Occurrence of retinal and 3-hydroxyretinal in a possible photoreceptor of the silkworm brain involved in photoperiodism

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Summary. Deficiency of dietary carotenoid and vitamin A caused an absence of photoperiodic response of diapause induction in the silkworm, *Bombyx mori*, and an addition of vitamin A to the diet restored the response. By high pressure liquid chromatography (HPLC) a possible photoperiodic receptor of the silkworm brain was found to contain both retinal and 3-hydroxyretinal which are chromophores of insect visual pigments. These pieces of evidence suggest that a retinoid protein might function in the photoperiodic response of the silkworm.
Key words. Photoperiodism; diapause; vitamin A; retinal; 3-hydroxyretinal; brain; *Bombyx mori*.

It is generally accepted that most insects use an extraocular system as the photoreceptor involved in photoperiodism, and that the receptors are brain-centered [1–6]. However, photoreceptor pigments have hardly been exploited as probes for studying the biochemistry of the photoperiodic clock system. Based on action spectra for the photoperiodic response of insects, some authors have suggested that a carotenoprotein might be the photoreceptor pigment [7, 8]. Functional involvement of carotenoid or vitamin A in the photoperiodic response have been recently reported in phytophagous and predacious mites (*Amblyseius potentillae, Tetranychus urticae*) [9–12], two lepidopteran species (*Diatraea grandiosella, Bombyx mori*) [13, 14], and a parasitoid wasp (*Apanteles glomeratus*) [15]. Some authors have discussed the possibility that a rhodopsin-like pigment might be involved in photoperiodic photoreception [10, 14]. However, no evidence for the presence of vitamin A or its derivative in the photoperiodic receptor of the insect brain has been obtained.

Recent progress of insect-vision research has revealed that the chromophore of an insect visual pigment is not retinal but 3-hydroxyretinal, with only a few exceptions [16–19]. Therefore, it is particularly interesting to know whether or not 3-hydroxyretinal and/or retinal occur in the insect brain. In the silkworm, *Bombyx mori*, which shows embryonic diapause dependent on maternal environmental conditions, the photoperiodic photoreceptor was demonstrated to be extraocular [20] and to reside in the larval brain [21]. In addition, possible involvement of a carotenoid (β-carotene) in the photoperiodic induction of diapause in the silkworm was shown by a dietary study [14]. We show here that vitamin A also functions in the photoperiodic response of the silkworm, and that retinal and 3-hydroxyretinal occur in the larval brain.

Materials and methods. A silkworm of a bivoltine hybrid race (Gunpo × Shugyoku) was used as the experimental animal. This insect shows a long-day type of photoperiodic response with a critical day length of 16.5 h during the larval stage [20]. The rearing of silkworms on a carotenoid- and vitamin A-free diet has already been described in detail [22]. The basal diet (called MO diet) was composed mainly of well-defatted soybean powder, cellulose powder, vitamins and growth-promoting ingredients. For the preparation of a vitamin A-supplemented diet 3 mg of vitamin A-palmitate (Nakarai Chemicals, LTD) was added to 10 g of the basal diet (MO). Eggs were incubated at 23 °C in continuous darkness (DD). As a control, some batches of silkworm larvae were reared on an artificial diet (M40) containing powdered mulberry leaves (40%, w/w) [20], and thus a sufficient amount of carotenoids. Some groups of larvae were kept at 25 °C under the light conditions shown in the table. The light intensity of each photophase was adjusted to 100 lux or 1.0 lux. Diapause percentage shows the percentage of females laying diapause eggs in each experiment.

Insects reared on mulberry leaves were kept in darkness for 24 h after eclosion and compound eyes were cut off under dim red light. Brains were extirpated from heads of 5th instar larvae reared on mulberry leaves. Twenty compound eyes were homogenized with phosphate buffer, and the homogenate was illuminated with intense white light to establish photoequilibrium of visual pigments. Oxime formation and extraction were carried out according to Suzuki and Makino-Tasaka [23] with a slight modification. The extraction of oximes was repeated twice with dichloromethane and *n*-hexane. The extract was evaporated and dissolved with solvents for HPLC. About 400 larval brains were used for analysis of retinals. Oxime formation and extraction was carried out as above. HPLC was performed with a Shimadzu IC-5A HPLC system equipped with a Zorbax-SIL column according to Tanimure et al. [19]. 3-Hydroxyretinal oxime and retinal oxime were detected by monitoring the absorbance at 350 nm. Authentic 3-hydroxyretinal (provided by Japan Roche Co. Ltd.) was dissolved in methanol, photoisomerized, and converted to oxime forms.

