Distribution of Rhodopsin and Retinochrome in the Squid Retina

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ABSTRACT The cephalopod retina contains two kinds of photopigments, rhodopsin and retinochrome. For many years retinochrome has been thought to be localized in the inner segments of the visual cells, whereas rhodopsin is in the outer segments. However, it is now clear that retinochrome can be extracted also from fragments of outer segments. In the dark-adapted retina of *Loligo pealei* retinochrome is distributed half-and-half in the inner and outer segments. *Todarodes pacificus* contains much more retinochrome than *Loligo*, and it is more abundant in the outer than in the inner segments. The outer segments of *Loligo* contain retinochrome and metarhodopsin in addition to rhodopsin, whether squids are kept in the dark or in the light. But there is extremely little metarhodopsin (about 3% of rhodopsin) even in light-adapted eyes. The inner segments contain only retinochrome, and much less in the light than in the dark. On the other hand, retinochrome in the outer segments increases markedly during light adaptation. These facts suggest the possibility that some retinochrome moves forward from the inner to the outer segments during light adaptation and there reacts with metarhodopsin to promote regeneration of rhodopsin.

INTRODUCTION

The retina of cephalopods contains two kinds of photosensitive chromoprotein with retinaldehyde as prosthetic group. One is the visual pigment, rhodopsin, which is located in the rhabdomal membranes of the outer segments of the visual cells. The other pigment is retinochrome, which was first found in retinal tissue that had been deprived completely of outer segments (Hara and Hara, 1965). It has been considered to be localized in the inner segments of the visual cells (Hara et al., 1967; Hara and Hara, 1972). When retinochrome is bleached by light, its prosthetic group is converted from all-*trans* to the 11-*cis* form (Hara and Hara, 1967, 1968). As this change of geometrical configuration is just the reverse of what occurs in the photoisomerization of rhodopsin, we suggested that the 11-*cis* retinal released from the photoproduct of retinochrome may be used to resynthesize rhodopsin (Hara and Hara, 1968).

In cephalopods light coming into the eye through the pupil is absorbed in the rhabdomes and black screening pigment in the anterior part of the retina so strongly that the posterior part, including the inner segments, is not exposed to light. However, the posterior parts of the retina *in situ* are not shielded from light that diffuses through the outer surface of the eyeball. In fact, when the

fresh, enucleated eye is irradiated after the pupil is covered so as not to allow light to enter the eye directly, the posterior part easily loses its color. In any case, on the assumption that retinochrome interacts with rhodopsin or opsin so as to maintain visual pigment, it was a serious question why retinochrome is localized far away from rhodopsin, on the other side of the basement membrane and the layer of black pigment. However, we noticed that some retinochrome was occasionally detected in rhodopsin extracts prepared from fragments of outer segments and confirmed, by different methods, that the outer segments in fact contain retinochrome (Hara and Hara, 1973 *a*; cf. Kito et al., 1973). We have therefore examined quantitatively the distribution of photopigments in different regions of dark-adapted retina of the Atlantic squid, *Loligo pealei,* and the Japanese common squid, *Todarodes pacificus*. We have also studied the way in which it depends on the adaptation of animals to light with *L. pealei,* which can be kept alive in seawater tanks in the laboratory. A preliminary account has been presented elsewhere (Hara and Hara, 1973 *b*), and discussed at the U.S.-Japan Cooperative Program Seminar on Visual Pigments held at the University of Illinois in August, 1975.

METHODS

L. pealei was collected in the daytime from shallow waters at Woods Hole by the Supply Department of the Marine Biological Laboratory. Some were dark adapted during the night in a laboratory tank of running seawater, and others fully light adapted by placing them in a white shallow pail soon after collection and exposing them to direct sunlight for half an hour. *T. pacificus* was captured during the night from deep waters of the Japan Sea and never exposed to light after collection. At the end of the adaptation period, all the animals were decapitated in the dark, and the excised eyes were immediately frozen and stored for 10–20 h to prevent changes in the distribution of retinal photopigments and to facilitate separation of the inner and outer segments of the visual cells. All the operations for preparing photopigment extracts were carried out under dim red light at temperatures as low as possible, using essentially the same techniques as previously reported (cf. Hara and Hara, 1972).

