

Retinochrome and Rhodopsin in the Extraocular Photoreceptor of the Squid, *Todarodes*

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ABSTRACT The deep-sea squid, *Todarodes pacificus*, possesses well-developed parolfactory vesicles as extraocular photoreceptors connected with the brain. The ventral set of vesicles forms a thread ~ 3mm long and looks orange owing to photopigments. The vesicle mainly consists of receptor cells, each of which is similar in structure to the visual cell, carrying rhabdomeres in the distal process and lamellated myeloid bodies in the proximal part. Recently we noticed that a crude extract of the vesicles is capable of isomerizing retinal from all-*trans* to the 11-*cis* form in the light, and confirmed that the vesicles in fact contained retinochrome in addition to rhodopsin. This is the first time that retinochrome has been detected in any place other than ocular tissues. The optical and chemical nature of these photopigments is the same as that we have observed in the *Todarodes* retina. Quantitative extractions have shown that the total yield of photopigments is ~ 0.0006 in absorbance at λ_{\max} (light path, 10 mm) per milliliter per thread of vesicles, and that the amount of retinochrome in the vesicles is roughly equivalent to that of rhodopsin. Whereas rhodopsin is located in the rhabdomal membranes, retinochrome is probably associated with lamellated structures and their derivatives in the cytoplasm. In the parolfactory vesicles, retinochrome may also cooperate with rhodopsin in the same way as has been discussed for retinal photoreception.

INTRODUCTION

Mollusca contain groups of small vesicles closely associated with the nervous system, as has been ascertained by J. Z. Young and many succeeding researchers. Especially remarkable are the epistellar body of octopods located at the posterior end of the stellate ganglion and the parolfactory vesicles of decapods attached to the surface of the optic tract just behind the optic lobe. On the basis of accumulated studies in histology, electron microscopy, biochemistry, and electrophysiology (cf. Mauro, 1977), at present there can be no doubt that they are capable of catching light and hence of acting as extraocular photoreceptors. Although nothing is yet clear about the biological significance of the presence of extraocular photoreceptors in the nervous system, they seem to be of importance in the physiological regulation and behavior of cephalo-

Pods, as suggested especially by the electrophysiological evidence that action potentials induced by irradiation are conducted down the axon toward the brain (Mauro and Baumann, 1968; Perrelet and Mauro, 1972; Mauro and Sten-Knudsen, 1972; Sperling et al., 1973).

The epistellar body and the parolfactory vesicles resemble each other in structure in that both contain many light receptor cells which send a process into the lumen of the vesicle (Nishioka et al., 1966 *b*; Messenger, 1967; Baumann et al., 1970). The process is provided with microvilli and forms a rhabdom (Nishioka et al., 1962; Nishioka et al., 1966 *b*; Perrelet and Mauro, 1972), similar to the outer segment of the visual cell in the cephalopod retina. In order to absorb light, these organs must contain photosensitive pigment, probably in the microvillar membranes of the rhabdomes. In fact, a photopigment was extracted from rhabdomeric materials of the epistellar body of *Eledone moschata* and the parolfactory vesicles of *Loligo vulgaris*, and was spectroscopically and biochemically identified as the same as rhodopsin in the eye (Nishioka et al., 1966 *a, b*).

It is well known that, in the cephalopod retina, the visual cells have a dual system of photosensitive chromoproteins with retinaldehyde as chromophores, rhodopsin, and retinochrome (Hara and Hara, 1965; Hara et al., 1967). Rhodopsin is associated only with the microvillar membranes of the outer segments of the visual cells, whereas retinochrome is found in both inner and outer segments (Hara and Hara, 1973 *a*, 1975 *a*, 1976). The two photopigments differ from each other in the stereoisomeric configuration of their chromophores, which is 11-*cis* in rhodopsin but all-*trans* in retinochrome. When they absorb light, their chromophores are changed respectively to the all-*trans* and the 11-*cis* configurations (Hara and Hara, 1967). In view of this difference, it was suggested that these photopigments could cooperate so that each, in the light, assists in the regeneration of the other (Hara and Hara, 1968, 1972). Since the discovery of rhodopsin in the extraocular photoreceptors by Nishioka et al. (1966 *a, b*), we have therefore suspected that they might also contain retinochrome. Particularly, the work with deep-sea squids such as *Todarodes* (Baumann et al., 1970; Perrelet and Mauro, 1972; Mauro and Sten-Knudsen, 1972) has stimulated us to investigate this in the parolfactory vesicles of the Japanese common squid, *Todarodes pacificus*. This paper presents conclusive evidence that retinochrome exists together with rhodopsin in other tissues than the retina. A preliminary account has been presented elsewhere (Hara and Hara, 1975 *b*), and discussed at the Third International Congress of Eye Research held in Osaka in May, 1978.

METHODS

The squids, *Todarodes pacificus*, were captured during the night in the southwestern waters of the Japan Sea. They have been familiar to us Japanese physiologists in studies on retinal photopigments (mantle length, ca. 22 cm; body wt, ca. 300 g). For morphological observations, the heads were removed as soon as possible after capture, and the parolfactory vesicles were excised and transferred into fixative for histology. For biochemical analyses, about 400 squids were decapitated at a time at the seaside before dawn, and their heads were brought to our laboratory in the dark at 0°C, and

frozen and stored at -20°C until required for the extraction of the photopigments of the parolfactory vesicles.

The vesicles were excised as quickly as possible under dim white light, because their orange color served as a guide for identifying them. When the head was cut open, the ventral set of parolfactory vesicles was found in a short thread hanging down from the optic tract between the optic lobe and the brain. A pair of such threads located on either side of the brain was lifted out with fine forceps and pooled in a lightproof, cold vessel. The present purpose was not always to prepare excellent solutions of photopigments, but to collect all the pigment. To this end, many threads of vesicles were cut in pieces with scissors, homogenized in 5 ml M/15 phosphate buffer, pH 6.5, with a teflon Potter-Elvehjem homogenizer, and centrifuged. The tissue fragments were suspended in 5 ml Weber-Edsall solution for 30 min, washed twice with water, and then treated with M/5 KH_2PO_4 for a short time. Finally the residue was washed once with phosphate buffer, mixed with 1.2 ml 2% digitonin solution buffered at pH 6.5, and extracted by gentle shaking. All the operations for preparing photopigment extracts were carried out under a deep red light, and at temperatures as low as 4°C except some extractions with digitonin.

