感桿型視細胞の構造と光受容の初期過程の分子機構

Study of the structure and the molecular mechanism of phototransduction of the rhabdomeric photoreceptor.

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要旨

本論文は、感桿型視細胞の微細構造と光受容の初期過程の分子機構について調べるために、おもにホタルイカを材料に行なった研究の報告である。

ホタルイカに吸収極大波長の異なる三種類の視物質が存在し、各々が異なる発色団（レチナール・3-デヒドロレチナール・4-ヒドロキシレチナール）を持つことが、松井らによって明らかにされた[付録1,2]。これらの三つの視物質は分子量や等電点やレクチンに対する結合能の差といった点では区別することができなかった。このことから、これらの視物質はアポ蛋白質部分が全く同じで、三つの異なる発色団分子を付け替えることによってできているかも知れないと考えられた。(chapter 1)では、視物質の部分一次構造を決めることによってこの点を明らかにしようとした実験について書いてある。 この結果ホタルイカの三種の視物質が、一種類のアポ蛋白質に異なる三種の発色団分子を結合することによって作られている可能性が高いことがわかった。また求めた一次構造を脊椎動物のものと比べることによって頭足類の視物質も脊椎動物のものと同じ祖先をもつものであることが明かとなった。

(chapter 2)では、ホタルイカで見つかった吸収極大の異なる三つの視物質が、イカにとってどのような意味を持つのかを考察するためにイカの網膜上での分布を調べた。この研究で4-ヒドロキシレチナールと3-デヒドロレチナールをもつ視物質は、網膜の腹側の限られた部分に存在していることが分かり、これらの視物質が色識別に使われている可能性が出てきた。これらの視物質の発色団分子のうち4-ヒドロキシレチナールは、視物質の発色団では初めて見つかったものである。この発色団がホタルイカ以外にどのような生物に使われているかを検索した。しかし4-ヒドロキシレチナールは、我々の調べた範囲ではホタルイカ以外には見つからなかった。またこのイカがどのようにして三種の発色団をつくっているのかを考察するために、ホタルイカの体内での視物質の発色団以外のレチノイドの分布を調べ、肝膵臓に大量の11－シス型レチノールエステルを見つけた。この11－シス型レチノールエステルは4-ヒドロキシレチナールや3-デヒドロレチナールの材料であろうと思われる。

(chapter 3)の実験は、ホタルイカが実際に4-ヒドロキシレチナールや3-デヒドロレチナールをつくることができることをin vitroで確認するために行なった。そして、腹側の網膜の側でだけ11-シス型4-ヒドロキシレチノールが11-シス型レチノールから作られることを示した。しかし3-デヒドロレチノイドについてはホタルイカ

の網膜でつくられているという証拠を得ることはできなかった。

感桿型の視細胞では、感桿分体を作っている規則正しく並んだ微絨毛の膜中に視物質が存在するために高い偏光識別能力を持つという特徴がある。この偏光識別能は、微絨毛がただ単に同じ方向に並んでいることだけによって起きるとしたときの予想値を大きく越えることから、微絨毛の膜中で視物質が結晶状に決まった方向に並んでいるのではないかと考えられてきた。この仮説が正しければX線回折法によって視物質の三次構造を決定することが可能である。我々が実験に用いているホタルイカの腹側の網膜は外節の長さが500μm以上ありX線を照射しやすく、X線の散乱を乱すと思われる色素顆粒を外節部にほとんど含んでいない。このイカを使えば、網膜にX線を照射して回折像を得ることができるのではないかと考え(chapter 4)に書いてある実験を行なった。

この実験で回折像の検出にイメージングプレートを用いシンクロトロン放射の強いX線を使うことで測定に必要な時間を数分のオーダーまで短縮し、固定していない生の網膜からの回折像を得ることが可能となった。得られた回折像から微絨毛の構造に関する情報が得られたが、残念ながら視物質の結晶性を示す反射は得られなかった。しかしこの実験で用いた方法は、光受容の初期過程を直接測定しうる可能性があることがわかった。得られた回折像は現在解析中である。

これらの研究に加えて光受容の細胞内情報伝達物質についての研究も同時に行なった。(chapter 5)の研究では脊椎動物の視細胞で細胞内情報伝達物質として働いているｃＧＭＰについて、無脊椎動物でも同様の役目を果たしているのかどうかを調べるためにヌクレオチドの定量を行なった。実験の材料には、イイダコとサルエビを用いた。イイダコについては、ｃＧＭＰが微絨毛一本当たりでは1.6分子しか存在しないことと、光刺激によって量が変わらないことから細胞内情報伝達物質である可能性はほとんどないことがわかった。サルエビではｃＧＭＰ・ｃＡＭＰとも光によって有意に減少したが、サンプル中に視細胞以外の神経細胞が含まれていることからこの減少の起きている細胞や反応を特定することはできなかった。

なお付録として、ホタルイカの三種の視物質の発見と視物質の発色団の同定に関する論文と、シュガーエステルの膜蛋白質に対する効果を調べた論文が載せてある。本研究では、このデタージェントを頭足類の視物質を安定に可溶化するために使っている。

Chapter 1

Amino acid sequence of the retinal binding site of squid visual pigment

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Amino acid sequence of the retinal binding site of squid visual pigment

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The retinylpeptides of visual pigments of two species of squid were identified in invertebrate visual pigments. Their primary structures were identical: H-Phe-Ala-Lys-Ala-Ser-Ala-Ile-His-Asn-pro-Hse(Met)-OH. The sequence was homologous to those of the corresponding region of other visual pigments, but the eighth amino acid, His, was found in squid visual pigments. In this experiment the retinylpeptides of eleven amino acid residues were isolated by monitoring the absorbance spectrum of the reduced retinal Schiff base without using radio-active [^{3}H]retinal. This method is valid for the isolation and identification of retinylpeptides of other invertebrate visual pigments in which the chromophore is not exchangeable.

The complete and partial primary structures of some vertebrate visual pigments have been determined to date [1–3]. High overall conservation between species is found particularly among mammalian visual pigments. However, the sequence in the vicinity of the retinal binding site is variable to some extent [3].

Invertebrate visual pigments are different from those of mammals in some features. For example, the molecular weight of a squid pigment, 47 kDa [4], is bigger than that of bovine pigment, 39 kDa [1], the photoproduct *meta* form of a squid visual pigment is stable at room temperature [5], and the molecular absorption coefficient of a squid pigment, 34000 [4], is considerably smaller than that of bovine pigment, 40600 [6]. This small molecular coefficient is especially interesting because it may result from the solvent effect of the protein structure [7] of squid visual pigment, which is different from those of mammalian forms. The solvent effect is ascribed in part to the environment surrounding the retinal Schiff base linkage. The putative structures of invertebrate visual pigments have been determined for four types of visual pigment of a fruitfly by DNA sequencing, and their retinal binding sites were proposed by analogy with those of mammals [8–11]. We determined the partial amino acid sequences of the portions of the peptides of squid visual pigments, which include the retinal binding site.

Visual pigments from two species of squids, *Todarodes pacificus* and *Watasenia scintillans*, were used in these experiments. Extraction and purification of the visual pigments of both squids were performed as in Ref. 12, using the detergent CT-1695 (1% (w/v) sucrose caprate, Mitsubishi Chemical Co., Yokohama). The reduction of visual pigment was carried out by using boranedimethylamine as in ref. 13 to obtain retinylopsin. The retinylopsin was subjected to CNBr cleavage (1 mg CNBr per mg visual pigment) in 0.1 M HCl for 12 h under N_2 atmosphere at 35°C. The reaction mixture was added to the same volume of ethanol and centrifuged at $1000 \times g$ for 10 min.

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The supernatant was applied to a column (24 × 100 mm) of HP-20SS (Mitsubishi Chemical Co.) and eluted with a gradient of 50–100% ethanol in water. The fraction containing retinylpeptides was applied to a Sephadex LH-20 column (27 × 800 mm) and eluted with ethanol. Each fraction containing retinylpeptides was further separated by HPLC using a Zorbax BP CN column with a gradient of 0–80% 2-propanol/acetonitrile (7 : 3, v/v) in 0.1% trifluoroacetic acid in water at a flow rate of 1 ml/min. The retinylpeptide fractions were collected by monitoring the absorbance at 330 nm and their absorbance spectra were measured. All these procedures were carried out under dim red light.

Sequence analysis was performed by stepwise Edman degradation, using a gas-phase automatic sequencer (Applied Biosystems, model 470A) coupled with a PTH-amino acid analyzer (Applied Biosystems, model 120A).

About 20% of absorbance at 330 nm of the retinylpeptides remained after CNBr treatment of *T. pacificus* visual pigment. The reaction mixture was dissolved in 50% ethanol in order to obtain monomeric dispersion of the detergent so as to avoid hindrance by the detergent in the purification of the retinylpeptides. More than 90% of these peptides were soluble in 50% ethanol.

The solution was applied to a HP-20SS column. Fig. 1A shows the presence of two peaks monitored at 280 nm. The first peak eluted with 50% ethanol contained the detergent and some peptides other than retinylpeptides. The second peak showed the λ_{max} at 330 nm characteristic of retinylpeptides, which was then separated by Sephadex LH-20 chromatography. Two major fractions of the retinylpeptides, CB-1 and CB-2 in Fig. 1B, showed the λ_{max} at 330 nm. Each fraction was subjected to HPLC using a Zorbax BP CN column. Fraction CB-1 contained not less than three different peptides and could not be further purified (data not shown). The retinylpeptide fractions CB-2-1 and CB-2-2 were separated from CB-2 (shown in Fig. 2). CB-2-1, a minor fraction of CB-2, contained some small retinylpeptides but was not further purified.

In these experiments, the sequences of the retinylpeptides of CB-1 and CB-2-1 fractions were not determined. However, as described below, it is probable that the sequences of these retinylpeptides more or less overlap the sequence of the retinylpeptide of CB-2-2. The retinylpeptides of CB-1 are larger than those of CB-2, as is shown in Fig. 1B, and probably have some more amino acids at the N- and/or C-terminal of the retinylpeptide of CB-2, due to the imperfect CNBr-cleavage. Since in some cases the amount of CB-1 was strongly dependent on the conditions of CNBr-cleavage (temperature and duration of exposure), and in some cases CNBr-cleaved retinylopsin of both squids yielded no CB-1, but only CB-2. As shown in Figure 2A, fraction CB-2-1 contained smaller retinylpeptides than fraction CB-2-, possibly resulting from non-specific hy-

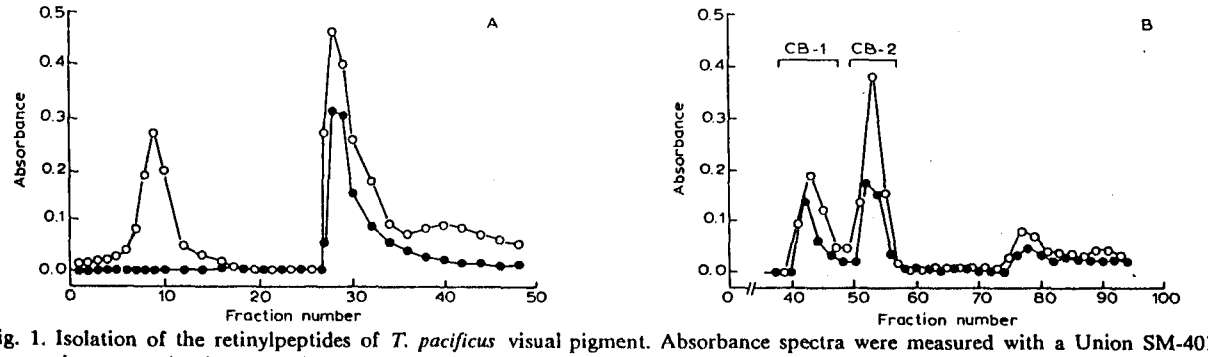

Fig. 1. Isolation of the retinylpeptides of *T. pacificus* visual pigment. Absorbance spectra were measured with a Union SM-401 spectrophotometer in the range 200 ≈ 400 nm. Absorbance at 280 nm; (○) and at 330 nm; (●). (A) Hydrophobic interaction chromatography profile of CNBr-cleaved retinylopsin on a HP-20SS column. Each fraction was 4 ml. (B) Gel-filtration chromatography profile of a retinylpeptide solution on a Sephadex-LH20 column. Each fraction was 3 ml. CB-1 and CB-2 were fractions with λ_{max} at 330 nm.

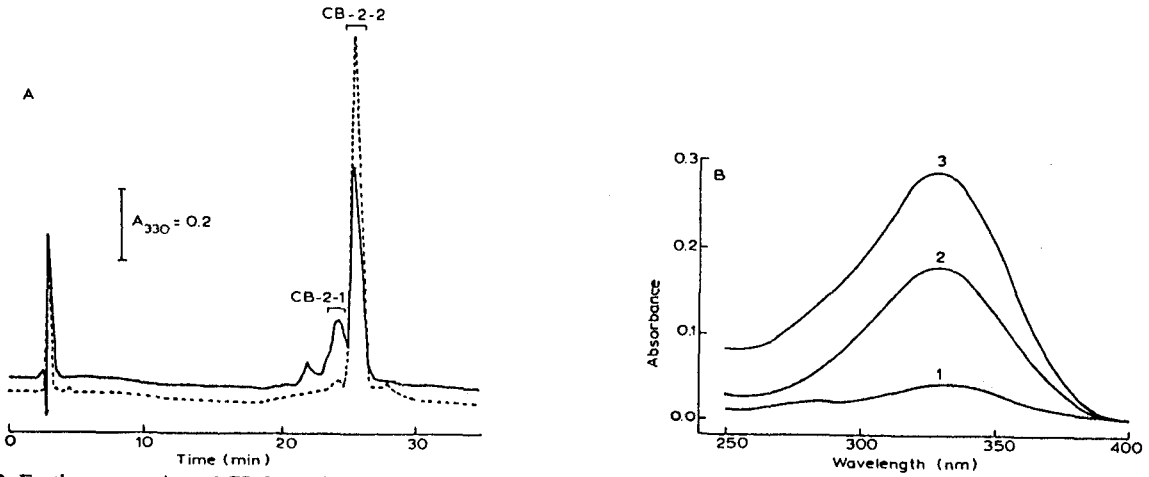

Fig. 2. Further separation of CB-2 fraction of retinylpeptides of both squids. (A) HPLC profile of CB-2 of *T. pacificus* (———) and *W. scintillans* (- - - - -) on a zorbax BP CN column monitored at 330 nm. CB-2-1 of *T. pacificus* and CB-2-2 of both squids were collected. (B) Absorbance spectra of fractions separated by the HPLC. Curve 1, CB-2-1 of *T. pacificus*; curve 2, CB-2-2 of *T. pacificus*; and curve 3, CB-2-2 of *W. scintillans*.

drolysis of the retinylpeptide in CB-2-2 under low pH condition for CNBr cleavage, as pointed out by Hofmann [14].

CB-2-2 had only one N-terminal amino acid, phenylalanine, as determined by amino acid sequencing. The amount of the PTH-phenylalanine was in agreement with the amount of the retinylpeptide estimated on the basis of the absorbance at 330 nm of the CB-2-2 fraction. The final yield of the retinylpeptide was only 1–5% of the initial visual pigment. However, it is certain that the chromophore retinal is linked to the lysine in the retinylpeptide, since the reduction of the retinal Schiff base was carried out by using boranedimethylamine at pH 1–2 which is far lower than the pK of the Schiff base in the dark where dislocation of the retinal dose not occur [7,13,15,16]. A retinylpeptide of *W. scintillans* visual pigment corresponding to fraction CB-2-2 of *T. pacificus* was obtained by the same procedure.

The amino acid sequences of the above retinylpeptides of visual pigments of both squids were identical:

H-Phe-Ala-Lys-Ala-Ser-Ala-Ile-His-Asn-Pro-Hse-OH

where Hse means homoserine derived from methionine. Table I shows the amount of PTH-amino acid from each cycle of Edman degradation. The low yield of PTH-lysine in the third cycle of Edman degradation suggests that the retinal should attach to the third lysine of the retinylpeptides. This is in accord with the fact that the retinal binding lysine was not detectable in the sequence analysis of bovine ^{3}H-labeled retinylpeptide [17].

The sequence surrounding the retinal binding lysine is very homologous between the squids and the other animals examined [18]. As has been discussed by Pappin et al. [3], amino acid substitu-

TABLE I

THE PARTIAL AMINO ACID SEQUENCES OF THE RETINYLPEPTIDES

After the 12th cycle, no amino acid was observed.