For each experiment 20–25 eyes were used. The frozen eyes were hemisected to remove the anterior part and the lens, and then the eyecups were shaken in three successive portions of M/15 phosphate buffer at pH 6.5, so as to detach the outer segments which enter the medium together with black pigment. The eyecup, which had lost the outer segments, still contained the retina consisting of the inner segments and nerve plexus. Such a rhabdome-free retina was lifted out with fine forceps, and treated by our routine method to extract the retinochrome that is located in the inner segments. The suspension of detached rhabdomes and pigment granules in phosphate buffer was centrifuged at 15,000 rpm to collect fragments of the outer segments in a black sediment (about 4.0 g per 25 eyes in *Loligo*). They were then ground in a mortar, washed with M/15 phosphate buffer at pH 6.2, and suspended in a 36% solution of sucrose in the same phosphate buffer. When this suspension was centrifuged for 15 min, the rhabdomal parts of the outer segments floated and the black debris sedimented. The supernatant was then diluted with an equal volume of phosphate buffer and centrifuged to obtain a residue, which was used to prepare rhodopsin. It was washed at least three times with M/10 Na_2HPO_4 (pH 9.2), washed again with phosphate buffer, and finally extracted with digitonin. The black debris that is left in the bottom of the tube after the centrifugation in

36% sucrose is usually discarded, though it is still rich in photopigments. However, in the present experiments, this black mass of cell fragments was divided into six or eight portions, which were washed repeatedly with M/10 Na_2HPO_4 to remove a large amount of ommochrome or ommin until successive supernatants were colorless and clear. These cleaning operations were most important for our present purpose and more than 10 washings were required to remove the soluble red pigment so as to make it possible to detect the photopigments in resultant digitonin extracts. Finally the dark precipitates, which still contained black insoluble pigment (melanin), were washed once more with phosphate buffer, and pooled before the addition of digitonin for extracting photopigments.

In this way, three different fractions were prepared from the retina: from the outer segments, (*a*) a light fraction, which floated on 36% sucrose, (*b*) a heavy fraction, which is sedimented, and (*c*) the fraction containing the inner segments. Each of them was then mixed with 1.5–3.0 ml 2% digitonin in M/15 phosphate buffer, pH 6.5, and the photopigments were extracted by gentle shaking for 20 min. The extraction with digitonin was carried out as many times as necessary until no further photopigment could be extracted. With fractions *a* and *b*, extraction was repeated in a refrigerator as long as thermolabile metarhodopsin appeared in the extract and thereafter at room temperature near 20°C. When the yield of photopigment gradually decreased as the extraction advanced, various means were tried to extract all the remaining photopigments: the period of extraction was prolonged up to 20 h, the temperature for extraction was raised, the material mixed with digitonin was frozen and thawed, or the pH of the mixture was changed up to 8.0 by addition of a trace of solid Na_2CO_3.

Absorption spectra of the extracts were measured with a Perkin-Elmer model 402 (Perkin-Elmer Corp., Mountain View, Calif.) or a Hitachi model 323 (Hitachi Corp., Tokyo, Japan) recording spectrophotometer. The amounts of rhodopsin, metarhodopsin, and retinochrome in an extract were determined from the absorbance at the absorption maximum of each by the methods described later. After calculation, the values presented in the tables are expressed as the absorbance which would be measured if the photopigment from 10 eyes were brought into a volume of 1 ml (absorbance per milliliter per 10 eyes). We chose these units because it was impossible to determine the dry weight of the material owing to the large increase of weight caused by the added digitonin.

RESULTS

Loligo Retinochrome

Until now retinochrome has been obtained by extracting retinas of various cephalopods after removing the rhabdomes (Hara and Hara, 1966; Takeuchi, 1966; Hara et al., 1967). In the squid, *L. pealei*, familiar to physiologists in Europe and America, retinochrome was extracted by a similar method (Sperling and Hubbard, 1971), and its chemical properties were examined (Hubbard and Sperling, 1973; Sperling and Hubbard, 1975). Fig. 1 shows the absorption characteristics of retinochrome in digitonin solution at pH 6.5, which we prepared from *L. pealei*. This was the first extract of the inner segments in the experiment with dark-adapted squid (dark 1), which appears in Table I. The optical purity of the extract was about 0.21 in $A_{400\,nm}/A_{500\,nm}$ and 3.0 in $A_{280\,nm}/A_{500\,nm}$. The absorption spectrum of retinochrome, unlike that of rhodopsin, depends on the pH of the medium (Hara and Hara, 1965, 1968; Hubbard and

Sperling, 1973; Sperling and Hubbard, 1975). At pH 6.5, the absorption maximum lies near 503 nm, at about 10-nm longer wavelength than that of *Loligo* rhodopsin, which has λ_{max} near 493 nm. This relationship is almost the same in the squid, *Todarodes*, whose retinochrome and rhodopsin, respectively, have λ_{max} near 490 nm and 480 nm. As shown in Fig. 1, when exposed to orange light (>530 nm), *Loligo* retinochrome bleaches to a photoproduct with a main peak at about 455 nm. This is a shorter wavelength than is λ_{max} of the photoproduct of *Todarodes* retinochrome with λ_{max} at about 465 nm. In the difference spectrum before and after irradiation at pH 6.5, *Loligo* retinochrome shows λ_{max} at about 515 nm.

