint in both the lens and retina. Non-specific fluorescence persisted in the epidermis and connective tissue around the DE (Figs. 7c–d, 8c–8d). The drastic fading of fluorescence within 30sec under UV irradiation characteristic of reduced photopigments was not detected in the DE, despite recording with a high-speed VTR system (Figs. 8c–8d).

DISCUSSION

I. Chromophores in the stalk and dorsal eyes of *Onchidium*

HPLC analyses in the present study revealed 11-*cis*- and all-*trans*-retinal in the SE of dark-adapted *Onchidium* and the amount of all-*trans*-retinal was higher than that of 11-*cis*-retinal. The all-*trans*-retinal is considered to be a chromophore of retinochrome (Hara and Hara, 1973; Ozaki *et al*., 1983, 1986). The rhabdomeric-type photoreceptor cells of gastropoda eyes, including *Onchidium* SE, contain abundantly photic vesicles in the cytoplasm (Eakin, 1972, 1990; Kataoka, 1975, Herman and Strumwasser, 1984; Katagiri *et al*, 1995). As photic vesicles contain retinochrome (Hara and Hara, 1973; Ozaki *et al*., 1986), the all-

Fig. 7. (**a**) Differential interference light micrograph of the dorsal eye corresponds to Figs. 7b-d. The dorsal eye (DE) consists of the pigmented layer (PL, lines), retina (R) and lens (L). To identify the location of fluorescence in the DE, the height of the PL is drawn with the lines and the outline of the lens is depicted with dots. The outer segment (*) of ciliary-type photoreceptor cell faces to the PL. An arrow shows paranuclei of ciliary-type photoreceptor cell. CT, connective tissue; E, epidermis; L, lens cell; M, microvilli of lens cell; ON, optic nerve. (**b**)–(**d**) Fluorescence micrographs of the dorsal eye treated with sodium borohydride. (**b**) Immediately after irradiation, faint, obscure yellow-green fluorescence was observed in the entire dorsal eye, including the retina and lens. Strong spotty autofluorescence (arrow) was frequently detected in the supranuclear regions of ciliary-type photoreceptor cells in the retina. (**c**) After 10 sec and (**d**) 30 sec irradiation, almost all fluorescence disappeared in the DE, except for non-specific fluorescence in the epidermis (E). **Fig. 8.** (**a**) Differential interference light micrograph of the dorsal eye corresponds to Figs. 8b–d. See abbreviations in Fig.7a. (**b**)–(**d**) Fluorescence micrographs of the dorsal eye (DE) treated with sodium borohydride after denaturation with formaldehyde and methanol. (**b**) Upon UV irradiation, the yellow-green fluorescence of N-retinyl proteins was observed indistinctly in the DE. Specific fluorescence derived from rhodopsin did not appear in the retina or microvilli (M) of lens cells. (**c**) After 10 sec and (**d**) 30 sec irradiation, non-specific fluorescence remained in the DE. Diminishing of specific fluorescence derived from photopigments was not detected in the DE, although endogeneous fluorescence remained in the epidermis (E) and connective tissue.

trans-retinal detected in SE by the present HPLC analyses is possibly derived from the retinochrome in photic vesicles of type 1 visual cells in the retina. Furthermore, 11-*cis*-retinal in the dark-adapted SE may be the chromophore of rhodopsin of type 1 visual cells of which the microvilli occupy almost all the villous layer (Katagiri *et al*., 1995). Numerous SEs were required for HPLC analyses due to their minute size. However, we could not obtain sufficient amounts of SE, and therefore could not examine the chromophores of the light-adapted SE. In the eye of the marine gastropoda, *Conomulex*, 11-*cis*- and all-*trans*-retinal were also detected by HPLC analyses (Ozaki *et al*., 1986). The 11-*cis*-retinal was dominant in extracts from the microvillar fraction, while all-*trans*-retinal was prevalent in extracts from the photic vesicle-containing fraction. These results indicate that the photopigment is rhodopsin in the former fraction and retinochrome in the latter. The amount of all-*trans*-retinal in the *Conomulex* eye is several times greater than that of 11-*cis*-retinal. The observed ratio of retinal isomers in chromophores of *Onchidium* SE is similar to that in *Conomulex* eye (Ozaki *et al*., 1986).

