# Adaptation of a Deep-Sea Cephalopod to the Photic Environment

## Evidence for Three Visual Pigments

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ABSTRACT   *Watasenia scintillans*, a bioluminescent deep-sea squid, has a specially developed eye with a large open pupil and three visual pigments. Photoreceptor cells (outer segment: 476 $\mu$m; inner segment: 99 $\mu$m) were long in the small area of the ventral retina receiving downwelling light, whereas they were short (outer segment: 207 $\mu$m; inner segment: 44 $\mu$m) in the other regions of the retina. The short photoreceptor cells contained the visual pigment with retinal ($\lambda_{max} \sim 484$ nm), probably for the purpose of adapting to their environmental light. The outer segment of the long photoreceptor cells consisted of two strata, a pinkish proximal area and a yellow distal area. The visual pigment with 3-dehydroretinal ($\lambda_{max} \sim 500$ nm) was located in the pinkish proximal area, giving high sensitivity at longer wavelengths. A newly found pigment ($\lambda_{max} \sim 471$ nm) was in the yellow distal area. The small area of the ventral retina containing two visual pigments is thought to have a high and broad spectral sensitivity, which is useful for distinguishing the bioluminescence of squids of the same species in their environmental downwelling light. These findings were obtained by partial bleaching of the extracted pigment from various areas of the retina and by high-performance liquid chromatographic analysis of the chromophore, complemented by microscopic observations.

## INTRODUCTION

Deep-sea residents adapt to their environment in various ways. It is known that visual pigments of many deep-sea fishes maximally absorb light at 470–480 nm, which corresponds to the blue light of their photic environment (Munz and McFarland, 1977). For example, in the deep-sea cephalopod *Watasenia scintillans*, the $\lambda_{max}$ of the visual pigment is reported to be 482 nm (Nashima et al., 1979). The eye of *W. scintillans* has a large open pupil to accommodate maximally photic information, and the retina has no equatorial strip, with a regional difference in the rhabdom

length, as described in some cephalopods by Young (1963). However, the rhabdom in the small area of the ventral retina receiving the downwelling light is more than two times as long as that in the other regions of the retina.

It was recently demonstrated that the squid eye contained not only the visual pigment with retinal, but also an additional pigment with 3-dehydroretinal in the ventral retina (Kito et al., 1986). This means that the eye does not adapt to environmental blue light alone, but can use a light of longer wavelength through the additional pigment. Here we report on the results of a further investigation of the 3-dehydroretinal pigment and a third type of visual pigment found in the retina of this squid.

## METHODS

### Materials

The "firefly" squid *W. scintillans* lives in the open ocean around Japan and is famous for its intense blue bioluminescence from a cluster of three photophores present at the tips of both arms of the fourth pair. The squid can also emit light from a number of small photophores distributed on the ventral surface of its whole body, probably for counterillumination. Furthermore, the squid has a row of five photophores on the ventral surface of the eye. Large groups of mature squids 5–7 cm in mantle size come to Toyama Bay of the Japan Sea in the spring to spawn. Squid were captured there at night and brought to our laboratory. They survived at <5°C for a few days. The eyes were ~10 mm in diameter. To extract visual pigments, eyes were isolated under dim red light and stored at −20°C until use. Fresh eyes from live squid were used in other experiments.

### Chromophore Analysis by High-Performance Liquid Chromatography

The lens was removed from the fresh eye, and the retina was incised radially, unfolded, and segmented, as shown in Fig. 1. The row of photophores on the ventral surface of the eye was a good marker in dissecting the eye.

The chromophore composition of the visual pigment in each retinal area was determined by high-performance liquid chromatography (HPLC) as described previously (Kito et al., 1986). Retinal was extracted as a retinal oxime, according to the technique described by Groenendijk et al. (1980) and Suzuki and Makino-Tasaka (1983). HPLC involved the use of a Zorbax BP SIL column (4.6 × 250 mm; DuPont Co., Wilmington, DE) with a solvent system of 8% diethylether and 0.4% ethanol in hexane. The absorbance at 360 nm was monitored. The standards of the respective isomers of authentic retinal and 3-dehydroretinal were a generous gift from Dr. T. Suzuki (Hyogo Medical College, Nishinomiya, Japan).

