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ENDOGENOUS RHYTHMS IN THE AMOUNTS OF 11-*cis* RETINAL IN THE COMPOUND EYE OF *LIGIA EXOTICA* (CRUSTACEA, ISOPODA)

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Summary

The volume of the rhabdom of *Ligia exotica* changes diurnally, with the rhythm persisting autogenically in continuous darkness. The morphological changes are accompanied by variations in the amounts of the different chromophores making up the visual pigment. The amount of 11-*cis* retinal was found to be high at night (26.2±3.5 pmol per eye) and low during the day (10.9±2.6 pmol per eye) and to display an endogenous rhythm. At dawn and dusk, the amount was 19.1±1.2 pmol. This rhythmicity persisted in continuous darkness, although the average amount of 11-*cis* retinal present gradually increased. In the case of all-*trans* retinal, changes in the amount were rhythmic in light–dark conditions. The amount of all-*trans* retinal present in the eye increased shortly after the onset of light and decreased soon after the end of illumination. There was about twice as much 11-*cis* retinyl ester as 11-*cis* retinal in the compound eye of *Ligia exotica*. Rhythmicity in the amount of 11-*cis* retinyl ester present during the light–dark cycle was observed, but it was opposite in phase to that of 11-*cis* retinal. Under conditions of continuous darkness a clear rhythm was not apparent: the amount of 11-*cis* retinyl ester increased not only during the subjective day but also during the following subjective night. Thus, a clear correlation exists between changes in structural organization of the rhabdom and the amounts of 11-*cis* retinal present. Both show features of a circadian rhythm.

Introduction

In the eyes of some vertebrates and invertebrates, the structure of the photoreceptor changes diurnally with the light–dark cycle. Since Young (1967) first discovered that in vertebrates the disc membranes of rod photoreceptor outer segments are renewed throughout life, many investigations have focused on membrane turnover, for example 'rod shedding' during the morning (Besharse, 1982). In many arthropods (such as insects, spiders and crustaceans) the volume of the rhabdom also changes diurnally (Waterman, 1982).

Arthropod visual pigments are proteins incorporated into the microvillar

Key words: circadian rhythm, chromophore, Crustacea, retinal, photoreceptor, *Ligia exotica*.

membrane of the rhabdomeres (see Laughlin *et al.* 1975). Diurnal changes in the total amounts of retinal isomers in the locust compound eye have been studied by high performance liquid chromatography (HPLC) (Isono *et al.* 1986). It was found that structural changes in the photoreceptors are paralleled by fluctuations in the amount of 11-*cis* retinal present. Under continuously dark conditions, following the first night, the volume of the rhabdom and the amount of 11-*cis* retinal both decrease during the first subjective day and remain low for several days. These results show that the rhythms operating in the locust eye are not controlled by an endogenous clock.

In contrast, the volume of the rhabdom in *Ligia exotica* changes rhythmically, not only in light–dark conditions but also in continuous darkness. During the day, the arrangement of microvilli is ordered and, except at the distal end, the rhabdom is of the open type. During the night, an irregular arrangement of microvilli appears, and the rhabdomeres touch each other. This rhythmicity persists in continuous darkness, indicating that it is endogenous to the eye of *Ligia exotica* (Hariyama *et al.* 1986).

Taking advantage of these features, this paper examines the extent to which the amounts of 11-*cis* retinal, all-*trans* retinal and 11-*cis* retinyl ester present in the eye are governed by endogenous mechanisms.

Materials and methods

Animals

Adult (approx. 4 cm) female and male *Ligia exotica* (Roux) were captured locally (Miura Peninsula, Kanagawa Prefecture, and Shichigahama, Miyagi Prefecture). They were reared under a 12 h:12 h L:D cycle (25 °C) in the laboratory for at least 1 month before being used in the experiments.

Sampling and oxime extraction

Groups of six animals were killed every 3 h throughout the day. The animals were quickly frozen in liquid nitrogen, and the eyes were removed with a razor blade under an infrared night viewer C3100 (Hamamatsu Photonics Co., Ltd). Four eyes were homogenized in a glass homogenizer. Three samples were taken for HPLC analysis at each time.