Cycle No.	PTH-amino acid identified	Amount recovered (pmol)	
		T. pacificus	*W. scintillans*
1	Phe	585	597
2	Ala	621	748
3	Lys	29	34
4	Ala	507	595
5	Ser	485	484
6	Ala	360	463
7	Ile	231	271
8	His	42	10
9	Asn	153	178
10	Pro	60	86
11	Hse (Met)	18	22

tion was conservative and but for histidine the total molecular volume occupied by the amino acids remained almost constant. The histidine in squid visual pigments at the fifth position following the retinal binding lysine is substituted by tyrosine in other visual pigments. This may be a significant substitution.

W. scintillans has three visual pigments, with λ_{max} values at 471, 484, and 500 nm, which could not be separated by column chromatographies utilizing concanavalin A affinity, isoelectric point, and/or molecular size [19]. Different sequences were not found in the retinylpeptides of CB-2 of the squid. Therefore, it may be that the protein structure of the three visual pigments of *W. scintillans* are not so different from each other, in contrast to the different protein structures of human visual pigments, as has been demonstrated recently [20].

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During the preparation of this report, a putative sequence of octopus visual pigment was reported [21]. Its deduced retinal binding site is similar to our result.

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Chapter 2

On the three visual pigments in the retina of the firefly squid, *Watasenia scintillans*

Summary

The deep-sea bioluminescent squid, Watasenia scintillans, has three visual pigments: the major pigment (A1 pigment) based on retinal has the λmax at 484 nm, and the second pigment (A2 pigment) based on 3-dehydroretinal has the λmax at 500 nm and the third pigment (A4 pigment) based on 4-hydroxyretinal has the λmax at 470 nm, respectively. The distribution of these three visual pigments in the retina was studied by HPLC analysis of the retinals in retina slices obtained by microdissection. It was found that A1 pigment was not located in the specific region of ventral retina receiving the downwelling light which contained very long photoreceptor cells, forming two strata. The A2 pigment and A4 pigment were found exclusively in the proximal pinkish stratum and in the distal yellowish stratum. The role of these pigments in the retina was discussed that they might function for the spectral discrimination. The extraction and analysis of retinoids to determine the origin of 3-dehydroretinal and 4-hydroxyretinal in the mature squid showed only a trace amount of 4-hydroxyretinol in the eggs. Similar analysis of other cephalopods collected near Japan showed the absence of A2 or A4 pigment in their eyes.

Introduction

Many deep-sea fishes and cephalopods have adapted the visual pigments sensitive to their photic environment (Dartnall 1975; Munz and McFarland 1977; Kito et al. 1979; Lythgoe 1980). Moreover, the eyes of these animals evolved to provide better visual sensitivity by the enlargement of the eye-size or the development of multiple banks of photoreceptor cells (Locket 1977).

Some deep-sea fishes have been known to have more than one visual pigment (Partridge et al. 1988). Most of them are bioluminescent and some of them can even emit red light from the photophore as a searchlight (Denton et al. 1970; O'Day and Fernandez 1974). The cephalopods also have well developed eyes, contained mostly a single visual pigment. Recently, we demonstrated that a deep-sea bioluminescent squid, Watasenia scintillans, has three kinds of visual pigments (Matsui et al. 1988a). A major pigment with the λmax of 484 nm based on retinal and the additional pigments with λmaxima of about 500 nm and about 470 nm based on 3-dehydroretinal and 4-hydroxyretinal, respectively (Matsui et al. 1988b). The amino-acid sequences of the eleven amino acid residues containing retinal binding site were identical in these three pigments (Seidou et al. 1988), although the corresponding peptides in this region were known to be the most variable part in the mammalian visual pigments which had many homologous primary structures (Pappin and Findlay 1984). Thus, we assumed that these three pigments

were composed of the same opsin and different chromophores, and that the differences in absorbance maxima was derived from the differences in the chromophore structures.

Previously we extracted the visual pigments from relatively large sections (2x2 mm) of the frozen retina, and the present HPLC condition allowed us to analyze the retinal composition in the far smaller slice of the live retina. In this paper the distribution of these pigments in Watasenia retina was investigated more precisely by microdissection and by HPLC analysis.

The fact that carotenoids with 3-dehydro or 4-hydroxy structure in the ionone ring have never been found in plants suggests that these retinoids are synthesized by this squid or come from other animals as their diet. In order to ascertain this possibility, the distribution of these retinoids in the squid body were investigated.

The squid, also known as the firefly squid due to its intense bioluminescence from large photophores at the tips of pair of the fourth arms, is unique among the cephalopods found around Japan. Some of the light emitted in the bioluminescence of the firefly squid is very different from the light found in the photic environment of the deep-sea, as illustrated in Figure 1. We discussed the relation between these lights and the vision of the firefly squid having three visual pigments.

To determine if this visual system is unique to the

firefly squid, other cephalopod found in the sea around Japan were studied to see if they contain A2 or A4 visual pigment. The implication of these findings will also discussed in this paper.

Materials and Methods

Materials

The firefly squids used in this study were captured at different times and places around Japan. Most of the firefly squids were captured at Namerikawa, Toyama of Japan Sea side on the spring nights when they came to spawn, and brought to our laboratory. These were mature squids of 5-7 cm mantle size which survived a few days in captivity below 10 ℃ in the dark. Young squids of about 2.5 cm mantle size were captured in the middle of October at Toyama Bay. The mature firefly squids living in the Pacific Ocean were captured in early February in the Sagami Bay, Shizuoka Japan and they were also used in this study. Other cephalopods were collected from Fisheries of Osaka Bay or some were supplied by Fukui and Toyama prefectural marine stations. All-trans 3-dehydroretinal was kindly supplied by F. Hoffman-La Roche (Basel). All-trans 4-hydroxyretinal was synthesized by the method of Renk et al.(1981).

Microdissection of Watasenia retina and HPLC analysis of

retinoids

The eye, about 10 mm diameter, was excised from live squid under red light. The lens was removed and the retina was incised radially and unfolded on the filter paper. A row of five photophores on the ventral surface of the eye served as a good marker for the dissection of the specific area of the retina situated to receive the downwelling light.

The diagram of the eye is illustrated in Figure 2. The thickest part of retina is located just behind the row of the eye photophores. The photoreceptor cells in this region have long outer segments (OS) of about 500 μm and inner segments (IS) of about 100 μm while the photoreceptor cells in other region have OS of about 200 μm and IS of about 50 μm. Two distinct strata, a pinkish proximal stratum and a yellowish distal stratum, can be discerned in the OS layer of the long photoreceptor cells (Matsui et al.1988 a).

The part of retina containing the two strata was cut into 1000x1000 μm blocks unfolded on a piece of filter paper using a guillotine equipped with a razor blade. Then the slices of the retina were horizontally dissected into 150 μm segments to separate the pinkish stratum from the yellowish stratum with the aid of a microscope illuminated with dim white light to visualize the two strata. The short OS layer of the retina appears yellowish. The larger block (5x5 mm) was prepared from the dorsal retina containing only thin OS.

The retinals present in these segments and block were extracted as the oxime derivatives (Groenendijk et al.1980;

Suzuki et al. 1983), and analyzed on a Nishio M type HPLC system with a 0.6x15 cm YMC silica gel column. For optimal separation, the oximes derived from retinal and 3-dehydroretinal were eluted with a mixture of ethylacetate, ethanol, and hexane, 60 v: 3 v: 937 v, and the oximes derived from 4-hydroxyretinal was eluted with a mixture of ethylacetate, ethanol,, and hexane, 200 v: 25 v: 775 v. Greater than 97 per cent of the retinals were chromophores of visual pigments, since this retina contained relatively little retinochrome and retinal binding protein (Matsui et al. 1988).

This squid has two pairs of extraocular photosensitive vesicles located in the ventral and dorsal side of the head. The dorsal vesicles when viewed through the windows where there were few integumental pigment cells appeared yellowish. They were dissected and collected for the retinal analysis. The eggs and the hepatopancreas (liver) were also collected for the analysis of retinoids. The retinoids (retinals, retinols and retinyl esters) were extractable with the same method for the retinal oximes. The retinyl esters were analysed as retinols after hydrolysis in KOH-ethanol (Bridges and Alvarez 1980).

The visual pigments of other cephalopod eyes were extracted and purified in a detergent solution of lauryl sucrose (L-1695, Mitsubishi chemicals, Tokyo) by the method reported previously (Nashima et al. 1979). After the determination of their absorbance maxima, the chromophore

compositions of the purified pigments were analyzed as the oxime-derivatives by HPLC.

Results

The results of the HPLC analysis of the oximes extracted from the 1000x1000x150 μm blocks of the Watasenia retina containing the long outer segment are shown in Figure 2. Only oximes derived from 3-dehydroretinal were extracted from the block composed mainly of the pinkish stratum and only oximes derived from 4-hydroxyretinal were extracted from the block composed of the yellowish stratum. The block of whole long OS containning both strata was shown to contain both 3-dehydroreinal oximes and 4-hydroxyretinal oximes but did not contain retinal oximes. The 5x5 mm block from the dorsal region of the retina with short OS contained only retinal oximes. This showed that visual pigments based on retinal (A1 pigment), 3-dehydroretinal (A2 pigment) and 4-hydroxyretinal (A4 pigment) occupy separate regions of the Watasenia retina, as shown in Figure 2.

The ratio of retinal to 3-dehydroretinal to 4-hydroxyretinal in the whole Watasenia retina was 67:8:25. This value was calculated from the respective peak areas of the oximes on the chromatogram monitored at 360 nm, assuming the molar extinction coefficients were the same for the oximes of retinal, 3-dehydroretinal and 4-hydroxyretinal. Similar ratio for the three retinals was found in the eyes of

young Watasenia. The analysis were performed on four eyes from two specimens of the young firefly squid and variations in the proportions of the three retinals from the four eyes were within 3 %. The mature squids caught in the spring were almost females and only few small male squids were found. They also had three visual pigments. The mature squids captured in Sagami Bay of Pacific Ocean had three pigments in a propotion similar to those of Japan Sea, although they had been isolated geographically.

In the dorsal photosensitive vesicles, only retinal oximes were found and after the vesicles were irradiated with light > 520 nm, the proportion of all-trans retinal oximes was considerably increased. Probably, this indicates the presence of the visusal pigment similar to the A1 pigment in the eye.

The hepatopancreas contained a large amount of 11-cis retinylester and trace amounts of all-trans retinylester but no other retinoids. This suggests that the retinoids with 3-dehydro or 4-hydroxy structure in the eye did not originate from their food, since those retinoids from the diet would be stored in the hepatopancreas. One animal had about 600-1000 eggs (Yuuki Y 1985) and they were used for retinoid analysis. They contained all-trans retinol and a smaller amount of 13-cis retinol and a trace amount of 4-hydroxyretinol. These results are summarized in Table 1.

Table 2 shows the absorbance maxima of the purified cephalopod visual pigments. They are listed in the order of

the wavelength of the maximum absorbance. These cephalopods listed in the table belong to several different families and most of them are limited to the pelagic and mesopelagic habitants.

Discussion

Careful microdissection of the specific region of the Watasenia retina receiving downwelling light allowed us to demonstrate by HPLC analysis of the extracted oximes that each of the three visual pigments found in this squid is located in the separate area of the retina. The proximal pinkish area of the long photoreceptor cells contained only the A2 pigment based on 3-dehydroretinal (λmax: about 500 nm) and the distal yellow area contained the A4 pigment based on 4-hydroxyretinal (λmax: about 470 nm). No A1 pigment was found in the specific area of the ventral retina with two strata, although the A1 pigment was extracted in the considerable proportion of 20-40% from the relatively larger section of the frozen retina in the previous experiment (Matsui et al. 1988b). Thus it is clear that the specific region of retina having thick OS layer contains only two kinds of visual pigments. The OS layer of this retina contained very few black pigment granules excepting in the dense pigmented layer near the basement membrane. The colour of each stratum was exactly corresponding to the colour of each visual pigment contained.

The relative area of the specific region with the two strata can be estimated from the ratio of the content of three retinals (67:8:25) in the whole retina. The yellow stratum with the A4 pigment was about twice as wide as the pinkish stratum with the A2 pigment, which was about 200 μm and comparable to the OS layer of other region of the retina with the A1 pigment. If we assume that each visual pigment exists in the same density throughout the OS layer of the retina, the area with the two strata might occupy about 13 per cent of the whole retina, the value of which was calculated using the relative absorbances of retinal oximes and 3-dehydroretinal oximes reported by Suzuki and Makino (1983).

We could not observe under light microscope the same special structure separating the two strata as seen in the multiple bank of photoreceptor cells in the retina of the deep-sea fish (Locket 1977). It is important to determine whether the two pigments are located in the OS of different photoreceptor cells or in the distal and proximal part of the same photoreceptor cell. It has been found in the Arthropod ommatidium that the long rhabdom is made up of a combination of the microvilli from the several surrounding photoreceptor cells (Eakin 1972). In this structure of the fused rhabdom, the microvilli of the individual photoreceptor cell are often indistinguishable from those of other cells when viewed through a light microscope.

Electron microscopy of the freeze-fractured or thinly sectioned preparations of Watasenia retina, (Masuda et al.

1988) revealed the existence of four types of photoreceptor cells in this specific region of the retina. One of these photoreceptor cells projects its microvilli upto 200 μm above the basement membrane and if the microvilli of this photoreceptor cell contain only the A2 pigment with the absorbance maximum of about 500 nm, the OS of these cells might form the proximal pinkish stratum. Another one of the photoreceptor cells found by electron microscopy had microvilli only at the distal layer of 300 μm from the vitreous side. The rest two types of the photoreceptor cells had their microvilli at the central zone of the OS layer. It may be considered that the thick OS layer of Watasenia retina constitutes another type of the multiple bank of photoreceptor cells, different from but fuctions similar to those in the deep-sea fish eyes.

Thus the specific region of Watasenia retina with the long OS is made of different photoreceptor cells with visual pigment with the absorbance maximum of 470 nm or 500 nm, which enables the squid to see the light with high and broad photosensitivity. Since the absorbance spectra of the two pigments overlaped considerably, the light which comes to the proximal OS layer would be largely modulated by passing through the yellow long OS layer. The proximal photoreceptor cells should respond to light that is not absorbed by the yellow layer and its action spectrum should appear red shifted.

Recently, Denton and Locket (1989) considered the

possibility of the spectral discrimination by the multiple bank of photoreceptors with a single visual pigment in the eye of the deep-sea fish. Each bank of the photoreceptor cells might have different spectral sensitivity according to the degree of modulation by the filter of the previous banks.

They also mentioned that if the multibank retina of the fish was composed of layers of the photoreceptor cells with different visual pigments in order of their absorbance maxima, the resulting structure should be more effective for colour discrimination. The visual system of firefly squid may be an interesting example of colour vision different from the system using the trichromatic cones in the vertebrate eyes.

As illustrated in Fig. 1, some of the lights emitted by the firefly squid contain longer wavelengths than the monochromatic blue light of the deep-sea environment. Since their eyes may be especially sensitive to the green light and may discriminate effectively between the light they generate and the light in the environment by the aid of the multiple OS mentioned above, their photophores can serve as a form of communication between the firefly squids that is invisible to its predators.

The young squid was also found to have three visual pigments in the eye. Much for their life cycle is not yet known, and it was assumed the A2 pigment was generated to enable the mature squid to come to the shallow water for spawning, similar to the seasonal variation in the visual

pigments of other animals (Bridges 1972, Makino et al. 1983). The existence of A2 pigment throughout the life of the firefly squid supports our hypothesis for its important role in color discrimination. The development of the three visual pigments throughout the life cycle of the firefly squid should be investigated. The larva should have A1 and A4 pigment because retinol and 4-hydroxyretinol were found in the eggs. Finding the stage when the squids develop A2 pigment may help us understand the function of this pigment. Unfortunately, our investigation are limited by our lack of understanding of the life cycle of this squid since they could only be caught at limited period throughout the year.

The retinoids with 3-dehydro and 4-hydroxy structure are considered to be the products of animal kingdoms, but we have not found another example of the occurrence of 3-dehydro-retinal and 4-hydroxyretinal in cephalopods (Table 2). Enoploteuthis chunii is a species phylogenetically closest to Watasenia, resembling in many features and often captured together with Watasenia scintillans. Yet we found only A1 pigment with the absorbance maximum of 484 nm and no specific region with the long OS layer in the ventral retina of the squid.

Since the 3-dehydro or 4-hydroxyretinoids were exclusively found in the Watasenia eye and not in hepatopancreas or photosensitive vesicles, some enzymatic systems in the special photoreceptor cells having the A2 or

A4 pigment may oxidize retinal to 4-hydroxyretinal and 3-dehydroretinal to supply them for the visual pigment synthesis.

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Figure 1.

Spectral distribution of light intensities in bioluminescence of the firefly squid (Kito et al. 1978) and at depths around 500 m in clear oceanic waters (Kampa 1970).

Solid line: bioluminescence, dotted line: deep-sea light.