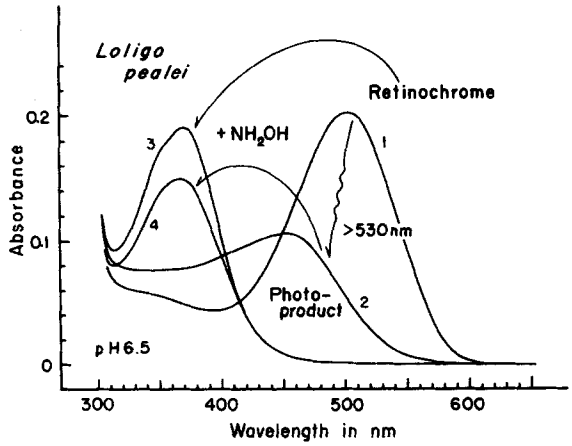

FIGURE 1. Absorption spectra at pH 6.5 of a digitonin extract of *Loligo* retinochrome before (1) and after (2) 5-min irradiation with orange light (>530 nm) from a tungsten filament microscope lamp shielded by a glass filter. The comparison between retinaldehyde oximes formed from retinochrome (3) and from its photoproduct (4) by addition of NH_2OH is also presented. The solutions were diluted with NH_2OH from 1.0 to 1.1 ml, and spectra 3 and 4 are not corrected for this dilution.

It is well known that, when hydroxylamine (NH_2OH) is mixed with retinochrome or its photoproduct, it is quickly decomposed forming, respectively, all-*trans* and 11-*cis* retinaldehyde oximes, which differ from each other in their absorption characteristics (Hara and Hara, 1967, 1972, 1973*c*). In such an extract of inner segments as shown in Fig. 1, the absorption in the visible range disappears completely after addition of NH_2OH, indicating that no other pigment than retinochrome is contained in the inner segments. This would be quite different, if rhodopsin or metarhodopsin, which are resistant to NH_2OH (cf. Hubbard and St. George, 1958), were contained in the extract. In fact, in the extracts obtained from the outer segments, rhodopsin, metarhodopsin, and retinochrome can often be seen. Fig. 2 gives such an example, derived from the

fourth extraction of the heavy fraction of the outer segments in experiment dark 1 of Table I. When 0.1 ml of freshly neutralized 1 M NH_2OH is added to 1 ml of the initial extract, spectrum 1 is changed into spectrum 2, the absorption near 500 nm being decreased far lower than the broken-line curve (spectrum 1′) that would be expected from the dilution to 1.1 ml. The observed decrease is due to the decomposition of retinochrome. On raising the pH of the solution to 10 by addition of a small amount of solid Na_2CO_3,[1] metarhodopsin is converted from the acid to the alkaline form (spectrum 3), showing a further decrease in

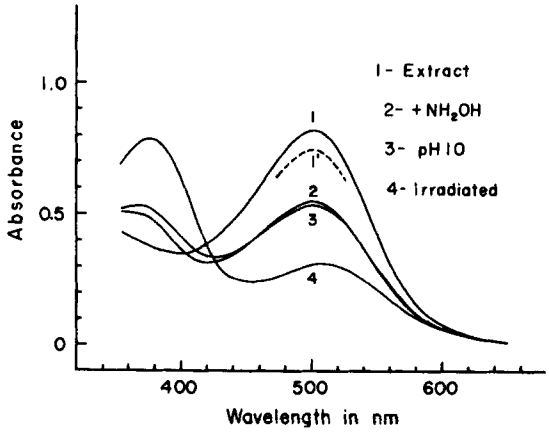

FIGURE 2. Identification of retinochrome, metarhodopsin, and rhodopsin in a digitonin extract of the outer segments of the visual cells. The extract is from the fourth extraction of the heavy fraction of the outer segments in exp. dark 1 shown in Table I. To 1 ml of the extract of photopigments at pH 6.5 (spectrum 1), 0.1 ml 1 M NH_2OH was added to detect retinochrome (spectrum 2). The pH of the mixture was then changed to 10 to convert metarhodopsin from the acid to the alkaline form (spectrum 3), and finally the mixture was irradiated 3 min with orange light to bleach rhodopsin (spectrum 4). Since spectra 2, 3, and 4 were from solutions diluted with NH_2OH, spectrum 1 was corrected for comparison (broken-line curve, spectrum 1′). The difference between spectra 1′ and 2 therefore corresponds to the net content of retinochrome.

absorbance, by which the metarhodopsin content is estimated (cf. Hubbard and St. George, 1958). Finally, when exposed to orange light for 3 min, any remaining rhodopsin is bleached to alkaline metarhodopsin, leaving red impurities which bear no relation to the photopigments (spectrum 4). By this technique the three different photopigments in an extract can be distinguished, as will be seen in the following sections. Thus we could show that retinochrome is present in the outer segments of the visual cells, together with rhodopsin and metarhodopsin.

