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Daily changes of structure, function and rhodopsin content in the compound eye of the crab *Hemigrapsus sanguineus*

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Summary. The compound eye of the crab *Hemigrapsus sanguineus* undergoes daily changes in morphology as determined by light and electron microscopy, both in the quantity of chromophore substances studied by HPLC and in visual sensitivity as shown by electrophysiological techniques.

1. At a temperature of 20 °C, the rhabdom occupation ratio (ROR) of an ommatidial retinula was 11.6% (maximum) at midnight, 8.0 times larger than the minimum value at midday (1.4%) (Figs. 2, 6).

2. Observations by freeze-fracture revealed that the densities of intra-membranous particles (9–11 nm in diameter) of rhabdomeric membrane were ca. 2000/μm^2 and ca. 3000/μm^2 for night and daytime compound eyes, respectively (Fig. 3).

3. Screening pigment granules migrated longitudinally and aggregated at night, but dispersed during the day. Reflecting pigment granules migrate transversally in the proximal half of the retinula layer i.e. cytoplasmic extensions containing reflecting pigment granules squeeze between neighbouring retinula cells causing optical isolation (Fig. 4). Thus the screening pigment granules within the retinula cells show longitudinal migration and radial movement so that the daytime rhabdoms are closely surrounded by the pigment granules (Fig. 2).

4. At 20 °C, the total amount of chromophore of the visual pigment (11-*cis* and all-*trans*-retinal) was 1.4 times larger at night than during the day i.e. 46.6 pmol/eye at midnight and 33.2 pmol/eye at midday (Fig. 9). Calculations of the total surface area of rhabdomeric membrane, total number of intra-membranous particles in rhabdomeric membrane and the total number of chromophore molecules in a compound eye, indicate that a considerable amount of chromophore-protein complex exists outside the rhabdom during the day.

5. The change in rhabdom size and quantity of chromophore were highly dependent on temperature. At 10 °C both rhabdom size and amount of chromophore stayed close to daytime levels throughout the 24 hours (Figs. 6, 9).

6. The intracellularly determined relative sensitivity of the dark adapted night eye to a point source of light was about twice as high as the dark-adapted day eye (Fig. 10). Most of the increase in the sensitivity is attributed primarily to the effect of reflecting pigment migration around the basement membrane (Fig. 4) and, secondarily, to the changes in the amount and properties of the photoreceptive membrane.

The results form the basis of a detailed discussion as to how an apposition eye can function possibly as a night-eye.

Introduction

Since the pioneering work on membrane turnover in larval mosquitoes (White 1967, 1968; White and Lord 1975) and the spider crab, *Libinia* (Eguchi and Waterman 1967), there have been numerous studies on the turnover mechanism of photoreceptive membranes in arthropods (see reviews by Blest 1980; Waterman 1982). Recently, the relationship between molecular aspects of visual pigment turnover and morphological changes in the photoreceptive membrane has been studied in detail for invertebrates, particularly in blowflies (Schwemer 1986). Daily changes in rhabdom structure have been studied in several crustacean species (Itaya

Abbreviations: *ROR* rhabdom occupation ratio; *MVB* multivesicular body; *HPLC* High-pressure liquid chromatography; *ER* endoplasmic reticulum

1976; Nässel and Waterman 1979; Stowe 1980a; Toh and Waterman 1982; Doughtie and Ranga Rao 1984; Hariyama et al. 1986a) and for the compound eyes of crabs, the turnover process consists of two separate phases. A phase of synthesis occurs at dusk and a breakdown phase around dawn with the consequence that the rhabdom is larger at night than during the day. The change in volume of the rhabdom is well documented, but the relationship to the amount of visual pigment contained within the rhabdomeric membrane is not known. Recently, the technique of high-pressure liquid chromatography (HPLC) has been used to measure accurately the amount of visual pigment chromophore in a single crayfish eye (Suzuki et al. 1984). This study reports the use of HPLC, with slight modifications, on the eye of a grapsid crab, *Hemigrapsus sanguineus*, to reveal the relationship between the amount of photoreceptive membrane and chromophore molecules. The photoreceptive membrane also shows a drastic change in volume. In addition, the response characteristics of a single retinula cell with both small and large rhabdoms was measured intracellularly.

In order to consider daily changes in ommatidial structure in detail, the general anatomy of the eye of *H. sanguineus* is described. This description includes the migration of screening and reflecting pigment granules in the retinula layer, which has been shown in other crustaceans (Stowe 1980b; Doughtie and Ranga Rao 1984). The relationship between the surface area of the photoreceptive membrane, the quantity of visual pigment chromophore, migration of screening and reflecting pigment granules and the physiological characteristics are discussed in detail.

Materials and methods

Subjects. Adult crabs. *Hemigrapsus sanguineus* of both sexes (carapace width 20–25 mm) were collected in the Nojima Park, Yokohama City, Japan. The crabs were kept in a 12:12 light/dark regime (L = 9:00–21:00) at 10 °C in a controlled close-environment system (Koitotron). At least two weeks prior to the experiments, the crabs were transferred to other chambers with the same light regime and at a temperature of 20 °C. The illumination intensity of the light period was about 2000 lux, a similar brightness to the natural habitat.

Transmission microscopy. Compound eyes were prefixed in 2% glutaraldehyde and 2% paraformaldehyde in 0.1 *M* cacodylate buffer at pH 7.2–7.4 for 12–14 h at 4 °C. After washing with the same buffer solution tissue was postfixed for 2–3 h in 2.0% OsO_4 in the same buffer, dehydrated in an acetone series and then embedded in Epon. For light microscopy, semi-thin sections were produced with glass knives and stained with toluidine blue. For electron microscopy ultra-thin sections were cut with a diamond knife, double stained with uranyl acetate and lead citrate for 5 min each and examined with a JEM 1200 EX electron microscope.

To compare the day-night change in rhabdom size, rhabdom occupation ratios of individual ommatidial retinulae (ROR) were measured using light micrographs. Cross-sections through 16–20 hexagonally arranged ommatidia were examined from the forward looking eye region at the nuclear layer of the retinula cells. Rhabdom area and inter-ommatidial distances (centre to centre) were measured by an image analyzing system connected to a computer (NEC PC9801E). The mean value of all inter-ommatidial distances for an eye was taken as the mean ommatidial eye diameter for the ROR calculation. The populations of multi-vesicular bodies (MVB) were quantified by the ratio of the MVB area to the total retinula cell area in transverse sections at the nuclear layer. Electron micrographic measurements were made on a Kontron-videoplan image analyzer.

Freeze fracture electron microscopy. Retinal tissues were fixed for 4 to 5 h at 4 °C with 0.1 *M* phosphate buffered 2.5% solution of glutaraldehyde at pH 7.3. Night eyes were fixed under a dim red light (> 600 nm). The tissues were washed and kept in the same buffer solution overnight, then immersed in 35% glycerol for 3 h before being rapidly frozen in liquid nitrogen and transferred to a JEOL freeze-fracture apparatus (JFD-7000) at a temperature of −120 °C and a vacuum of 10^{-6} Torr.

Immediately after the tissue was fractured by a razor blade, platinum was evaporated onto the fractured surface at an angle of 45°, followed by a carbon from above.

High-pressure liquid chromatography. The quantity of the chromophore of the visual pigment was measured by HPLC and extracted with an excesss of NH_2OH (Groenendijk et al. 1980). A compound eye was isolated from the eye stalk and homogenized, freeze-dried and washed several times with petroleum ether. Retinal was extracted as oxime and analyzed by a JASCO HPLC-system equipped with a column of Zorbax SIL (7 μm, 2.1 × 250 mm) or Nucleosil (5 μm, 2.1 × 250 mm). The absorbance at 360 nm was monitored by a UV-detector (UVIDEC 100-III) and the peak area was determined with an integrator (Chromatopac CR-3A, Shimadzu). Retinal was quantified from the peak areas and standard curves determined with authentic compounds. Details of the analytical procedure have been reported by Makino-Tasaka and Suzuki (1986).