Homogenates were treated with 0.2 ml of 1 mol l^{-1} hydroxylamine (pH 6.0), to which 0.5 ml of methanol had been added to obtain oximes of isomers of the pigment chromophores. The oximes were extracted using 0.5 ml of dichloromethane and 1 ml of *n*-hexane. The solvent was then removed in an evaporator. The extraction with dichloromethane/hexane was repeated three times. The final extracts were dissolved in 50 μl of elution solvent for HPLC.

Saponification of retinyl ester

The fractions around the solvent front eluted during HPLC analysis for retinal were separated and evaporated. 1 ml of KOH/ethanol (0.6 ml of 33 %

KOH + 10 ml ethanol) and 1 mg of ascorbic acid were added. Each solution was incubated at 55 °C for 30 min under nitrogen. The retinol, made by saponification of retinyl esters, was extracted three times with petroleum ether. The evaporated extracts were dissolved in 50 μl of elution solvent for HPLC.

HPLC analysis

For retinal oximes, the elution solvent was 7 % ether in *n*-hexane containing 0.075 % ethanol, and the flow rate was kept constant at 1.2 ml min^{-1}. For retinol (saponified retinyl ester), the elution solvent was 5 % dioxane in *n*-hexane and the flow rate was the same as for retinal oximes. The HPLC system consisted of a YMCA-012 SIL column, Personal Pump NP-D (Nihon Seimitsu Kagaku Co., Ltd) and a monitor (UNIDEC-100-V; JASCO Co., Ltd). The peak area was determined by integrating the absorbance at 360 nm (retinal oxime) or at 330 nm (retinol, saponified retinyl ester; Chromatocorder II, System Instruments Corp.). A spectro-multichannel photodetector MCPD-350 (Otuka Electronics Co. Ltd, Osaka, Japan) was used for multi-scan HPLC analysis.

Results

The amounts of 11-*cis* and all-*trans* retinal present in the eye were measured over a period of about 3 days. Examples of the chromatograms are shown in Fig. 1; these were taken at midnight (6 h after dusk, 0:00 h), at midday (6 h after dawn, 12:00 h), at midnight, and, in the ensuing continuous darkness, at subjective midday (18 h after dusk, S12:00 h) and at subjective midnight (30 h after dusk, S0:00 h). There was more 11-*cis* retinal present during the night. During the day, the amount of 11-*cis* retinal decreased, while that of all-*trans* retinal increased with time after 'sunrise'. During the subjective day, the amount of all-*trans* retinal did not change significantly, but that of 11-*cis* retinal decreased. The amount of all-*trans* retinal also showed little change during the subjective night, whereas the amount of 11-*cis* retinal increased.

The chromatogram of the multiscanning HPLC (Fig. 2) suggested that one of the solvent front peaks was a kind of carotenoid because of its λ_{max} in the blue (450 nm) and the similarity in the form of the spectra (Zechmeister, 1962). A large peak (peak number 3 in Figs 1 and 2) between the peaks for all-*trans* retinal syn and 11-*cis* anti was also thought to be due to carotenoids. The amounts of 11-*cis* retinal and all-*trans* retinal, measured every 3 h for about 3 days, are shown in Fig. 3. Under a light–dark cycle, the amount of 11-*cis* retinal displayed a minimum value of 9.2±2.3 pmol per eye (mean±S.D., N=12) at midday. After midday, the amount gradually increased, despite the fact that the light was still on, to 19.1±1.3 pmol per eye at dusk. After dusk, the amount continued to increase until midnight (maximum value 26.0±2.1 pmol per eye), and then gradually decreased, to about 20 pmol per eye at dawn. These changes persisted in continuous darkness, though it should be noted that the average amount present gradually increased in darkness.