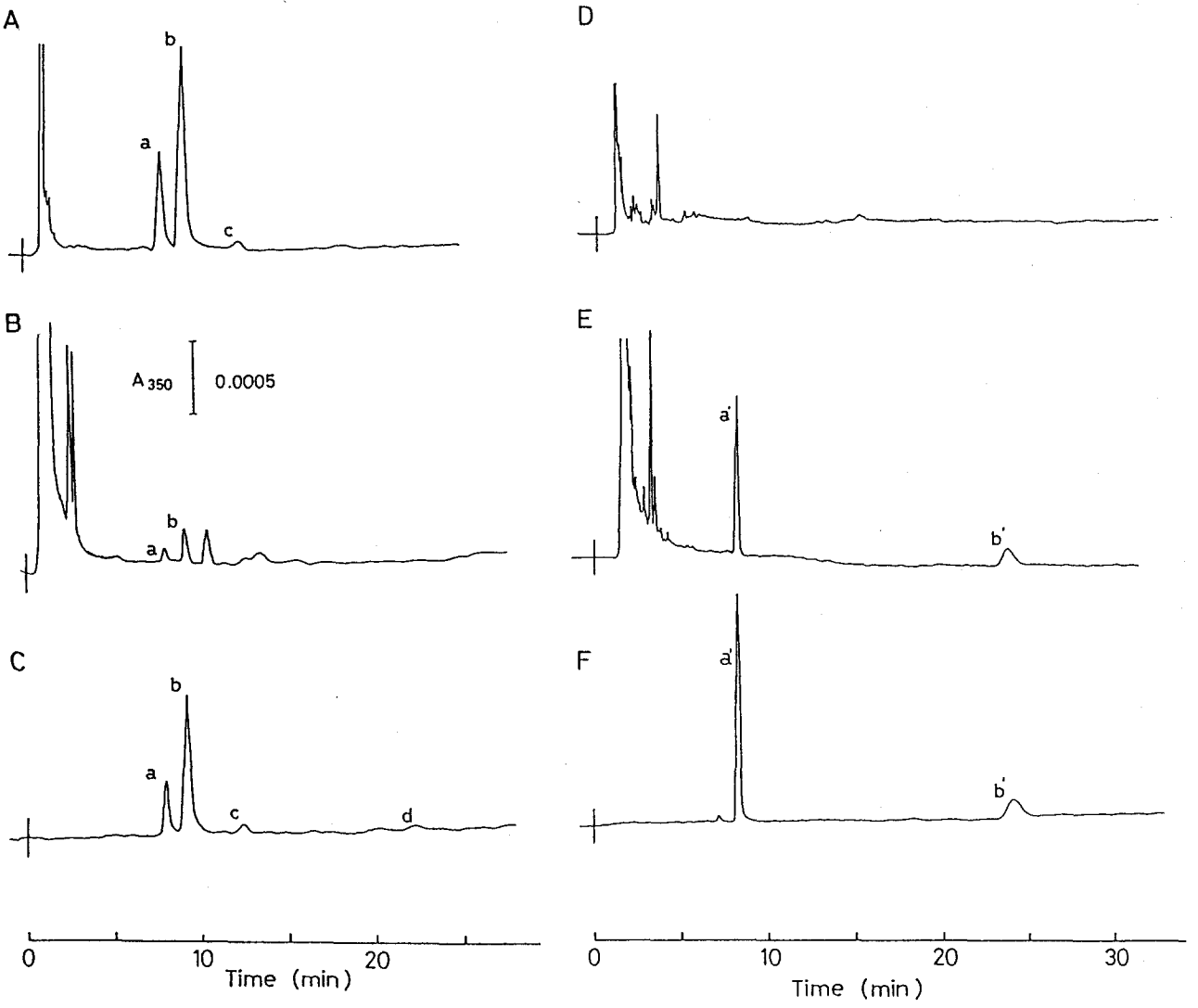
Results and discussion. A vitamin A-free artificial diet, brought about a severe vitamin A-deficiency in the silkworm [22]; the effect of this deficiency, and of supplementing the diet with vitamin A, on the diapause incidence was investigated.

The absence of photoperiodic signals in larvae kept during 4th and 5th instar in DD condition resulted in high diapause incidences irrespective of diets, although young larvae were reared under LD 20:4 at high intensity of light (100 lux) (table, 1st column).