[1] In experiments with *Todarodes*, the pH had to be raised to 10.5 because its metarhodopsin has a higher pK than *Loligo* metarhodopsin (Takeuchi, 1966).

TABLE I

SUCCESSIVE EXTRACTIONS OF PHOTOPIGMENTS, RHODOPSIN (R), METARHODOPSIN (MR), AND RETINOCHROME (Ret), FROM THE THREE RETINAL FRACTIONS FROM DARK- AND LIGHT-ADAPTED SQUIDS, *Loligo* (ABSORBANCE PER MILLILITER PER 10 EYES)

Experiment	Extraction no.	Outer segments: Light fraction R	Light fraction MR	Light fraction Ret	Heavy fraction R	Heavy fraction MR	Heavy fraction Ret	Inner segments, Ret
Dark 1	1	0.686	–	0.017	0.586	0.035	–	0.393
	2	1.503	0.065	0.031	2.186	0.085	0.123	0.207
	3	0.685	0.043	0.005	1.509	0.026	0.232	0.077
	4	0.490	0.027	0.008	0.319	0.022	0.276	0.026
	5	0.416	0.026	0.003	0.110	0.003	0.201	0.026
	6	0.152	0.011	0.002	0.035	–	0.078	0.019
	7	0.073	0.005	0.001	0.016	–	0.040	0.241*
	8	0.027	–	–	0.011	–	0.045	0.115*
	9				–	–	0.007	–
Light 1	1	0.096	0.006	–	0.081	0.008	0.009	0.318
	2	0.818	0.032	0.017	1.633	0.090	0.149	0.228
	3	1.360	0.035	0.010	1.343	0.039	0.245	0.111
	4	0.710	0.026	0.007	1.453	0.037	0.331	0.036
	5	0.330	0.014	0.004	0.614	0.005	0.424	0.024
	6	0.110	0.010	0.002	0.155	–	0.258	0.015
	7	0.126	0.004	0.004	0.054	–	0.223	0.080*
	8	0.038	0.004	0.002	0.014	–	0.092	–
	9	0.018	–	0.001	0.010	–	0.102	
	10				0.002	–	0.136	
	11				–	–	0.025	
Totals								
Dark 1		4.03	0.18	0.07	4.77	0.17	1.00	1.11 ‡
Dark 2		5.01	0.28	0.11	6.81	0.45	1.34	1.34
Totals								
Light 1		3.61	0.13	0.05	5.36	0.18	1.99	0.81
Light 2		3.36	0.21	0.05	5.83	0.28	2.11	0.70

* Extraction performed at pH 8.0, where λ_{max} of retinochrome was not very different from that at pH 6.5 (cf. Sperling and Hubbard, 1975).

‡ In a separate experiment with dark-adapted squids, we obtained 1.35 per milliliter per 10 eyes as the content of retinochrome in the inner segments.

Retinal Photopigments in Loligo

Two sets of experiments in which the photopigments were extracted from the three different fractions of the retina (the light and heavy fractions of the outer segments and the fraction that included the inner segments of the visual cells) were carried out with dark-adapted and light-adapted *Loligo*. These are labeled dark 1 and 2, and light 1 and 2. Table I (upper part) shows the results of experiments dark 1 and light 1; those of experiments dark 2 and light 2 confirmed their general features. Dashes mean that we could not detect any

photopigment. As seen in the table, metarhodopsin and retinochrome could be extracted in addition to rhodopsin from both the light and heavy fractions of the outer segments, whereas only retinochrome was extracted from the inner segments. With each photopigment, the yield tends to decrease gradually with repeated extractions, as might be expected. However, it should be noted that rhodopsin is extracted more easily or quickly than retinochrome. This will be clear, for instance, from Fig. 3, which is based on data from the heavy fraction of the outer segments in experiment light 1. In the figure, the amounts of the three photopigments in each extract (no. 1 ~ 11) are expressed as the division of a circle and change as the number of extractions increases. The first extracts are very rich in rhodopsin, but contain little metarhodopsin and retinochrome.