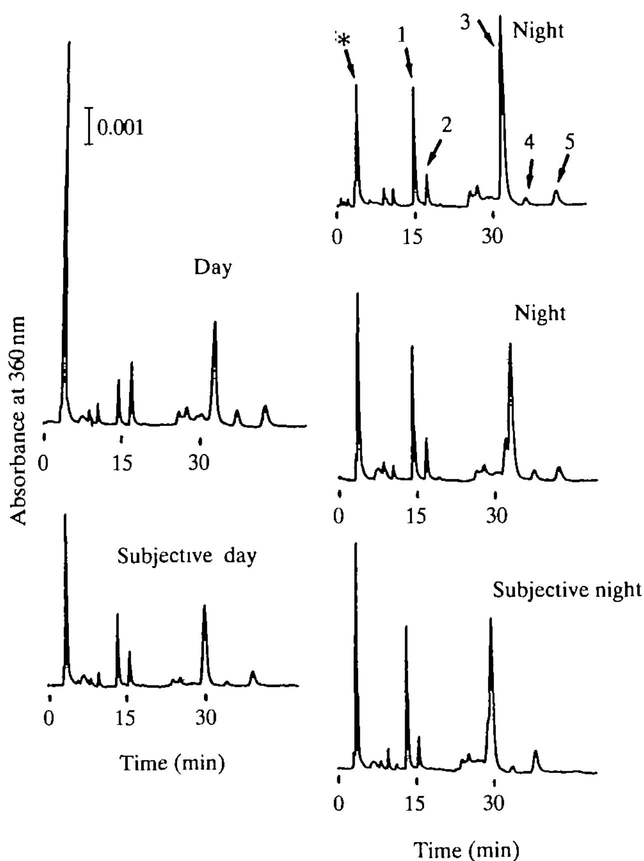

Fig. 1. Examples of chromatograms showing diurnal changes in the amounts of 11-*cis* retinal and all-*trans* retinal. The amount of 11-*cis* retinal oxime (1) was high at night (26.2±3.5 pmol per eye) and low during the day (10.9±2.6 pmol per eye). This rhythmicity persisted under conditions of continuous darkness. Rhythmicity in the amount of all-*trans* retinal was observed in L:D 12 h:12 h conditions, but not in conditions of continuous darkness. *, solvent front (including carotenoid); 1, 11-*cis* retinal oxime (syn); 2, all-*trans* retinal oxime (syn); 3, carotenoid; 4, 11-*cis* retinal oxime (anti); 5, all-*trans* retinal oxime (anti).

The amount of all-*trans* retinal showed a different diurnal pattern, commencing with an increase, following the onset of light, to a level that remained more or less constant throughout the day. There was a rapid decrease following the onset of darkness, and this low level was maintained until dawn. In contrast to the changes in 11-*cis* retinal, no peaks were observed at midday or midnight. In continuous darkness, the amount of all-*trans* retinal remained constant at a low level, and no rhythmical change related to a 24-h cycle was observed.

The magnitude of the solvent front seemed to show a diurnal rhythm under the light–dark cycle. The amount of retinyl ester was measured after saponification. The average amount of the retinyl ester under the light–dark cycle was about

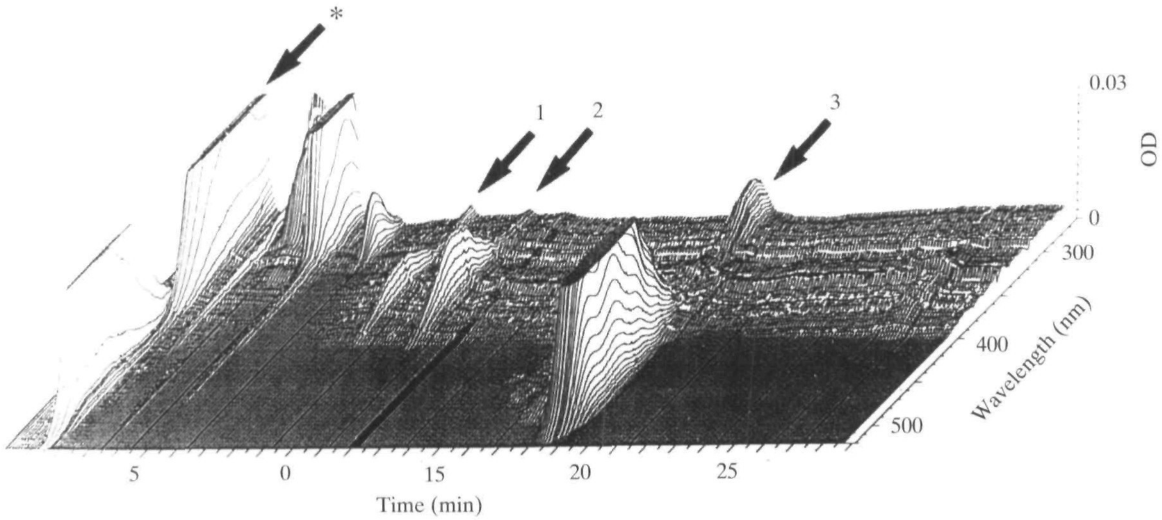

Fig. 2. A chromatogram of retinal oxime analysis using multiscanning HPLC. The typical pattern and λ_{max} (approximately 450 nm) of carotenoid were observed at the solvent front and at the place (retention time 18 min) between syn and anti peaks. *, solvent front; 1, 11-*cis* retinal oxime; 2, all-*trans* retinal oxime; 3, carotenoid; OD, optical density.