Even when silkworm larvae were reared on a diet which was severely deficient in carotenoid and vitamin A, young larvae still maintained their visual photosensitivity owing to the presence of these compounds derived from eggs [22]. But grad-

Effect of a deficiency of dietary vitamin A, and photoperiod, on diapause incidence of the silkworm

Dietary condition	Photoperiodic conditions during larval stage					
	1–3 LD 20:4 (100 lux) 4,5 DD		LD 20:4 (100 lux)		LD 20:4 (1.0 lux)	
	Diapause %	n	Diapause %	n	Diapause %	n
M40 (carotenoid-rich)	100	39	0	44	30	20
MO (carotenoid-depleted)	78	18	9	34	75	61
MO + vitamin A	100	11	8	13	26	34



HPLC elution profiles of 3-hydroxyretinal oximes and retinal oximes. *A* 3-Hydroxyretinal oximes isolated from 6 compound eyes; *B* 3-Hydroxyretinal oximes from 400 larval brains; *C* Authentic 3-hydroxyretinal oximes; peak a: *syn* 11-*cis* 3-hydroxyretinal oxime; peak b: *syn* all-*trans* 3-hydroxyretinal oxime; peak c: *anti* all-*trans* 3-hydroxyretinal oxime; peak d: *anti* 11-*cis* 3-hydroxyretinal oxime. *D* Retinal oximes from 6 compound eyes; *E* Retinal oximes from 350 larval brains; *F* Authentic retinal oximes; peak a': *syn* all-*trans* retinal oxime; peak b': *anti* all-*trans* retinal oxime. Solvents: 20% ethyl acetate and 15% dichloromethane in *n*-hexane for 3-hydroxyretinal oxime, and 7% ether and 0.075% ethanol in *n*-hexane for retinal oxime. Flow rate: 3 ml/min.

ual decrease and eventual loss of the photosensitivity occurred in the vitamin A-deprived larvae by its dilution [22]. If these compounds operate in the photoperiodic induction, an increase in the diapause incidence even under a long-day regimen should occur because of a failure to perceive photoperiodic signals in later instars.

When larvae were reared under LD 20 : 4 (100 lux) throughout the larval stage, most of the resultant females responded to a long-day photoperiodic regime and produced non-diapause eggs, regardless of the dietary conditions (table, 2nd column). However, under LD 20 : 4 with the photophase of low light intensity (1.0 lux) an effect of vitamin A-deficiency on the diapause incidence was observed: only 30% of the resultant females reared on M40 diet laid diapause eggs, whereas 75% reared on MO diet laid diapause eggs ($p < 0.005$). Addition of vitamine A-palmitate to MO diet reduced the diapause incidence to that of the group reared on M40 diet; dietary vitamin A restored the photoperiodic response to the long-day regime ($p < 0.005$) (table, 3rd column). These pieces of evidence suggest that maternally-derived vitamin A (or carotenoid) still functions in photoperiodic perception at the intensity of 100 lux. The concentration appears, however, to be too small to detect sufficiently the photoperiodic cycles under the dim light condition (1.0 lux). As vitamin A is not metabolically converted into carotenoid in animals, these results suggest functional involvement of retinoid compounds in photoperiodic perception in the silkworm.

Next, we tried to identify 3-hydroxyretinal and retinal in a possible photoperiodic receptor of the larval brain [21]. The figure shows chromatograms obtained by HPLC analysis of adult compound eyes and larval brains. In addition of *cis* and *trans* isomers about C=C bonds, retinal oximes exist in *syn* and *anti* conformations at the C=N linkage [23]. In light-adapted compound eyes peaks corresponding to *syn* 11-*cis* and *syn* and *anti* all-*trans* 3-hydroxyretinal oximes were detected (fig., A). All-*trans* isomer was dominant in light-adapted eyes as shown in the figure, whereas 11-*cis* isomer was dominant in dark-adapted eyes (data not shown). No retinal oxime was found in compound eyes (fig., D).

In the brain of 5th instar larvae both 3-hydroxyretinal and retinal occurred (fig., B, E). Peaks corresponding to *syn* 11-

cis and *syn* all-*trans* 3-hydroxyretinal oximes were observed in the chromatogram obtained by analysis of about 400 larval brains (fig, B). Peaks of *anti* 11-*cis* and *anti* all-*trans* 3-hydroxyretinal oxime were not clear in this chromatogram possibly because of the minor amount. In addition to 3-hydroxyretinal, *syn* and *anti* all-*trans* retinal oximes were detected in the larval brain (fig. E). Contents of 3-hydroxyretinal and retinal were estimated as follows; one compound eye contains about 5 pmol of 3-hydroxyretinal and one larval brain contains about 0.01 pmol of 3-hydroxyretinal and about 0.03 pmol of retinal, respectively.

3-Hydroxyretinal is reported to be the chromophore of the insect rhodopsin[16–19]. The present HPLC analysis demonstrates that the *Bombyx* compound eye contains 3-hydroxyretinal. Geometrical isomers of 3-hydroxyretinal are also found in the larval brain, while only all-*trans* retinal was detected in this organ. Though we have not examined the reversible interchange in the amount between these isomers, which might reflect the photoconversion of a chromophore in the photoreceptor pigment in the brain, the presence of both 11-*cis* and all-*trans* isomers in the brain suggests that 3-hydroxyretinal in the larval brain is also the chromophore of a functional pigment involved in the photoperiodic response. A plausible assumption is that the pigment actually involved in photoperiodic light perception forms a complex with an apoprotein. It is unlikely that free 3-hydroxyretinal and retinal, the absorption spectra of which fall mainly in the UV-light region, function in photoreception. The photoreceptor pigment is probably a retinoid protein complex, with its main absorption in the blue-green region[7]. Further identification of the photopigment active in the photoperiodic response of the silkworm is now under way in our laboratory.