Crystalline all-*trans*-retinal was made by our routine method from all-*trans*-vitamin A alcohol: the alcohol was oxidized to retinal on a column of active manganese dioxide, and retinal was crystallized from a concentrated solution in petroleum ether at about -70°C . After recrystallization, all-*trans*-retinal was dissolved in digitonin solution at about 43°C and diluted before use as a test reagent. For preparing opsin, cattle retinas were shaken in neutral phosphate buffer, spread on a plate, and exposed to white light for 1 h. The same procedure as used for preparing rhodopsin was then carried out in the light. The outer segments were suspended in a 36% solution of sucrose and floated by centrifuging. After two such flotations, the residue was washed with phosphate buffer, hardened in 4% potassium alum, lyophilized and extracted with petroleum ether, and then extracted with 2% digitonin. The opsin solution thus prepared was adjusted so as to yield rhodopsin with absorbance of about 3.0^1 or 1.0 (in Fig. 4) at 500 nm in a 10-mm light path, when incubated in the dark with an excess of 11-*cis*-retinal.

In the present study, irradiation was carried out with orange light (>560 nm) from a 100 W tungsten filament projector lamp equipped with a glass filter (Toshiba VO-56, Toshiba Corp., Tokyo). All absorption measurements were performed with a Shimadzu model MPS-5000 recording spectrophotometer (Shimadzu Corp., Kyoto, Japan). The photopigments in the extracts were distinguished from one another by the methods described later, and their amounts were determined from the absorbance at the absorption maximum of each. The composition of retinal isomers was examined by a Hitachi model 635 high performance liquid chromatograph (Hitachi Corp., Tokyo), and printed out in a molar ratio by a Takeda-Riken model TR-2200 integrator (Takeda-Riken Co. Ltd., Tokyo).

For microanatomy, the parolfactory vesicles were fixed in 10% neutral formalin, sectioned in paraplast at $6\ \mu\text{m}$, and stained with haematoxylin and eosin. For electron microscopy, the vesicles were fixed in 2.5% glutaraldehyde in 0.18 M cacodylate buffer (pH 7.2), washed with the buffer, and postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer. After fixation they were stained with uranium acetate, dehydrated through a graded series of alcohols, and embedded in Epon 812 resin (Shell Chemical Co., New York). Epon sections were further stained with lead citrate and examined in a Hitachi HS-7 electron microscope. In addition, thick sections ($1\ \mu\text{m}$),

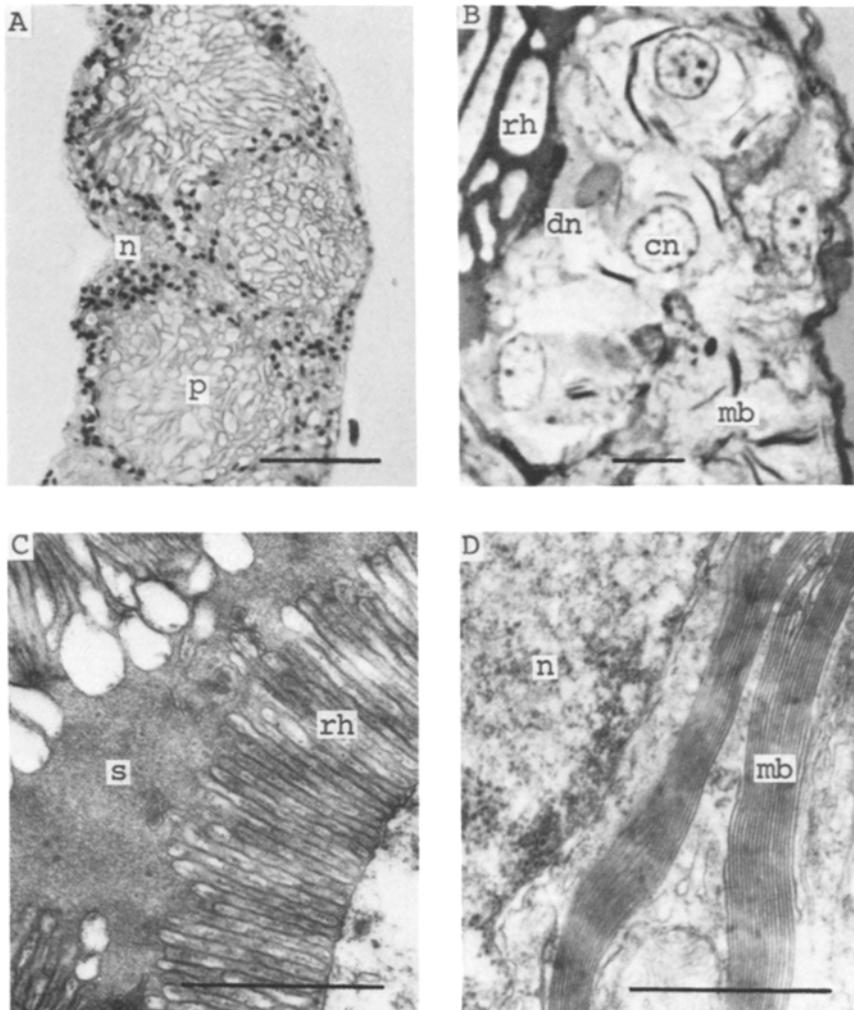
¹ This sample was used in the experiment matching that of Fig. 2 described in Results.

derived from the same material, were stained with toluidine blue for examination with the light microscope.

RESULTS

Morphological Observations on Parolfactory Vesicles

The parolfactory vesicles of decapods are orange-pigmented small sacs found near the optic tract. In the deep-sea squids such as *Todarodes*, they are much better developed than in *Loligo*, and particularly the ventral set of vesicles forms a thread, extending down from the optic tract (Baumann et al., 1970). In *Todarodes pacificus*, the thread is 2–3 mm long and about 0.5 mm wide, and contains probably 20–30 vesicular sacs that are 150–200 μm in diameter. As shown in histological sections (Fig. 1 A and B), the wall of the vesicular sacs is composed of supporting cells and of the nucleated cell bodies of photorecep-



tors; the central lumen of the sacs is filled with the tapering and crooked processes of the photoreceptor cells. The processes differ in size and are randomly arranged in the lumen. However, each of them is entirely surrounded by a brush of microvilli (Fig. 1 C) to form a somewhat different type of rhabdom from that seen in the distal segments of the visual cells. Unlike the visual cell, this photoreceptor cell contains no screening pigment of ommin granules and shows no distinct basement membrane between the process and the cell body. Its cytoplasm contains a number of lamellated bundles of membranes (Fig. 1 D) in the basal region of the process as well as in the nucleated cell body (Fig. 1 B). These structures are very similar to the myeloid bodies revealed by electron microscopy in the proximal segments of visual cells of various cephalopods, such as octopus (Yamamoto et al., 1965) and squid (Zonana, 1961; Hara and Hara, 1975 a). The parolfactory vesicles are originally orange in color and slightly fade on exposure to light, indicating the presence of photopigment. Since cephalopod rhodopsin is not readily bleached by light at ordinary temperatures (Hubbard and St. George, 1958; Hara and Hara, 1972), this fact suggests that the parolfactory vesicles contain a different photopigment from rhodopsin. We therefore considered that the photolabile component might be retinochrome.