### Extraction of the Visual Pigment and Partial Bleaching

About 50 fresh retinas were sectioned horizontally into three parts, the ventral, central, and dorsal regions. The ventral region was limited in the center area (24 and 46% areas in Fig. 1) just behind the photophores. The preparation of rhabdomeric membranes and extraction of the visual pigment were performed as described previously (Nashima et al., 1978). The rhabdomeric membranes were obtained by flotation in 40% sucrose solution. The visual pigment was extracted with 5% L-1695 solution and purified in 0.2% L-1695 solution by column chromatography with DEAE-cellulose and concanavalin A–Sepharose 4B.

The visual pigment solution thus obtained from different regions of the retina was analyzed by the method of partial bleaching (Dartnall, 1957). Since the metaform, the photoproduct of the cephalopod visual pigment, exists in both acid and alkaline forms (Hubbard and St.

George, 1958) and is stable and relatively resistant against hydroxylamine at room temperature (Nashima et al., 1980), the visual pigment was bleached by light at 4°C and pH 10.5 to obtain an alkaline metaform.

First, the visual pigment solution was irradiated with light of $\lambda > 660$ nm using a filter (V-R66, Toshiba, Tokyo, Japan) and a 500-W projection lamp until spectral changes discontinued, and the difference spectrum before and after irradiation was recorded. The pH of the solution was adjusted to 7.3 by the addition of a small amount of $KH_2PO_4$ to convert the alkaline metaform of the pigment into the acid metaform. The difference spectrum was also recorded after this conversion. Second, further partial bleaching was performed with a light of $\lambda > 640$, 620, 600, or 580 nm using different filters (V-R64, V-R62, V-R60, and V-O58, Toshiba) and the same lamp. In each step, the chromophore composition of the solution was determined by HPLC. The absorbance spectrum was measured with a spectrophotometer (SM 401, Union Co., Ltd., Osaka, Japan) equipped with a data processor.

### Microdissection

The fresh eye was fixed with 2% glutaraldehyde in artificial seawater (ASW) for 1 h, washed twice with ASW containing 7% sucrose, and frozen with dry ice. Frozen sections, 100 $\mu$m thick, were prepared at $-30°C$ and placed on a slide, and the pinkish area of the outer segment in the 24 and 46% areas of the ventral retina was dissected under microscope and pooled for HPLC analysis.

### Microscopic Observation

The fresh eye was fixed with Bouin's fluid and dehydrated in graded concentrations of ethanol. Sections 4 $\mu$m thick were prepared from the retina embedded in Paraplast and stained with Ehrlich hematoxylin. The stained sections were observed with an Olympus BH microscope. The sections were photographed, and the lengths of the outer segment (OS) and the inner segment (IS) of photoreceptor cells were measured.

### Chemicals

The detergent L-1695 (sucrose laurate) was a gift from Mitsubishi Chemical Co. (Yokohama, Japan). Concanavalin A–Sepharose 4B was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). The other chemicals used were of reagent grade.

## RESULTS

### Localization of 3-Dehydroretinal in the Squid Retina

Fig. 1 shows the localization of 3-dehydroretinal in the retina, with the percentages indicated. A 46% retinal area, containing 46% of the total 3-dehydroretinal in the retina, was found in the ventral region of the eye just behind the row of photophores, but the dorsal region contained no 3-dehydroretinal. The retinoids in this squid were preliminarily studied. Only 2–4% of the retinal and 3-dehydroretinal was in the all-*trans* form, originating mostly from the metaform of visual pigment. The retinochrome bearing all-*trans* retinal (Hara and Hara, 1965) was contained in <0.5% of the visual pigment content. The retinal-binding protein detectable in the supernatant of the retinal homogenate (Ozaki et al., 1983) amounted to 0.4% of the total retinal in the eye. A trace of retinol was detected in the eye, whereas there was a large retinoid stock ($\sim$10-fold retinal in the eye) in the form of 11-*cis* retinol ester in the digestive gland (liver). Thus, 3-dehydroretinal (Fig. 1), based on extraction of

the entire retinal section, seemed to be derived from the visual pigment based on 3-dehydroretinal in the respective retinal area.

