The Role of Retinal Photoisomerase in the Visual Cycle of the Honeybee

W. CLAY SMITH and TIMOTHY H. GOLDSMITH

From the Department of Biology, Yale University, New Haven, Connecticut 06511

ABSTRACT The compound eye of the honeybee has previously been shown to contain a soluble retinal photoisomerase which, in vitro, is able to catalyze stereospecifically the photoconversion of all-*trans* retinal to 11-*cis* retinal. In this study we combine in vivo and in vitro techniques to demonstrate how the retinal photoisomerase is involved in the visual cycle, creating 11-*cis* retinal for the generation of visual pigment. Honeybees have ~2.5 pmol/eye of retinal associated with visual pigments, but larger amounts (4–12 pmol/eye) of both retinal and retinol bound to soluble proteins. When bees are dark adapted for 24 h or longer, >80% of the endogenous retinal, mostly in the all-*trans* configuration, is associated with the retinal photoisomerase. On exposure to blue light the retinal is isomerized to 11-*cis*, which makes it available to an alcohol dehydrogenase. Most of it is then reduced to 11-*cis* retinol. The retinol is not esterified and remains associated with a soluble protein, serving as a reservoir of 11-*cis* retinoid available for renewal of visual pigment. Alternatively, 11-*cis* retinal can be transferred directly to opsin to regenerate rhodopsin, as shown by synthesis of rhodopsin in bleached frog rod outer segments. This retinaldehyde cycle from the honeybee is the third to be described. It appears very similar to the system in another group of arthropods, flies, and differs from the isomerization processes in vertebrates and cephalopod mollusks.

INTRODUCTION

In most species, the pathway by which 11-*cis* retinal is made available for the synthesis of visual pigments has been one of the last aspects of the visual cycle to be understood. In the squid, an invertebrate with a thermally stable metarhodopsin, 11-*cis* retinal is formed by retinochrome, an intrinsic membrane protein that binds all-*trans* retinal and mediates its photoisomerization to the 11-*cis* configuration (Hara and Hara, 1972). In vertebrates, however, the critical reaction occurs in the pigment epithelium, where 11-*cis* retinol is formed in the dark from all-*trans* retinyl esters, driven by the energy liberated on hydrolysis (Fulton and Rando, 1987; Deigner et al., 1989; Rando et al., 1989; Trehan et al., 1990). In this paper we describe a third system, that of the honeybee (*Apis mellifera*), in which the visual cycle is built around a soluble retinal photoisomerase (RalPI).

An unusual feature of the honeybee is that 80% of its retinal can be extracted with

Address reprint requests to Dr. Timothy H. Goldsmith, Kline Biology Tower, P.O. Box 6666, New Haven, CT 06511-8112.

aqueous buffers bound to protein absorbing maximally at 440 nm (Goldsmith, 1958). More recent studies have shown that this aqueous-extractable retinal is bound via a Schiff-base linkage (Pepe et al., 1982). When irradiated with violet light in vitro, the protein stereospecifically catalyzes the formation of 11-*cis* retinal (Schwemer et al., 1984). Such photoisomerase activity is reminiscent of the cephalopod membrane-bound retinochrome system (Hara and Hara, 1972), which generates 11-*cis* retinal for use in visual pigment (Ozaki et al., 1987; Hara, 1988).

There is, however, no information on the role of this soluble RalPI in the visual cycle of the bee. Furthermore, Goldsmith and Warner (1964) found that in bees retinol increased during light adaptation. The significance of this observation in an animal with a presumably stable metarhodopsin has not been clear. Due to the large amount of retinal photoisomerase in the compound eye, the honeybee offers the advantage of using both in vivo and in vitro techniques to analyze the visual cycle. In this report we demonstrate the role of the RalPI in vivo and examine its probable relation to other steps in the biochemical pathway for cycling retinoids. The only other insect visual cycle to have received any detailed attention is that of the fly (Schwemer, 1983, 1984, 1988, 1989; Isono et al., 1988), where there is also evidence for the presence of a violet-sensitive retinoid photoisomerase in vivo.