Electrophysiology. For electrophysiological recordings, only crabs with hard carapaces were used. Legs and chelae were autotomized by heating with a soldering iron. The eye stalk was glued with dental cement to prevent movement, and each crab preparation was fixed on an experimental stage, and then positioned at the centre of a Cardan arm perimeter device. A small triangular hole was made into the forward looking region of the right eye by a fresh chip of a razor blade. A 3 *M* KCl filled glass microelectrode (80–100 MΩ) was inserted into the small hole before the blood coagulated and an indifferent electrode was placed at the base of the cemented eye stalk. All experiments were conducted with a room temperature of 20–25 °C.

A white-light stimulus was provided by a 500 W Xenon arc lamp and led into a Faraday cage by an fibre optic. The tip of the fibre optic was attached to the Cardan arm of the perimeter device to provide a point source of light. Tip diameter of the fibre optic was 1.6° which is much smaller than the angular sensitivity of a single retinula cell in the light-adapted day-eye. Light intensities were adjusted by neutral density filters and an optical wedge over a range of seven log units. Night-eye specimens were prepared for recordings under dim red light

(>600 nm). After electrode impalement, the optical axis of a retinula cell was located by moving the tip of the fibre optic until dim flashes of constant intensity light produced a maximum response. All animals were left in the dark for about 30 min prior to the commencement of recordings. The responses were recorded through a pre-amplifier (MEZ-8201, Nihon-Koden), an oscilloscope (VC-10, Nihon-Koden) and a continuous recording camera (RLG-6201, Nihon-Koden).

Receptor potentials were measured at 21–29 different light intensities over a range of 7 log units. Light flashes of 100 ms duration were used for stimulation. The resulting stimulus-response (V-log I) curve was fitted to the Naka and Rushton equation (as modified by Norman and Werblin 1974),

$$V/V_{max} = I^n/(I^n + K^n)$$

where I is the stimulus intensity, V is the amplitude of the response. V_{max} is the maximum response amplitude, K is the light intensity yielding a response of 50% V_{max} and n represents the slope of linear part of the curve. Set of V_{max} and n values which closest fits the theoretical curve determined by computer (NEC PC9801E), using a spline function, was taken as the result.

Results

General anatomy

The eye-stalks are ca. 3.8 mm in length in *H. sanguineus* with carapace width of 20–25 mm. The compound eye is composed of ca. 5000 ommatidia; each ommatidium is an 'apposition type' (Fig. 1) and the retinal volume of a single eye is ca. 4.2×10^8 μm^3. Located immediately below the hexagonally packed corneal facets are the crystalline cones, composed of four cone cells, which occupy the layer between cornea and retinula layer. In each ommatidium, four thin proximal processes, one from each cone cell, run separately along the lateral or basal surface of the retinula cells. These processes enlarge before reuniting at the basement membrane. Distal and proximal screening pigment cells surround the crystalline cone at each end. Cells containing numerous reflecting granules occupy the inter-ommatidial space in the retinula layer mainly surrounding the proximal part of the ommatidial retinula. There are slender processes however, extending more distally, surrounding the crystalline cone. The nuclei of the reflecting cells are located 20–40 μm distal to the basement membrane.

As in other decapod compound eyes (Shaw and Stowe 1982), the ommatidial retinula of *H. sanguineus* consists of a distal retinula cell with four lobes (R8) and seven regular retinula cells (R1–7). In transverse section (Fig. 2a, b), R1 is twice the size of the other six retinula cells (R2–7). The nuclei of all retinula cells are located distally near the tip of the crystalline cone. The rhabdom occupies the centre of an ommatidial group of retinula cells, with an interdigitated, banded structure in longitudinal section (ca. 200–250 bands). A single rhabdom microvillus is ca. 0.08 μm in diameter and the thickness of a single rhabdom band is ca. 1μm. The length and thickness of a single rhabdom band, including the microvillus diameters, do not change between day and night. Using the freeze-fracture technique, the cleaved rhabdom microvillus reveals prominent intra-membranous particles (diameter 9–11 nm) on a convex protoplasmic leaflet (P-face) with a density of ca. 2000/μm^2 (night) and ca. 3000/μm^2 (day). In comparison, the concave exoplasmic leaflet (E-face) does not contain as many particles and has a relatively smooth surface (Fig. 3).

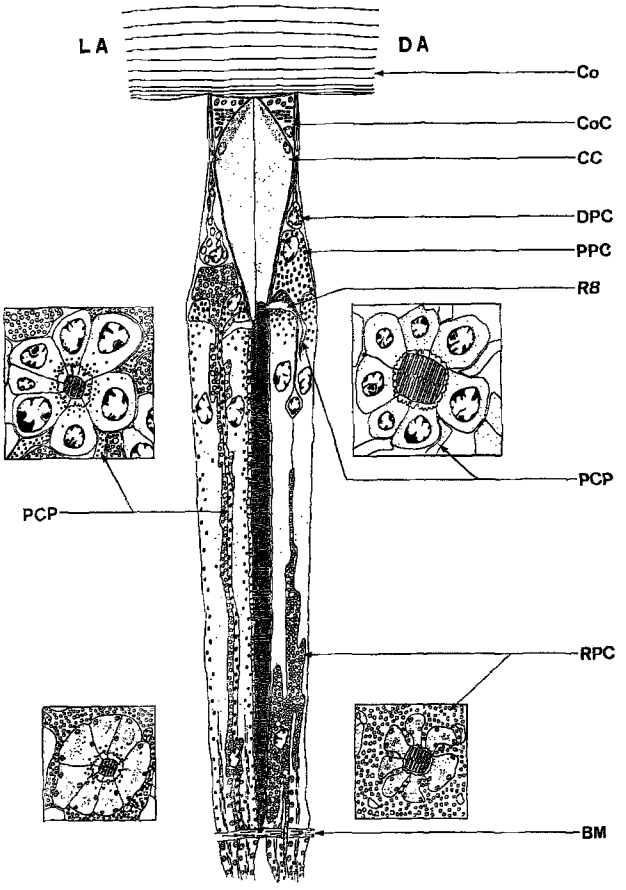

Fig. 1. Schematic diagram of ommatidia from the eye of *Hemigrapsus sanguineus* showing day (left half) and night (right half) conditions. Inset drawings are cross-sections of day and night eyes through the nuclear regions of retinula, reflecting pigment cells, the change of rhabdom size and geometrical arrangement of retinula cells particularly at the base of the ommatidial retinula. *BM* basement membrane; *CC* crystalline cone; *Co* cornea; *CoC* corneagenous cell; *DPC* distal pigment cell; *PCP* proximal pigment cell process; *PPC* proximal pigment cell; *R8* retinular cell 8; *RPC* reflecting pigment cell