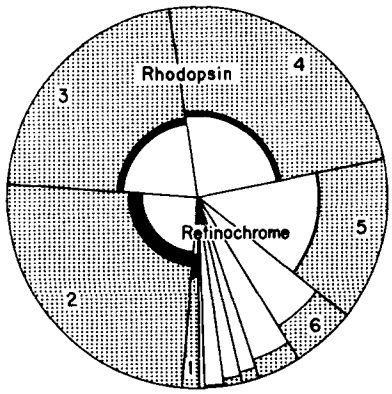

FIGURE 3. Amounts of rhodopsin, metarhodopsin, and retinochrome in successive extracts (no. 1 ~ 11) obtained from the heavy fraction of outer segments. The area of each triangular slice of a circle equals the total photopigments in the extract, and the slice is then subdivided to show the amount of each photopigment in the particular extract. The stippled area represents rhodopsin, the black area metarhodopsin, and the white area retinochrome. The initial extracts contain high percentages of rhodopsin, the final ones, of retinochrome. Metarhodopsin is found only in the early extracts. Rhodopsin therefore is more easily extracted than retinochrome from fragments of the outer segments.

Metarhodopsin disappears after the fifth extraction; the proportion of retinochrome increases as the extraction advances, and the last extract contains only retinochrome and no rhodopsin. Such a reversal in the proportions of rhodopsin and retinochrome could be ascribed to a difference in the intracellular location of the two photopigments. Thus, whereas the former is located in the rhabdomal membranes (microvilli), which are at the cell surface, the latter may be contained in the cytoplasm, perhaps in the cores of the outer segments or in their basal regions.

The amounts of each photopigment extracted from the retinal fractions in the dark and light experiments are also shown in Table I (lower part). In general, the heavy fraction of the outer segments contains about as much, or a little more, rhodopsin than the light fraction, and the proportion of rhodopsin to metarhodopsin is nearly the same in all cases. This is probably because a large part of the

rhabdomeres was carried into the heavy fraction due to imperfect separation of the cell fragments during the single sugar flotation. If this is so, retinochrome is the main constituent by which the heavy fraction should be characterized. This also suggests that retinochrome, found in the outer segments, is contained in other places than the rhabdomeres (cf. Table IV).

Comparing the dark and light experiments, it will be noted that less retinochrome is extracted from the inner segments of the light-adapted than the dark-adapted eyes. This may be because the retinochrome in the inner segments is bleached to its photoproduct during light adaptation. However, in the extracts examined throughout the present study, we could not recognize any contamination with the photoproduct, which is extractable. If it exists it should show λ_{max} at shorter wavelengths. Consequently the fact that there is less retinochrome in the inner segments of light-adapted eyes suggests that some of it has been transferred from the inner segment to another place in the course of light adaptation. This may be somewhere in the outer segment. Another point that can be seen from comparing extracts from light- and dark-adapted eyes is the fact that the light-adapted animals have much more retinochrome in the heavy fraction of the outer segments than do dark-adapted animals. This increase in retinochrome in the outer segments appears to be associated with its decrease in the inner segments. On the other hand, the outer segments retain large amounts of rhodopsin even in the light-adapted animals. This is surprising, and a distinguishing trait of the invertebrate retina. But it is possible that rhodopsin is quickly regenerated from metarhodopsin by contact with retinochrome under our experimental conditions (cf. Hara and Hara, 1972).

Though we used medium-sized squids in all our experiments, small variations in size were inevitable. To facilitate comparison among experimental results, the yields of photopigments from the outer segments shown in Table I are converted to percentages in Table II. Regardless of adaptation, the light fraction contains a high percentage of rhodopsin, about 94%, accompanied by small amounts of metarhodopsin and retinochrome. This is not reflected in the data on the total outer segments, shown in the right column of the table, in which the features of the heavy fraction predominate. Assuming that the amount of rhodopsin in the outer segments is constant in dark or light, these data tell us that when animals are light adapted the amount of retinochrome in the outer segments almost doubles.

The distribution of photopigments in the whole retina is summarized in Table III, which is based on Table I. In the dark-adapted eye, almost equal amounts of retinochrome are contained in the inner and the outer segments, and the total amount corresponds to 24% of rhodopsin. In the light-adapted eye, retinochrome decreases in the inner and increases in the outer segments. This table suggests that the amounts of rhodopsin and retinochrome are essentially the same in dark- and light-adapted eyes and that the most important change is that during light adaptation retinochrome shifts from the inner to the outer segments. Based upon absorbance, the percentage of metarhodopsin is, on the average, 5.2% of rhodopsin in the dark and 4.4% in the light, an average of 4.8%. This reflects a molar ratio of 0.03, since the extinction coefficient of

metarhodopsin is about 1.5 times larger than that of rhodopsin (Hubbard and St. George, 1958).