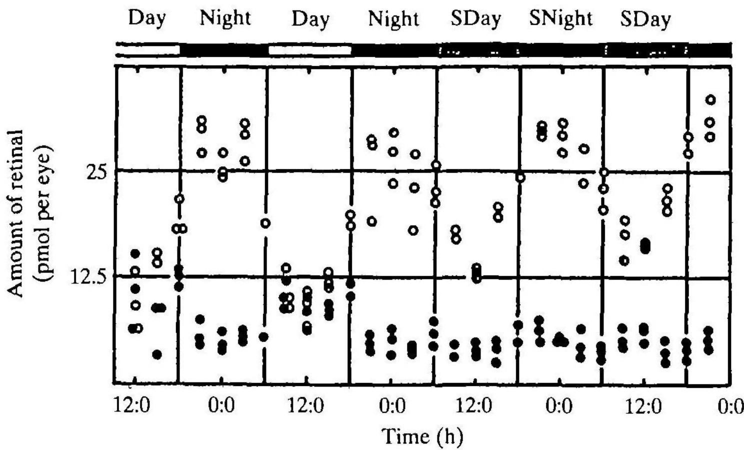

Fig. 3. The diurnal changes in the amounts of 11-*cis* retinal (○) and all-*trans* retinal (●). Groups of six animals were killed every 3 h and three samples (each sample included four eyes) were taken for HPLC analysis at each time. The amounts of 11-*cis* retinal during night and subjective night (SNight) were about 30 pmol per eye, at dawn and at dusk they were about 20 pmol per eye. During day and subjective day (SDay), the amounts were about 10 pmol per eye. The amounts of all-*trans* retinal changed only under changing light–dark conditions.

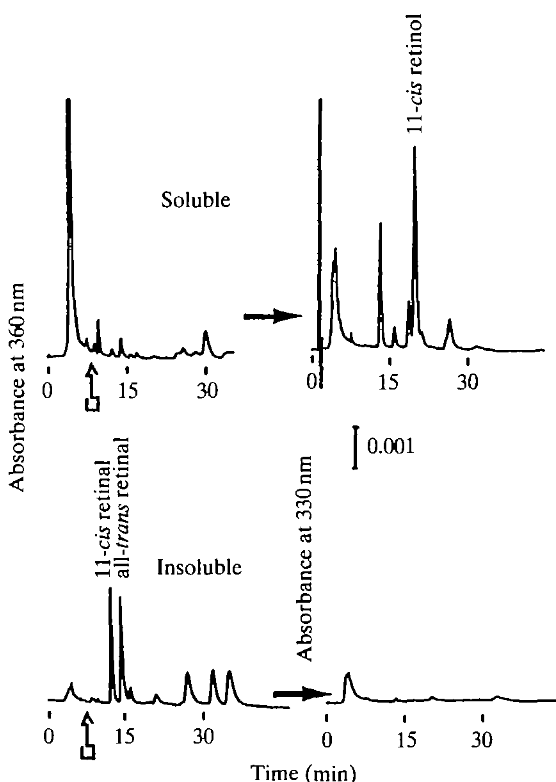

Fig. 4. Chromatograms to distinguish the soluble and insoluble components in petroleum ether. (For details, see Results.) The soluble fraction contains retinyl ester but not retinal; the insoluble fraction contains retinal but not retinyl ester. □, solvent front.

45 pmol per eye. In contrast to the diurnal changes in 11-*cis* retinal, the amount increased during the day (to approximately 55 pmol per eye) and decreased during the night (to approximately 35 pmol per eye). In continuous darkness, the amount increased during the first subjective day and continued to increase under continuous darkness. The difference between midday and midnight levels of retinyl ester was about 20 pmol per eye. This value is almost the same as the midday and midnight difference in 11-*cis* retinal content.