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1 Williams, C. M., and Adkisson, P. L., Biol. Bull. *127* (1964) 511.
2 Claret, J., Ann. Endocr. (Paris) *27* (1966) 311.
3 Kono, Y., Jap. J. appl. Ent. Zool. *17* (1973) 203.
4 Seuge, J., and Veith, K., J. Insect Physiol. *22* (1976) 1229.
5 Bowen, M. F., Saunders, D. S., Bollenbacher, W. E., and Gilbert, L. I., Proc. natl Acad. Sci. USA *81* (1984) 5881.
6 Lees, A. D., J. exp. Biol. *41* (1964) 119.
7 Lees, A. D., J. Insect Physiol. *11* (1981) 761.
8 Adams, A. J., J. Insect Physiol. *32* (1986) 71.
9 Van Zon, A. Q., Overmeer, W. P. J., and Veerman, A., Science *213* (1981) 1131.
10 Veerman, A., Overmeer, W. P. J., van Zon, A. Q., de Boer, J. M., de Waard, E. R., and Huisman, H. O., Nature *302* (1983) 248.
11 Veerman, A., and Helle, W., Nature *275* (1978) 234.
12 Veerman, A., Physiol. Ent. *5* (1980) 291.
13 Takeda, M., Ph. D. Thesis, Univ. Missouri 1978.
14 Shimizu, I., and Kato, M., Photobiochem. Photobiophys. *7* (1984) 47.
15 Veerman, A., Slagt, M. E., Alderliste, M. F. K., and Veenendaal, R. L., Experientia *41* (1985) 1194.
16 Vogt, K., Z. Naturforsch. *38c* (1983) 329.
17 Vogt, K., and Kirschfeld, K., Naturwissenschaften *71* (1984) 211.
18 Vogt, K., Z. Naturforsch. *39c* (1984) 196.
19 Tanimura, T., Isono, K., and Tsukahara, Y., Photochem. Photobiol. *43* (1986) 225.
20 Shimizu, I., J. Insect Physiol. *28* (1982) 841.
21 Hasegawa, K., and Shimizu, I., J. Insect Physiol. (1987) in press: Using isolated brain-subesophageal ganglion complex of the silkworm, in vivo and in vitro photoperiodic induction of diapause were performed, and it was demonstrated that components (photoreceptor, clock, and counter) of the photoperiodic clock are located in the brain.
22 Shimizu, I., Kitabatake, S., and Kato, M., J. Insect Physiol. *17* (1981) 593.
23 Suzuki, T., and Makino-Tasaka, M., Analyt. Biochem. *129* (1983) 111.

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Age-related perseveration of the precopulatory behaviour in male *Drosophila melanogaster*

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Summary. In response to an interruption of their courtship, males of *D. melanogaster* exhibit a lasting sexual arousal (up to 30–60 min), expressed behaviourally by characteristic wing displays. A study of this effect centered on two 'memory mutants' of different ages suggests that it can be related to an ageing-dependent perseveration, rather than to modifications in memory processing.
Key words. Drosophila melanogaster; sexual behaviour; ageing; memory mutants.

The courtship of male *Drosophila melanogaster*[3] is normally performed in close contact with the female. However, in small observation chambers it can commonly be observed that, when a female moves off, the male does not always follow immediately. Remaining behind, he subsequently performs a few courtship movements in her absence before moving off. Brief wing displays can sometimes be seen as the male moves around the chamber and before he makes contact with the female again.

Such observations strongly suggest that, following stimulation, a male's sexual arousal does not immediately decline. It might be possible to regard this phenomenon as expressing a sexual 'central excitatory state', analogous to that described for food in *Phormia*[4] and in *Drosophila*[5] itself. We have now begun to study this persistent courtship in order to discover its extent and control.

To make reasonable measurements, we require a situation in which a male can be given limited contact with a female, who is then removed whilst observations continue on the male. We used an observation chamber made in two adjacent circular compartments, each 25 mm in diameter and 5 mm deep, separated by an opaque moveable partition. Our basic procedure was to introduce a young, virgin female (24–36 h from eclosion) into one compartment and a mature, but inexperienced male into the other. The partition was kept closed whilst the male was allowed 3 min to settle down before his locomotor activity was measured each minute, for a further 5 min. This was done by dividing the floor of the compartment into quadrants and counting the number of quadrants entered with all six legs.

Any wing movements related to courtship (i.e. scissoring or vibration) were also recorded. At the end of this period, the