Retinal Photopigments in Todarodes

Todarodes is a little larger than *Loligo* and has larger eyes. Two series of experiments, dark I and dark II, were performed to examine the distribution of

TABLE II

RELATIVE PERCENTAGES OF RHODOPSIN (R), METARHODOPSIN (MR), AND RETINOCHROME (Ret) CONTAINED IN THE OUTER SEGMENTS OF THE VISUAL CELLS OF DARK- AND LIGHT-ADAPTED SQUIDS, *Loligo*

	Light fraction			Heavy fraction			Total (light + heavy)		
Experiment	R	MR	Ret	R	MR	Ret	R	MR	Ret
Average of dark 1 and 2	93.5	4.7	1.8	79.8	4.1	16.2	85.3	4.3	10.4
Average of light 1 and 2	94.1	4.6	1.4	71.1	2.9	26.1	78.4	3.4	18.1

TABLE III

DISTRIBUTION OF PHOTOPIGMENTS IN THE WHOLE RETINA OF DARK- AND LIGHT-ADAPTED SQUIDS, *Loligo* (ABSORBANCE PER MILLILITER PER 10 EYES)

	Outer segments			Inner segments, retinochrome	Total retinochrome	Total pigments
Experiment	Rhodopsin	Metarhodopsin	Retinochrome			
Dark 1	8.80	0.35	1.07	1.11	2.18	11.33
Dark 2	11.82	0.73	1.45	1.34	2.79	15.34
Average	10.31	0.54	1.26	1.23	2.49	13.34
	(77.3)	(4.0)	(9.4)	(9.2)	(18.7)	(100%)
Light 1	8.97	0.31	2.04	0.81	2.85	12.13
Light 2	9.19	0.49	2.16	0.70	2.86	12.54
Average	9.08	0.40	2.10	0.76	2.86	12.34
	(73.6)	(3.2)	(17.0)	(6.2)	(23.2)	(100%)

Percentages are given in parentheses.

photopigments in the retinas of animals, dark adapted under natural conditions, that were collected at night. The yield of photopigments from the different retinal fractions is shown in Table IV, which itemizes the many extractions performed in our procedure. The abundance of retinochrome in the *Todarodes* retina is mainly found in the outer segments, especially in their heavy fraction, although there is also a good deal of retinochrome in the inner segments. Retinochrome tends to be extracted more slowly than rhodopsin from *Todarodes* as well as from *Loligo* (cf. Fig. 3). This again suggests that retinochrome is not located in the rhabdomeres, but mostly in other parts of the outer segments.

The distribution of photopigments in the dark-adapted, whole retina of *Todarodes* is summarized in Table V. A rough comparison with the dark experiments in Table III shows that the total amount of photopigment per eye is 2.0–2.5 times greater in *Todarodes* than in *Loligo*. *Todarodes* yields approximately 1.3

TABLE IV

SUCCESSIVE EXTRACTIONS OF PHOTOPIGMENTS, RHODOPSIN (R), METARHODOPSIN (MR), AND RETINOCHROME (Ret), FROM THE THREE RETINAL FRACTIONS FROM DARK-ADAPTED *Todarodes* (ABSORBANCE PER MILLILITER PER 10 EYES)

		Outer segments						
		Light fraction			Heavy fraction			
Experiment	Extraction no.	R	MR	Ret	R	MR	Ret	Inner segments, Ret
Dark I	1	0.924	0.020	0.012	1.594	0.073	0.198	0.275
	2	1.122	0.022	0.044	3.055	0.030	1.190	0.315
	3	1.065	0.044	0.062	1.249	0.061	1.067	0.511
	4	1.168	0.037	0.070	0.484	0.041	0.693	0.227
	5	0.972	0.031	0.073	0.267	0.019	0.481	0.361
	6	0.532	0.035	0.099	0.184	0.008	0.908	0.209
	7	0.142	0.014	0.023	0.050	0.005	0.328	0.120
	8	0.087	0.007	0.070	0.036	–	0.756	0.050
	9	0.053	0.003	0.072	0.055	–	1.196	0.292*
	10	0.014	–	0.021	0.022	–	0.402	0.392*
	11	0.017	–	0.050	0.023	–	1.762	0.156
	12	0.011	–	0.032	0.003	–	0.653	0.111
	13	–	–	0.014	0.014	–	0.726	0.084
	14				0.008	–	0.564	0.058
	15				–	–	0.195	0.044
	16				–	–	0.118	0.029
	17				–	–	0.083	
Totals								
Dark I		6.11	0.21	0.64	7.04	0.24	11.37	3.23
Dark II		7.22	0.47	0.61	6.05	0.26	9.59	3.96

* Extraction performed at pH 8.0.