Isomerization of Retinal with Crude Extract of Parolfactory Vesicles

When retinochrome is irradiated with visible light, its chromophore is isomerized into the 11-*cis* configuration. In the presence of all-*trans*-retinal, the protein moiety of bleached retinochrome (metaretinochrome) immediately regenerates retinochrome in the dark. The rapidity of this resynthesis is one of

FIGURE 1 (*opposite*). (A) Light micrograph of the ventral parolfactory vesicles of *Todarodes pacificus*. A 6- μ m paraplast section stained with hematoxylin and eosin. A series of the vesicles including photoreceptor cells forms a short thread-like organ 2-3 mm in length. The nuclei of photoreceptor cells (n) are arranged in the wall of the vesicle, and their processes (p) protrude irregularly into the central lumen. The bar represents 100 μ m (\times 160). (B) Light micrograph showing a portion of the wall of the parolfactory vesicle. The material, prepared for electron microscopy, was sectioned at 1 μ m and stained with toluidine blue. Dark nuclei (dn) belong to supporting cells; clear nuclei (cn) are of photoreceptor cells. In the cytoplasm of the photoreceptor cell, lamellated membrane systems (myeloid bodies, mb), which look like dark pieces of tape, are rich around the nucleus and in the basal region of the process. The surface of the process is covered with rhabdomeres (rh), densely dyed with toluidine blue. The bar represents 10 μ m (\times 960). (C) Electron micrograph of the rhabdomere in the photoreceptor cell. Many microvilli project perpendicularly from the process of the photoreceptor cell, to form the rhabdomere (rh) that entirely surrounds the process. The rhabdomeres of adjacent processes usually fuse in a type of rhabdom, but sometimes extracellular space (s) can be seen between the processes. The bar represents 1 μ m (\times 27,000). (D) Electron micrograph showing the lamellated membranes of the myeloid bodies (mb) present near the nucleus (n). Such structures composed of many layers are very similar to those found in the inner segments of the visual cells of cephalopods and perhaps contain the retinochrome that has been extracted from the parolfactory vesicles (\times 27,000).

the characteristic properties of retinochrome protein (Hara and Hara, 1968, 1973 *a*). Consequently, when all-*trans*-retinal is mixed with a small amount of retinochrome and irradiated with an orange light which retinal does not absorb, 11-*cis*-retinal accumulates during the irradiation, because the retinochrome is photolyzed and resynthesized repeatedly until most of the retinal has been converted from all-*trans* to the 11-*cis* configuration. In this process, the absorption maximum of all-*trans*-retinal in digitonin solution (near 390 nm) is shifted toward 380 nm, and this is accompanied by a characteristic marked decrease in absorbance. Such a reaction does not proceed without retinochrome protein, so that the catalytic specificity gives us a useful method to detect a trace of retinochrome. The experiment illustrated in Fig. 2 was

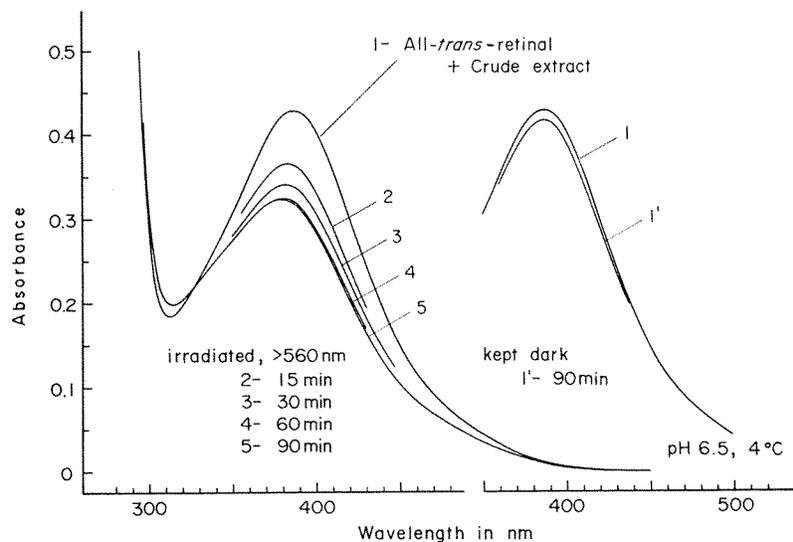


FIGURE 2. Light isomerization of retinal with a crude extract of the parolfactory vesicles of *Todarodes pacificus*. 30 pieces of the organ were mashed in a solution of all-*trans*-retinal, and the supernate was prepared by centrifugation (spectrum 1). On irradiating the supernate with orange light (>560 nm) at 4°C, the absorption around 390 nm gradually decreased and λ_{\max} shifted toward shorter wavelengths, so that spectra 2, 3, 4, and 5 were recorded after successive irradiations of 15, 15, 30, and 30 min. The fall in absorbance was almost complete 1 h after the start of irradiation. An aliquot in the dark showed no marked spectral change, as indicated by spectrum 1' recorded after 90 min. This experiment suggests that the parolfactory vesicles contain a light-sensitive component that promotes the isomerization of retinal from all-*trans* to *cis*.

therefore designed in order to test the presence of retinochrome in the parolfactory vesicles.

30 pieces of the excised thread of parolfactory vesicles were placed in 2.5 ml of ~0.3% digitonin, pH 6.5, that contained all-*trans*-retinal with an absorbance of 1.0 at λ_{\max} . They were crushed down into fine fragments with a teflon homogenizer, and centrifuged at 12,000 rpm for 20 min. So much of the all-*trans*-retinal was adsorbed by the tissue fragments and sedimented with them

that the absorbance of the supernate was reduced to 0.425 at 390 nm (spectrum 1). Of course, this preparation contained some digitonin extract of parolfactory vesicles, showing strong absorption at wavelengths shorter than 300 nm. When a 1-ml aliquot of it was irradiated at 4°C with orange light successively for 15, 15, 30, and 30 min, the near ultraviolet absorption peak of retinal was gradually lowered and slightly shifted toward shorter wavelengths (spectra 2, 3, 4, and 5).² The last two spectra nearly overlapped each other, showing that the reaction was almost completed 1 h after irradiation. Another 1-ml aliquot (spectrum 1) was kept dark at 4°C as an unirradiated control, and its absorption was monitored at the same intervals. No marked change was observed even after 90 min (spectrum 1').