Figure 2.

Left; Diagram of Watasenia scintillans eye. l: lens, y: yellowish OS layer, p: pinkish OS layer, ep; eye photophore, b; black pigment layer, i: IS layer.

Right; HPLC profile of the chromophore extracted from the eye in the oxime-derivatives. Trace A represents the extract of the block A in the dorsal retina. Trace B represents the extract of the segment B microdissected from the pinkish stratum of the ventral retina. Trace C represents the extract of the segment C from the yellowish stratum of the ventral retina. The positions of A, B and C are shown in Diagram. The peaks were assigned by comparison with the respective authentic compounds. The labels on the peaks, syn 11-1, anti at-1, syn 11-2, syn 11-4 and anti 11-4, should be read as syn 11-cis retinal oxime, anti all-trans retinal oxime, syn 11-cis 3-dehydroretinal oxime, syn 11-cis 4-hydroxyretinal oxime and anti 11-cis 4-hydroxyretinal oxime, respectively. The HPLC was carried out by the following step elution: initially the solvent system for the retinal-oximes until appearance of anti all-trans 3-dehydroretinal oxime and then the second solvent system for the 4-hydroxyretinal-oximes. Ghost is a peak due to the solvent exchange. Flow rate: 1 ml/min.

Table 1.

Retinoids of the firefly squid, Watasenia scintillans.

	content (nmol)			
	Eye	Eggs*	Dorsal P.V.	Hepatopancreas
retinal	26.8±5.3		$14.2 \pm 0.6 (\times 10^{-3})$	
3-dehydroretinal	4.2±1.1			
4-hydroxyretinal	10.4±2.6			
retinylester				420±6.0
retinol		2.7±0.3		
4-hydroxyretinol		$30\text{-}400 (\times 10^{-3})$		

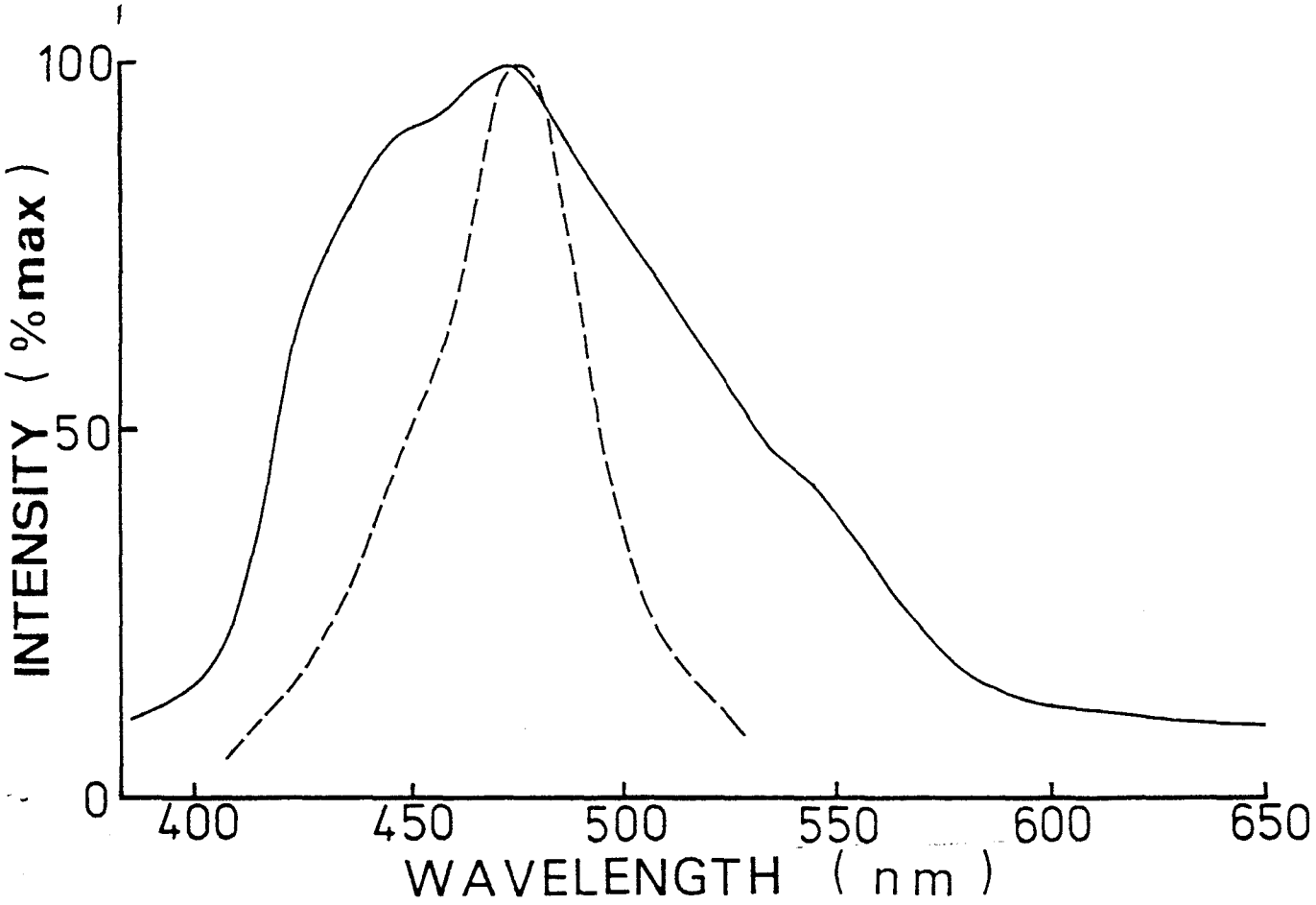
*Most of retinoids in the eggs were all-trans and little 13-cis, and retinoids in other parts were mostly 11-cis with a small amount of all-trans. One animal contained about 600-1000 eggs and the content of 4-hydroxyretinol was largely varied. P.V. denotes the extraocular photosensitive vesicle.

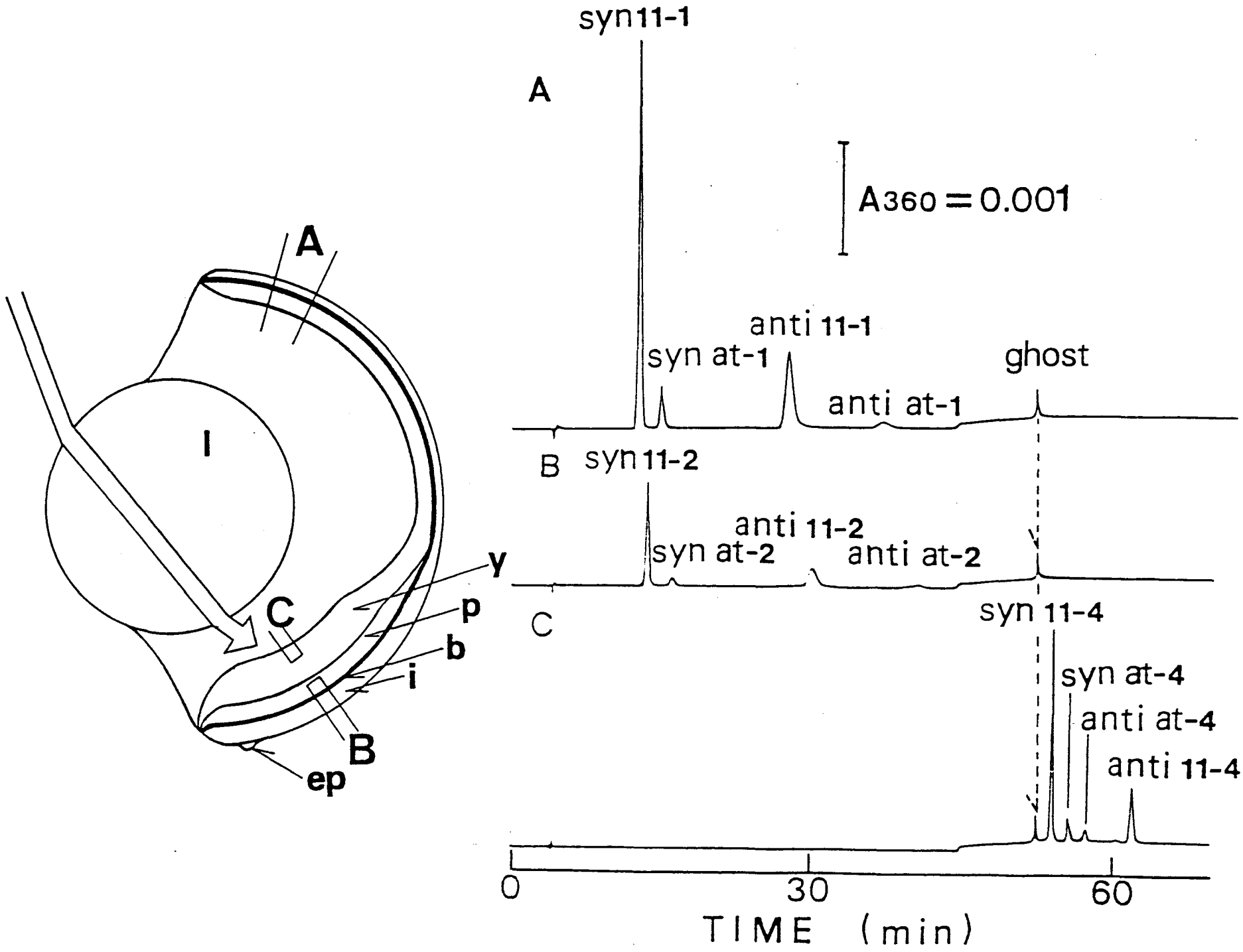
Table 2.

Chromophore and Absorbance Maximum of Visual Pigment of Cephalopods collected near Japan

species	nm	chromophore
Sepiella japonica	504	A1
Loligo japonica	496	A1
Loligo beka	496	A1
Doryteuthis bleekeri	494	A1
Euprymna morsei*	494	A1
Loligo edulis*	491	A1
Sepia lycidas	491	A1
Sepia esculenta	490	A1
Todarodes pacificus	482	A1
Ommastrephes bartrami	482	A1
Berryteuthis magister	484	A1
Watasenia scintillans*	484	A1
	500	A2
	470	A4
Enoploteuthis chunii*	484	A1
Sepioteuthis lessoniana	ND	A1
Idiosepius pygmaeus	ND	A1
Thysanoteuthis rhombus	ND	A1
Eucleoteuthis luminosa*	ND	A1
Chiroteuthis imperator*	ND	A1
Gonatopsis boreulis	ND	A1
Octopus vulgaris	480	A1
Octopus minor	480	A1
Octopus ocellatus	480	A1
Paroctopus dofleini	480	A1
Paroctopus sp.(amadako)	471	A1

A1: retinal, A2: 3-dehydroretinal, A4: 4-hydroxyretinal.
* denotes bioluminescent species. ND: not determined.


100
50
0
INTENSITY (%max)
400
450
500
550
600
650
WAVELENGTH (nm)


A
I
C
B
ep
y
p
b
i
syn 11-1
A
A360 = 0.001
anti 11-1
syn at-1
ghost
anti at-1
B
syn 11-2
anti 11-2
syn at-2
anti at-2
C
syn 11-4
syn at-4
anti at-4
anti 11-4
0
30
60
TIME (min)

Chapter 3

The synthesis of the 4-hydroxyretinol in the eye of the firefly squid, *Watasenia scintillans*

Summary

The 4-hydroxyretinal is a novel chromophore of visual pigment recently found and it forms a blue sensitive visual pigment in the eye of the firefly squid, Watasenia scintillans. This squid has three visual pigment its retina, each visual pigment being segregated in different parts of the retina and being based on a different chromophore. This paper describes the 4-hydroxyretinol is produced in the specific area of the ventral retina containing the blue sensitive visual pigment. The homogenate of the retina was incubated with 11-cis retinol and the 4-hydroxyretinoids production was examined with HPLC. The specific area dissected from the ventral retina can convert about 3 % of the added 11-cis retinol to the 4-hydroxyretinol. The specific part of the retina is also considered to produced 3-dehydroretinal, the chromophore of another visual pigment contained in the part. However the evidence of formation of 3-dehydroretinal is not detected in this experiment.

Key words: 4-hydroxyretinal; 4-hydroxyretinol; A_4 pigment; (squid)

Introduction

Watasenia scintillans, a bioluminescent deep-sea squid has three kinds of visual pigments. The visual pigments with λ_{max} values at 484 nm, 500 nm and 471 nm are based on

retinal, 3-dehydroretinal and 4-hydroxyretinal, respectively [1,2]. Some of the lights emitted by the squid contain longer wavelengths than the monochromatic blue light of the deep-sea photic environment where they live [3]. By use of the three visual pigments, the squid probably utilize the bioluminescent light for their communication or making shoal.

A large amount of 11-cis retinylester was stored in the hepatopancreas but 3-dehydroretinal, 4-hydroxyretinal and their derivatives were not found in any other tissues of the squid, except the specific area of the retina [3]. The absence of 3-dehydroretinoids and 4-hydroxyretinoids in the hepatopancreas suggested that these retinoids do not originate in the diet of the squid, because dietary retinoids would be stocked in the hepatopancreas. It is reasonable to consider that in the special photoreceptor cells containing the 3-dehydroretinal and 4-hydroxyretinal based on the visual pigments, an emzymatic system may oxidize retinal to 4-hydroxyretinal or 3-dehydroretinal to supply for visual pigment synthesis. Here we report the synthesis of the 11-cis-4-hydroxyretinol from 11-cis retinol in vitro.

Materials and methods

The firefly squid, Watasenia scintillans, was captured at Toyama Bay of Japan Sea at spring night and brought to our laboratory. The eye of about 10 mm diameter was excised from squid under dim red light and stored at below -20 ℃. There was a row of five photophores on the ventral surface of

the eye. It was a good marker for dissection of the retina. The eye was cut into three parts, the ventral, central and dorsal retina, on the dry ice block. About 50 pieces of ventral or dorsal parts of retinae were collected in glass homogenizers. They were homogenized with 20 ml of 100 mM Tris-acetate buffer (pH 7.5). The homogenized retina was filtered through a net of 0.5 mm mesh to remove the sclera and divided into three portions. 11-cis retinal or 11-cis retinol dissolving in ethanol was added the homogenate. The volume of ethanol added was below 0.5 % of the homogenate. In some experiment, reduction cofactors, reduced nicotinamide-adenine-dinucleotide (NADH, Oriental yeast Co. Ltd., Tokyo, Japan) and reduced nicotinamide-adenine-dinucleotide phosphate (NADPH, Oriental yeast Co. Ltd., Tokyo, Japan) was added. The homogenates were stirred for 2 hours and then retinoids were extracted according to the technique described by Suzuki et al. [4].

The retinoid composition was determined by high-performance-liquid-chromatography (HPLC). The HPLC involved the use of a YMC silica gel column (6 × 150 mm, YMC Co. Ltd., Kyoto, Japan) with two solvent systems. One of them is an eluent of 3 % ethylacetate and 0.03 % ethanol in hexane for the analysis of retinoids and 3-dehydroretinoids and another is an eluent of 15 % ethylacetate and 1.5 % ethanol in hexane for the analysis of 4-hydroxyretinoids. The absorbance at 380 nm was monitored

to determine the amounts of aldehyde form of retinoids and that of 320 nm was monitored to determine the amounts of alcohol form of retinoids. The peak fraction which had the same retention time as 11-cis 4-hydroxyretinol was collected and rechromatographed with the same condition. The absorbance spectrum of the eluted peak fraction was measured with a spectro multi channel detector DP-L340 (Japan Spectroscopic Co. Ltd., Tokyo, Japan).

The standards of the respective isomers of authentic retinal and 3-dehydroretinal for HPLC were a generous gift of Dr. T Suzuki. The all-trans 4-hydroxyretinal was synthesized according to the method of Renk et al. [5] and other isomers of 4-hydroxyretinal were prepared as described previously [2]. The standards of retinol, 3-dehydroretinol and 4-hydroxyretinol were made by $NaBH_4$ reduction of the above aldehyde forms.

Result and discussion

The homogenate of the ventral retina had originally three visual pigment chromophores of retinal, 3-dehydroretinal, 4-hydroxyretinal in the ratio of 1.0 / 1.4 / 2.0. The homogenate of the dorsal retina had only retinal. These homogenates did not contain any other retinoids such as retinols and retinylesters.

11-cis retinal was added to the homogenate of the ventral retina to give a final concentration of about 200 μM which was about 50 times higher than the original retinal

content. The amounts of 3-dehydroretinoids and 4-hydroxyretinoids were not changed.