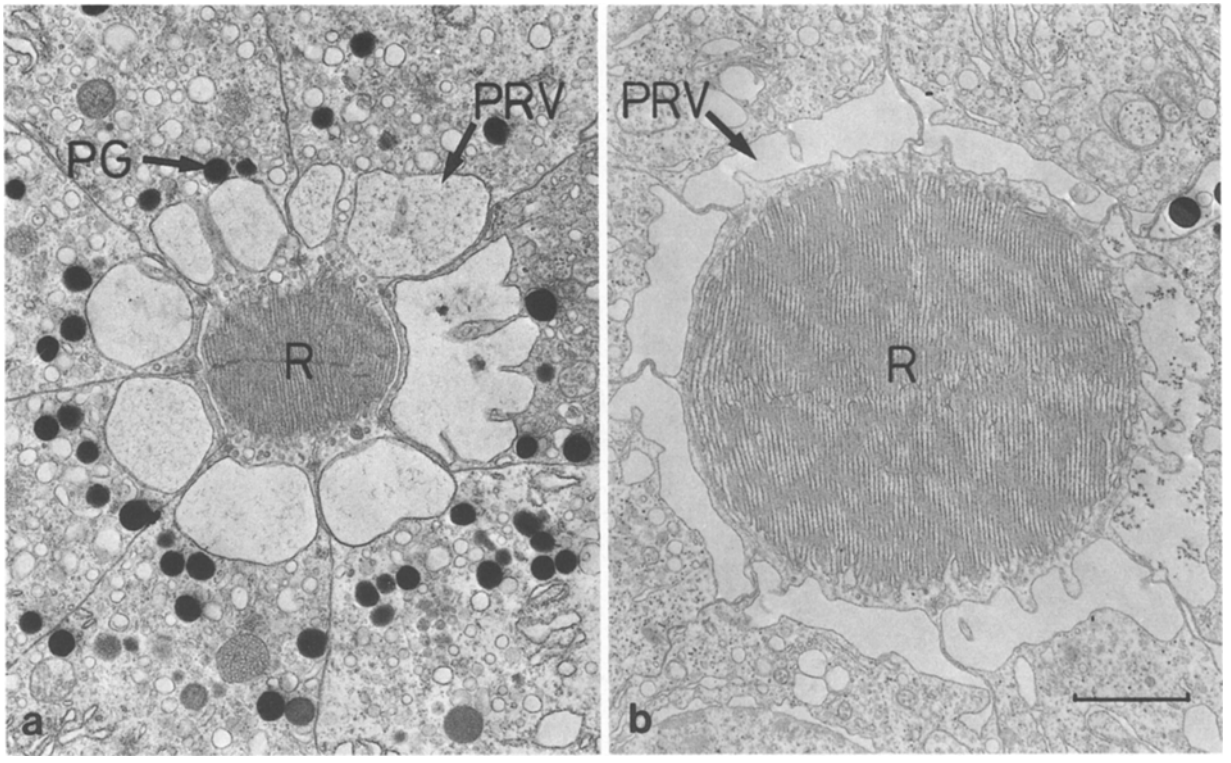

Fig. 2. Electron micrographs taken at same magnification (scale 1 μm), to show cross-sections of rhabdoms at the retinula cell nuclear layer: **a** 15:00 (midday), **b** 3:00 (midnight). In both micrographs, rhabdoms are composed of regularly-arranged microvilli, belonging to four retinula cells (R 2, 3, 6, 7). At midday the rhabdom is significantly smaller compared to midnight and the pigment granules in the retinula cells surround the peri-rhabdomal vacuole in the midday eye only. Volume of the peri-rhabdomal vacuoles appears similar in both conditions. *R* rhabdom; *PRV* peri-rhabdomal vacuole (palisade); *PG* pigment granules in retinula cells

Daily morphological changes of the compound eyes

Pigment migrations. The behaviour of the pigment cells in the compound eye of *H. sanguineus* is comparable to that of another grapsid crab, *Leptograpsus* (Stowe 1980b) and a grass shrimp *Palaemonetes* (Doughtie and Ranga Rao 1984). The large pigment granules of the distal screening pigment cells do not show a distinct migration along a crystalline cone during the day, with only a slight shift towards the proximal region. However, the pigment granules of the proximal screening pigment cells, do show a marked migration between day and night. In the daytime, the main bodies of the cells occupy the narrow space around the proximal end of the crystalline cone. The pigment granules mostly shift to the cytoplasmic processes and only a few granules remain in the cell bodies. At night, the main cell bodies move distally and occupy the space around the proximal halves of the crystalline cone. The pigment granules are confined to the cytoplasm surrounding the crystalline cone and are almost totally absent in the cytoplasmic processes projecting into the retinula cell layer.

In addition to the screening pigment cells, pigment granules within the retinula cells also migrate between day and night. Based on longitudinal sections, the pigment granules are scattered throughout the cytoplasm of the retinula cell during the day except for the extreme distal region. However, transverse sections reveal that most of the pigment granules are restricted to the area just outside the peri-rhabdomal palisade of vacuoles belonging to the endoplasmic reticulum (ER) that surrounds a rhabdom. At night, the pigment granules are seen to occupy only the extreme proximal or distal parts of a retinula cell. Unlike in the locust (Williams 1982) and grass shrimp (Doughtie and Ranga Rao 1984), the palisade in the crab shows no notable changes in morphology between day and night. The reflecting pigment cells fill the space around the proximal half of the retinula cell layer during the night only. At night the cytoplasmic extensions containing reflecting pigment granules squeeze between neighbouring retinula cells and isolate them at the apex (separated, Fig. 4d). During the day the reflecting pigment cells elongate in a distal direction, beyond the proximal screening pigment

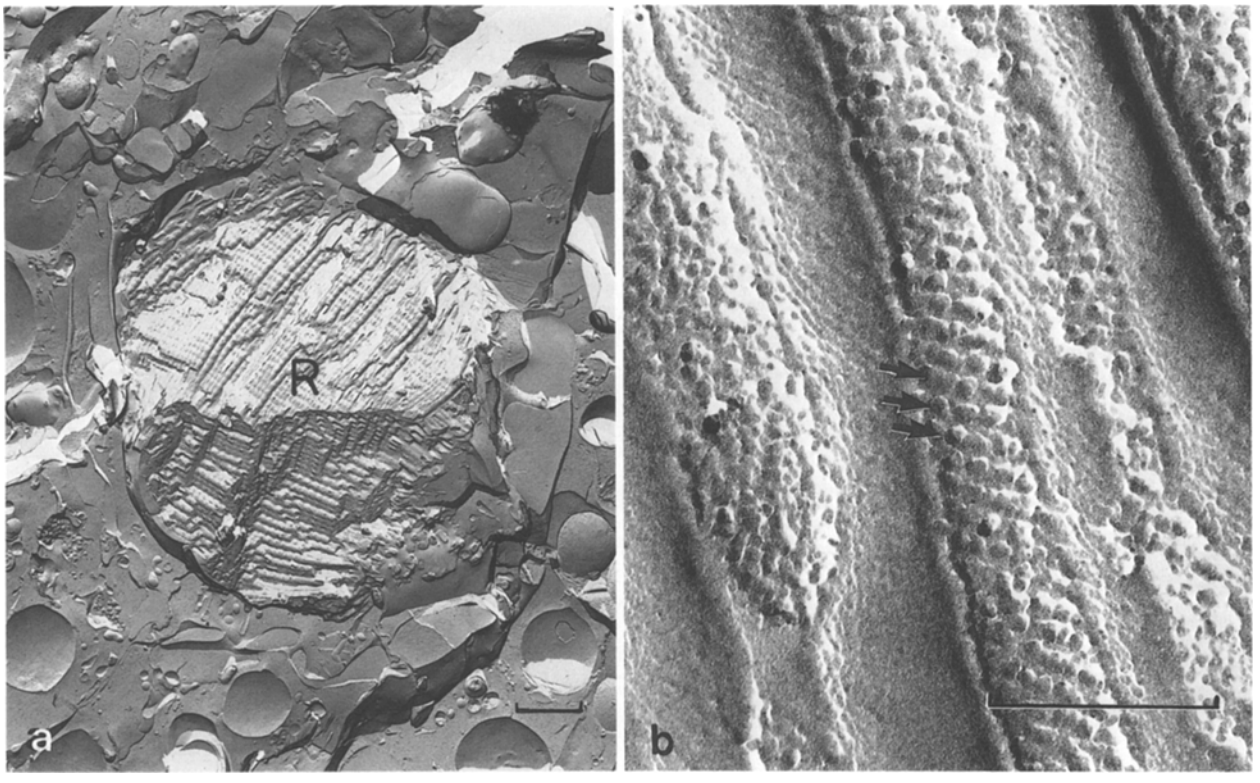

Fig. 3a Lower magnification electronmicrograph of a cross-cleaved rhabdom from a freeze-fractured compound eye, fixed at 3:00 (midnight). Within the rhabdom, tightly packed microvilli are arranged in two perpendicular directions. Scale 1 μm. **b** Higher magnification of a part of the freeze-fractured rhabdom in a compound eye, fixed at 3:00 (midnight), showing longitudinally cleaved microvilli. Convex protoplasmic faces have numerous particles 9–11 nm in diameter. A microvillus at the centre of the picture contains particles arranged in a spiral fashion (arrows). Concave exoplasmic faces without particles appear smooth. Scale 0.1 μm