In order to confirm that the fall in absorbance induced by the irradiation was due not to destruction of retinal but to its isomerization, the irradiated and unirradiated aliquots (spectra 5 and 1') were mixed with 0.5 ml cattle opsin, incubated dark for 2 h at 20°C, and examined for the formation of photosensitive pigment. After freshly neutralized hydroxylamine (NH₂OH) was added to the mixtures, spectra before and after irradiation with orange light were measured. The unirradiated mixture hardly showed any formation of photopigment. In contrast, the irradiated mixture yielded cattle rhodopsin with λ_{max} at 500 nm, showing that 11-*cis*-retinal had been formed in the aliquot that has spectrum 5. The concentration of all-*trans*-retinal in the aliquot in spectrum 1 was estimated to be 0.399 in absorbance at 390 nm. If it had been photoisomerized completely to 11-*cis*-retinal, it should have yielded cattle rhodopsin with an absorbance of 0.423 at 500 nm. This corresponds to 0.282 (= 0.423 $\times \frac{2}{3}$) in the mixture with opsin, whereas the absorption of synthesized rhodopsin was 0.201. Hence, 71% of the all-*trans*-retinal was converted to the 11-*cis* form during the irradiation in the presence of a crude extract of parolfactory vesicles.

A separate identification was carried out also by means of high-performance liquid chromatography. Another preparation consisting of all-*trans*-retinal and the extract of vesicles was made by the same procedures as used before, and two aliquots of it were kept at 4°C for 90 min, one in the dark and the other in orange light. Hexane was then added to both, the samples were shaken vigorously and centrifuged to separate the hexane layers, and the retinal isomers in the hexane extracts were analyzed. The isomeric composition is shown in Table I. All-*trans*-retinal in the unirradiated sample was scarcely changed. In the irradiated sample, however, all-*trans* was decreased to 17%, whereas 67% had been converted to 11-*cis*. These results were consistent with the previous experiment in which the amount of 11-*cis*-retinal was determined with cattle opsin. Hence, the parolfactory vesicles undoubtedly contain a photosensitive component—perhaps retinochrome—that can promote the *trans*-to-*cis* isomerization of retinal in the light.

Photopigments Extracted from Parolfactory Vesicles

Further experiments were aimed at elucidating whether the parolfactory vesicles have the same photopigments as we have observed in the squid eye.

² A similar spectral change could be observed even with only a few pieces of the thread of parolfactory vesicles.

Extracts from ~ 50 threads were too dilute to analyze their composition, although active enough to isomerize large quantities of retinal. To identify the photopigment we had to collect many threads of parolfactory vesicles, as shown in Table II. The identification of photopigments was based upon the spectral and biochemical properties of rhodopsin and retinochrome found in the visual cells of the squid, *Todarodes pacificus* (cf. Hara and Hara, 1972, 1973 b).

Rhodopsin and retinochrome have λ_{\max} near 480 and 490 nm, respectively. When irradiated with orange light at alkaline pH (e.g., 10.5), rhodopsin is bleached to alkaline metarhodopsin with λ_{\max} at 378 nm. However, on irradiation at weakly acid pH (e.g., 6.5), it does not bleach and slightly deepens in color, resulting in a one-to-one photochemical equilibrium mixture of rhodopsin and acid metarhodopsin. Acid metarhodopsin has λ_{\max} at 488

TABLE I
ANALYSIS OF THE MIXTURE PRODUCED BY LIGHT
ISOMERIZATION OF ALL-*trans*-RETINAL WITH A CRUDE
EXTRACT OF PAROLFFACTORY VESICLES

Isomer	Retinal solution %	Retinal solution + crude extract*	
		Dark	Irradiated >560 nm %
13- <i>cis</i>	1	3	10
11- <i>cis</i>	—	—	67
9- <i>cis</i>	—	1	4
7- <i>cis</i>	—	—	2
All- <i>trans</i>	99	97	17

* The mixtures were kept dark or light at 4°C for 90 min.

nm and about 1.5 times the maximum absorbance of rhodopsin. On the other hand, retinochrome is bleached by exposure to orange light, irrespective of the pH of the medium. At acid and neutral pH, the photoproduct metaretinochrome shows a low peak near 470 nm. Spectra of both retinochrome and metaretinochrome are dependent on pH: at alkaline pH, the absorbance of retinochrome at 490 nm decreases and that of metaretinochrome disappears. Unlike rhodopsin and metarhodopsin, retinochrome and metaretinochrome are instantly destroyed by hydroxylamine, so that the absorption in the visible disappears completely.

In view of these findings, photopigment extracts were irradiated under the following circumstances: (a) at alkaline pH, (b) at weakly acid pH, and (c) in the presence of NH_2OH . A set of results is shown in Fig. 3. The three samples are derived from the first extraction in experiment IV shown in Table II. Their spectrum was characterized by two absorption maxima near 482 nm and 275 nm, as shown in spectrum 1 in Fig. 3 C.

IRRADIATION AT ALKALINE pH The sample was irradiated at pH 10.5 (see Fig. 3 A), in the same way as used by Nishioka et al. (1966 b) for identifying rhodopsin in extraocular photoreceptors. When the pH of the

sample was raised from 6.5 to 10.5 by addition of a trace of 2 M sodium hydroxide, spectrum 1 changed to 2, as the absorption peak decreased. When exposed to orange light for 5 min, the remaining photopigment was bleached (spectrum 3). These results were essentially the same as those observed by Nishioka et al. (1966 *b*). The fall in absorbance after the alkalization might be due to the conversion of metarhodopsin from the acid to the alkaline form; the fall after irradiation might be due to rhodopsin bleaching. Even if retinochrome were contained in the extract, it would be difficult to detect in this type of experiment, because retinochrome loses part of its visible absorp-

TABLE II
EXTRACTIONS OF RHODOPSIN, METARHODOPSIN, AND
RETINOCHROME FROM PAROLFACTORY VESICLES OF THE
SQUID, *Todarodes pacificus*