Then, about 30 μM of 11-cis retinol was added to the homogenate of the ventral retina. After 2 hours incubation at 10 ℃, 5 - 10 % of added 11-cis retinol was converted to the retinal form. But the amount of 3-dehydroretinal and 4-dehydroretinal were not changed in the homogenate. In this experiment, we found a peak which had the same retention as 11-cis 4-hydroxyretinol on the HPLC chromatogram as shown in Fig. 1 (A). In order to ascertain that the peak is ascribed to 11-cis 4-hydroxyretinol, the absorbance spectrum of the peak fraction collected from several runs were measured with spectro multi channel detector (Fig. 1 (B)). The absorption maxima locates around 320 nm and the absorbance spectrum is similar to that of retinol. The absorbance spectrum and the retention time of the new peak suggested that the substance was 11-cis 4-hydroxyretinol.

The 11-cis 4-hydroxyretinol was amounted to 2.6 % of the added11-cis retinol. Possibly the inner segment portion of the ventral retina is the site of production of 4-hydroxyretinol. In this experiment, we used the homogenate of the ventral retina without separating the outer segment portion because we were afraid of the loss of some cofactors participating in the oxidation reaction. Since the large portion of the added 11-cis retinol was absorbed by the membrane of outer segment, the small amount of 11-cis

retinol were oxidized to the 11-cis 4-hydroxyretinol in the membrane of the inner segment. The reduction cofactors, 1mM NADH and 1mM NADPH, and temperature, 0 ℃, or 10 ℃ or 20 ℃, did not affect this reaction.

To the homogenate from dorsal retina, final concentration of 200 μM 11-cis retinol was added but the 4-hydroxyretinol was not detected. The result that 11-cis 4-hydroxyretinol was produced from 11-cis retinol at the specific ventral area of the retina indicates that the 4-hydroxyretinal does not come from the diet, but it was produced by the cell which has the the visual pigment based on 4-hydroxyretinal in the ventral retina.

The amount of 11-cis 4-hydroxyretinal did not increase in this experiment. It is probably due to that the newly produced 4-hydroxyretinal is far small to vary the amount of 4-hydroxyretinal in the homogenate. If the oxidation happened with the same ratio of 5 - 10 % as in the case of 11-cis retinol to the retinal, the amount of newly produced 11-cis 4-hydroxyretinal will be only 0.3 -1 % of the amount of the 11-cis 4-hydroxyretinal originally contained in the homogenate.

In all experiments, we noticed the increase of 3-dehydroretinoids, especially the appearance of 3-dehydroretinol but we could not detected. It was not clear whether 3-dehydroretinoids synthesis did not occur in such a homogenate used in this study. In the crayfish eye, Suzuki proposed that 3-dehydroretinol may be the intermediate of the

synthesis of 3-dehydroretinal from retinal [personal communication]. The 4-hydroxyretinol in the squid eye may play such a role in the 3-dehydroretinal synthesis as 3-hydroxyretinol in the crayfish. In this *in vitro* experiment, the amount of newly produced 4-hydroxyretinol was probably too little to be the intermediate of the reaction in the homogenate.

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Figure 1

(A) HPLC profile of the extract from the homogenate of the ventral retina mixed with 11-*cis* retinol.
Peak 1, 2 and 3 are isomers of 4-hydroxyretinal. Peak 1 corresponds to 13-*cis*; peak 2, all-*trans*; peak 3, 11-*cis*. Peak 4 is 11-*cis* 4-hydroxyretinol.

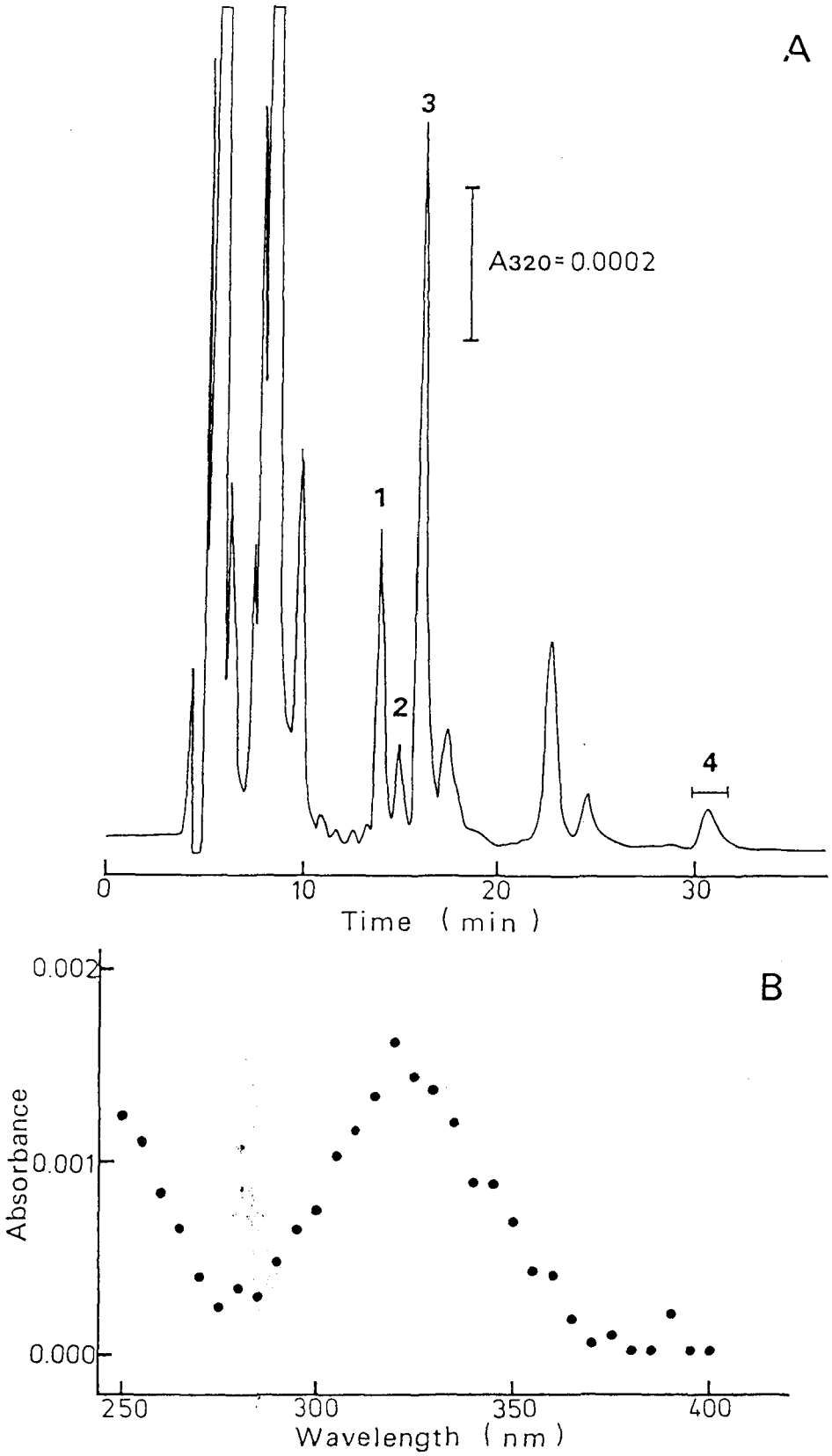
(B) Absorbance spectrum of the peak 4 in figure 1 (A). The spectrum is the mean of 6 times measurements repeated at 3 sec intervals.

Table 1

Normalized contents of 4-hydroxyretinoid in the homogenates of the ventral and the dorsal retina.

	ventral (nmol)			dorsal
	−retinoid (n=3)	+11-cis-A_1-retinal (n=3)	+11-cis-A_1-retinol (n=9)	+11-cis-A_1-retinol (n=3)
11-cis-A_1 retinal	11.3 ± 1.6	11.7 ± 2.1	11.6 ± 3.4	N.D.
11-cis-A_1 retinol	N.D.	N.D.	0.79 ± 0.51	N.D.

N.D. means "not detectable".


A
3
A320= 0.0002
1
2
4
0
10
20
30
Time (min)
B
0.002
0.001
0.000
Absorbance
250
300
350
400
Wavelength (nm)

Chapter 4

X-ray diffraction of the live squid retina

X-RAY DIFFRACTION OF THE LIVE SQUID RETINA

Introduction

The initial step of the visual process is the absorption of light by the visual pigment. The squid visual pigment is located in microvilli which are cylindrical extensions of the cell membrane, arranged hexagonally within the rhabdomes. Until now, only a few papers have been published on the structural study of invertebrate rhabdomes by x-ray diffraction. In those, however, the retina fixed by glutaraldehyde was used, because this tissue disintegrated within 1 hour of dissection. In the present study, we could succeed in recording the x-ray diffraction pattern from unfixed retina by the use of the synchrotron radiation and a storage phosphor screen, the imaging plate. The result suggests that it will be possible to investigate the structural response of photoreceptors to the light stimulation.

Experimental

Living, active specimens of the squid, *Watasenia scintillans* were captured at Toyama Bay of the Japan Sea and brought to Tsukuba within several hours. The squids were decapitated and their retinas dissected in dim red light. For the x-ray experiments, 1-mm thick slices of retina were kept in an artificial seawater chamber with Mylar windows at 4 °C. Schematic diagram of a slice of squid retina was shown in Fig. 1.

X-ray experiments have been performed with a mirror-monochromator optics (the Muscle Diffractometer) at BL-15A1.[1] The wavelength of the radiation was 1.50 Å. The sample-to-detector distance was 2196 mm. X-ray scattering intensity was recorded on the imaging plate and stored on magnetic tape after converting to the digital signals with the image reader and the image processor.[2] The exposure time was 5-10 minutes and each recording finished within 30 minutes after the decapitation.

Results

Figure 2 shows the x-ray diffraction pattern from outer segments of live squid retina, which was reproduced and photographed from the stored image with the image writer. The low angle x-ray diffraction spots are due to the 570-Å hexagonal lattice of microvilli. The six diffuse maxima centered around 1/40 $Å^{-1}$ may originate from the bilayer structure of microvillar membranes. The intensities are different among equivalent Bragg reflections and stronger near the vertical axis of the pattern. This result suggests that rhabdomes are variously oriented around the vertical axis of retina in the sample as a whole.

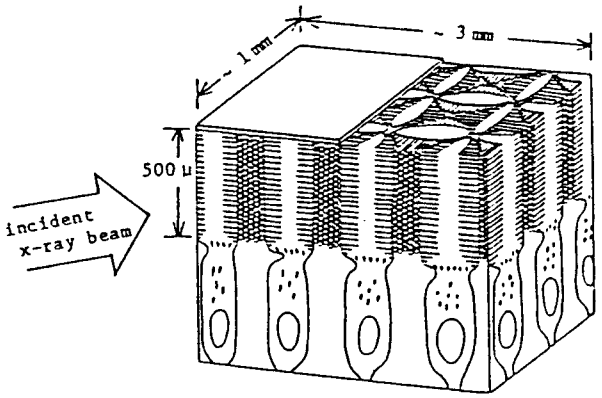


Figure 1. Schematic diagram of a vertical slice of squid retina. The retina consists almost entirely of photoreceptor cells, which are vertically separated into inner and outer segments. The photoreceptive outer segments are in the upper layer and consist of microvilli, which are cylindrical extensions of the cell membrane, packed hexagonally in the rhabdomes. The microvilli are 600 Å in diameter and 1 μm long.

Figure 2. The x-ray diffraction pattern from a slice of live squid retina in artificial seawater. The storage ring was operated at 2.5 GeV with a beam current of 188 mA. The exposure time was 5 minutes.

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X-RAY DIFFRACTION OF THE LIVE SQUID RETINA IN THE DARK AND LIGHT

Introduction

The initial step of the visual process is the absorption of light by the visual pigment. The squid visual pigment is located in microvilli which are cylindrical extensions of the cell membrane, arranged hexagonally within the rhabdomes. Until now, only a few papers have been published on the structural study of invertebrate rhabdomes by x-ray diffraction. In those, however, the retina fixed by glutaraldehyde was used, because this tissue disintegrated within 1 hour of dissection. In the previous report[1], it was described that we could succeed in recording the x-ray diffraction pattern from unfixed retina by the use of the synchrotron radiation and a storage phosphor screen, the imaging plate. In the present study, we have investigated the structural response of photoreceptors to the light stimulation.

Experimental

Living, active specimens of the squid, *Watasenia scintillans* were captured at Toyama Bay of the Japan Sea and brought to Tsukuba within several hours. The squids were decapitated and their retinas dissected in dim red light. For the x-ray experiment, a 1-mm thick slice of retina was kept in an artificial seawater chamber with Mylar windows at 4°C. Schematic diagram of a slice of squid retina was shown in the previous report[1]. The artificial seawater containing D-glucose was oxygenated and gently circulated through the sample chamber during the experiment. A 100-W halogen lamp in conjunction with an interference filter and a heat filter, was used for light stimulation (about 500 nm in wavelength).

X-ray experiments have been performed with a mirror-monochromator optics (the Muscle Diffractometer) at BL-15A1[2]. The wavelength of the radiation was 0.150 nm. The sample-to-detector distance was 2276 mm. X-ray diffraction intensity was recorded on the imaging plate and stored on magnetic tape after converting to the digital signals with the image reader and the image processor[3]. The exposure time was 4 minutes and each recording finished within 40 minutes after the decapitation.

Results

Figure 1 shows the x-ray diffraction patterns from outer segments of a live squid retina in the dark (*left*) and light (*right*), which were reproduced and photographed from the stored images with the image writer. The low angle x-ray diffraction spots are due to the oblique lattice of microvilli. Upon light illumination, the prominent changes have been observed in the lattice constants and the x-ray intensity distribution. The analysis of these data by model calculations suggests that the diameters of microvilli increase as well as the spacings among microvilli in response to the light stimulation.

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3) Y. Amemiya et al., Nucl. Instrum. Methods, A266, 645 (1988)

Figure 1. The x-ray diffraction patterns from a live squid retina in the dark (*left*) and light (*right*). The x-ray diffraction spots are due to the oblique lattice of microvilli. The lattice constants are a=60.0 nm, b=59.0 nm and γ=118° in the dark, and a=65.5 nm, b=64.0 nm and γ=118° in light, respectively. The storage ring was operated at 2.5 GeV with a beam current of 300 mA. The exposure time was 4 minutes for each pattern. The recording of both patterns has finished within 40 minutes after the decapitation.

Chapter 5

Light-induced variation in the contents of cyclic nucleotides in the rhabdomeric photoreceptors

Summary

The amounts of cyclic GMP and cyclic AMP contained in the octopus, Octopus ocellatus, retina and the shrimp, Trachypenaeus curvirostris, eye were measured by competitive radioimmunoassay under dark adapted and light stimulated conditions. Retinal content in each retina was used for the correction of the amounts of nucleotides. In the octopus retina, 2.4 ± 0.5 pmol cGMP and 36 ± 7.0 pmol of cAMP were found in the dark adapted and under light stimulated conditions. The shrimp eye contained 22 ± 8.0 pmol of cGMP and 3.4 ± 0.7 pmol of cAMP in the dark and 2.3 ± 6.0 pmol of cGMP and 7.0 ± 1.4 pmol of cAMP under light stimulated conditions. Cyclic nucleotides may be important in mediating the phototransduction in the shrimp eye and not in the octopus eye, since their contents are not different in the octopus retinas in the dark adapted or under light stimulation.

Key words: Transduction; Cyclic GMP; (Squid); (Shrimp)

Introduction

In many invertebrate photoreceptors, light stimulation produces the depolarizing receptor potential, which is generated by the opening of the ion channel [1]. In the phototransduction process, intracellular messengers mediate between the photon absorption by a visual pigment and the gating of the ion channel which controls the ion conductance

of the photoreceptor cell membrane. Cyclic GMP [2,3] and inositol trisphosphate [4-7] have been proposed as the candidates for the messenger. Particularly it has been speculated that cGMP is the cytosolic messenger of phototransduction in the invertebrate photoreceptor cell [2,3] based on analogy to the better-known vertebrate phototransduction mechanism. The analogy was introduced as a result of the high homology in the primary structure of the visual pigments of vertebrate and invertebrate.

However this problem is still open to the question for the following reason. The employment of the total protein content in the retina as an internal standard for quantification of cGMP content [3], did not take into account the contents of the proteins that participate in the phototransduction mechanism. The cGMP content measured in the previous experiment could not be compared with the content of visual pigment or channel molecule and so on. For example, changes in cGMP content upon light stimulation, may not be enough to affect the light activated conductance. If the cGMP level is constant before and after the light stimulation, it would not be possible to distinguish between the two following explanations: the cGMP content is not affected by the light stimulation or the cGMP which participates in phototransduction is a small portion of the cGMP measured in the experiment so that the variation in the cGMP content can not be detected. We measured both amounts

of cGMP and chromophore of the visual pigment in the photoreceptor in order to allow us to distinguish between the possibilities. The ratio of cGMP to retinal is a good estimate of the ratio of cGMP to the visual pigment. Our results showed that cGMP content is too little to participate in the phototransduction in the octopus photoreceptor cells, but sufficient to participate in the eye of shrimp.