On the other hand, in the dark-adapted *Onchidium*, the total amount of retinal in one SE was 2.4 times higher than that in one DE. A pair of SEs is situated in the tentacles. In contrast, more than 50 DEs are distributed on the dorsal mantle surface (Katagiri *et al*., 1985). Therefore, DE appears to be superior in terms of total numbers and consequently, total amount of retinal in an individual *Onchidium*. However, the SE is regarded as the principal eye within the multiple photoreceptive system (Hisano *et al*., 1972, Katagiri *et al*., 1985, 1990) on the basis of the presence of sufficient retinal per SE, existence of a rhodopsin–retinochrome system (Katagiri *et al*., 2001) and the typical structure of the gastropoda eye (Eakin, 1972, 1990; Kataoka, 1975; Jacklet and Colquhoun, 1983; Herman and Strumwasser, 1984; Katagiri *et al*., 1995).

The present study is the first significant report showing that the *Onchidium* DE contains 11-*cis*- and all-*trans*-retinal as well as SE. The DE comprises two different types of photoreceptor cells, specifically, ciliary-type in the retina and rhabdomeric-type in the lens. Since it is very difficult to separate the retina and lens in the DE, HPLC analysis was performed on all photopigments derived from both ciliary- and rhabdomeric-type photoreceptor cells. It is proposed that as the retina is the essential receptive site in DE, the HPLC data mainly represent the chromophores of photopigments in ciliary-type photoreceptor cells. The proportion of 11-cis-retinal (59.5%) to total retinal in dark-adapted DE was characteristically greater than that in dark-adapted SE (41.3%). Light-adapted DE contained a decreased proportion of 11-*cis*-retinal to all-*trans*-retinal compared to dark-adapted DE, suggesting the recycling of retinals in the DE. Notably, total retinal in light-adapted DE was far less (one-fourth) than that in dark-adapted DE. This decreased amount of total retinal could be explained on the assumption that predominant ciliary-type photoreceptor cells in DE contain vertebrate-type visual pigments. In vertebrate eyes containing ciliary-type photoreceptors, all-*trans*-retinal is released from the protein opsin during light adaptation, which is converted into retinol and subsequently stored as retinylesters in pigment epithelial cells (Saari, 1994). It is possible that all-*trans*-retinal in the ciliary-type photoreceptor cells of light-adapted DE is released from protein and stored as retinylesters in adjacent pigmented cells that directly face the outer segments of ciliary-type cells. At the same time, retinylester might be stored in the paranuclei of ciliary-type photoreceptor cells, similar to the oil droplets reported as the storage site of retinylester in crayfish retina (Suzuki *et al*., 1988). Unfortunately, our HPLC analyses did not encompass data on retinylesters, since these are eluted at the solvent front and therefore not quantified with the present experimental conditions (Suzuki *et al*., 1988). Retinals detected in the light-adapted DE may be derived mainly from the photopigments (rhodopsin and retinochrome) of rhabdomeric-type lens cells in the DE. Rhabdomeric-type lens cells have massive microvilli, and contain no photic vesicles but abundant endoplasmic reticulum, which is the storage site of retinochrome (Katagiri *et al*., 1998). The photic vesicle is a special form of endoplasmic reticulum (Whittle, 1976). Photic vesicle and endoplasmic reticulum were impregnated by prolonged osmification that demonstrates the presence of lipids, such as vitamin A (Eakin and Brandenburger, 1970; Pourcho and Bernstein, 1975; Katagiri 1984, 1998). In the *Onchidium* DE, several coordinating systems for photopigment recycling may be present, such as those in ciliary-type and rhabdomeric-type photoreceptor cells. The rhodopsin-retinochrome system was reported in the SE (Katagiri *et al*., 2001). Since 11-*cis* and all-*trans*-retinal were detected in both SE and DE, the rhodopsin-retinochrome system may be present in the DE similarly.