### Visual Pigment in the Dorsal Retina and the 24 and 46% Areas of the Ventral Retina

In order to examine the visual pigment in different retinal regions, the retina was dissected horizontally into three parts, and the ventral region was further limited to the 24 and 46% areas in Fig. 1, from which the visual pigment was extracted and purified as described in the Methods. Fig. 2 shows the absorbance spectra of visual pigment solutions from dorsal retina and the 24 and 46% areas. Because the chromophore of the visual pigment from the dorsal retina was ascertained by HPLC to be composed of retinal alone, curve 1 in Fig. 2 was regarded as the absorbance spectrum of a pure pigment with retinal that had a $\lambda_{max}$ of 484 nm. About 50% of the chromophore of the pigment extracted from the 24 and 46% areas was found to
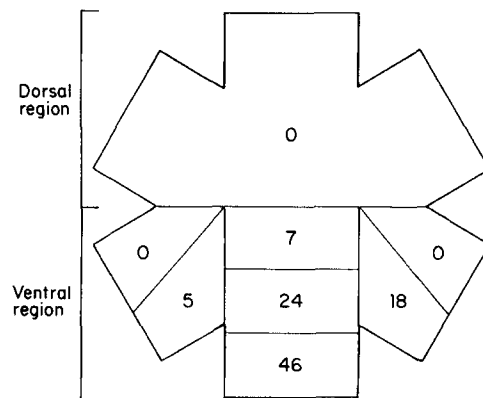


FIGURE 1. The distribution of 3-dehydroretinal in the retina of *W. scintillans*. The numbers indicate the percentage of 3-dehydroretinal in each area. The 46% area is just behind the row of five photophores.

be 3-dehydroretinal, and the remaining 50% was found to be retinal by HPLC analysis. The absorbance spectrum of the pigment solution from the 24 and 46% areas (curve 2 in Fig. 2) showed marked tailing in the region of wavelengths longer than 560 nm and higher absorbance in the region below 400 nm, as compared with that from the dorsal retina (curve 1 in Fig. 2). The visual pigment with 3-dehydroretinal was reported to have a $\lambda_{max}$ at 500 nm (Kito et al., 1986), and the visual pigment with retinal had a $\lambda_{max}$ at 484 nm, as mentioned above. Therefore, if the 24 and 46% areas contained both the retinal and 3-dehydroretinal pigments, the $\lambda_{max}$ of the mixture should have been located at a longer wavelength than 484 nm, namely an intermediate value between the $\lambda_{max}$ values of the respective pigments. However, the absorbance maximum is located at 473 nm in curve 2 in Fig. 2. This suggests the presence of another pigment in the 24 and 46% areas, the absorbance maximum of which may be at a considerably shorter wavelength than 484 nm.

### Partial Bleaching of the Extracts

To examine the visual pigment in question in the extract of the 24 and 46% areas, partial bleaching was performed. The visual pigment solution at pH 10.5 from the

area was irradiated for 1 min with light of $\lambda > 660$ nm to obtain an alkaline meta-form of the pigment, and the difference spectrum before and after irradiation indicated a maximum decrease in absorbance at 500 nm and a maximum increase at 400 nm (curve 5 in Fig. 3). The residual pigment had a $\lambda_{max}$ at 471 nm (curve 2 in Fig. 3). At the same time, the chromophore composition of the solution was analyzed by HPLC before and after irradiation. About 90% of the 11-*cis* 3-dehydroreti-nal was isomerized to an all-*trans* configuration, whereas isomerization of only a fragment of 11-*cis* retinal to the all-*trans* configuration was observed. Thus, the bleached pigment was based mainly on 3-dehydroretinal and its $\lambda_{max}$ was ~500 nm, as reported previously (Kito et al., 1986).
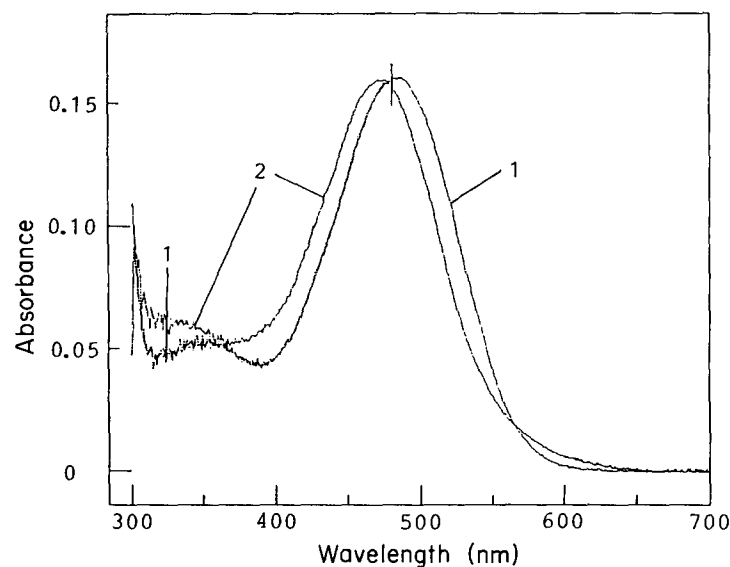