cells, to reach the distal screening pigment cells. Cell elongation probably causes the intervening processes between adjoining retinula cells seen during the night to withdraw, so that the retinula cells can contact each other laterally (compact pattern, Fig. 4a). The shift from a compact to separated pattern takes 2 h following the onset of light.

Rhabdom and rhabdom occupation ratio. Figure 5 shows the difference in rhabdom size and rhabdom occupation ratio for one ommatidium (ROR) in relation to the depth of the retinula. The length of one ommatidial retinula (ca. 230 μm) does not change between day and night. At midday, the rhabdom area is almost constant throughout the length of the ommatidium and the increase in ROR at the deeper level of the day eye is due to the decrease in the overall ommatidial diameter. At midnight the volume of the rhabdom is relatively small just above the basement membrane, possibly due to space limitations imposed by the constant pore size of the basement membrane. The following measurements of ROR refer to the level where the nuclei of retinula cells appeared (*n* in Fig. 5).

Changes of the ROR were examined throughout a light regime of LD 12:12 (light phase: 9:00 to 21:00) (Fig. 6). At 20 °C, the ROR is about eight times larger at night ($11.6 \pm 0.9\%$, at 3:00) compared to the day ($1.4 \pm 0.1\%$, at 15:00). However, a similar ROR change cannot be detected at 10 °C and the ROR remains close to the daytime level ($3.7 \pm 0.7\%$ at 3:00, $1.0 \pm 0.1\%$ at 15:00).

Under the 20 °C condition and 30 min before lights-on at 8:30 the ROR is already significantly smaller than at midnight ($5.6 \pm 0.3\%$). After exposure to light, there is a further rapid decrease in rhabdom size, reaching a minimum after 2 h of light ($0.9 \pm 0.1\%$). On the other hand, rhabdom regeneration, does not start until the light is off, since the ROR is still at the minimum level at 20:30 i.e. 30 min prior to lights-off ($0.9 \pm 0.04\%$). Subsequently the ROR increases rapidly in the dark and reaches $7.5 \pm 1.0\%$ within 30 min of lights-off (21:30), which is about 65% of the maximum

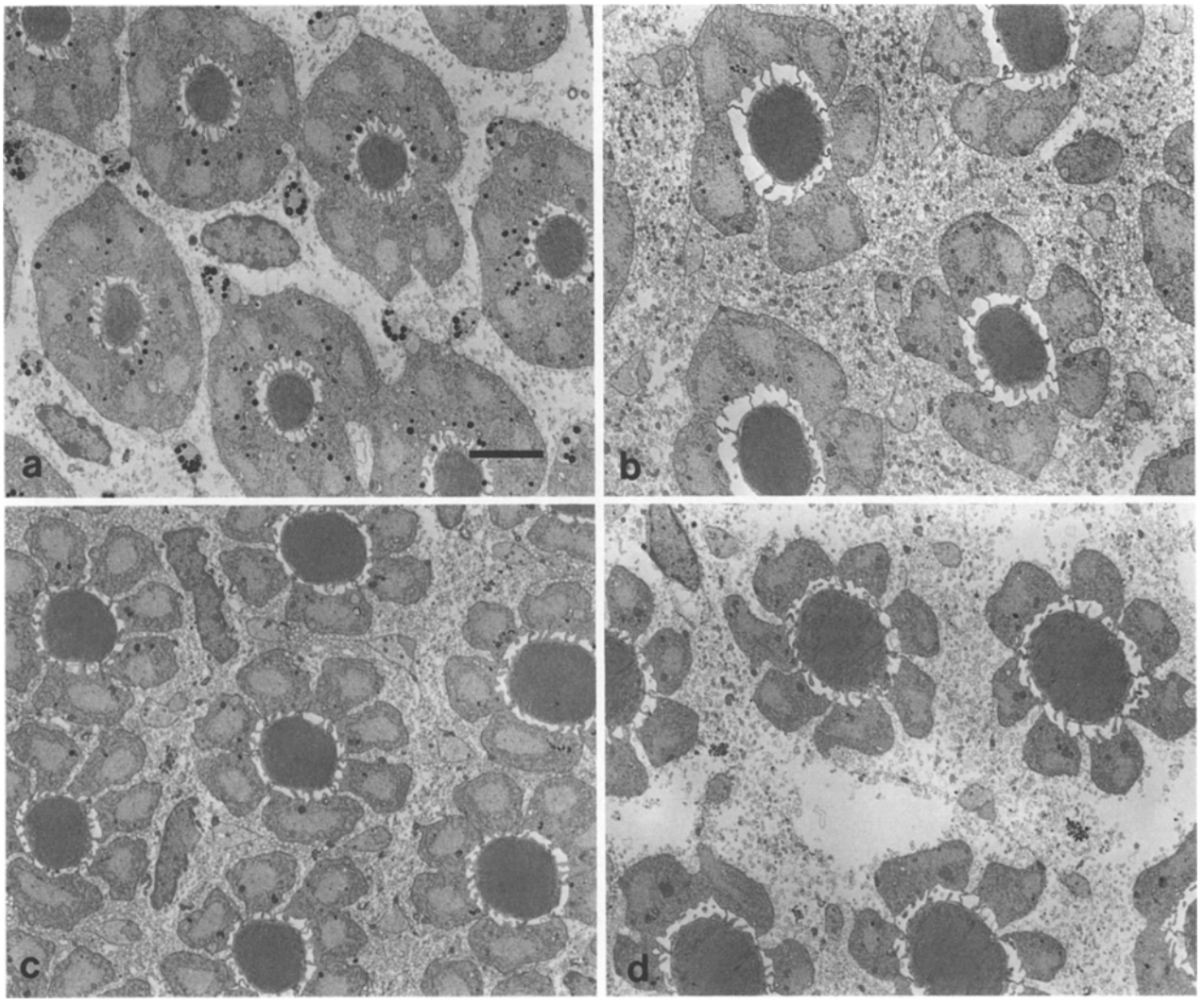

Fig. 4a–d. Cross sections through the nuclear region of reflecting pigment cells fixed at different times of the day. **a** 20:30 (30 min before lights off, showing the compact pattern); **b** 21:30 (30 min after lights off); **c** 22:00; **d** 23:00 (showing the separated pattern). The shift from the compact to the separated pattern requires about two hours following lights off. Scale 5 μm

ROR at midnight. The morphological results described above are summarized in Table 1.

Cell organelles. The rhabdom microvilli are regularly arranged throughout the day, except for the first 60 min after the onset of light. In retinula cells fixed 15 min after the onset of light (9:15), the rhabdom membranes are severely disorganized and the microvilli are short, variable in width and irregularly shaped. This phenomenon is regarded as an artefact caused by the unnatural abrupt onset of bright light at dawn. Resumption of an ordered appearance of the rhabdoms requires an additional 45 min. During this period, pinocytotic vesicles increase remarkably in number which may be closely related to the mechanism of rhabdom breakdown.