II. Fluorescence histochemistry

The localization of retinal protein in the DE was examined by fluorescence histochemistry and compared with that of SE. Fluorescence histochemistry is a convenient and valuable method for detecting photopigments, since the method only requires treatment with reducing agent and not antibodies (Ozaki *et al*., 1983). Fluorescence of N-retinyl protein that was reduced by borane dimethylamine was reported formerly in the snail eye (Eakin and Brandenburger, 1978). Ozaki *et al*. (1983, 1986) and Hara *et al*. (1992) demonstrated the localization of rhodopsin and retinochrome, based on differences in their reactivity to $NaBH_4$ in several invertebrate eyes. In *Onchidium* SE, fluorescence derived from rhodopsin and retinochrome was observed in villous and somatic layers of the retina. This fluorescence faded rapidly, in concurrence with earlier observations on the retinas of several gastropods (Ozaki *et al*., 1983, 1986). The stronger fluorescence in the somatic layer of *Onchidium* SE than that in the villous layer confirmed that the amount of retinochrome is greater than rhodopsin, as shown by HPLC analyses.

The SE from dark-adapted *Onchidium* contained sufficient retinal (0.41 pmol/ SE) to analyze emitted fluorescence after $NaBH_4$ treatment. However, in the DE (0.17 pmol/ DE) from dark-adapted animals, it is difficult to identify the presence or absence of photopigment-specific fluorescence. In SE, the localization of photopigments was confirmed by both immunohistochemistry and fluorescence histochemistry (Katagiri *et al*., 1995, 2001). Therefore, SE may be employed as a standard positive control in current fluorescence histochemistry analyses. We simultaneously treated DE and SE mounted on the same glass slide using similar procedures to examine the localization of fluorescence in DE. In SE, the fluorescence from rhodopsin and retinochrome was observed in specific regions after reducing treatment with or without denaturation, whereas in DE, faint diffuse fluorescence was observed and did not always correspond to the photoreceptive sites, such as the outer segments of ciliary-type photoreceptor cells and microvilli of the lens cells. Ohkuma and Tsuda (2000) developed a time-resolved fluorescence difference imaging method to visualize retinal proteins in tissues containing low quantities of retinal proteins but strong endogenous fluorescence. Octopus retina and ocellus of ascidian larva were treated according to the method of Ozaki *et al*. (1983). The subtracted fluorescence of N-retinyl protein before and after UV irradiation effectively distinguished the real change in fluorescence of bleached photopigments in these specimens (Ohkuma and Tsuda, 2000). Using the time-resolved difference fluorescence imaging method**,** real fluorescence changes in *Onchidium* SE were visualized in the villous and somatic layers, although the fluorescence of the connective tissue did not change (Katagiri *et al*., 2000). However, even this method was unsuccessful in visualizing fluorescence in specific regions of DE (Katagiri *et al*., unpublished data), similar to the present study.

It is very difficult to explain clearly why the fluorescence histochemistry technique was unsuccessful in visualizing the fluorescence of photopigments in the DE. There are a number of conceivable reasons for the unexpected obscure fluorescence of DE. Particularly, the amount of photopigment in the DE may be too low for fluorescence microscopy as shown by the present HPLC analysis. Non-specific unknown fluorescence found in the dorsal mantle tissue may cover the intrinsic fluorescence derived from photopigments. It is presumed that the nature of photopigments in DE may be different from that of SE. While fluorescence derived from rhodopsin was distinctly recognized in the rhabdomeric-type visual cells of the SE, it was hardly observed in ciliary-type photoreceptor cells in the DE. In DE, rhodopsin may be reduced only by $NaBH_4$ treatment without denaturation and may therefore diminish rapidly. It is presumed that the fluorescence in the lens cell of DE that appears after $NaBH_4$ treatment (Fig. 7b) is partially derived from rhodopsin. It should be noted that the chromophore of rhodopsin in both photoreceptors of the DE may be lost by formaldehyde/ methanol treatment. On the other hand, the spot-like autofluorescence in the retina of DE was observed in the paranuclei of ciliary-type photoreceptor cells. This specific autofluorescence disappeared after methanol treatment, indicating that the autofluorescence derived from methanol soluble substance such as retinyl-ester. It is known that retinyl-ester emits the fluorescence in the tissue without $NaBH_4$ treatment, although rhodopsin and retinochrome emit the yellow-green fluorescence after treatment with $NaBH_4$ with or without denaturation, respectively (Ozaki *et al*. 1983). The spot-like autofluorescence in Fig. 7b is possibly due to retinyl-ester. The paranucleus is a membrane-bound heterogeneous structure including an electron dense portion, and is one of the presumed storage sites of retinyl-ester. Upon $NaBH_4$ treatment, fluorescence in the spot-like structure partly decreased. The ciliary-type photoreceptor cells contain a small number of endoplasmic reticulum distributed around the paranucleus. Therefore, we suppose that the retinochrome in the endoplasmic reticulum participates in fading fluorescence.