FIGURE 2.   The absorbance spectra of visual pigment solutions from the dorsal retina and the 24 and 46% areas of the ventral retina in Fig. 1. Curve 1: the spectrum of the solution from the dorsal retina, with $\lambda_{max}$ at 484 nm. Curve 2: the spectrum of the solution from the 24 and 46% areas with $\lambda_{max}$ at 473 nm.

In order to detect residual pigment, further partial bleaching was performed by irradiating the solution with light of $\lambda > 640$, 620, 600, or 580 nm, and only one pigment was identified. Curve 7 in Fig. 3 shows the difference spectrum before and after irradiation with light of $\lambda > 580$ nm, with a maximum decrease at 471 nm and a maximum increase at 379 nm.

After selective bleaching of the pigment with 3-dehydroretinal, the pH of the solution was returned to 7.3 to obtain an acid metaform of the pigment. The maximum of the difference spectrum (curve 6 in Fig. 3) was at 520 nm, which indicates a possible absorbance maximum for the acid metaform of the 3-dehydroretinal pigment. After irradiation with light of $\lambda > 580$ nm, the pH of the solution was adjusted to 7.3, and the difference spectrum was recorded (curve 8 in Fig. 3), which

indicates the absorbance spectrum of the mixture of the acid metaforms of the 3-dehydroretinal pigment and the possible pigment contained in the 24 and 46% areas. Curve 9 in Fig. 3 represents the difference spectrum between curves 6 and 8, which indicates that the absorbance spectrum of the acid metaform of the possible pigment has a $\lambda_{max}$ of ~483 nm.
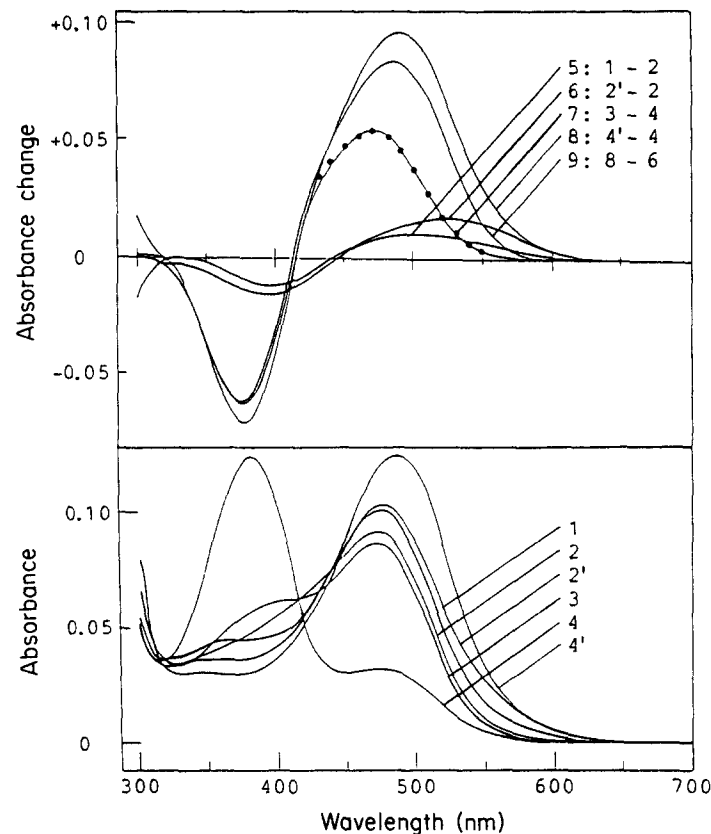
FIGURE 3.   Partial bleaching of the pigment solution from the 24 and 46% areas of the ventral retina. The solution at pH 10.5 (curve 1) was irradiated with light of $\lambda > 660$ nm for 1 min (curve 2). Further irradiation with light of $\lambda > 580$ nm was performed for 2 s (curve 3). Furthermore, irradiation with the same light was performed for 4 min (curve 4). The pH of the solution for curves 2 and 4 was adjusted to 7.3 (curves 2' and 4'). The upper panel shows the difference spectra between the pigment and its alkaline metaform, and between its alkaline metaform and its acid metaform (curves 5–9). The filled circles were derived from Dartnall's nomogram for visual pigment with $\lambda_{max}$ at 471 nm.