TABLE V

DISTRIBUTION OF PHOTOPIGMENTS IN THE WHOLE RETINA OF DARK-ADAPTED *Todarodes* (ABSORBANCE PER MILLILITER PER 10 EYES)

	Outer segments			Inner segments, retinochrome	Total retinochrome	Total pigments
Experiment	Rhodopsin	Metarhodopsin	Retinochrome			
Dark I	13.15	0.45	12.01	3.23	15.24	28.84
Dark II	13.27	0.73	10.20	3.96	14.16	28.16
Average	13.21	0.59	11.11	3.60	14.70	28.50
	(46.4)	(2.1)	(39.0)	(12.6)	(51.6)	(100%)

Percentages are given in parentheses.

times as much rhodopsin and 5.9 times as much retinochrome as *Loligo*. Metarhodopsin amounts to about 4.5% of rhodopsin on the average, similar to *Loligo*. In *Todarodes* the total amount of retinochrome is a little larger than that of rhodopsin, 1.16 and 1.07 times, respectively, in experiments dark I and dark II. This abundance of retinochrome distinguishes *Todarodes* from *Loligo*.

DISCUSSION AND CONCLUSIONS

Retinal Photopigments in Loligo and Todarodes

In order to prepare experimental samples for studies on photopigments, we previously devised a method to collect them separately, rhodopsin from the outer segments of visual cells and retinochrome from the rhabdome-free retinas including the inner segments (Hara and Hara, 1965). This procedure fostered the false impression that retinochrome is resticted to the inner segments. It is now clear that retinochrome is contained also in the outer segments, and that the traditional method of extraction of rhabdome-free retinas yields only one-quarter to one-half the retinochrome in the whole retina. On the other hand, rhodopsin is localized entirely in the outer segments, where it always exists together with metarhodopsin in the molar ratio of about 100:3. If one does many sugar flotations in order to prepare pure samples of rhodopsin, one loses the major part of the cell fragments desired for collecting rhodopsin, and although other contaminating pigments are removed, the final yield of rhodopsin is reduced to half or much less. The results of the present experiments, in which we quantitatively extracted all the photopigments, show the concentration of rhodopsin in the outer segments not to be very different in *Loligo* and *Todarodes*. However, the concentration of retinochrome is clearly much greater in both the inner and outer segments of *Todarodes* than of *Loligo*. There is not yet a sufficient explanation for this difference, which may be due to differences in their phylogenetic and ecological characteristics. We wish to bear in mind that *L. pealei* is younger phylogenetically and lives in shallow waters of less than 100-m depth, whereas *T. pacificus* is rather old and lives in far deeper waters.

Photopigments during Light Adaptation

When rhabdome-free retinas are treated under a white light by our usual method for preparing retinochrome, an extract of the photoproduct of retinochrome is obtained. Throughout the present experiments with the inner and outer segments, we never observed any evidence of such a substance in a spectrum of photopigment extracts. The rhabdome-free retinas of light-adapted squid contained much more unbleached retinochrome than we expected. But we must remember that retinochrome bleached by light can be regenerated slowly to retinochrome when kept in the dark (Hara and Hara, 1969, in *Todarodes*; Sperling and Hubbard, 1975, in *Loligo*). Consequently there is a possibility that the retinochrome extracted from the inner segments of light-adapted animals partly includes a fraction of retinochrome regenerated during the extraction procedures in the dark after decapitation. Anyhow the difference in the distribution of retinochrome between light and dark experiments is so distinct that it

cannot be denied that the difference is caused by migration of retinochrome or its photoproduct from the inner to the outer segments during light adaptation of the animals. Strictly speaking, the increase of retinochrome in the outer segments during light adaptation seemed to be a little larger than its decrease in the inner segments, but the reason is not yet clear.

As previously described, the outer segments always contain much rhodopsin with just a little metarhodopsin. The amounts of each do not markedly change even during light adaptation. There must therefore be a mechanism by which the metarhodopsin that is formed from rhodopsin in the light is continuously converted back to rhodopsin, perhaps with the aid of retinochrome. About this we would suggest the following: At the first stage of light adaptation, some retinochrome is drawn up from the inner segments, and joins the retinochrome that was originally located in the outer segments. Thus retinochrome is accumulated there and its concentration remains high. At the following step, an interaction between retinochrome and metarhodopsin is developed in the light, that promotes the regeneration of rhodopsin.