Just before the onset of light at 9:00, numerous pinocytotic vesicles have been produced from the bases of the rhabdomeric microvilli, and many small vesicles are present in the narrow cytoplasmic area (peri-rhabdomal cytoplasm) between the microvillar bases and the peri-rhabdomal palisade of vacuoles of the ER. The small vesicles are 50–100 nm in diameter and the outer surface is covered with an electron dense coat (coated vesicle). Many MVB containing numerous naked vesicles (without coat), which are supposed to originate from pinocytotic vesicles, are also found around the palisade of the 9:00 preparation.

At 9:15 (15 min after the onset of light) both coated vesicles and MVB have increased in number. In specimens fixed at 10:00 the rhabdo-

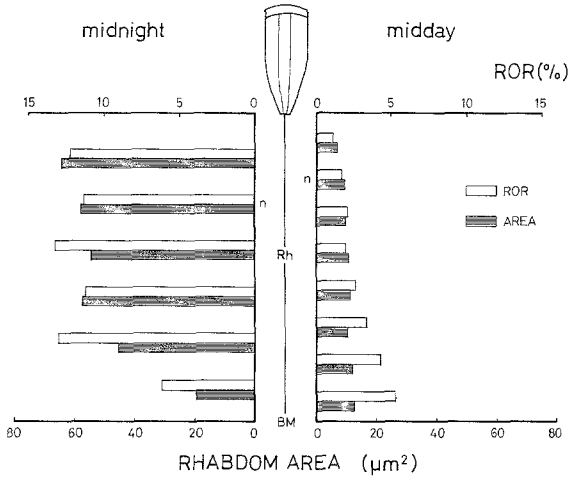

Fig. 5. Change in rhabdom size (area in μm^2, and ROR in %) as a function of the depth of the retinula, and time of day (midday and midnight). *BM*, *n* and *Rh* represent the position of the basement membrane, the retinula cell nuclei and the rhabdom respectively

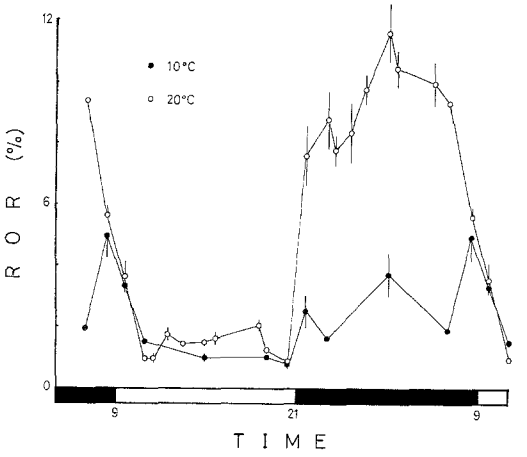

Fig. 6. Change of ROR throughout the day at 20 °C and 10 °C. Each data point represents mean value of 5 individuals, with standard error bars. ROR was measured from cross-sections at the nuclear region of the retinula cells

Table 1. Results of morphological and HPLC measurements

		Midnight	Midday
Number of ommatidia		5000	
Volume of retinula (μm^3)		4.2×10^8	
Diameter of microvilli (μm)		0.08	
Rhabdom occupation ratio (%)	20 °C	11.6± 0.9	1.4± 0.1
	10 °C	3.7± 0.7	1.0± 0.1
Total amount of chromophore (pmol/eye)	20 °C	46.6± 3.3	33.2± 2.7
(11-*cis*-retinaloxime %)		(91.1)	(76.7)
	10 °C	22.9± 2.9	22.5± 2.2
		(74.7)	(50.3)
Density of intramembranous particles (μm^{-2})		2008 ±706	3032 ±208

Number of ommatidia and the retinula volume are based on values obtained from crabs with carapace widths near 23 mm

meric microvilli are short but regularly arranged and there are a few coated vesicles in the peri-rhabdomal cytoplasm; however, numerous larger MVBs compared to those at 9:15 are scattered throughout the cytoplasm (Fig. 7a).

The change in MVB quantity of MVBs throughout the day was plotted in Fig. 8 which shows a gradual increase before the onset of light. The MVB continue to increase rapidly during the initial few hours after the onset of light, but both MVB and coated vesicles are almost non-existent in retinula cells fixed around dusk. Other lysosomal structures in the cytoplasm such as lamellated bodies and dense bodies increase in number during the light phase but do not peak as sharply as the MVB during a restricted time of the day.

In retinula cells fixed at 20:30 (30 min before lights-off), and for the first few hours of the dark phase rough ER is abundant in the cytoplasm while smooth ER (vacuoles) is distributed around the cytoplasm, especially the palisades.

Retinula cells fixed after 60 min in the dark (22:00), when elongation of the rhabdomeric microvilli has already commenced, show irregularly shaped saccules appearing in the palisade bridges and peri-rhabdomal cytoplasm (Fig. 7b).

Vesicular rough ER increases for a few hours after the onset of light, at a time when it is assumed that the ingestion of the rhabdomeric membrane is occurring vigorously.

Rhythmic alterations in the size or number of other cell organelles (e.g. mitochondria and Golgi