A similar procedure was performed on the pigment solution from the dorsal retina. Partial bleaching with light of $\lambda > 660, 640, 620, 600,$ or 580 nm resulted in a maximum decrease in absorbance at 484 nm and a maximum increase at 382 nm. Curve 3 in Fig. 4 shows the difference spectrum before and after irradiation with light of $\lambda > 580$ nm. The pH of the visual pigment solution was then adjusted to 7.3

to obtain an acid metaform of the visual pigment with retinal, and the difference spectrum was recorded. The absorbance maximum of the acid metaform of the pigment with retinal was ~501 nm (curve 4 in Fig. 4). Thus, the possible pigment in the 24 and 46% areas could also be distinguished from the visual pigment with retinal in the dorsal retina on the basis of the absorbance maximum of its acid metaform.

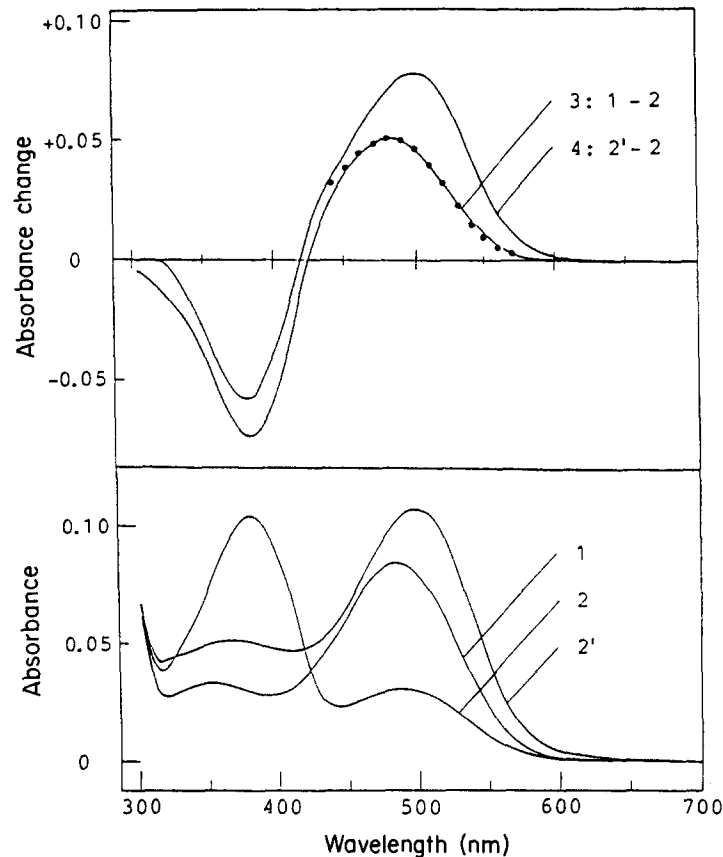In this experiment, complete bleaching could not be achieved and the solution of



FIGURE 4. Bleaching of the pigment solution from the dorsal retina. The solution at pH 10.5 (curve 1) was irradiated with light of $\lambda < 580$ nm for 4 min (curve 2). The pH of the solution was adjusted to 7.3 (curve 2′). Curves 3 and 4 represent the difference spectra between curves 1 and 2 and between curves 2′ and 2, respectively. In the upper panel, a nomogram match for visual pigment with $\lambda_{max}$ at 484 nm is also shown as filled circles.

curve 2 in Fig. 4 contained 16% original pigment, 6% acid metaform, and 78% alkaline metaform. The pK of the metaform of the pigment in the detergent L-1695 was 9.1, which is higher than that in digitonin (Nashima et al., 1980), preventing further bleaching at pH 10.5 in this detergent. If the pH of the solution had been raised further, part of the pigment would have been denatured.