Retinal Location of Retinochrome

In an earlier paper, we suggested that retinochrome extracted from rhabdome-free retinas was present in a region between the basement membrane corresponding to the constrictions of the visual cells and the nucleated cell bodies, that is, in the so-called inner segment (Hara and Hara, 1965). Before our report it was already revealed in *L. pealei* by electron microscopy that this region contains lamellated bundles of membranes (Zonana, 1961; cf. Cohen, 1973), and we inclined to the idea that retinochrome is located in these structures, often called the myeloid bodies. At present, similar structures are also known in other cephalopods, such as *Octopus vulgaris* (Yamamoto et al., 1965) and *T. pacificus* (Hara and Hara, 1975). Each membrane of those structures often divides into two sheets to form a cistern and often makes contact with the neighboring mitochondria and the Golgi lamellae. The outermost membrane of the myeloid bodies is sometimes peeled off and sometimes carries ribosomes on its surface which turns into rough endoplasmic reticulum. These observations give us an impression that the lamellated structures cannot be functionally static, and we think it likely that they constantly play active parts in relation to visual function or, more specifically, to the synthesis of photopigment proteins, the storage of retinochrome, and so on.

To examine the distribution of these myeloid bodies, light microscopy can be rather useful, as shown in Fig. 4. This is a 1-μm section stained with methylene blue and Azur II, which is prepared from material that has been OsO_4 fixed and Epon embedded for electron microscopy. It clearly shows the inner segments filled with many myeloid bodies. Special attention should also be paid to the fact that some myeloid bodies are scattered within the basal regions of the outer segments. They must have moved forward from the inner segments across the basement membrane, and presumably carry retinochrome. Although retinochrome has now been found in the outer segments, there is so much of it and there are so few myeloid bodies that they cannot be associated with all the retino-

chrome that is found there. As we cannot find any lamellated structures in the distal parts of the outer segments beyond the layer of black pigment, it seems likely that the myeloid bodies are transformed in the outer segments into smaller kinds of vesicles convenient for carrying retinochrome. However, it is still obscure what type of structure is the site of retinochrome, especially in the outer segments. The most probable conclusion from the evidence obtained so far is that retinochrome, synthesized in the inner segments, is brought forward by the myeloid bodies into the basal regions of the outer segments, and, when neces-

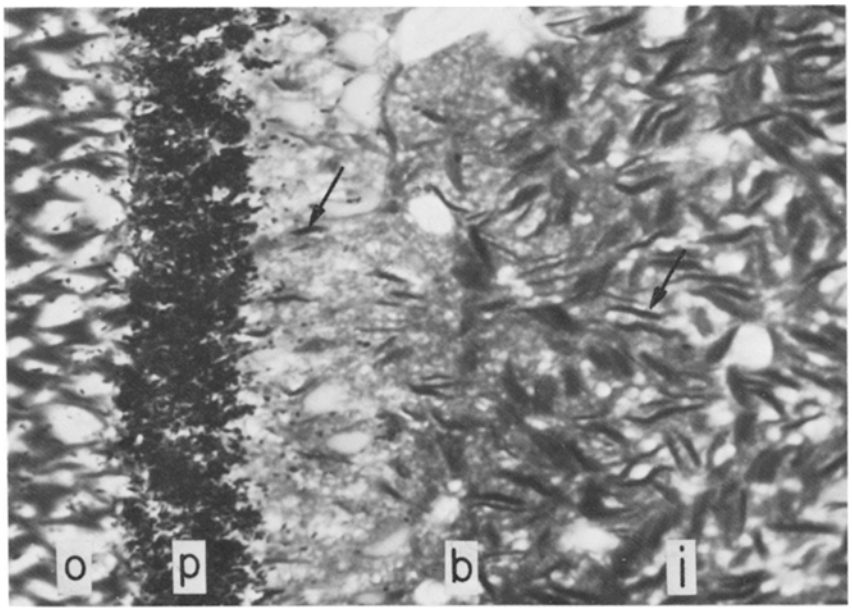

FIGURE 4. Light micrograph showing the distribution of myeloid bodies in the inner portions of the visual cells in the *Todarodes* retina (× 660). The material, prepared for electron microscopy, was sectioned at 1 μm and stained with methylene blue and Azur II. Myeloid bodies, which look like dark pieces of tape (arrow), are very abundant in the inner segments (*i*). Note some myeloid bodies also scattered between the layer of black pigment (*p*) and the basement membrane (*b*), that is, in the basal regions of the outer segments (*o*).

sary, moves through the cytoplasm towards the rhabdomeres, where it interacts with visual pigment. It is not unreasonable to postulate such movement of retinochrome, in view of the fact that ommin granules can migrate from one end of the outer segment to the other as a function of the state of adaptation (Cohen, 1973; Daw and Pearlman, 1974).

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