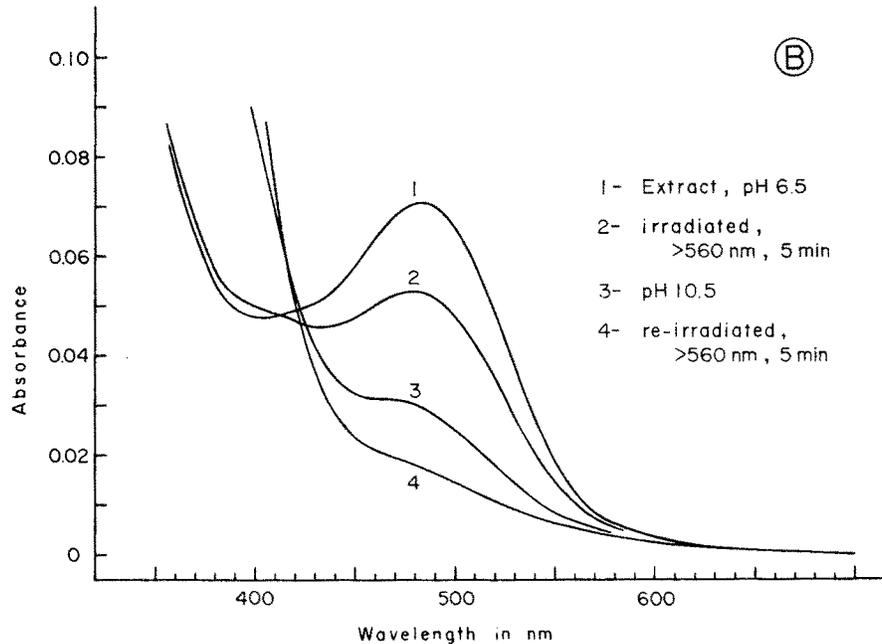
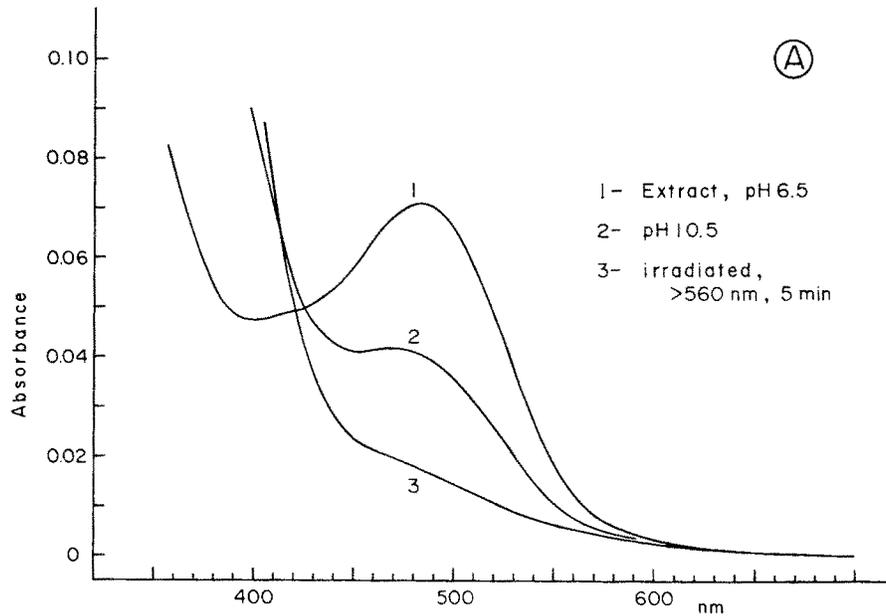
Experiment	Extraction			R	MR	Ret	Total pig- ment
	No.	Time	Temp.				
	<i>n</i>	<i>h</i>	°C	absorbance at λ_{\max} per milliliter			
I (235)	1	1	20	0.0451	0.0150	0.0183	0.0784
II (130)	1	3	4	0.0304	0.0213	0.0191	0.0708
III (571)	1	1	15	0.0615	0.0247	0.0681	0.1543
	2	2	18	0.0318	0.0210	0.0465	0.0993
	3	8	18	0.0084	0.0102	0.0240	0.0426
	4	16	18	0.0024	0.0048	0.0255	0.0327
	5	16	18	—	—	0.0105	0.0105
Totals				0.1041	0.0607	0.1746	0.3394
IV (515)	1	1.5	4	0.0508	0.0421	0.0976	0.1905
	2	3.5	4	0.0134	0.0076	0.0524	0.0734
	3	20	4	0.0019	0.0032	0.0246	0.0297
	4	24	20	—	—	0.0045	0.0045
Totals				0.0661	0.0529	0.1791	0.2981

Abbreviations: R, rhodopsin; MR, metarhodopsin; Ret, retinochrome. In parentheses are shown the numbers of threads used in the four experiments.

tion on raising pH and further bleaches on irradiation. However, λ_{\max} in the difference spectrum before and after irradiation (spectra 3-4) was shifted slightly toward longer wavelengths compared with that of rhodopsin (480 nm), giving a hint of the presence of retinochrome in the extract.

IRRADIATION AT WEAKLY ACID pH As seen in Fig. 3 B, the sample at pH 6.5 (spectrum 1) was partly bleached on exposure to orange light (spectrum 2). By changing the pH to 10.5 the absorbance was further decreased (spectrum 3), and irradiation with orange light bleached this sample completely (spectrum 4). If the initial extract had contained predominantly rhodopsin, the absorbance would have increased in the first irradiation, owing

to the formation of acid metarhodopsin with a higher extinction coefficient. And, if it had been a photoequilibrium mixture of rhodopsin and acid metarhodopsin, the absorbance would have remained unchanged on irradiation. Furthermore, if the original extract consists only of rhodopsin with its photoproduct, it cannot be bleached by irradiation at pH 6.5. The partial bleaching caused by the first irradiation must mean that the extract also



contained another photopigment (retinochrome) which could be bleached readily by light at weakly acid pH. The further bleaching on alkalization therefore is due to absorbance decreases of acid metarhodopsin and metaretnochrome, and the final decrease in absorbance on irradiation is due to