Although the pigment from the 24 and 46% areas contained 50% retinal and 50%

3-dehydroretinal as its chromophores, the absorbance of the 3-dehydroretinal pigment (curve 5 in Fig. 3) was one-fifth of the total absorbance of the pigments contained in the solution (curve 1 in Fig. 3). This cannot be explained, even if the molar extinction coefficient of the 3-dehydroretinal pigment is smaller than that of the retinal pigment (Bridges, 1967). It may be that the molar extinction coefficient of the possible pigment is much larger than that of 3-dehydroretinal pigment. This issue needs to be clarified as soon as possible.

### Microdissection of the Ventral Region of the Retina

The OS layer of the photoreceptor cells in the 24 and 46% areas of ventral retina consisted of two distinct strata (Fig. 5). The distal area of the OS was yellow, and the
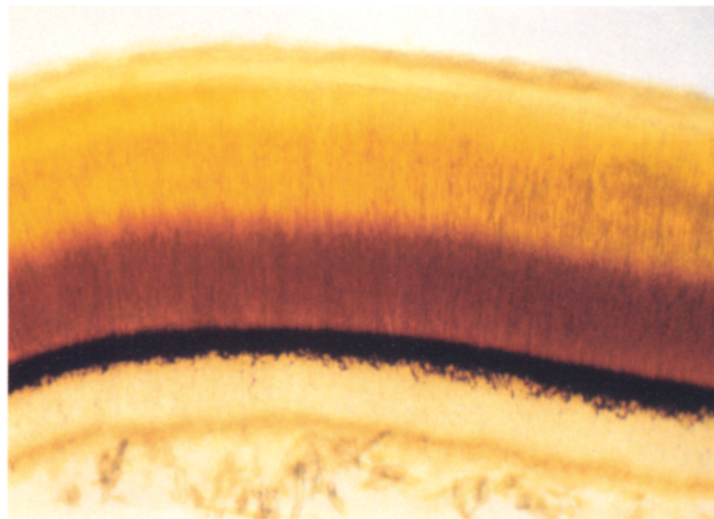


FIGURE 5.  Vertical section ~330 μm thick of the 46% area of the squid retina. The fresh retina was cut on the filter paper with the razor blade of a guillotine (×100).

proximal area was pinkish. The pinkish area was dissected in the light from ~20 frozen sections (100 μm thick) under the microscope and pooled for HPLC analysis. Fig. 6 is the HPLC pattern of the pooled fraction. The ratio of the sum of 11-*cis* and all-*trans* retinal oximes to that of 3-dehydroretinal oximes was estimated to be ~1:20. This indicates that the pinkish area of the OS layer of photoreceptor cells mainly contains 3-dehydroretinal pigment.

### Microscopic Observation

Sections (4 μm thick) of the retina were prepared for microscopic observation (Fig. 7). The lengths of the OS and IS in both the ventral and dorsal retinas were measured. The average lengths of the OS and IS of the 24 and 46% areas in Fig. 1 were 476 and 99 μm, respectively. While the average lengths in the dorsal region were

207 μm (OS) and 44 μm (IS), the central, anterior, and posterior regions of the retina showed values similar to those of the dorsal region. The diameter of the photoreceptor cell could not be measured precisely with the light microscope.

### DISCUSSION

Although the squid *W. scintillans* was believed to have only a single visual pigment with a $\lambda_{max}$ at 482 nm (Nashima et al., 1979), the present study demonstrates the presence of three visual pigments.