Materials and methods

The octopus, Octopus ocellatus, and the shrimp, Trachypenaeus curvirostris, were used in this study. These animals were kept alive in the dark for more than 24 hours prior to the experiment.

The octopus eye were excised from live animals. For each eye, the lens was removed and the retina with sclera was incised radially, unfolded on the filter paper, then attached on a specimen holder made of a plastic block, and rapidly frozen by push it face down on a copper block previously cooled in liquid nitrogen to obtain the dark adapted retina.

The dark adapted shrimp eyes were obtained as follows: The eyes were cut off at the eyestalk. The eye facing upward was put on a polystyrene foam board. The eye was frozen by being smashed with a hammer cooled with liquid nitrogen.

The light stimulated retinae and eyes were obtained under the same conditions except that less than 300 ms before the retinae and eyes frozen they were irradiated with a

strobe light (200 μs duration). The frozen eyes and retinae were kept at below -80 ℃ until extraction. Above procedures were carried out under infrared light except light irradiation.

The following procedures were carried out under dim red light. The frozen retina or eye was homogenized in 1.5 ml of ice cold 6 % trichloroacetic acid. The homogenate was centrifuged at 10000 rpm for 30 min and the supernatant was collected. This extraction procedure was repeated twice. The collected supernatant was washed 3 times with 10 ml of ethylether to remove trichloroacetic acid, freeze-dried and dissolved in 2 ml of water. 100 μl of this solution was used for the measurement of cyclic nucleotides after being succinylated to facilitate to assay the contents of cyclic nucleotides by radioimmunoassay kits (Yamasa shoyu Co. Ltd., Chiba, Japan). The remainder of the solution was used for the measurement of the guanosine triphosphate (GTP), guanosine diphoshate (GDP), guanosine monophosphate (GMP), adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) by HPLC analysis. The HPLC analysis was performed on a SUMIPAX ODS column (6 × 150 mm, Sumitomo Chemical. Co. Ltd., Tokyo, Japan) and eluted with a solvent system composed of 22.5 % (v/v) methanol in 4 mM tetrabutylammonium phosphate and 15.5 mM potasium phosphate buffer (pH 5.6) at 15 ℃. The flow rate was 0.5 ml/min for initial 30 min after the sample injection and then changed to

1.0 ml/min. The absorbance at 260 nm was monitored with UV-detector (JASCO UVIDEC 100-V , Japan Spectroscopic CO. Ltd., Tokyo, Japan).

The precipitate of trichloroacetic acid treatment was homogenized in 1 ml of 1 M Tris-HCl at pH 7.3. The chromophore of visual pigment in the homogenate was extracted as retinal oximes, according to the technique described by Groenendijk et al [10] and Suzuki and Makino-Tasaka [11]. The extracted retinal oximes were applied to a YMC silica gel column (6 × 150 mm, YMC. Co. Ltd., Japan) and eluted with a solvent system composed of 10 % (v/v) ethylacetate in 0.4 % (v/v) ethanol in hexane at a flow rate of 1 ml/min. The absorbance at 360 nm was monitored.

Result and discussion

Table 1 shows the amounts of nucleotides and retinal in the one of shrimp eye or the one of the octopus retina. In the case of O. ocellatus, the whole eye contained average of 5.23 ± 0.26 nmol of retinal, out of four eyes used in this study. The unfolded retina (n=9) had 4.3 ± 0.50 nmol of retinal. So 18 % of retina was lost during the removal of the lens. The 27.6 % of 11-cis retinal was isomerized to all-trans form by the illumination, indicating that at least 27.6 % visual pigment in the retina was activated. Since the illumination should also isomerize all-trans retinal to 11-cis form in the meta form of the visual pigment as well as in the retinochrome, the actual amount of the activated

visual pigments was greater than the calculated.

Significant differences in the amount of cGMP, cAMP, ATP, ADP and GTP, were not found between light and dark samples. AMP, GMP and GDP were not able to be measured because of impurities which had absorption at 260 nm as shown in Fig.1.

It has been thought that cGMP may be the second messenger of phototransduction in invertebrate photoreceptor as well as in vertebrate rod cell [7 and 8]. But the data showed that the amount of cGMP was not changed by illumination. We are calculated the number of cGMP molecules in one microvillus to distinguish between the two following explanations: the cGMP content is not affected by the light stimulation or the variation in the cGMP content can not be detected because the cGMP which participates in phototransduction is a small portion of the cGMP measured in the experiment. The dimensions of O. ocellatus retinal photoreceptors were obtained from the photograph of electronmicroscopy of cross sectioned or tangential sectioned octopus retina by Yamamoto [personal communication]. The average area of a cross section of a photoreceptor was $3.9 \times 10 \mu m^2$. One cell had 3.5×10^5 of microvilli. The average area of the retina used in this experiment was 1.0 cm^2. So the retina had 9.1×10^{11} of microvilli. If all of cGMP exist in the microvilli, one microvillus had 1.6 molecule of cGMP in it. The ratio cGMP to visual pigment

is 1 / 1.8×10^3 and the area of the surface of the microvillus, which has an average length of the 1.6 μm and an average of diameter of 0.075 μm, is $3.8 \times 10^{-1} \mu m^2$, so the microvillus has 7.6×10^3 molecules / μm^2 of the visual pigment in the membrane. The density of the visual pigment is one twelfth of that of the bacterio rhodopsin in the purple membrane [12]. If the actual number of the microvilli in the retina is less than the value calculated here, the number of cGMP in one microvillus has to be less than 19 because the density of the visual pigment should be less than that of the bacterio rhodopsin. The illumination used in this experiment was strong enough to activate all of the microvilli. The above estimation led us to conclude that if cGMP content was affected by light, the variation in the amount of cGMP had to be detectable. The possibility that cGMP may be the second messenger was little in the eye of O. ocellatus. In the same manner, the possibility that cAMP may be participate in phototransduction is small. Table 1 shows that the paucity of cGMP and cAMP was not caused by lack of their source materials, GTP and ATP, in the tissues. Because we could measure the ERG from the retina which was prepared in the same manner, the result that the content of nucleotides were not affected by light stimulation was not induced by the damage from the unfolding of the retina.

Since the visual pigment of T. curvirostris was more unstable than that of O. ocellatus, after trichloroacetic

acid treatment, the ratio of 11-*cis* retinal to all-*trans* retinal extracted from the dark adapted retina was identical with the one from illuminated retina. Unless the eye was treated with trichloroacetic acid, it was shown that the illumination could convert 10-30 % of visual pigment into meta form in the same procedure. So the stimulating light could also activate sufficient number of visual pigments in the shrimp eye. In the dark adapted sample, there are one cGMP per 32 visual pigments and one cAMP per 200 visual pigment. As shown in table 1, the illumination reduced the amount of cGMP and cAMP to about one tenth and one fifth of the dark level, respectively. So in the case of *T. curvirostris*, cGMP and also cAMP could be candidates of the second messenger. Unfortunately, since the sample used in this study had nervous tissues in its eyestalk, we can not be sure that the reduction of the cyclic nucleotide levels happened in the photoreceptor cells or cells in other nervous tissues. Anyhow it may be considered that cGMP and cAMP are involved in either phototransduction or adaptation in the eye of *T. curvirostris*.

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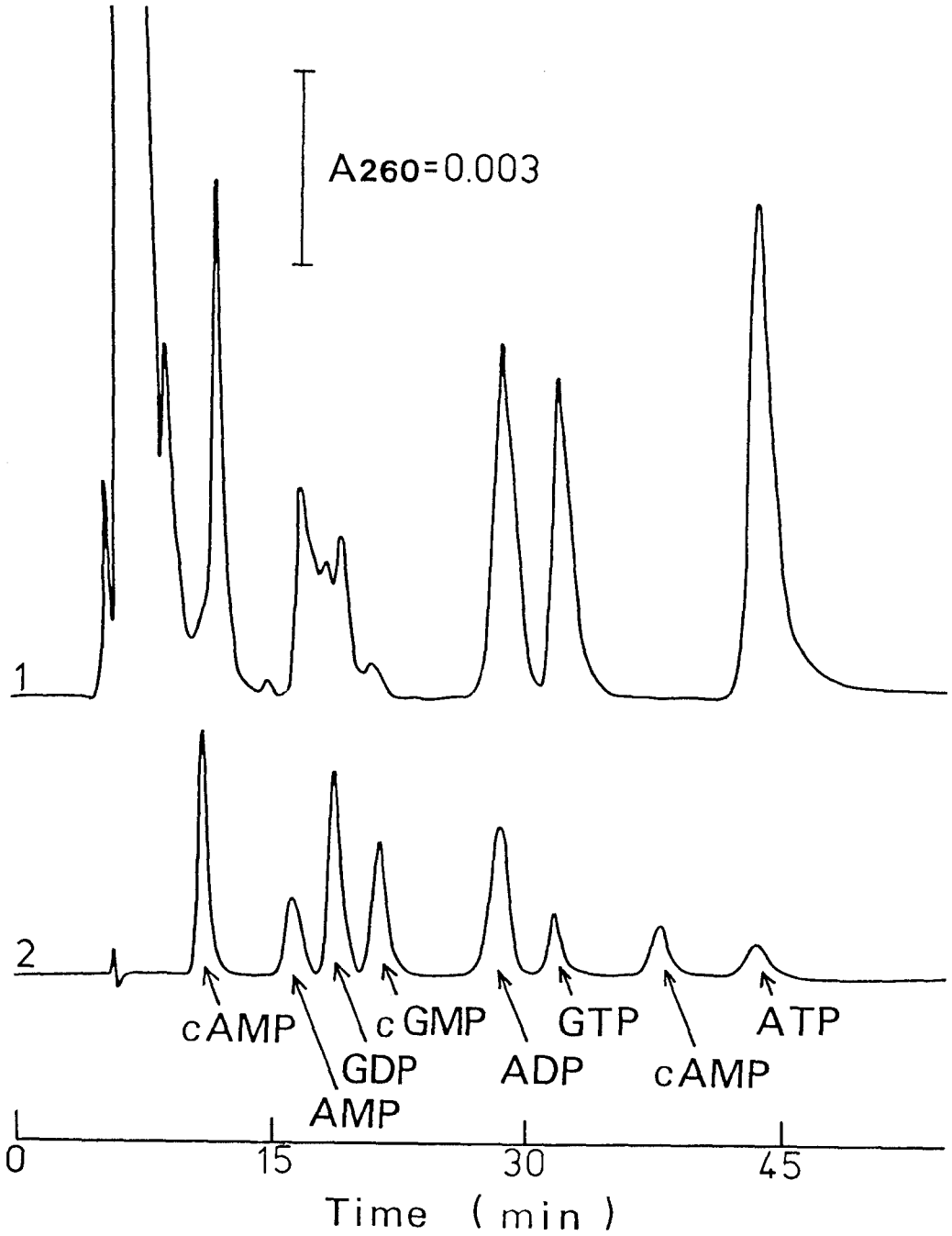
Figure.1

HPLC analysis of nucleotides in the retina of O. ocellatus. Trace 1 is a chromatogram of the TCA extract from an octopus retina. Trace 2 is one of a mixture of authentic compounds. Flow rate was 0.5 ml/min for first 30 min, after then it was 1ml/min.

Table 1

Contents of chromophore and nucleotides in one eye ($x10^{-9}$ mol)

	Octopus ocellatus		Trachypenaeus curvirostris	
	dark (n=4)	light (n=5)	dark (n=3)	light (n=3)
retinal	4.4 ± 0.7	4.2 ± 0.4	0.69 ± 0.17	0.76 ± 0.15
cGMP	0.0024 ± 0.0005	0.0023 ± 0.0009	0.022 ± 0.008	0.0023 ± 0.0006
cAMP	0.036 ± 0.007	0.032 ± 0.004	0.0034 ± 0.0007	0.00070 ± 0.00014
GTP	11 ± 3	11 ± 4		
ATP	17 ± 8	17 ± 6		
ADP	4.0 ± 1.3	5.8 ± 1.7		


A260=0.003
1
2
cAMP
GDP
AMP
cGMP
ADP
GTP
cAMP
ATP
0
15
30
45
Time (min)

付録

1. Adaptation of a deep-sea cephalopod to the photic environment evidence for three visual pigments
   Journal of General Physiology, Vol.92, pp.55-66.

2. 4-Hydroxyretinal a new visual pigment chromophore found in the bioluminescent squid, Watasenia scintillans
   Biochimica et Biophysica Acta, Vol.966, pp.370-374.

3. On some physicochemical properties of sucrose esters and the stability, they confer to membrane proteins
   Journal of Colloid and Interface Science, Vol.128, pp.230-236.

Adaptation of a Deep-Sea Cephalopod to the Photic Environment

Evidence for Three Visual Pigments

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 μm; inner segment: 99 μm) were long in the small area of the ventral retina receiving downwelling light, whereas they were short (outer segment: 207 μm; inner segment: 44 μm) in the other regions of the retina. The short photoreceptor cells contained the visual pigment with retinal (λ_{max} ~ 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 (λ_{max} ~ 500 nm) was located in the pinkish proximal area, giving high sensitivity at longer wavelengths. A newly found pigment (λ_{max} ~ 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 λ_{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

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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 μm 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 μm 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 (~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 λ_{max} of 484 nm. About 50% of the chromophore of the pigment extracted from the 24 and 46% areas was found to

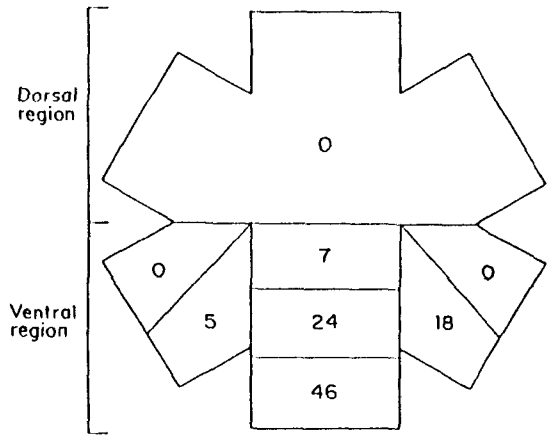

FIGURE 1. The distribution of 3-dehydroretinal in the retina of *W. scintillans*. The numbers indicate the percentage of 3-dehydroretinal in each area. The 46% area is just behind the row of five photophores.

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 λ_{max} at 500 nm (Kito et al., 1986), and the visual pigment with retinal had a λ_{max} at 484 nm, as mentioned above. Therefore, if the 24 and 46% areas contained both the retinal and 3-dehydroretinal pigments, the λ_{max} of the mixture should have been located at a longer wavelength than 484 nm, namely an intermediate value between the λ_{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 metaform 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 λ_{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-dehydroretinal 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 λ_{max} was ~500 nm, as reported previously (Kito et al., 1986).

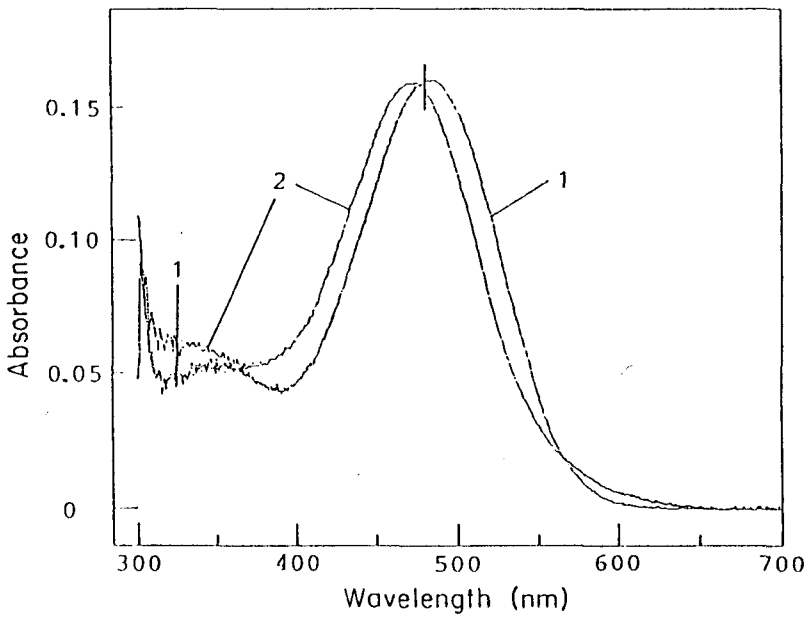

FIGURE 2. The absorbance spectra of visual pigment solutions from the dorsal retina and the 24 and 46% areas of the ventral retina in Fig. 1. Curve 1: the spectrum of the solution from the dorsal retina, with λ_{max} at 484 nm. Curve 2: the spectrum of the solution from the 24 and 46% areas with λ_{max} 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 λ_{max} of ~483 nm.