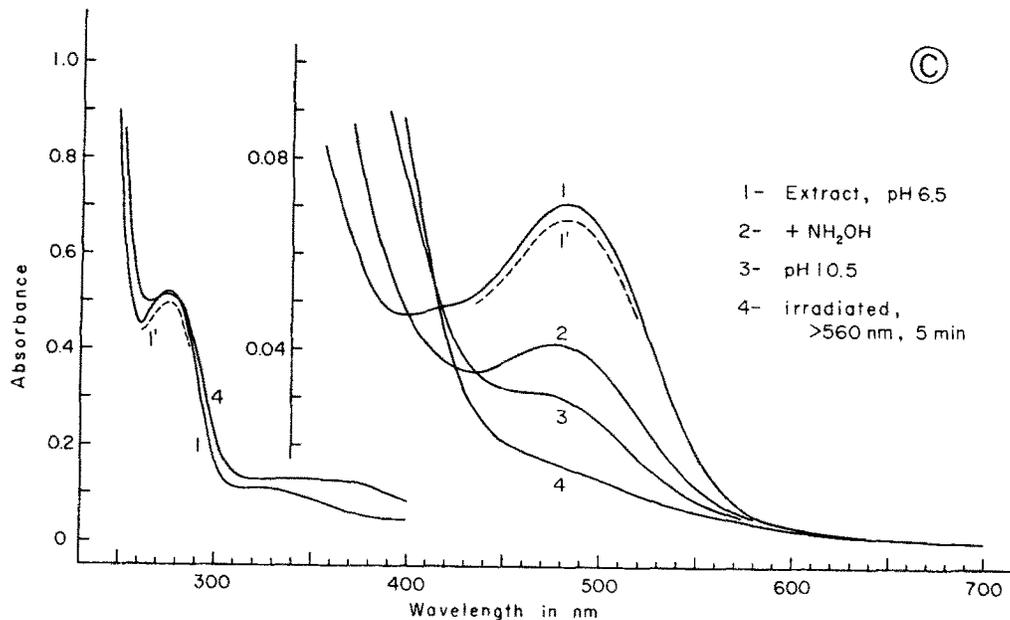


FIGURE 3. Absorption spectrum of a digitonin extract from the parolfactory vesicles, and identification of retinochrome, metarhodopsin, and rhodopsin. Samples were derived from the first extraction in experiment IV of Table II, and showed two main peaks of absorption near 482 and 275 nm, as indicated by spectrum 1 in panel C. (Opposite) (A) Irradiation at pH 10.5. After the pH was raised from 6.5 to 10.5 (spectrum 2), the extract was bleached by irradiation with orange light (spectrum 3). λ_{\max} of the difference spectrum for bleaching lay at somewhat longer wavelength than that of rhodopsin (480 nm), suggesting that retinochrome may be contained in the extract. (B) Irradiation at pH 6.5. When irradiated at pH 6.5, the spectrum of the extract was readily changed to spectrum 2, which shows that the extract contained retinochrome. After changing the pH to 10.5 (spectrum 3), the extract could be bleached further by re-irradiation with orange light, owing to the photolysis of rhodopsin which had remained after the previous irradiation at pH 6.5. It was therefore clear that both retinochrome and rhodopsin were present in the extract. (Above) (C) Irradiation in the presence of NH_2OH . To 1 ml of the extract at pH 6.5 (spectrum 1), 50 μl of 1 M NH_2OH were added to detect retinochrome (spectrum 2). The pH of the mixture was then raised to 10.5 to convert acid metarhodopsin to the alkaline form (spectrum 3), and finally the mixture was irradiated 5 min with orange light to bleach rhodopsin (spectrum 4). Since spectra 2, 3, and 4 were derived from the extract diluted with NH_2OH , spectrum 1 was corrected for comparison (broken line, spectrum 1'). The stepwise decrease in absorbance in the visible range that was caused by these procedures gave a method to estimate the amount of each photopigment in an extract. See text for further details.

bleaching of the rhodopsin which remained in equilibrium with metarhodopsin during the first irradiation.

IRRADIATION IN THE PRESENCE OF HYDROXYLAMINE Retinochrome and metaretinochrome are quickly decomposed by addition of low concentrations of hydroxylamine, with which they form retinaldehyde oximes (Hara and Hara, 1967, 1972, 1973 *b*). This gives us a useful method to discriminate retinochrome from rhodopsin and metarhodopsin. Fig. 3 C shows such an experiment, using the same sample as described above. Actually, when 50 μ l of 1 M NH_2OH were added to 1 ml of the sample at pH 6.5 (spectrum 1), the absorbance was greatly decreased in the visible range (spectrum 2), far lower than would result from the dilution (spectrum 1'). On raising the pH to 10.5, the absorption decreased further (spectrum 3). Finally, the sample was bleached by exposure to orange light (spectrum 4). The decrease in absorbance caused by adding NH_2OH is due to the decomposition of retinochrome.³ Rhodopsin and metarhodopsin remain unchanged. The further decrease in absorbance on alkalization is due to the conversion of acid metarhodopsin to alkaline metarhodopsin, and the final decrease on irradiation is due to the bleaching of rhodopsin to alkaline metarhodopsin. In fact, the absorption maxima of the difference spectra obtained by subtracting spectrum 2 from 1', spectrum 3 from 2, and spectrum 4 from 3 were identical with the known values: near 490 nm for retinochrome, 488 nm for acid metarhodopsin, and 480 nm for rhodopsin (cf. Hara and Hara, 1972). From each difference in absorbance, we could therefore measure the amounts of the three different photopigments.

Photopigment extracts usually were bleached to different degrees by direct irradiation with orange light (cf. Fig. 3 B) or by addition of NH_2OH (cf. Fig. 3 C), depending on the proportions of the different components. In any case, the observed bleaching is fully explained by the assumption that retinochrome is present in the parolfactory vesicles together with rhodopsin and metarhodopsin.

Contents of Photopigments in Parolfactory Vesicles

In order to estimate how much photopigment is contained in the parolfactory vesicles, the amounts of rhodopsin, metarhodopsin, and retinochrome in each extract were determined from the absorbance at their absorption maxima as described in Fig. 3 C. The results are summarized in Table II, where they are expressed as the absorbance that would be measured if the volume of the extract throughout were 1 ml. In experiments III and IV, the extraction with digitonin was repeated until no further photopigment was released. Dashes indicate that we could not detect any photopigment.

The total amounts of photopigment in a thread of parolfactory vesicles are calculated to be 0.00033, 0.00054, 0.00058, and 0.00059 per milliliter in

³ Metaretinochrome was not present in the extract. In fact, λ_{max} of the difference spectrum before and after addition of NH_2OH was not at 470 nm as for metaretinochrome, but at 490 nm as for retinochrome. Metaretinochrome spontaneously regenerates to retinochrome when kept in the dark (cf. Hara and Hara, 1976).

experiments I, II, III, and IV. Based upon the successive extractions in experiments III and IV, it is better estimated as about 0.0006 per milliliter. In experiment I (single extraction for a short time), the yield is smaller than in the others, with more rhodopsin than retinochrome. In this case, about half the photopigment, and perhaps most of the retinochrome may have remained in the tissue. As seen in experiments III and IV, the proportion of retinochrome to rhodopsin increases as the extraction advances, and the last two extracts mainly contain retinochrome. This tendency of rhodopsin to be more readily extracted than retinochrome was quite similar to what we had observed with the *Todarodes* retina (Hara and Hara, 1976.) It can perhaps be ascribed to a difference in the intracellular location of the two photopigments: in the parolfactory vesicles, rhodopsin probably is located in the microvillar membranes, whereas retinochrome may be combined with such structures as the myeloid bodies in the cytoplasm.

In the two quantitative experiments, III and IV, the ratios of rhodopsin, metarhodopsin, and retinochrome were approximately 10:6:17 and 7:5:18. This is equivalent to molar ratios of 10:4:12 and 7:3:12, assuming that the extinction coefficients of metarhodopsin and retinochrome are about 1.5 times that of rhodopsin (Hara and Hara, 1972, 1973 *a*). High proportion of metarhodopsin seem to be caused by the inevitable exposure of tissues to a dim white light during dissection. In any case, since metarhodopsin is derived from the photolysis of rhodopsin, the ratio of rhodopsin to retinochrome in the vesicles can be estimated as 14:12 or 10:12. In other words, their amounts are roughly equal in the parolfactory vesicles. For comparison, the corresponding proportion in the *Todarodes* retina is calculated at 14:10 from our previous date (Hara and Hara, 1976).