MATERIALS AND METHODS

Extraction and Identification of Retinoid Isomers

Samples were extracted according to the oxime method of Groenendijk et al. (1979, 1980). 1 M hydroxylamine (pH 6.5) and methanol were added in equal volumes to each sample and mixed. Retinoids were recovered by vortexing and centrifuging with two washes of methylene chloride. This extract was evaporated to dryness with a nitrogen stream and the residue was taken up in 1% isopropanol in hexane.

Retinal oxime and retinol isomers were separated on a normal-phase silica column (Rainin Microsorb, 5 μm; Rainin Instrument Co. Inc., Woburn, MA) using an isocratic eluant of 9% dioxane in hexane at 1 ml/min (method of Goldsmith et al., 1986). Retinal oxime isomers were detected by monitoring absorbance at 357 nm; retinol isomers were detected by absorbance at 325 nm and confirmed by monitoring fluorescence. The system was calibrated using known quantities of retinal oxime and retinol standards (Sigma Chemical Co., St. Louis, MO).

Retinyl esters were recovered from the column outflow between the solvent front and the first oxime peak. This sample was evaporated to dryness and saponified in 3% KOH in methanol (30 min, 30°C). The retinol generated by this procedure was extracted with several washes of hexane, washed with distilled water to remove traces of KOH, and then analyzed for retinol isomers as described above.

Irradiation In Vivo

Live honeybees (*Apis mellifera*) were obtained from a local hive and brought to the laboratory. The bees were kept up to 8 d in a small cage of Plexiglas and aluminum screening on an 18-h light, 6-h dark cycle with sugar water (15–20% sucrose) continuously available.

Illumination was provided by a Fiber-Lite (Dolan-Jenner Industries, Inc., Woburn, MA) fiber optic illuminator with a tungsten halogen lamp (150 W, 21 V). Various filters provided the desired illumination wavelengths, and light energies were measured with a calibrated photodiode (United Detector Technology Inc., Culver City, CA) (violet: Spectrocoat 4403C [Optics Technology Inc., Palo Alto, CA], 440-nm interference filter with a 10-nm half-bandwidth, 7.6 × 10^{13} quanta·cm^{-2}·s^{-1} at corneal surface; green: Spectrocoat 5103C, 510-nm interference filter

with a 10-nm half-bandwidth, 2.7×10^{14} quanta·cm^{-2}·s^{-1} at corneal surface; orange: Corning CS3-66 long-pass, 1% transmission at 555 nm, 1.7×10^{16} quanta·cm^{-2}·s^{-1} between 555 and 700 nm at corneal surface). The cage was evenly illuminated and the bees were allowed to move freely about the cage.

Retinoids were extracted as follows. 10–15 bees were removed from the cage and immediately frozen on dry ice. All subsequent procedures were conducted in the dark or in dim red lighting. The heads were bisected dorsoventrally with a razor blade and crushed in 1 ml cold insect Ringer's solution (Pringle, 1938) with a glass rod. The homogenate was centrifuged for 3 min (17,000 *g*), the supernatant was removed, and the pellet was suspended in 1 ml Ringer's and centrifuged. The supernatants were combined, and both the pellet and supernatant fractions were assayed for retinoids as described previously. Statistical comparison of averaged data was conducted using Student's *t* test.

Extraction of RalPI

A crude extract of RalPI was obtained by crushing the desired number of bee heads in cold insect Ringer's solution (1 ml/25 heads) with a mortar and pestle, and centrifuging as above. In some experiments the extract was filtered with a 0.3-μm filter (type PH; Millipore Continental Water Systems, Bedford, MA). Desired final volumes were obtained by ultrafiltration (Amicon Diaflo [Amicon Corp., Danvers, MA] with Millipore ultrafiltration membrane, 10-kD exclusion, 55 psi) under nitrogen. The extract could be enriched with 11-*cis* retinal by irradiation with blue-green light (510-nm narrow band interference filter as above) for 1 h on ice.