The present study demonstrates that both SE and DE of *Onchidium* have a common photopigment system, a rhodopsin-retinochrome system, containing the 11-cis- and the all-trans-retinal as chromophore. However, there are several important differences between the two eyes, specifically in terms of photoreceptor cell type, photoresponse, spectral sensitivity (Katagiri *et al*., 1985) and the appearance of fluorescence derived from photopigments. Photopigments in photoreceptor cells that comprise the *Onchidium* multiple photoreceptive system may differ in chemical nature.

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REFERENCES

Bownds D and Wald G (1965) Reaction of the rhodopsin chromophore with sodium borohydride. Nature 205: 254–257

Eakin RM (1990) Photic vesicles. Veliger 33: 209–214

Eakin RM and Brandenburger JL (1978) Autofluorescence in the retina of a snail, *Helix aspersa*. Vision Res 18: 1541–1543

Eakin RM (1972) Structure of invertebrate photoreceptors. In "Handbook of Sensory Physiology: Photochemistry of Vision Vol. 7/1" Ed by Dartnall HJA, Springer-Verlag, Berlin, pp 625–684

Eakin RM and Brandenburger JL (1970) Osmic staining of amphibian and gastropod photoreceptors. J Ultrastruct Res 30: 619–641

Hara T, Hara R (1991) Retinal-binding protein: Function in a chromophore exchange system in the squid visual cell. In "Progress in Retinal Research Vol. 10" Eds by Osborne N, Chader J, Pergamon Press, Oxford, pp 179–206

Hara T, Hara R (1973) Biochemical properties of retinochrome. In "Biochemistry and Physiology of Visual Pigments" Ed by Langer H, Springer-Verlag, Berlin, pp 181–191

Hara T, Hara R, Kishigami A, Koshida Y, Horiuchi S, Raj U (1995)

Rhodopsin and retinochrome in the retina of a Tetrabranchiate Cephalopod, *Nautilus pompilus*. Zool Sci 12: 195–201

Hara T, Hara R, Kishigami A, Koshida Y, Horiuchi S, Yoshida M, Yamamoto M, Goto T, Raj U (1992) Localization of retinochrome in the retina of a Tetrabranchiate Cephalopod, *Nautilus pompilius*. Zool Sci 9: 211–217

Herman KG, Strumwasser F (1984) Regional specializations in the eye of *Aplysia*, a neuronal circadian oscillator. J Comp Neurol 230: 593–613

Hisano N, Tateda H. Kuwabara M (1972) Photosensitive neurones in the marine pulmonate mollusc *Onchidium verruculatum*. J Exp Biol 57: 651–660

Jacklet JW, Colquhoun W (1983) Ultrastructure of photoreceptors and circadian pacemaker neurons in the eye of a gastropod, *Bulla*. J Neurocytol 12: 673–696

Katagiri N, Terakita A, Shichida Y and Katagiri Y (2001) Demonstration of a rhodopsin-retinochrome system in the stalk eye of a marine gastropod, *Onchidium,* by immunohistochemistry. J Comp Neurol 433: 380–389