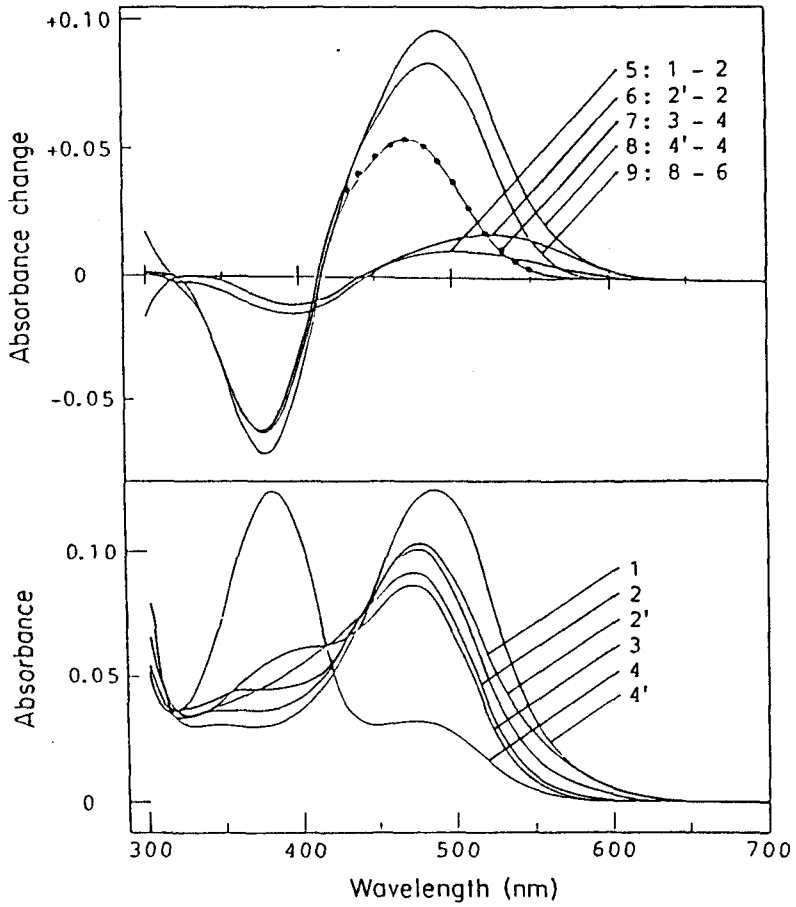

FIGURE 3. Partial bleaching of the pigment solution from the 24 and 46% areas of the ventral retina. The solution at pH 10.5 (curve 1) was irradiated with light of $\lambda > 660$ nm for 1 min (curve 2). Further irradiation with light of $\lambda > 580$ nm was performed for 2 s (curve 3). Furthermore, 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 λ_{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

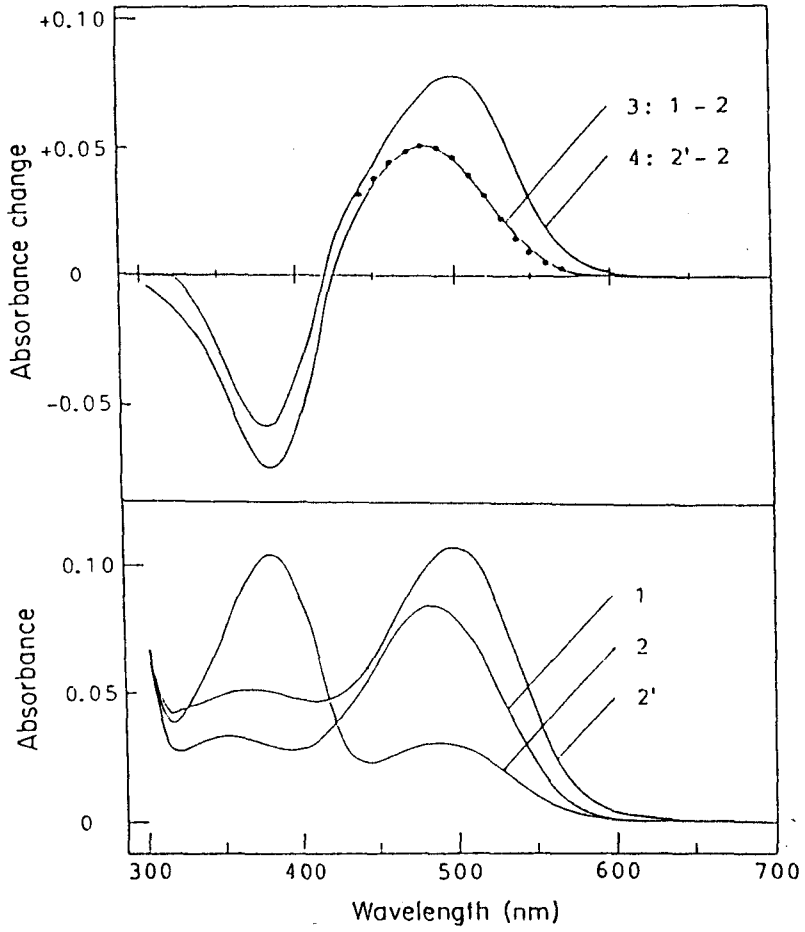

FIGURE 4. Bleaching of the pigment solution from the dorsal retina. The solution at pH 10.5 (curve 1) was irradiated with light of $\lambda < 580$ nm for 4 min (curve 2). The pH of the solution was adjusted to 7.3 (curve 2'). Curves 3 and 4 represent the difference spectra between curves 1 and 2 and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

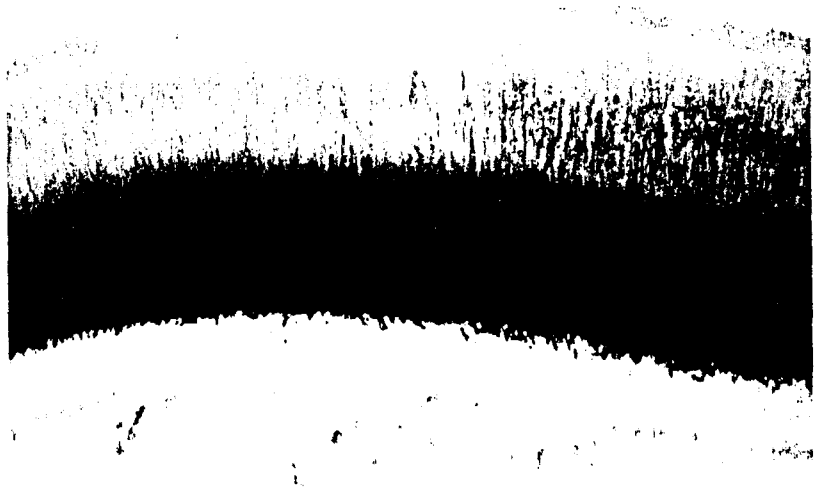

FIGURE 5. Vertical section ~330 μm thick of the 46% area of the squid retina. The fresh retina was cut on the filter paper with the razor blade of a guillotine (×100).

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 λ_{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

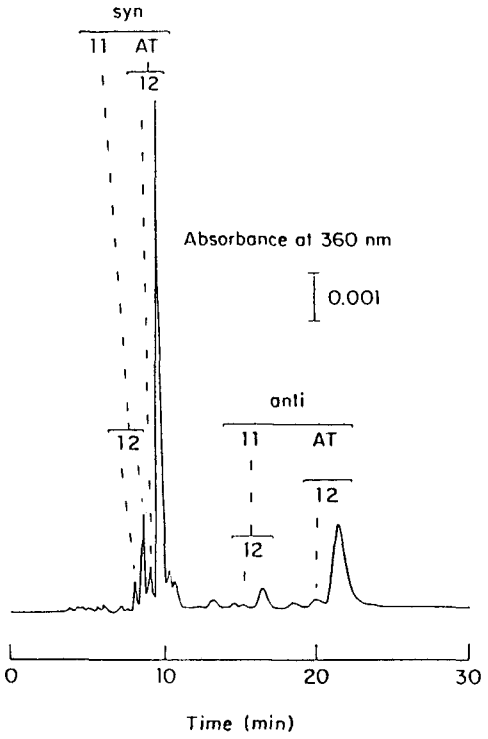

FIGURE 6. HPLC analysis of the chromophore involved in the pinkish proximal region in the outer segment layer of the photoreceptor cells in the 24 and 46% area of the retina of *W. scintillans*. Only the peaks of 11-*cis* (11) and all-*trans* (AT) are indicated on the syn and anti forms of the respective 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 λ_{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

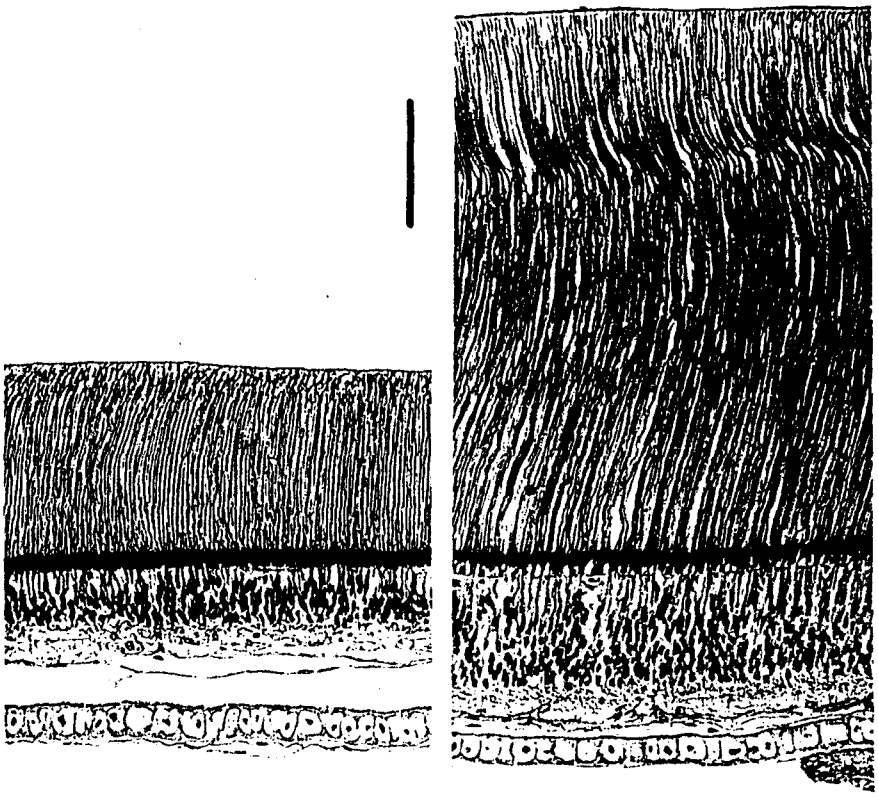

FIGURE 7. The vertical sections (4 μm thick) of the dorsal region (left) and the 46% area in Fig. 1 (right) of the squid retina. The other retinal areas were very similar to the dorsal retina. Bar: 100 μm.

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 λ_{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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4-Hydroxyretinal, a new visual pigment chromophore found in the bioluminescent squid, *Watasenia scintillans*

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Key words: Retinal; 3-Dehydroretinal; 4-Hydroxyretinal; Visual pigment; Bioluminescence; (Squid)

The bioluminescent squid, *Watasenia scintillans* has three visual pigments. The major pigment, based on retinal (λ_{max} 484 nm), is distributed over the whole retina. Another pigment based on 3-dehydroretinal ($\lambda_{max} \approx 500$ nm) and the third pigment ($\lambda_{max} \approx 470$ nm) are localized in the specific area of the ventral retina just receiving the downwelling light. Visual pigment was extracted and purified from the dissected retina. The chromophores were then extracted and analyzed with HPLC, NMR, infrared and mass spectroscopy, being compared with the synthetic 4-hydroxyretinal. A new retinal derivative, 11-*cis*-4-hydroxyretinal, is identified as the chromophore of the third visual pigment of the squid.

Introduction

The 'firefly squid', *Watasenia scintillans* lives in the open sea around Japan and is well known for the intense bioluminescence from large photophores at the tips of its fourth arms. It has numerous small photophores distributed over the ventral surface of the body and five relatively large photophores forming an array at the ventral surface of the eye. In a previous paper [1], it was reported that photoreceptors located just behind the array of photophores have extraordinarily long rhabdomes and receive the downwelling light. The major pigment (λ_{max} 484 nm) is based on retinal and is distributed over the whole retina. A second pigment ($\lambda_{max} \approx 500$ nm), based on 3-dehydroretinal, is contained in the proximal part of the long rhabdome. A third one ($\lambda_{max} \approx 470$ nm) is in the distal part and was previously thought to be based on retinal. However, the quantitative analysis of the chromophore's composition by HPLC was not consistent with the results of the partial bleaching experiments on the extracted and purified pigments from this specific area of the ventral retina. The conditions of the HPLC analysis were thus reevaluated in view of the possibility that the chromophores of the visual pigments in the specific area of the retina might be more polar than retinal and 3-dehydroretinal, such as the 3-hydroxyretinal found in the insect orders [2,3,4], and thus not detectable in the solvent system used for the analysis. This paper reports that the third pigment is based on 4-hydroxyretinal.

Materials and Methods

In spring, the squid comes into Toyama Bay, Japan Sea, for spawning. The squids of 5–7 cm mantle size used in this experiment were captured there at night. 100 eyes each were used to prepare

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the visual pigment solutions from the dorsal retina and the specific area of the ventral retina, according to the method reported previously [1,5]. Rhabdomeric membranes were floated in 40% sucrose solution by centrifugation. The visual pigments were extracted in 2% solution of the detergent L-1695 (Mitsubishi Chemical Company, Yokohama) and were purified in 0.2% L-1695 and 50 mM Tris-HCl buffer solution at pH 7.4 by DEAE-cellulose and concanavalin A-Sepharose 4B column chromatography. All the procedures were carried out under dim red light.

The chromophores were extracted and analysed according to Groenendijk et al. [6] and Suzuki et al. [7] on a Nishio HPLC system M type, equipped with a YMC silica gel column (6 × 150 mm). The HPLC analysis of the visual pigment chromophores was performed mainly on their oxime derivatives, monitoring absorbance at 360 nm and using an initial solvent system of 6% ethyl acetate and 0.3% ethanol in hexane for oximes of retinal and 3-dehydroretinal and a second solvent system of 20% ethyl acetate and 2.5% ethanol in hexane for the more polar chromophore. The standards of the respective isomers of authentic retinal and 3-dehydroretinal for HPLC were a generous gift of Dr. T. Suzuki. The all-*trans*-3-hydroxyretinal was kindly supplied by F. Hoffman-La Roche (Basel). All-*trans*-4-hydroxyretinal was synthesized according to the method of Renk et al. [8]. Spectroscopic analyses were carried out with a spectrophotometer (Union SM 401), a circular dichrograph (Jobin Yvone-Union J), a NMR spectrometer (Nicolet NT 360), an infrared spectrometer (Nicolet FTIR-7000), and a mass spectrograph (Hitachi 80-B).

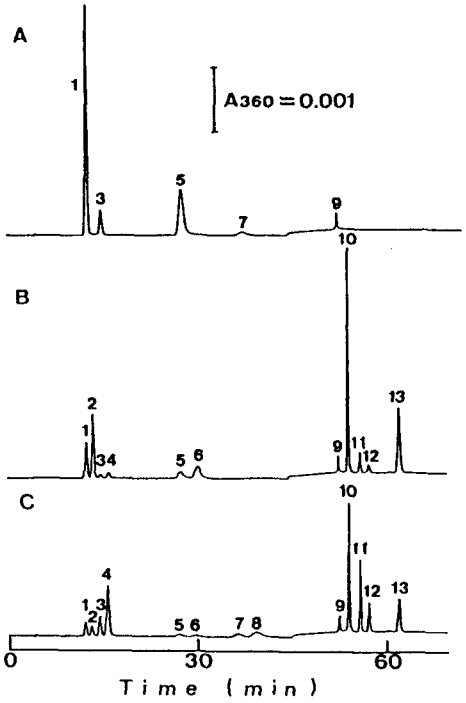


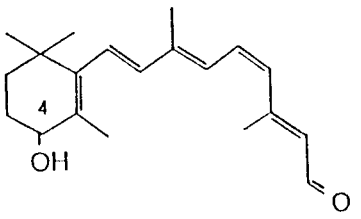
Fig. 1. HPLC analysis of chromophores of *W. scintillans* visual pigments. Chromatogram (A) of the pigment from the dorsal retina, (B) of the pigment from the specific area of the ventral retina, and (C) the latter pigment irradiated with light of $\lambda > 560$ nm. Peaks 1, 3, 5, and 7 are isomers of retinal oxime. Peak 1 corresponds to 11-*cis-syn*; peak 3, all-*trans-syn*; peak 5, 11-*cis-anti*; peak 7, all-*trans-anti*. Peaks 2, 4, 6, and 8 are isomers of 3-dehydroretinal oxime. Peak 2 corresponds to 11-*cis-syn*; peak 4, all-*trans*-syn; peak 6, 11-*cis-anti*; peak 8, all-*trans-anti*. These peaks were assigned by comparison with the respective authentic compounds. Peaks 10, 11, 12 and 13 are isomers of 4-hydroxyretinal oxime, as described in the text. Peak 10 corresponds to 11-*cis-syn*; peak 11, all-*trans-syn*; peak 12, all-*trans-anti*; peak 13, 11-*cis-anti*. The HPLC was carried out by the following step elution: initially the solvent system of 5% ethyl acetate and 0.3% ethanol in hexane until appearance of peak 8 and then the second solvent system of 20% ethyl acetate and 2.5% ethanol in hexane. Peak 9 is a ghost peak due to the solvent change. Flow rate: 1 ml/min.