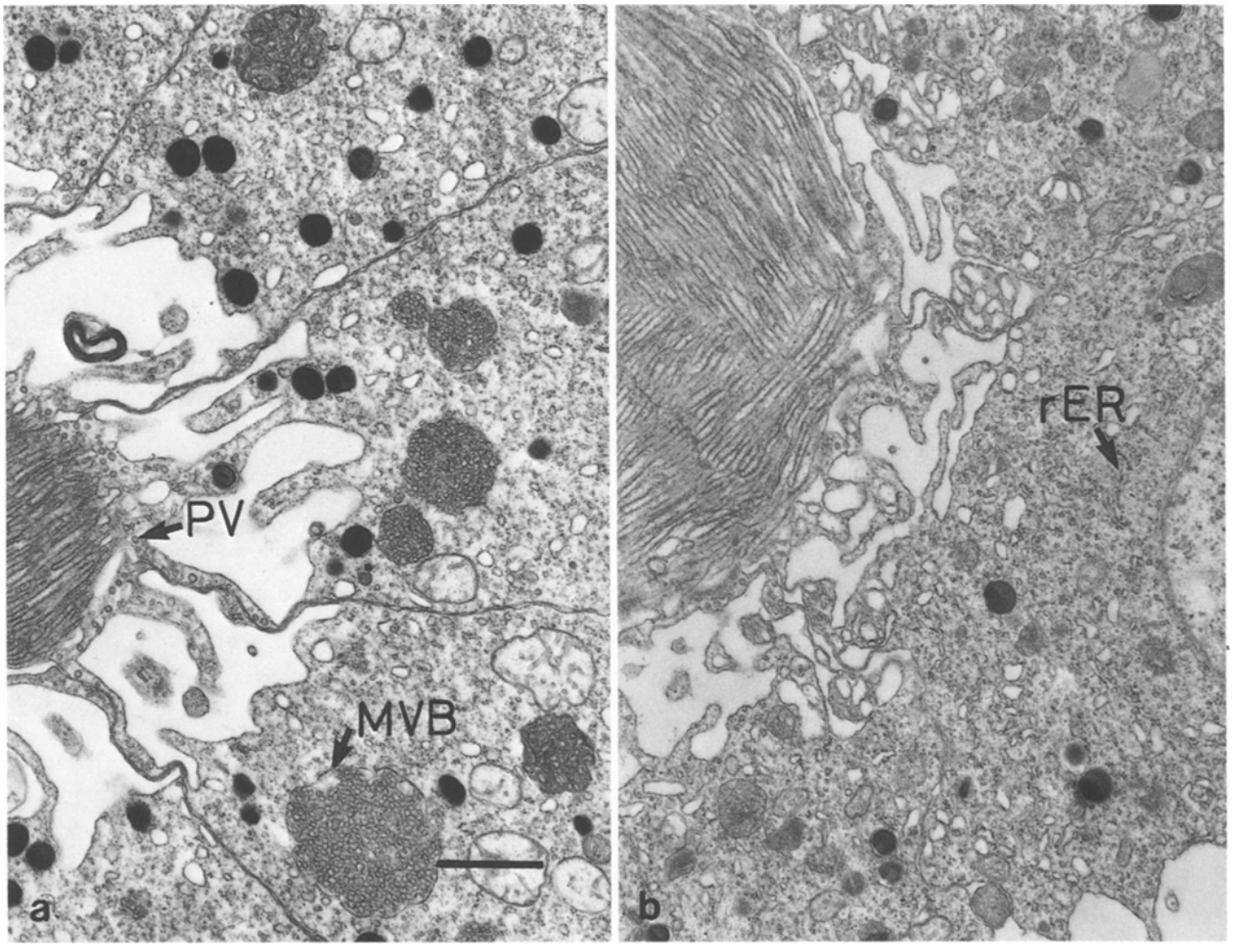

Fig. 7a, b. Cross-sections through the nuclear region of retinula cells. **a** Fixed at 10:00 (60 min after light onset). Numerous pinocytotic vesicles (*PV*) and multivesicular bodies (*MVB*) are seen within the cytoplasm. **b** Fixed at 22:00 (60 min after lights off). Rough ER is prominent in the cytoplasm instead of MVB found in the 9:15 preparation. Scale 1 μm

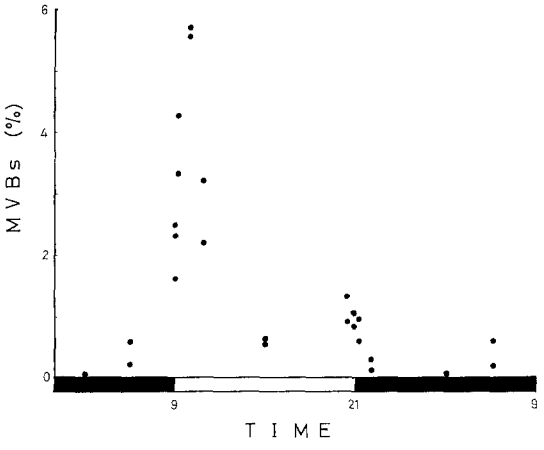

Fig. 8. Change in amount of MVB in the retinula cell cytoplasm during day. Each data point represents the percentage (%) of the MVB cross-sectional area to the cytoplasmic area. A single very sharp increase in MVB is observed around dawn

apparatus), which would parallel the changes in rhabdom organization, could not be observed.

Chromophore contents

The visual pigment of *H. sanguineus* is a complex of chromophore 11-*cis*-retinal and the protein opsin, termed rhodopsin. Vitamin A_2-based visual pigment which has been demonstrated in crayfish during winter (Suzuki et al. 1984) was not detected in the crab (Fig. 9a). 11-*cis*-retinal is isomerized to all-*trans*-retinal by light absorption and in this experiment, the relative proportions of 11-*cis*- and all-*trans*-retinal were measured. The two compounds determined here are regarded as the chromophore combined with opsin because any free retinal in the eye was removed with petroleum ether in the washing process. The results are shown

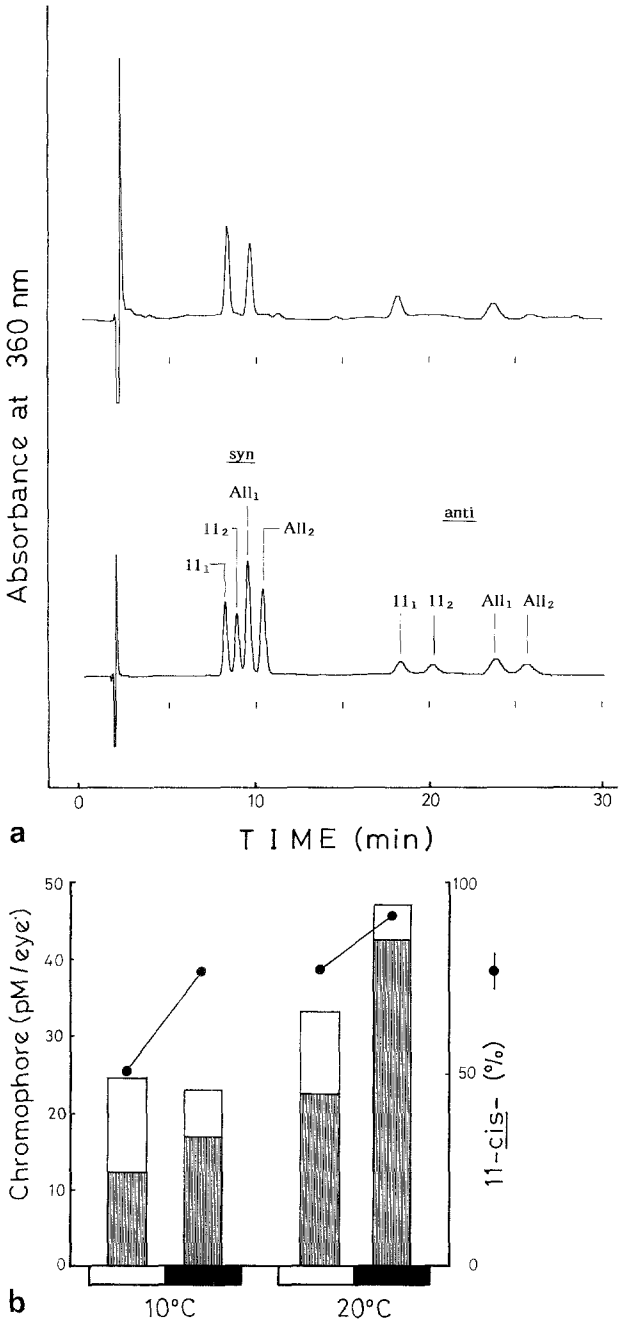

Fig. 9. a Chromatogram of extracts obtained from a day crab. Lower trace are the standard oximes of 10 pmol each. 11_1, 11-*cis*-retinaloxime; 11_2, 11-*cis*-3dehydroretinaloxime; All_1, all-*trans*-retinaloxime; All_2, all-*trans*-3dehydroretinaloxime. **b** The amount of visual pigment chromophore in a compound eye at 10 °C and 20 °C. Each column represents the total amount of chromophore containing both 11-*cis*-retinal (hatched) and all-*trans*-retinal (unfilled). Percentage of 11-cis-retinal to total chromophore for each condition is plotted (filled circles)

diagrammatically in Fig. 9b and summarized in Table 1, together with other relevant morphological data. Specimens of crab eye for HPLC analyses were collected at 15:00 (midday) and 3:00 (midnight). In Fig. 9b each column shows an average

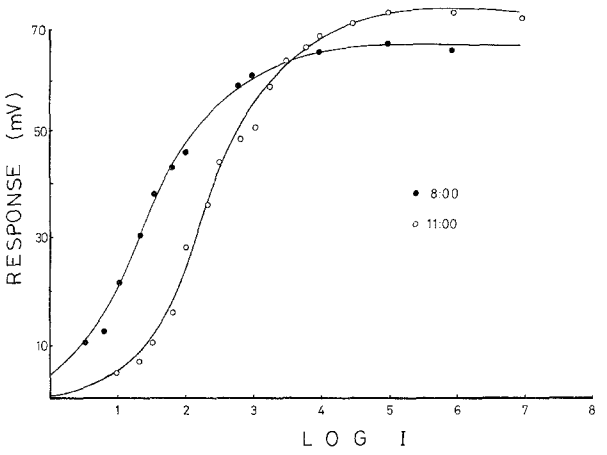

Fig. 10. Two V-log I curves recorded from a single cell at 8:00 (one h before onset of light) and 11:00 (2 h after onset of light, but maintained in the dark)

of the data from five eyes of different individuals. At 10 °C, the total amount of chromophore is not significantly different between day (24.5 pmol/eye) and night (22.9 pmol/eye). On the other hand, for crabs kept at 20 °C, the amount of chromophore was much greater at night (46.6 pmol/eye) than during the day (33.2 pmol/eye). These observations agree with the daily changes of ROR at both 10 °C and 20 °C. At both temperatures the amount of all-*trans*-retinal was significantly higher during the day than at night (in the dark-adapted state).