Retinal Isomerase Activity of Photopigment Extract

As stated before, a crude extract of parolfactory vesicles was able to catalyze the isomerization of retinal toward the *cis* form in the light. The following experiments with better extracts were performed to examine whether the retinochrome contained in the vesicles is equivalent in isomerase activity to that found in the eye. Coupling with cattle opsin was used to determine the amount of 11-*cis*-retinal, and high-performance liquid chromatography (HPLC) to determine the isomeric composition of retinal.

Fig. 4 shows an experiment with part of the second extract obtained in experiment III of Table II. The photopigment extract at pH 6.5 (spectrum 1) was irradiated at 4°C with orange light (spectrum 2). This bleached extract contained a photoequilibrium mixture of rhodopsin and acid metarhodopsin in addition to the photoproduct, metaretinochrome, which was desired for the experiment. Although this equilibrium mixture was not removed from the extract, it exerted no bad effect in view of the experimental results so far achieved. When 1.0 ml of the bleached extract was mixed with 0.2 ml all-*trans*-retinal (spectrum 3), the net concentration of all-*trans*-retinal in the mixture was estimated to be 0.408 in absorbance at its λ_{\max} . This mixture was then irradiated with orange light at 4°C, and spectral changes were recorded

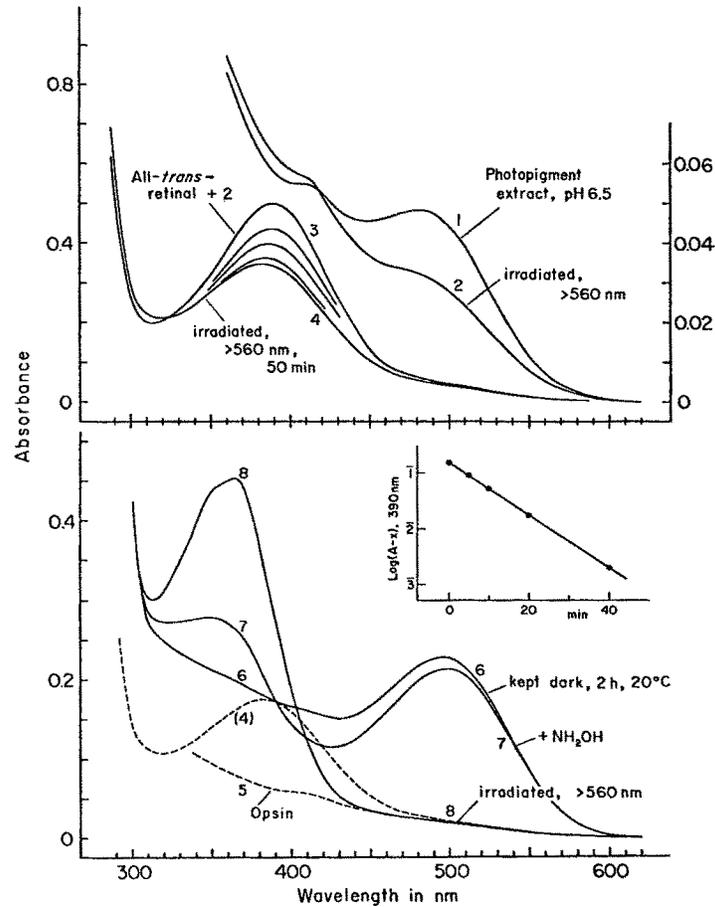


FIGURE 4. Retinal isomerase activity of photopigment extract from the parol-factory vesicles. The extract at pH 6.5 from the second extraction in experiment III of Table II (spectrum 1, ordinate at right) was bleached at 4°C by a 5-min exposure to orange light (spectrum 2), and 1 ml of the bleached extract was mixed with 0.2 ml all-*trans*-retinal (spectrum 3, ordinate at left). When this mixture was irradiated at 4°C with orange light (>560 nm, not absorbed by retinal), the near-ultraviolet absorption peak of retinal decreased greatly (spectrum 4), as revealed by the spectral changes observed at intervals. As shown in the *inset*, the time-course of the fall in absorbance is linear when $\log(A - x)$ is plotted against time, where A is the absorbance at 390 nm of the sample at time t , and x is its minimal value after prolonged irradiation. In order to determine what kinds of retinal isomers were produced during the irradiation, a part of the sample (spectrum 4) was mixed with the same volume of cattle opsin (spectrum 5), and kept in the dark 2 h at 20°C to test for the formation of cattle photopigment (spectrum 6). After 10 μ l of 1 M NH_2OH were added to the mixture (spectrum 7), the synthesized pigment was bleached by a 5-min irradiation with orange light (spectrum 8). The difference spectrum before and after bleaching was that of cattle rhodopsin with λ_{max} 500 nm, and showed that the all-*trans*-retinal shown in spectrum 3 had been isomerized by irradiation almost completely to the 11-*cis* form in spectrum 4.

after 5, 10, 20, 40, and 50 min. The last two spectra fully overlapped, showing that the isomerization was finished after 50 min (spectrum 4). The time-course of the fall in absorbance at 390 nm (isomerization) apparently followed first-order kinetics, as shown by a straight line on the inserted graph with the semilogarithmic scale in Fig. 4.

Half a volume (0.6 ml) of the isomerate produced by the irradiation was then mixed with the same volume of cattle opsin (spectrum 5), and the mixture was kept in the dark 2 h at 20°C. During the incubation the absorbance near 500 nm increased, indicating the synthesis of a pigment (spectrum 6). When the synthesized pigment was irradiated 5 min with orange light after addition of 10 μ l of 1 M NH_2OH , spectrum 7 was changed into spectrum 8. In the difference spectrum before and after bleaching, λ_{max} was at 500 nm, showing that cattle rhodopsin had been formed. Since the opsin was present in excess, the all-*trans*-retinal present in the initial mixture (spectrum 3; absorbance at 390 nm, $0.408 \times \frac{1}{2} = 0.204$) was almost completely recovered as the synthesized rhodopsin in the final mixture (spectrum 7; absorbance at 500 nm, 0.192). This means that in visible light nearly all the all-*trans*-retinal (~90%) was isomerized to 11-*cis* by retinochrome. In another experiment with about double the amount of the bleached extract of photopigment, the isomerate derived from all-*trans*-retinal had an absorbance of 0.242 at 390 nm and yielded a rhodopsin absorbance of 0.238 at 500 nm, the recovery being as much as 93%.