Results

The purified visual pigment from the dorsal retina had its λ_{max} at 484 nm. Fig. 1A indicates that this visual pigment from the dorsal retina has only one single chromophore, retinal, as reported previously [1]. The purified visual pigment from the specific area of the ventral retina had its λ_{max} at 471 nm. This pigment contained a certain amount of 3-dehydroretinal pigment with λ_{max} at about 500 nm, and the absorbance at wavelengths greater than 560 nm was a little higher than that of the pigment from the dorsal retina. These spectral data were similar to those presented previously [1]. In Fig. 1B, oxime derivatives of retinal and 3-dehydroretinal chromophores of the pigments in this specific area of the ventral retina were eluted in the initial solvent system and subsequently some more polar compounds were eluted in the second solvent system. Visual pigment was extracted after irradiation at 4° C for 2 min with a

light of $\lambda > 560$ nm using a filter (Toshiba VO-56) and a 500 W projection lamp in order to convert a substantial amount of it to the *meta* form. Fig. 1-C indicates that the relative amounts of all-*trans* forms of oximes of retinal and 3-dehydroretinal increase upon irradiation, and that the elution profile of the polar compounds was also altered. Synthetic 11-*cis*-*syn*-3-hydroxyretinal oxime prepared as described in Ref. 4 eluted after peak 12 in this HPLC system.

In order to determine the molecular structure, the compound representing the last peak in Fig. 1B was isolated by HPLC from 500 eyes. The substance was chiral (−) and its absorbance spectrum (λ_{max} 355 nm in ethanol) was similar to those of oximes of 11-*cis*-retinal (Fig. 2). Structural analysis of the compound by ^{1}H-NMR (Table I), infrared spectroscopy (3350 cm^{-1}, hydroxyl group), and mass spectrometry (dehydration peaks at *m/z* 297 and 279) indicated the oxime of 11-*cis*-4-hydroxyretinal illustrated in Scheme I as a possible structure. Furthermore, conversion of the compound to the methyl ether derivative by HCl methanol showed the presence of an allylic alcohol group [9].



Scheme I. 11-*cis*-4-Hydroxyretinal.

The chromophores of the visual pigment were then extracted in retinal form according to Suzuki et al. [10], and the fractions of the extract with higher polarity were collected by HPLC (15% ethyl acetate and 0.5% ethanol in hexane). Racemic all-*trans*-4-hydroxyretinal (λ_{max} 377 nm; ϵ_{max} 41 300 in ethanol) was synthesized by the method of Renk et al. [8]. Fig. 3 shows the chromatogram of the retinal-isomers. The peaks near the solvent

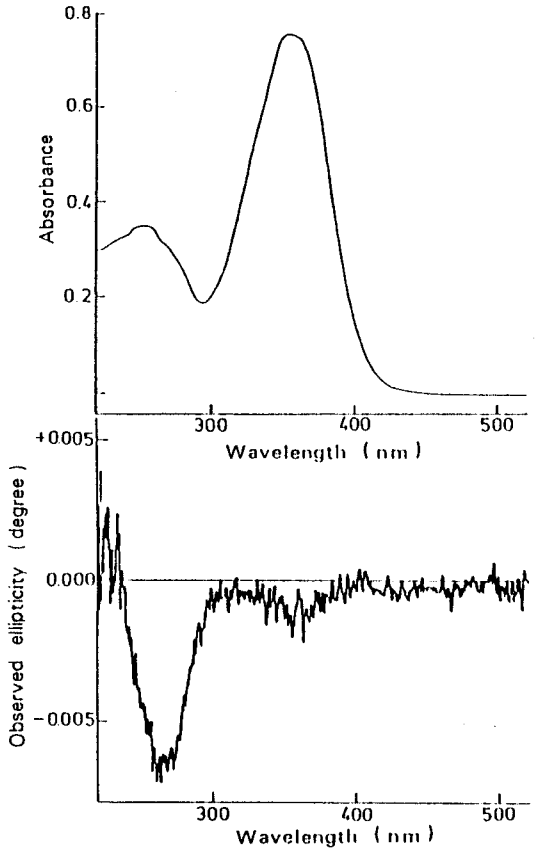


Fig. 2. Absorbance spectrum and circular dichroic spectrum of a chromophore extracted from *W. scintillans* visual pigment in oxime form. The fraction was collected from peak 13 in Fig. 1 and the measurements were performed at 15°C with a 1 cm light-path (solvent: ethanol).

TABLE I

^{1}H-NMR DATA (NICOLET NT 360) OF THE OXIME EXTRACT OF A CHROMOPHORE OF *W. SCINTILLANS* VISUAL PIGMENT COLLECTED FROM PEAK 13 IN FIG. 1, AS ASSIGNED TO 11-*cis*-4-HYDROXYRETINAL OXIME

Values are chemical shifts in ppm. Coupling constants (in Hz) are: $J_{3,4} = 4.5$; $J_{7,8,trans} = 16$; $J_{10,11} = 12$; $J_{11,12,cis} = 12.5$; $J_{14,15} = 9.5$.

3H two C-1 methyls	3H C-5 methyl	3H C-9 methyl	3H C-13 methyl	H C-4	H C-10	H C-7	H C-8	H C-11	H C-12	H C-14	H C-15
1.01 s 1.04 s	1.83 s	1.96 s	2.10 s	4.00 t	6.00 d	6.16 d	6.25 d	6.54 dd	6.63 d	6.73 d	7.49 br.d

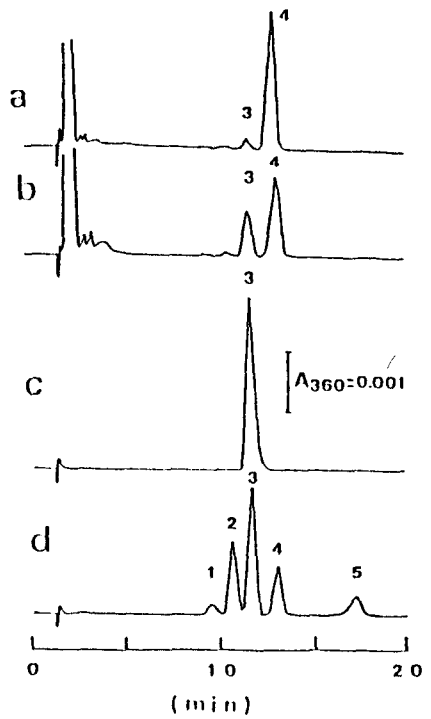


Fig. 3. HPLC profile of the chromophore extracted from *W. scintillans* eye in retinal forms (a,b) and synthetic 4-hydroxyretinal (c,d). Trace a represents the extract from the dark retina after formaldehyde treatment according to Suzuki et al. [10] and trace b the extract from the retina irradiated for 1 min with light of $\lambda > 560$ nm. The solvent system was 15% ethyl acetate and 0.5% ethanol in hexane and the peaks near the solvent front derive from isomers of retinal and 3-dehydroretinal. Traces c and d represent synthetic all-*trans*-4-hydroxyretinal before (c) and after (d) irradiation with light of $\lambda > 440$ nm in ethanol. Peak 3 is all-*trans* and peak 4 is 11-*cis*-4-hydroxyretinal. Peaks 1, 2 and 5 are not yet assigned. Absorbance was monitored at 360 nm. Flow rate: 1 ml/min.

front in Fig. 3a and b derive from retinal and 3-dehydroretinal. The fraction (λ_{max} 370 nm) collected from peak 4 in Fig. 3a should be a 11-*cis* form and, as expected, yielded oximes corresponding fairly well to peak 10 (*syn*) and peak 13 (*anti*) in Fig. 1. Peak 3 in Fig. 3c represents synthetic all-*trans*-4-hydroxyretinal. The oximes of the all-*trans*-4-hydroxyretinal corresponded to peak 11 (*syn*) and peak 12 (*anti*) in Fig. 1. Fig. 3b represents the extract from the retina irradiated for 1 min with light of $\lambda > 560$ nm. Peak 4 (11-*cis*) was reduced and peak 3 (all-*trans*) was increased. The synthetic all-*trans*-4-hydroxyretinal was irradiated in ethanol for 1 min with light of $\lambda > 440$ nm, yielding additional peaks, 1, 2, 4 and 5 in Fig. 3d. The compound of peak 4 was isolated by HPLC and derivatized to its oximes, the *anti* form of which was characterized as 11-*cis* in view of the coupling constant in ^{1}H-NMR, $J_{11\text{-}12,cis} = 12.5$ Hz ($J_{11\text{-}12,trans} = 15.5$ Hz) [11]. Its NMR, infrared spectroscopy and mass spectrometry data were consistent with those of the component extracted from the firefly squid, peak 13 in Fig. 1.

Discussion

The methodology of extraction of visual pigment chromophores in native configuration was greatly advanced by Groenendijk et al. [6] and Suzuki et al. [7,10]. In this paper, we used mainly the oxime method, since the oxime derivatives of retinals are thermally stable. Both methods were effective for the new type of chromophore, as shown in Figs. 1 and 3. From the detergent solution of the purified visual pigment, more than 90% of the chromophore population was obtained in the native configuration by a single extraction. In our experience, these methods could be even more effective for native photoreceptor membranes than for detergent solution. In a late stage of this study, chromophores were extracted and separated in the retinal form and then converted to more polar oximes. In the first HPLC step, some materials with the same polarity and with no absorbance at 360 nm contaminated the retinal fraction. In the second HPLC of the oximes, the samples were further purified and this was most helpful for the spectroscopic characterizations.

The NMR, HPLC and ultraviolet absorbance data of 2-hydroxyretinal and 3-hydroxyretinal have been reported by Ito et al. [11]. The molar absorption coefficient of all-*trans*-4-hydroxyretinal (41 300 in this study) is slightly lower than those of other retinals (43 000 for retinal, 42 000 for 2-hydroxyretinal and 44 000 for 3-hydroxyretinal). If we tentatively assume the same molar absorption coefficient for 11-*cis*-4-hydroxyretinal as for 11-cis retinal, the 4-hydroxyretinal content in the pigment from the specific area of the ventral retina amounts to more than 60% of the total chromophore. This would explain the discrepancy, in the previous paper [1], of the HPLC data and the

partial bleaching experiments of the visual pigment extracted from this specific area of the ventral retina. The high content of the new chromophore in the firefly squid eye preparation (over 15% from the above assumption) enabled us to identify the new chromophore as 11-*cis*-4-hydroxyretinal from NMR spectroscopy. With HPLC, the 4-hydroxyretinal is easily distinguishable from 2-hydroxyretinal and 3-hydroxyretinal after the transformation of the allylic hydroxyl group (OH at C-4) to the methyl ether.

Another point to be noted in Fig. 1 is that after irradiation with light of $\lambda > 560$ nm, the decrease in 11-*cis*-4-hydroxyretinal was smaller (about 30%) than in the case of 11-*cis*-retinal (55%) and 3-dehydroretinal (70%). This is further evidence that the third pigment with the shortest absorbance maximum is based on the 11-*cis*-4-hydroxyretinal.

Thus 4-hydroxyretinal is a new chromophore of visual pigment and the fourth type found in the animal kingdom. Apparently, this particular squid has a unique trichromatic photoreceptor system using three visual pigments with different spectral sensitivities which have been attained by the modification of the chromophore molecule, probably in combination with only one opsin. Chromatographic separation of three pigment proteins could not be accomplished and the amino-acid sequences of the peptides of the retinal-binding site of these pigments were similar (unpublished data). The use of 3-dehydroretinal by crayfish in the invertebrate family was recently discussed by Suzuki et al. [12] as providing the animal with photosensitivity at longer wavelengths. Similarly, the use of 4-hydroxyretinal allows the squid to respond to shorter wavelength light.

Here, we propose to call 4-hydroxyretinal-based visual pigments A4 pigments, following the convention of A1 and A2 pigments [13], since this is the fourth of visual pigment discovered and its chromophore has a hydroxyl group at C-4. Firefly squid seems to be unique among the cephalopods to possess this A4 pigment, because our search for the A4 pigment in any other species among the more than 20 cephalopods living near Japan has been unsuccessful.

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On Some Physicochemical Properties of Sucrose Esters and the Stability They Confer to Membrane Proteins

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A homologous series of fatty acid monoesters of sucrose whose chain length varies from 8 to 22 carbon atoms has been examined with respect to some physicochemical properties. In this series, the critical micelle concentration is comparable to that of lysophospholipids having similar hydrophobic moiety. The behavior of a spin probe dispersed in micelles of sucrose esters and their surface pressure–area isotherms at the air/water interface indicate that, despite a relatively large hydrophilic head, they form increasingly compact structures as their chain length increases. Finally, when used as dispersing medium for the purified intrinsic protein rhodopsin, long-chain esters confer to the pigment a thermal stability which is close to that of its membrane-bound state. We conclude that these detergents may be among those best suited to purified intrinsic membrane protein studies.

INTRODUCTION

Isolation, purification, and characterization of intrinsic membrane proteins always require the use of detergents. Over the years, a wide variety of them, including both ionic and nonionic species, with different aliphatic, cyclic, or aromatic hydrophobic moieties has been used with more or less success because many detergents, although efficient to disperse membrane lipids, strongly affect protein structure and function. In search of an ideal mild medium to suspend purified membrane proteins, new detergents have been synthesized in the past 15 years (1). Among them, the fatty acid esters of sucrose, initially designed for food and cosmetic industries, were proposed as mild detergents, specially owing to the stabilizing effect of sucrose toward protein structure (2). As predicted by the theory of micelle formation in aqueous solutions (3), fatty acid esters of sucrose form ellipsoid micelles whose size increases with chain length (4). In addition, they bind noncooperatively without apparent specific interactions between sucrose esters and proteins (2).

Among membrane proteins which can be used as a reference in stabilization/destabilization studies of proteins by detergents, visual rhodopsin is very convenient since its 500-nm absorption band due to the retinal chromophore, through sensitive interaction with the apoprotein opsin, enables one to easily analyze its stability, regenerability, and photosensitivity by absorption spectroscopy. Its properties are known to be sensitive to the extractant used for its purification (5) and it already has been studied in a dispersed state using several kind of natural or synthetic detergents, namely, cholic acid (6), digitonin (7), alkyltrimethylammonium bromide (8), Triton X-100 (9), Emulphogene BC-720 (10), Ammonyx LO

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(11), alkyl glucosides (12), and dodecyl maltose (13), making several reference points available.

The relative stability of visual pigments in sucrose esters was first exemplified by their use in the purification of the very fragile cephalopod rhodopsin (14). More recently, we have found that they also could stabilize bacterial rhodopsin in the purified state (15, 16). Sucrose esters becoming available as a homologous series of increasingly long hydrophobic chains, we used them to study the effect of chain length on the photochemistry of the purified pigment (17), its regenerability, and absorption characteristics (18). These properties were found closer to the membrane-bound state as the length of the hydrophobic moiety increased. In order to determine whether sucrose esters behave as predicted theoretically (3) with respect to micellar rigidity, despite a relatively large polar head which could prevent compact self-association, and to see to what extent they could mimic lipidic membrane components, we have measured their surface pressure–area isotherms at the air/water interface and the relative mobility of a spin probe dispersed in the micelles they form. The results show that, as expected, these detergents form increasingly rigid structures as their chain length increases. In addition, analysis of the thermal stability of bovine rhodopsin dispersed in these micelles reveals that in the long-chain esters, the pigment stability is very close to that of its native membrane-bound state.