Electrophysiology

Receptor potentials were recorded intracellularly from retinula cells of the forward looking eye region. Resting potentials varied in each preparation between about −60 mV to −80 mV and only recording with maximal response amplitudes (V_{max}) greater than 50 mV were used.

Receptor potentials were recorded under constant darkness to minimize the effect of extensive pigment migration caused by the onset of background illumination. Measurements were made from a single retinula cell in the dark one hour before dawn (8:00) and also 2 h after dawn (11:00) under constant darkness. Measured under constant darkness the estimated ROR of the compound eyes at 8:00 is about 8.0% (Fig. 6) and 5.0% at 11:00 (Arikawa et al., in preparation). Examples of V-log I curves are shown in Fig. 10 for a cell with a maximum response amplitude (V_{max} = 73.0 mV) of the dark-adapted day eye slightly higher than that of the dark-adapted night eye (V_{max} = 67.4 mV). Since V_{max} was slightly higher in the night eye in other cells, the differences in V_{max}

Table 2. Summary of electrophysiological data (mean ± SE of seven separate data)

	8:00	11:00
V_{max} (mV)	62.14 ± 2.96	62.94 ± 2.89
n	0.75 ± 0.02	0.77 ± 0.03
Sensitivity ratio	2.04	1.00
ROR ratio	1.6 (ROR = 8.0%)	1.00 (ROR = 5.0%)

Measurements carried out at 8:00 and 11:00. ROR-value for each time was determined from measurements of ROR change under constant darkness (unpublished data)

amplitudes between the two eye conditions was not considered significant (Table 2). The value of n, representing the slope of the linear part of the V-log I curve, was about 0.8 in both day and night dark-adapated eyes. Changes of relative sensitivity are represented by log K values (see Methods); high K values correspond to low sensitivity. In this case, the V-log I curve recorded from a dark adapted day eye (11:00) is shifted about 0.6 log units (Table 2) to the right along the abscissa, indicating lower sensitivity. Mean values of V_{max}, log K and n are shown in Table 2.

Discussion

Membrane turnover

In *H. sanguineus* the morphological differences between day and night eyes, in particular rhabdom and cytoplasmic organelles such as the lysosomal components are in close agreement with observations in *Libinia* (Eguchi and Waterman 1967), *Grapsus* (Nässel and Waterman 1979), *Leptograpsus* (Stowe 1980a) and *Callinectes* (Toh and Waterman 1982).

It has been reported in crustacean compound eyes, that morphological and physiological changes are strongly influenced by environmental temperature (Meyer-Rochow and Tiang 1979; Meyer-Rochow and Eguchi 1984) and such an effect was found in *H. sanguineus* as well (Figs. 6, 9). At 10 °C the size of the night eye rhabdom was maintained close to the level of the 20 °C daytime eye, indicating that some steps, in the process of rhabdom synthesis, probably enzymatic, are either inhibited at lower temperatures or accelerated at higher ones.

It has been observed repeatedly in crustacean eyes that microvillar membranes are pinched off into the cytoplasm as small vesicles at the time of rhabdom degradation, and aggregate into MVB (Eguchi and Waterman 1976; Itaya 1976; Meyer-Rochow and Tiang 1979). In the present study numerous pinocytotic vesicles and MVB appear when the rhabdoms decrease in size around dawn (Fig. 7a). Therefore, the extent of membrane ingestion can be estimated roughly by the number of coated vesicles and MVB present (Fig. 8). A similar result has been obtained in crayfish retinula cells (Hafner et al. 1980).

The calculations in Table 1, indicate that the rhabdomeric membrane area of any R2–7 retinula cells at midnight is ca. 5.56×10^4 μm^2 (maximum) compared to only ca. 0.69×10^4 μm^2 (minimum) at midday. Degeneration of the rhabdomeric membrane takes only a few hours although the biological clock starts the process slightly before dawn. If reduction of rhabdomeric membrane is due solely to the ingestion of pinocytotic vesciles, then 3.25×10^6 vesicles are required to reduce 4.87×10^4 μm^2 of membrane in a single retinula cell, since the surface area of a single pinocytotic vesicle is 0.015 μm^2 (0.07 μm in diameter). As the reduction in rhabdom size is completed within the first few hours, the speed of pinocytotic vesicle production is very high (451 vesicles/s per cell).

According to Stowe (1980a), the formation of rhabdomeric membrane in the crab *Leptograpsus variegatus*, proceeds as follows: (1) Rough ER becomes swollen and forms tubules of new smooth ER. (2) The smooth ER tubules form a doublet ER. (3) The doublet ER moves into the rhabdomal region and assembles into the rhabdomal microvilli. In *H. sanguineus*, swollen rough ER and smooth irregular saccules appear at the time of normal dusk, when the formation of new rhabdomal membranes begins (Fig. 7b), but no structures resembling Stowe's doublet ER could be found. From the present study, the formation process of rhabdomal membranes can be divided into two steps: (a) synthesis of rough ER membranes, (b) incorporation of rough ER into microvilli by transformation into irregular saccules. The amount of rough ER is small from midnight to dawn but large around dusk. Changes in rhabdom size may reflect variation of incorporating activity during that period since the microvillar membranes are not vigorously ingested between midday and midnight in the normal light cycle. In contrast to activity during synthesis, the incorporating activity is strongly influenced by ambient light, because under a normal dark-light regime, it increases immediately after dark onset.

Change in the amount of chromophore

There are three kinds of chromophores of the invertebrate visual pigments (retinal, 3-dehydroret-

inal, 3-hydroxyretinal; see Kirschfeld 1986 for review), but only retinal was found in the eye of *H. sanguineus*. In a similar fashion to the ROR value, the total amount of chromophore remained near the daytime level throughout a 24 h cycle at 10 °C. However at 20 °C, chromophore was 1.4 times higher at midnight compared to midday. This suggests a strong relationship between the turnover processes of the photoreceptive membrane and the amount of visual pigment, and that new rhodopsin molecules are synthesized at night. The source of the chromophore molecules of newly synthesized rhodopsin remains unknown. In the isopod crustacean *Ligia exotica*, an increase in retinal is accompanied by a decrease in retinyl ester (Hariyama et al. 1986b). Unfortunately, the behaviour of retinol and retinyl ester are not known because these retinoids were rinsed out in the present experiments (see Methods and Results).

In contrast to a 1.4 times change in the total amount of chromophore between day and night, photoreceptive membrane area increased by 8.0 times at night. The difference between these values can be explained by assuming that 1) rhodopsin density per unit area of photoreceptive membrane is much lower at night than during the day, and/or 2) a considerable amount of chromophore exists outside the rhabdom during the day.