The dissected eyes were vacuum-desiccated and washed with petroleum ether. After centrifugation, the petroleum ether extract and the residue were analyzed by the oxime method using HPLC, and the solvent fronts were isolated and saponified. The soluble fraction contained retinyl ester but no retinal. The insoluble fraction contained retinal but no retinyl ester (Fig. 4).

Discussion

Paulsen and Schwemer (1983) reported that the 11-*cis* form of retinal induced

the synthesis of opsin in the retinula cells of the blowfly. T. Suzuki (personal communication) found that light adaptation caused a reduction in the amount of opsin and in the number of chromophores in the eyes of crayfish. These findings indicate a correspondence between the 11-*cis* retinal and opsin contents of the retina, so the amount of 11-*cis* retinal could, therefore, be an index of the total amount of rhodopsin.

The volume of the rhabdom during the night was about three times the volume found during the day, and these cyclic fluctuations continued even under constant darkness (Hariyama *et al.* 1986). The amount of 11-*cis* retinal changed from approximately 10 pmol (midday) to approximately 30 pmol per eye (midnight). These changes persisted under conditions of subsequent continuous darkness. The change in the amount of 11-*cis* retinal corresponds with the change in the volume of the rhabdom, suggesting that the concentration of the chromophore in the retinula cell remains constant during day and night. The change in the amount of 11-*cis* retinal could be related to the renewal of visual pigment accompanying the renewal of the rhabdom membrane. Rhythmicity in the amount of all-*trans* retinal present was observed in L:D 12 h:12 h conditions but not in continuous darkness. The amount increased after the onset of light, but decreased at the onset of darkness. These results suggest that all-*trans* retinal is isomerized from 11-*cis* retinal by the response of rhodopsin to light. The difference in the content of all-*trans* retinal between day and night was smaller than that of 11-*cis* retinal, suggesting that all-*trans* retinal is not a direct precursor of 11-*cis* retinal.

The difference between midday and midnight levels of the retinyl ester, under light–dark conditions, was approximately 20 pmol per eye and the changes were opposite in phase to those of 11-*cis* retinal. This could be taken as evidence that 11-*cis* retinyl ester is a candidate precursor of 11-*cis* retinal in the normal light–dark cycle. Under conditions of continuous darkness, however, the amount of 11-*cis* retinyl ester showed no circadian rhythmicity.

We conclude that the content of 11-*cis* retinal is controlled by an endogenous rhythm, but that the amounts of all-*trans* retinal and of retinyl ester are governed by the prevailing light conditions.

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References

BESHARSE, J. C. (1982). The daily light–dark cycle and rhythmic metabolism in the photoreceptor–pigment epithelial complex. In *Retinal Research*, vol. 1 (ed. N. N. Osborne and G. J. Chader), pp. 81–124. Oxford, New York: Pergamon Press.

HARIYAMA, T., EGUCHI, E. AND MEYER-ROCHOW, V. B. (1986). Diurnal changes in structure and function of the compound eye of *Ligia exotica* (Crustacea, Isopoda). *J. exp. Biol.* **123**, 1–26.

ISONO, K., HARIYAMA, T., KITO, Y. AND TSUKAHARA, Y. (1986). Exogenous and diurnal rhythms of chromophore turnover of visual pigment in the locust analyzed by HPLC. *Neurosci. Res.* (Suppl.) **4**, 1–10.

LAUGHLIN, S. B., MENZEL, R. AND SNYDER, A. W. (1975). Membranes, dichroism and receptor sensitivity. In *Photoreceptor Optics* (ed. A. W. Snyder and R. Menzel), pp. 237–259. Berlin, Heidelberg, New York: Springer-Verlag.

PAULSEN, R. AND SCHWEMER, J. (1983). Biogenesis of blowfly photoreceptor membranes is regulated by 11-*cis* retinal. *Eur. J. Biochem.* **137**, 609–614.

WATERMAN, T. H. (1982). Fine structure and turnover of photoreceptor membranes. In *Visual Cells in Evolution* (ed. J. A. Wastfall), pp. 23–41. New York: Raven Press.

YOUNG, R. W. (1967). The renewal of photoreceptor outer segments. *J. Cell Biol.* **33**, 61–72.

ZECHMEISTER, L. (1962). Cis-trans *Isomeric Carotenoids Vitamins A and Arylpolyenes*. Wien: Springer-Verlag.