The pigment with retinal was the sole pigment in the dorsal retina that was present throughout the retina. The absorbance maxima of the pigment and its acid
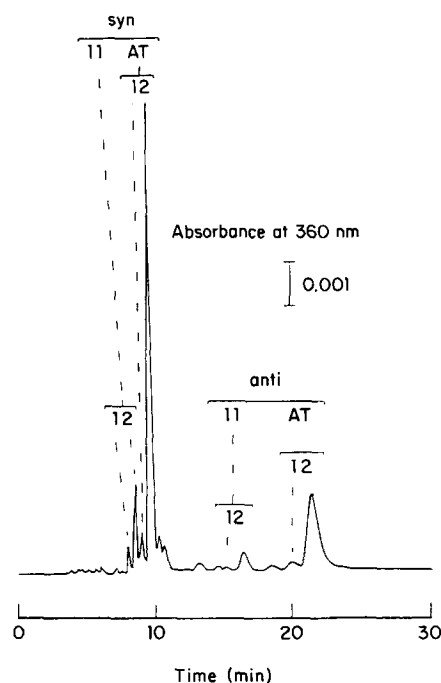


FIGURE 6. HPLC analysis of the chromophore involved in the pinkish proximal region in the outer segment layer of the photoreceptor cells in the 24 and 46% area of the retina of *W. scintillans*. Only the peaks of 11-*cis* (11) and all-*trans* (AT) are indicated on the syn and anti forms of the respective oximes. Microdissection of the retina was performed in the light, and so the all-*trans* configurations of both retinal (1) and 3-dehydroretinal (2) were dominant, and minor fractions of other isomers (9-*cis* and 13-*cis;* not indicated) appeared.

metaform were at ~484 and ~501 nm, respectively (Fig. 4). The second pigment was the 3-dehydroretinal pigment; the absorbance maxima of the pigment and its acid metaform were at ~500 and ~520 nm, respectively (Fig. 3). The 3-dehydroretinal pigment was localized in the 24 and 46% areas of ventral retina (Fig. 1) and it accounted for ~15% of the total visual pigment (Kito et al., 1986). The pinkish proximal region of the OS of the 24 and 46% areas contained only 3-dehydroretinal pigment, as demonstrated by microdissection and HPLC. Judging from its color distribution in frozen sections, the 3-dehydroretinal pigment appears to be located in such restricted regions of the ventral retina as the 24 and 46% areas in Fig. 1.

Contamination with the main pigment with retinal must be taken into account in identifying the newly found third pigment. Absorbance peaks of several extracts

from the 24 and 46% areas deviated within 2 nm. Curve 2 in Fig. 2 was one of those with the shortest $\lambda_{max}$. Even if the absorbance spectrum represented by curve 7 in Fig. 3 was due to the mixture of the main retinal pigment and the third pigment, and if the solution contained the main pigment with retinal, for example, in an amount <20%, we could not have clearly separated them by the method of partial bleaching used in this experiment because of the proximity of their respective absorbance spectra. This suggests that the absorbance maxima of the third pigment and its acid metaform are shorter than 471 and 483 nm, respectively. The three
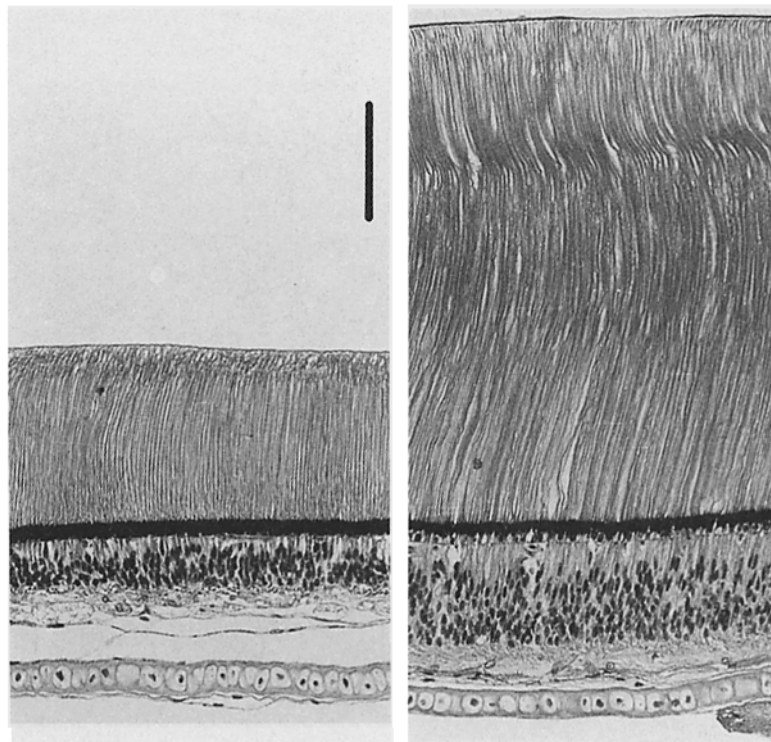


FIGURE 7.  The vertical sections (4 $\mu$m thick) of the dorsal region (left) and the 46% area in Fig. 1 (right) of the squid retina. The other retinal areas were very similar to the dorsal retina. Bar: 100 $\mu$m.

visual pigments in *W. scintillans* may each have apoproteins, but the chromatographic separation in our laboratory of the three pigments remains unsuccessful.