Rhodopsin in the microvillus membrane can be visualized by freeze fracture as intra-membranous particles in both vertebrates (Jan and Revel 1974; Yamamoto et al. 1974; Yamada 1979) and invertebrates (Perrelet et al. 1972; Nickel and Menzel 1976; Fernandez and Nickel 1976; Brandenburger et al. 1976). The idea that most intramembranous particles are rhodopsin molecules is widely accepted (Nickel and Menzel 1976; Williams 1982, 1983; Yamamoto and Takasu 1984). However the number of rhodopsin molecules which correspond to a single intra-membranous particle is still uncertain although several studies addressed this problem: at least 2 rhodopsin molecules corresponding to a particle (Clarke 1975), 2–3 molecules (Boschek and Hamdorf 1976), 4 molecules (Usukura and Yamada 1981), 4–5 molecules (Besharse and Pfenninger 1980). Although the diameter of the intra-membranous particle of *H. sanguineus* is constant (9–11 nm) between day and night, the density is about 1.5 times higher during the day than at night (Table 2). If the difference in values between ROR (8.0 times) and chromophore change (1.4 times) depends solely on the rhodopsin density in the photoreceptive membrane, then the particle density during the day should be about 6 times higher than at night, but this is not the case.

The total number of chromophore molecules contained in a midday eye is calculated as ca. 1.99×10^{11}/eye, while the total number of intramembranous particles is ca. 9.0×10^{11}/eye (Table 1). If all rhodopsin molecules are visualized as the particles, then one intra-membranous particle corresponds to 22 molecules of rhodopsin in the midday eye. Blasie et al. (1969) predicted theoretically that the diameter of a rhodopsin molecule was ca. 4 nm. So that 9–11 nm particle of the *H. sanguineus* rhabdom does not appear to correspond to a single rhodopsin molecule. The very high value of 22 molecules per particle in the day eye indicates that a considerable number of chromophore molecules exist outside the rhabdom. Even if it is assumed that one particle corresponds to 5 rhodopsin molecules (the highest value reported), extra-rhabdomeric chromophore exceeds 70% of the total chromophore.

In the midnight eye, a similar calculation indicates about 7 rhodopsin molecules to a single particle but this value is still higher than has been reported, and so extra-rhabdomeric chromophore probably still exists. Recently, it has been shown that about 10% of intra-membranous particles may correspond to other membranous proteins such as GTP-binding protein in toad retina (Roof and Heuser 1982; cephalopod, Tsuda, personal communication). If this is also true in *H. sanguineus* extra-rhabdomeric chromophore may be proportionately higher than calculated above.

As stated in the Results, the retinoids measured here bind opsin and other retinoid binding proteins. The MVB in retinula cells of the spider crab, *Libinia*, contain the intra-membranous particles of similar size and density to those found in rhabdomeric membranes (Eguchi and Waterman 1976) and in addition *Drosophila* MVB react positively to the monoclonal antibody of R1–6 opsin (Tominaga et al. 1986). These observations suggest that MVB and related lysosomal structures are possible candidates which may contain extra-rhabdomeric chromophore. In the vertebrate retina, it is well-known that the outer segment tips of visual cells are phagocytosed by the adjacent pigment epithelial cells. In the crab compound eyes, however, such a phagocytotic function for the pigment cells surrounding ommatidial retinula has not been observed. Even if there are some processes involving chromophore exchange between retinula and pigment cells, most of the extra-rhabdomeric chromophore binding protein would not be membrane bound but exist freely in the cytoplasm. This is

because there are not as many membranous structures in the cytoplasm, such as the myeloid bodies found in the visual cells of cephalopods (Yamamoto et al. 1965) which could accommodate all of the extra-rhabomeric chromophores. Of interest is the existence of all-*trans*-retinal both during the day (23.3%) and night (8.9%) and can be detected in a crab kept in the complete darkness for several days (Arikawa et al., in preparation). The origin of such all-*trans*-retinal e.g. metarhodopsin and/or retinochrome at dark period is not clear yet.

Recently, the molecular aspects of visual pigment turnover in relation to membrane turnover have been studied in detail (Schwemer 1986). The identification of location sites, origin of the large amount of extra-rhabdomeric chromophore and dark all-*trans*-retinal in *H. sanguineus* will contribute greatly to the understanding of the molecular processes of membrane turnover.

Significance of sensitivity change

The relative sensitivity of a single dark-adapted retinula cell increases about two fold at night (ROR = 8.0%) compared to the day (ROR = 5.0%) (Table 2). Ignoring changes in the nature of the optics and the photoreceptive membrane, retinula cell sensitivity to an extended light source whose diameter is larger than the receptive field of a single retinula cell increases in proportion to the area of the rhabdom tip because the acceptance angle increases (Snyder 1979). However in the present experiments, the increase in retinula cell sensitivity is not due to the increase in rhabdom tip area because a point source of light was used as a stimulus. Furthermore, the nightly decrease in the density of intra-membranous particles suggests that the sensitivity of the retinula cell to a point source is much lower than during the day. The present results suggest the opposite effect, so it is pertinent to ask what factors contribute to making the night eye more sensitive?

One major factor is the increase in reflected light at the proximal region of the retinula, due to the marked movement of the reflecting pigment cells as described in this study (Figs. 1 and 4). Another factor is migration of the screening pigment which eliminates shielding between neighbouring ommatidia and may increase stimulus contamination by the stray light from a point source. In addition, the radial movement of the screening pigment granules within a retinula cell may be important, although the palisade morphology is constant between day and night. An increase in the refractive index of the retinula cell cytoplasm during the day may cause the loss of the incident light, which eventually lowers sensitivity (Miller 1979). Constant values of V_{max} and n indicate a constant photoreceptor gain between day and night. In the locust, Williams (1983) has emphasized that the changes in sensitivity to an extended light source are achieved by changing the amount of exposed receptor membrane rather than the nature of the photoreceptive membrane, but more detailed analyses are required. Eguchi and Horikoshi (1984) have shown that the value of n changes in proportion to the ROR based on electroretinographic recording in various species of Lepidoptera. Studies on the possible contribution of the wide variety of optics in compound eyes would be interesting also.

What is the functional significance of the increase in eye sensitivity during the night in *H. sanguineus*? In general, the apposition compound eye is adapted for diurnal behaviour while the superposition eye fits to nocturnal and diurnal activities. It is reasonable to assume that the increase in the size of the rhabdom at night in *H. sanguineus* represents an adaptation to a dark environment although previously this crab has been considered primarily as a diurnal species. In *Leptograpsus*, the absolute sensitivity during the night is high enough to see stars, and behavioural results correspond well to the electrophysiological sensitivity measured by quantum bumps (Doujak 1985). In *H. sanguineus*, such high sensitivity may be expected also, at night for example, by rhabdom enlargement. In this case, spatial resolution will be reduced due to the increasing acceptance angle caused by the enlargement of the cross-sectional area of the rhabdom tip, as well as the position of the screening pigments. Although the behaviour of *H. sanguineus* has not been studied in detail, it is known that they move actively in an aquarium with ca. 30 cm depth of water during both day and night, but in the field at low tide, most of the crabs stay under rocks and do not move. The activity of crabs may turn out to be controlled by light and tidal conditions, so it would be advantageous to have eyes maximally sensitive at high tide during the night. In *Scylla*, it has been demonstrated that dark adaptation during the day is always imperfect (Leggett and Stavenga 1981), and this phenomenon may also occur in *H. sanguineus*. The advantage of pre-adaptation to daylight is evident if a crab rests under a stone and then is attacked by a predator. The crab must escape and seek a new refuge in bright daylight. In conclusion, the biological implications of a night-adapted apposition eye,

as in *H. sanguineus* are far from clear and require more detailed behavioural investigations.

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