MATERIALS AND METHODS

Purification of Sucrose Esters

The sucrose esters used throughout this work are the caprylic (C_8), capric (C_{10}), lauric (C_{12}), myristic (C_{14}), palmitic (C_{16}), stearic (C_{18}), and behenic (C_{22}) monoesters of sucrose (Fig. 1). These products were synthesized at the Mitsubishi Chemical Co. Ltd. (Yokohama, Japan). Depending on different batches received, they can contain up to 20% of di- and triesters; they were thus purified prior to use. For this purpose, a sucrose ester sample (ca. 0.5g) was dissolved in chloroform and adsorbed on a 2.5 × 20-cm silica gel column. After washing with 10 ml of chloroform, the mono-, di-, and triesters were separated by elution with a mixture of chloroform:methanol:water (20:5:0.7, v/v). Separation could easily be evaluated by analysis of refractive index of the fractions. The monoester fractions (slowest and most abundant) were pooled and recovered by solvent evaporation in a rotoevaporator. Thin-layer chromatography of these fractions on silica plates lacked the typical di- and triester spots when developed in the same solvent mixture. These samples thus contained only monoesters, but probably linked at different methoxyl position on the sucrose molecule.

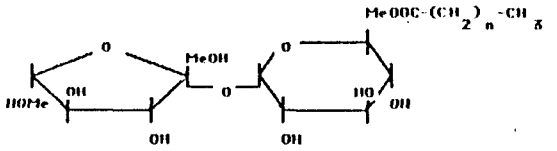

FIG. 1. Structure of sucrose monoester.

Preparation of Rhodopsin and Analysis of Its Thermal Stability

Bovine rod outer segment membranes were prepared in the same manner as that in Ref. (17) and rhodopsin was purified as previously described, giving samples containing less than two phospholipids per rhodopsin (18). Purified rhodopsin dispersed in the different detergents was obtained by eluting the pigment in the presence of 0.2% (w/v) of the desired detergent (except for the C_{10} and C_8 esters which were used at 0.5 and 2.0%, respectively) during the last purification step: the affinity chromatography on concanavalin A–Sepharose 4B (Pharmacia, Upsala, Sweden). After chromatography, samples were dialyzed overnight against 10 m*M* Tris–Cl buffer (pH 7.3) and then used for thermal stability analysis. This was achieved by incubating samples in a controlled temperature spectrophotometer cell. Practically, rhodopsin samples were placed in the thermostated cell/cell holder assembly of

a Union SM-401 or a Pye Unicam SP8-100 spectrophotometer whose temperature was controlled by water circulating from a thermostatic pump. During that time, sample absorbance at 530 nm was monitored every 10 min. This wavelength was chosen to avoid possible interference from denaturation products which could have residual absorbance at 500 nm. From time to time, a complete spectrum was measured to make sure that absorbance lost corresponded to rhodopsin denaturation and not to baseline drifts. In all the samples analyzed, a semilogarithmic plot of the relative residual absorbance ($A_{\text{at time } t}$ / $A_{\text{at time zero}}$) versus time always gave straight lines, indicative of a first-order kinetic.

Physicochemical Techniques

The critical micelle concentration (CMC) of the sucrose esters has been determined at 22°C by plotting surface tension as a function of detergent concentration in pure water, using a Nouy tensiometer. A break was observed in these curves at the CMC.

To estimate the relative molecular mobility in sucrose esters micelles, we have used electron spin resonance (ESR) spectroscopy. A spin probe (12-(4,4-dimethyloxazolidine-*N*-oxyl)stearic acid), obtained from Serdary Research Laboratories (London, Canada), was incorporated in sucrose ester micelles. This was achieved by first drying a known amount of chloroform-solubilized probe on the walls of a volumetric flask under a nitrogen stream followed by its solubilization in 0.2% (w/v) detergent. The amount of probe used was adjusted to give a final probe:detergent ratio of 1:150. ESR spectra were recorded at 22°C with a Varian E-231 spectrometer at a magnetic field of 3356 G (scan range, 80 G; frequency, 100 kHz) with a microwave power of 2 mW (frequency, 9.4 GHz). From these spectra, the amplitude of motion of the long molecular axis of the probe has been estimated by calculation of the order parameter (S) in the manner suggested by Marsh (19) for this kind of probe.

Surface pressure–area isotherms of the detergents spread at the air/water interface have been measured on the fully automated system previously described (20), using a 61.9 × 13.2 × 1.5-cm^3 trough where the surface pressure was measured by means of a Langmuir–Adam–Harkins Mylar float system. Purified sucrose esters were precisely weighted with a Cahn electrobalance (Model RG 2000, Ventron Instrum. Corp., CA) and dissolved in a 9:1 (v/v) mixture of distilled hexane and ethanol. Depending on the solution concentration, from 10 to 100 μl was deposited dropwise from a microsyringe at the surface of the monolayer trough; solvent was allowed to evaporate for 15 min after which compression was started by pushing a Teflon moving barrier at the rate 1.9 $cm \cdot min^{-1}$. The aqueous subphase consisted of 1 m*M* phosphate buffer (pH 8.0) prepared from quartz distilled water having a specific resistivity better than 17×10^6 ohm · cm and a surface tension higher than 70 $mN \cdot m^{-1}$. Its temperature and that of the stainless-steel glovebox enclosing the monolayer trough were kept at 20.0 ∓ 0.5°C. Isotherm reproducibility was carefully checked by making five sets of measurements with each sucrose ester.

RESULTS

In an homologous series of amphiphiles, the critical micelle concentration and the HLB number (hydrophilic–lipophilic balance) decrease as chain length increases. As already observed by other investigators, we have also found that the CMC of sucrose esters is not different from that of other nonionic detergents having comparable chain length (e.g., alkyl glucose). Indeed, Makino *et al.* (2) reported CMCs of 24, 2.5, and 0.4 m*M* for C_8, C_{10}, and C_{12} sucrose esters, respectively. Our measurements agree with these values. In addition, we observed that CMC decreased to 0.06 and 0.05 m*M* for the C_{16} and C_{18} esters, respectively. The lower solubility of the C_{22} ester in water made it difficult to measure accurately its CMC.

Increasing chain length which leads to lower CMCs and higher micellar size should also increase the micellar rigidity in terms of chain compaction, provided that molecular packing is not sterically prevented by a large polar head group (3). In order to verify that point for sucrose esters, we have incorporated a spin probe label in their micelles. We have used the 12-(4,4-dimethyloxazolidine-*N*-oxyl)stearic acid. This probe has a longer chain length than that of most of the detergents used; nevertheless, it was chosen precisely because the position of the nitroxide label in its chain was sufficiently far from the polar head to give information relevant to the environment experimented by the hydrophobic part of the intrinsic proteins which also extends beyond the length of the hydrophobic chains of the sucrose esters. The ESR spectra obtained with that probe in the different detergents are shown in Fig. 2. By comparison with the isotropic spectrum observed for the solubilized state, the ESR spectra show an increasing immobilization of the probe when it is dispersed in sucrose esters of increasing chain length. The order parameters

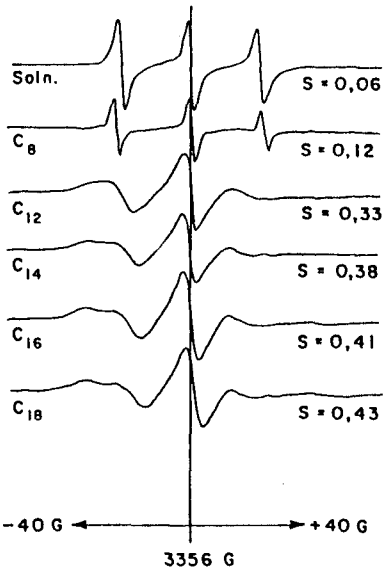

FIG. 2. ESR spectra observed at 22°C for the stearic acid label incorporated in micelles of different chain length (C_8 to C_{18}) sucrose esters. The spectrum of the same concentration of probe dissolved in chloroform (Soln.) is also shown. Order parameters (S) measured from the spectra are indicated.

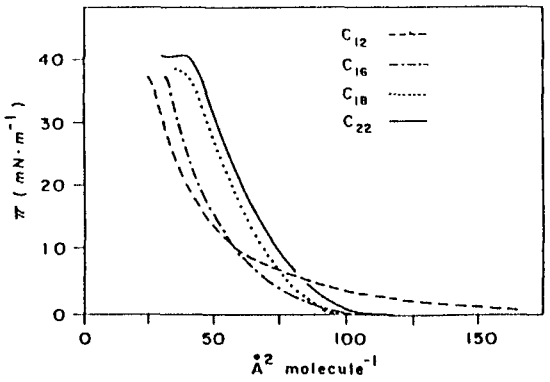

FIG. 3. Surface pressure–area isotherms measured at 20 ∓ 0.5°C for C_{12} (- - -), C_{16} (-·-), C_{18} (· · ·), and C_{22} (—) sucrose esters.

(S) calculated from these spectra range from 0.12 to 0.41 when the acyl chain of the detergent grows from 8 to 18 carbon atoms. This significant increase in the order parameter is an indication of restricted mobility (molecular rigidity) experimented by the probe. It is noteworthy that the same spin probe dispersed in micelles of Ammonyx LO (dodecyl dimethyl amine oxide), a zwitterionic detergent with a C_{12} alkyl chain, gives an ESR spectrum identical with that observed in the C_8 sucrose ester.

Self-organization of amphiphilic molecules like detergents can also be examined by spreading them at the air/water interface, provided they form stable monolayers. The sucrose esters of fatty acids do form stable monolayers, as seen in Fig. 3, where surface pressure–area isotherms of the C_{12}, C_{16}, C_{18}, and C_{22} esters are represented. All the sucrose esters tested showed surface pressure–area isotherms with collapses in the 40 mN · m^{-1} region, irrespectively of the chain length of the ester. Obviously, this is the apparent collapse pressure, not the equilibrium pressure. The main difference between isotherms is their tendency to form liquid-condensed films as they hydrophobic tail grows. For instance, the isotherm observed with the C_{12} ester extends to very large molecular area (>150 Å^2 · molecule^{-1}) at low surface pressure. This effect is still more pronounced in the C_8 ester (result not shown) but it gradually disappears in the

longer chain esters. This transition from high to low compressivity, in short-chain esters, as pressure is increased, is the well-characterized liquid-expanded to liquid-condensed state transition observed for amphiphilic molecules at the air/water interface (21). A similar transition should occur in longer chain esters but at a higher temperature. The difference between isotherms thus only reflects the strength of chain–chain hydrophobic interactions with increases with chain length and enables molecules to form a more condensed structure. With respect to this, one could expect the longer chain esters to show smaller molecular area than short-chain ones in the 20–40 $mN \cdot m^{-1}$ range. Contrary to such an expectation, short-chain esters show the smallest molecular area, most probably due to their higher solubility in the subphase.

According to their physicochemical properties, the different detergents used to purify proteins must more or less maintain their structural integrity. The effect of a given detergent on the stability of a purified protein is probably best estimated by its thermal stability. In Fig. 4, the time dependence of denaturation of rhodopsin by heat is plotted. It is clear from the figure that, as expected, thermal stability increases with the sucrose ester chain length. At 40°C, it takes 5 h to denature 90% of the rhodopsin when it is dispersed in the C_8 ester, while in the C_{18} ester, incubation under the same conditions does not allow observation of any denaturation. In the same time period, significant (40%) denaturation is observed at 50°C in the C_{18} ester. At this temperature, rhodopsin is completely destroyed in 20 min when dispersed in the C_8 ester. For comparative purposes, the thermal denaturation curves of rhodopsin contained in its native membranes are also plotted in Fig. 4. It is seen that in the C_{16} and C_{18} esters, the thermal stability of rhodopsin at 50°C is better than that in rod outer segment membranes suspended in 67% (v/v) aqueous glycerol. At the same temperature, the stability of the pigment in the C_{18} ester is only three times less than its stability in pure rod outer segment membranes. Thermal stability thus does not only correlate with the ester chain length; long-chain esters can stabilize the purified pigment almost as efficiently as the native membrane environment.

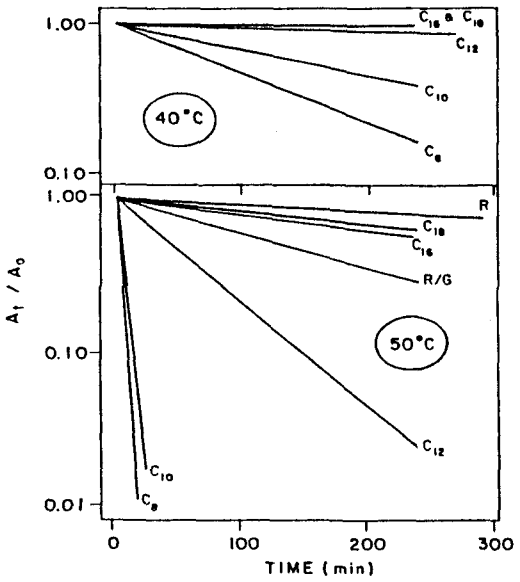

FIG. 4. Rate of thermal denaturation of rhodopsin in the different sucrose esters at 40 and 50°C. The fraction of residual absorbance (A_t/A_0), measured at 530 nm, is plotted on a logarithmic scale versus time. The lines are identified (C_8 to C_{18}) according to the length of the sucrose ester chain in which purified rhodopsin was dispersed. "R" stands for sonicated rod outer segment membranes in water while "R/G" stands for the same membranes in 67% (v/v) aqueous glycerol (as in Ref. (5)).

DISCUSSION

The alkyl esters of sucrose series behave essentially in the same manner as that of other homologous series of detergents. The critical micelle concentration of the sucrose esters is not much different from that of related molecules. For instance, dodecyl maltose, which differs from lauryl sucrose by a small structural change in the polar head and by the presence of an ether link between its hydrophilic and hydrophobic parts, has a CMC of 0.6 m*M* (13) compared to 0.4 m*M* for the equivalent sucrose ester. Moreover, the series obey the empirical linear relation between ln(CMC) and the alkyl chain length whose slope gives the

free energy of transfer (−680 cal/mol.) of the methylene group from water to the micellar state (2, 3). Interestingly, the CMC of the sucrose esters is also similar to that of lysophospholipids of comparable chain length. Indeed, palmitoyl lysophosphatidyl choline has its CMC between 0.1 and 0.06 m*M* (22) while we found palmityl sucrose to have its CMC also at 0.06 m*M*. In addition, the number of molecules per micelle which range from 83 to 151 in the C_{12} to C_{18} sucrose esters (4) is not far from the value of 180 observed for the above-mentioned phospholipid (22). From this point of view, sucrose esters thus behave like monoacylated membrane components.

As predicted theoretically (3) and experimentally observed in other homologous series (13), chain elongation and growth of micellar size should also be accompanied by an increase in molecular packing density. The ESR spectra observed with the spin probe that we have dispersed in sucrose esters micelles indicate that this series does not behave differently. The tendency of these detergents to form more condensed structures as their hydrophobic tail length increases is also well exemplified by the measurement of their surface pressure–area isotherms. At room temperature, short-chain esters form highly compressible monomolecular films. Under conditions where a large area is available for the molecules, shorter esters seem to occupy all the surface and show measurable surface pressure while longer esters, under the same conditions, do not occupy all the surface and thus already form isolated condensed structure which will create a measureable surface pressure only when the available area will be reduced. Since all sucrose esters have identical polar heads, this effect is a direct measure of the strength of the hydrophobic interactions which take place between alkyl chains and progressively induce the water repulsion and molecular compaction required for stabilization of membrane proteins.

Rhodopsin exists as a monomeric membrane protein for which interaction with lipidic membrane components does not seem to imply chemical specificity. The properties of this pigment which are determined by the molecular environment most probably rely on its physicochemical properties and "long-range" interactions (23–25). On this basis, it was previously concluded that its native stability, by comparison with the detergent-solubilized pigment, depended on appropriate solvation by amphiphiles of sufficiently large hydrophobic domains (26) and that its native configuration was maintained as long as water was prevented from penetrating it (27). The results presented in this paper show that long-chain sucrose esters fulfill the requirements for stabilization of rhodopsin. They have a sufficiently long hydrophobic tail to achieve, by hydrophobic interaction, the required degree of packing and their hydrophilic head is still sufficiently small to prevent steric repulsion from taking place in the micelles. In fact, in the C_{18} ester, the thermal stability of purified rhodopsin is only three times less than that measured for rhodopsin contained in its discal membranes. It corresponds to a significant improvement over more frequently used detergents in which rhodopsin thermal stability is orders of magnitude less (5).

In conclusion, we believe that stabilization of visual rhodopsin in a state very close to its membrane-bound state by sucrose esters should also apply to other membrane proteins, provided they do not require chemical specificity for a particular lipidic group, and that these detergents may be best suited to the study of intrinsic proteins.

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