Another noticeable point was that the 24 and 46% areas bearing both the 3-dehydroretinal pigment and the third pigment were morphologically different from other regions of the retina. The lengths of the OS and IS of these areas were more than twice those of the other regions of the retina. Young (1963) and Muntz (1977) described the regional difference in the length of photoreceptor cells corresponding to the shapes of their pupils in cephalopods and stated that the equatorial strip would function somewhat like the fovea in the vertebrate eye. Some deep-sea fishes have long or multibank photoreceptor cells that are highly sensitive (Locket, 1977).

In *W. scintillans*, a cluster of long photoreceptor cells is built up in the small area of the ventral retina receiving downwelling light (Figs. 5 and 7), which probably increases the sensitivity.

However, it is not so simple. The OS of the long photoreceptor cells in the specific area consists of two strata, the 3-dehydroretinal pigment in the proximal region and the third pigment in the distal region. We searched for a membrane-like structure separating the two strata, i.e., we tried to determine whether or not the different pigments belong to different respective photoreceptor cells. However, this could not be observed directly by light microscopy.

All the squid used in the present study were captured in shallow water when they came to spawn. They may have acquired the 3-dehydroretinal pigment in response to changes in the photic environment, when they emerged from the deep sea to the shallow shore. The proximal layer containing the 3-dehydroretinal pigment may have been generated from the base of the OS. It is necessary to examine whether the pigment is present throughout the entire life cycle of the squid.

It may be considered that the small area of the ventral retina having the two pigments develops to provide the squid with a broad spectral sensitivity. The photic environment of the deep sea where the squid lives must be rather simple, since in the open clear ocean, the downwelling light from the sun is rapidly attenuated with depth, leaving only the blue light that penetrates to deeper levels (Munz and McFarland, 1977). Most of animals living in this environment are known to possess a visual pigment with an absorbance maximum of 470–480 nm (Lythgoe, 1980). The third pigment and the main visual pigment with retinal described in this article have $\lambda_{max}$ values of ~471 and ~484 nm, respectively, which are within the range of the environmental light.

*W. scintillans* is a bioluminescent squid, having photophores in the fourth pair of arms, the ventral part of the eye, and the whole body. The latter two areas seem to be involved in counterillumination. Even if predators look up, they may not see the silhouette of the squid illuminated with a light similar to the downwelling light. Actually, Young and Roper (1976) observed the countershading of the squid *Abraliopsis*. The squid *W. scintillans* was first named *Abraliopsis scintillans* (Berry) and might be phylogenetically close to the squid investigated by Young and Roper (1976). A similar experiment was performed on *W. scintillans* in our laboratory. A weak red light was given from above for 2–5 min, and after the cessation of illumination, intensified bioluminescence from the ventral body was seen for 5–15 min. This happened repeatedly as long as they were alive. The color of the luminescence was blue, and judging from the spectrograph, the maximum of its spectrum was at 475 nm (Kito et al., 1979). However, the bandwidth of the spectrum of the luminescence was very broad (half-bandwidth, 4,600 cm$^{-1}$) compared with that of their environmental light. The specific area of ventral retina has a broad spectral sensitivity, which probably also promotes mutual recognition by luminescence. This suggests that the bioluminescence of this tiny squid is aimed at not only countershading against their predators, but also at communication, which is facilitated by the 3-dehydroretinal pigment in the specific area of retina of the eye and by the polarized light analyzer of the rhabdomeric structure. This mechanism may play a role in preserving a large population density convenient for life in the open deep sea. The recent finding by Tsuji (1985) that the luminescence of this squid is dependent on

$Mg^{++}$ and ATP in a manner very similar to that in fireflies is interesting and may contribute much to the elucidation of the life of this squid.

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