The product of the photoisomerization also was examined by HPLC as follows. One-half of the extract obtained in experiment II of Table II was similarly irradiated with orange light and mixed with all-*trans*-retinal. The all-*trans*-retinal in this mixture had an absorbance of 0.344 at 390 nm. The mixture was divided into two 1 ml portions, and one was irradiated with orange light at 4°C, until the decrease in absorbance was completed (after 50 min). The other was kept dark at 4°C as a control and showed no change in spectrum during the same period. The resulting isomerate in each mixture was extracted with hexane and analyzed for its isomeric composition, as shown in Table III. In the control mixture in the dark, there was no isomerization of retinal.⁴ In contrast, when the mixture was irradiated, all-*trans*-retinal was almost entirely isomerized to the 11-*cis* form (98%). Of course, the all-*trans*-retinal solution showed neither spectral change nor isomerization on exposure to orange light, unless mixed with the bleached extract of vesicles. It was therefore evident that the observed photoisomerization was promoted by the retinochrome protein in the extract. These results with HPLC were consistent with those obtained with opsin. We conclude that the parolfactory vesicles contain retinochrome with the same capacity to isomerize retinal specifically from all-*trans* to the 11-*cis* form as that in the squid eye.

When a crude extract of vesicles was used in the experiment shown in Fig. 2, the percentage of 11-*cis*-retinal produced from all-*trans* was as low as 70%. In this case, it seems likely that much of the all-*trans*-retinal was trapped by

⁴ A small amount of 11-*cis*-retinal (2%) must be derived from metaretinochrome introduced into the mixture. The chromophores of rhodopsin and metarhodopsin are not extracted into hexane by the present treatment.

lipids and proteins other than retinochrome, and that it was not accessible for isomerization by retinochrome. Table III also includes results obtained with an extract from the optic lobe. 10 pieces of tissue, about 1.2 g, were withdrawn from the optic lobes of five animals, treated in the same way as the vesicles and extracted with digitonin. To facilitate comparison, the extract was diluted to the same absorbance near 280 nm as that of the parolfactory vesicles in the above experiment, and mixed with all-*trans*-retinal. The final absorbance of retinal was 0.316 at 390 nm. In the dark, there was hardly any isomerization of retinal, and even after a 50-min irradiation, the percentage of 11-*cis* isomer was only 12%. This suggests that the optic lobe may not contain a component like retinochrome.

TABLE III
ANALYSIS OF THE MIXTURE PRODUCED BY LIGHT
ISOMERIZATION OF ALL-*trans*-RETINAL WITH EXTRACTS OF
THE PAROLFATORY VESICLES AND THE OPTIC LOBE

Isomer	Retinal solution %	Retinal solution + ex- tract of parolfactory vesi- cles*		Retinal solution + ex- tract of optic lobe*	
		Dark %	Irradiated >560 nm %	Dark %	Irradiated >560 nm %
13- <i>cis</i>	1	1	1	4	7
11- <i>cis</i>	—	2	98	—	12
9- <i>cis</i>	—	—	—	—	2
7- <i>cis</i>	—	—	—	—	2
All- <i>trans</i>	99	97	1	96	77

* The mixtures were kept dark or light at 4°C for 50 min.

DISCUSSION AND CONCLUSIONS

Retinochrome is a photosensitive pigment that was originally found in the cephalopod retina. Since the all-*trans* chromophore of retinochrome is isomerized by light to 11-*cis*, the form that is required to resynthesize rhodopsin, we have suggested that retinochrome interacts with rhodopsin in the visual cycle so that the irradiation of one promotes the regeneration of the other (Hara and Hara, 1968, 1972). The present experiments have shown that retinochrome and rhodopsin are also contained in the parolfactory vesicles located near the brain. This is the first instance in which retinochrome has been detected in extraocular, photosensitive tissues. More than 10 yr ago, Nishioka et al. (1966 *a, b*) reported the existence of rhodopsin in the parolfactory vesicles and the epistellar body, but they overlooked retinochrome. Their photopigment extracts were prepared from cell fragments which floated in a 36% solution of sucrose, and after raising the pH of the extracts to above 9, they were bleached by irradiation with room light. These procedures are adequate to detect rhodopsin, but not retinochrome for the following reasons. First, most retinochrome-bearing cell fragments are sedimented by this type

of centrifuging and therefore would have been discarded. Secondly, it would hardly be possible to distinguish retinochrome from rhodopsin by means of irradiation, even if retinochrome were contained in the extracts, because the spectral change that accompanies the photolysis of rhodopsin at alkaline pHs is very similar to that of retinochrome. To identify retinochrome, the extracts must be irradiated at neutral pH or tested with NH_2OH . Therefore, these experiments showed that rhodopsin was present in the rhabdomeres of the photoreceptor cells, but did not demonstrate that it was the only photosensitive pigment in those tissues. Our present experiments show that these cells contain about as much retinochrome as rhodopsin, and that the retinochrome is not located in the rhabdomeres.

In visual cells, the outer segments contain rhodopsin and retinochrome, and the inner segments retinochrome alone. Rhodopsin is restricted to the microvillar membranes of the rhabdomes, whereas retinochrome is present in the cytoplasm of both inner and outer segments. Although the further details of retinochrome distribution are not yet clear, we have suggested that retinochrome is located in the inner segments in close association with membrane stacks often called myeloid bodies, which are very abundant between the basement membrane and the layer of nuclei. We think that it is also contained in the protoplasmic cores of the outer segments, perhaps associated with some myeloid bodies that protrude into their basal regions and with minute vesicles that are derived from these bodies (Hara and Hara, 1976). As shown in Fig. 1B and D, the photoreceptor cells of the parolfactory vesicles, like the visual cells, include many-layered membrane stacks of the myeloid type, and these presumably carry the retinochrome. Furthermore, retinochrome may be associated with some other vesicular reticulum as well. In any case, many questions remain concerning the micro-environment of rhodopsin and retinochrome as well as their functional interrelationship. As reviewed by Whittle (1976), so far much of the knowledge that has been accumulated concerns the form, occurrence, and probable function of reticular specializations in visual photoreceptors; most of our questions will be made clear through further work on the photopigments in the visual cells. We think that our understanding of the photoreceptor mechanism in vision may be fully applicable to understanding that in extraocular organs.

Extraocular photoreceptors are much better developed in deep-sea cephalopods such as *Todarodes* and *Eledone* than in *Loligo* and *Octopus*, which may be related to their need to detect light changes in deep waters (Baumann et al., 1970). Although these organs are extremely small compared with the eye, they can be effective for responding to light diffusing from all possible directions, whereas the retina is highly sensitive to direct light coming through the pupil. For the life of *Todarodes pacificus* that is characterized by spawning migrations, the well-developed parolfactory system may be important as a light intensity detector. In any case, we can now expect that the pair, rhodopsin and retinochrome, so far known only in vision, may participate more generally in photoreception by organs other than the eye. However, in those organs, photopigments may often be so scanty that they are difficult to examine in extracts. In such cases, the technique used in the experiment in Fig. 2 will be

useful to find out whether there is photopigment. In fact, in these experiments we were able to establish the presence of retinochrome or aporetinochrome in the parolfactory vesicles by detecting their retinal isomerase activity, before the extraction experiments were carried out.

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