Katagiri N, Katagiri Y, Shimatani Y, Hashimoto Y (1998) 'Lens cell' in the dorsal eye of *Onchidium* is a rhabdomeric-type photoreceptor cell that depolarizes in response to light. Invest Ophthalomol Vis Sci 39: S1059

Katagiri N, Katagiri Y, Shimatani Y, Hashimoto Y (1995) Cell type and fine structure of the retina of *Onchidium* stalk-eye. J Electron Microsc 44: 219–230

Katagiri N, Hama K, Katagiri Y, Shimatani Y, Hashimoto Y, Aikawa E (1990) High-voltage electron microscopic study on the axon of the dermal photoreceptor cell in the dorsal mantle of *Onchidium verruculatum*. J Electron Microsc 39: 363–371

Katagiri N (1984) Cytoplasmic characteristics of three different rhabdomeric photoreceptor cells in a marine gastropod, *Onchidium verruculatum*. J Electron Microsc 33: 142–150

Katagiri N, Fujimoto K, Katagiri Y (1983) Cellular composition and photoresponse of the lens in the *Onchidium* dorsal eye. Zool Mag 92: 199–206

Katagiri Y, Katagiri N, Shimatani Y, Terakita A, Ohkuma M, and Tsuda M. (2000) A rhodopsin-retinochrome system in the *Onchidium* stalk eye. Invest Ophthalomol Vis Sci 41: S599

Katagiri Y, Katagiri N, Fujimoto K (1985) Morphological and electrophysiological studies of a multiple photoreceptive system in a marine gastropod, *Onchidium*. Neurosci Res 2 (suppl): S1–S15

Kataoka S (1975) Fine structure of the retina of a slug, *Limax flavus* L. Vision Res 15: 681–686

Makino-Tasaka, M and Suzuki, T (1986) Quantitative analysis of retinal and 3-dehydroretinal by high-pressure liquid chromatography. Method Enzymol 123: 53–61

Ohkuma M, Tsuda M (2000) Visualization of retinal proteins in the cerebral ganglion of Ascidian, *Halocynthia roretzi*. Zool Sci 17: 161–170

Ozaki K, Terakita A, Hara R, Hara T (1986) Rhodopsin and retinochrome in the retina of a marine gastropod, *Conomulex luhuanus*. Vision Res 26: 691–705

Ozaki K, Hara R, Hara T (1983) Histochemical localization of retinochrome and rhodopsin studied by fluorescence microscopy. Cell Tissue Res 233: 335–345

Pourcho RG, Bernstein MH (1975) Light dependence of osmium reactivity in mouse photoreceptor cells. Am J Anat 143: 371–386

Robles LJ, Camacho JL, Torres SC, Flores A, Fariss RN, Matsumoto B (1995) Retinoid cycling proteins redistribute in light-/dark-adapted Octopus retinas. J Comp Neurol 358: 605–614

Robles LJ, Watanabe A, Kremer NE, Wong F, Bok D (1987) Immunocytochemical localization of photopigments in cephalopod retinae. J Nerurocytol 16: 403–415

Saari JC (1994) Retinoids in photosensitive systems. In "The retinoids: Biology, Chemistry and Medicine 2nd ed", Eds by Sporn MB, Roberts AB, Goodman DS, Raven Press, New York, pp351–385

Stantschinsky W (1908) Über den Bau der Rückenaugen und die Histologie der Rückenregion der Oncidien. Zeit wiss Zool 90: 137–178

Suzuki T, Maeda Y, Toh Y, Eguchi E (1988) Retinyl and 3-dehydroretinyl esters in the crayfish retina. Vision Res 28: 1061–1070

Terakita A, Hara R, Hara T (1989) Retinal-binding protein as a shuttle for retinal in the rhodopsin-retinochrome system of the squid visual cells. Vision Res 29: 639–652

Whittle AC (1976) Reticular specializations in photoreceptors: A review. Zool Scr 5: 191–206

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