RalPI was purified using a modification of the method of Schwemer et al. (1984). In brief, the crude extract was fractionated by nondenaturing electrophoresis on 7% polyacrylamide gel and the band containing RalPI was eluted (Elutrap; Schleicher & Schuell, Inc., Keene, NH). The protein was further purified using two passes on an anion exchange column (TSK-DEAE-5-PW; Bio-Rad Laboratories; Cambridge, MA 0.1–0.5 M NaCl gradient, first pass; 0.2–0.35 M NaCl gradient, second pass) followed by size-exclusion chromatography (TSK 250; Bio-Rad Laboratories) as a final purification step. Homogeneity was assayed by 13.5% SDS-PAGE, which showed a single band by silver stain with an apparent molecular mass of 27 kD.

Regeneration of Frog Visual Pigment

Frog (*Rana pipiens*) rod outer segments (ROS) were obtained by the method of Fong et al. (1982), using a sucrose density gradient. For regeneration experiments, ROSs from two retinas were bleached with yellow light (30 min, Corning CS3-68 long pass, 1% transmission at 525 nm to prevent isomerization of free retinal) on ice in the presence of 1 mM hydroxylamine. Bleached ROSs were pelleted (12,000 *g*, 30 min) and washed twice with 67 mM phosphate buffer (pH 7.0).

Bleached ROSs were suspended in crude bee RalPI extract from 500 bees, irradiated with green light to enrich the 11-*cis* retinal concentration (3 nmol), and gently stirred on ice for 1 h. Alternatively, ROSs were suspended in purified RalPI obtained from 3,000 bees (also containing ~3 nmol 11-*cis* retinal). After incubation, ROSs were pelleted and washed with phosphate buffer twice to remove the soluble bee retinoids. Visual pigment extracts of ROSs were made with 1% digitonin (Sigma Chemical Co.) or 1% L1695 (Mitsubishi-Kasei Co., Tokyo, Japan), extracting for 1 h. The extracts were cleared by centrifugation (19,000 *g*, 30 min) and absorption spectra were measured on a dual-beam spectrophotometer (Shimadzu UV-260, Columbia, MD). Bleaching spectra were obtained by irradiation with yellow light (Kodak Wratten #22 long-pass, 1% transmission at 550 nm) in the presence of 1 mM hydroxylamine, and difference spectra were calculated.

Bleached frog ROSs were also incubated with a phosphate buffer suspension of lipid vesicles containing 11-*cis* retinal. Vesicles were prepared by sonicating 2.5 mg/ml L-α phosphatidylcholine (type V-E; Sigma Chemical Co.) in phosphate buffer. 11-*cis* retinal (HPLC-purified) was

added to the vesicles by gently layering the retinoid in hexane on the vesicle suspension, evaporating the hexane with nitrogen, and then briefly vortexing to suspend the retinoid in the vesicles. Frog ROSs and opsin were used because there is not a method for readily obtaining bee opsin in sufficient quantity for these experiments, and there is no reason to expect the metarhodopsin in rhabdoms to exchange its chromophore.

Frog Alcohol Dehydrogenase

Whole frog eyes were homogenized in cold distilled water and centrifuged (27,000 *g*, 1 h), and the supernatant was collected to obtain alcohol dehydrogenase activity. Endogenous, free retinoids were removed by washing the extract with equal volumes of hexane until HPLC analysis of an aliquot showed that little retinoid was present (usually three to five washes).

All of the above procedures were conducted in darkness or in dim red lighting unless otherwise stated.

RESULTS

Changes in Retinoids In Vivo

Effects of prolonged light and dark adaptation. Bees were dark adapted or light adapted for 24 h, and retinoids were extracted for analysis (Fig. 1). Irradiation caused three significant changes: (*a*) Retinal in the pellet (most of which is presumably bound to opsins) shifted from 11-*cis* as the more abundant isomer to all-*trans* ($P < 0.01$), as would be expected for the establishment of photosteady states between the several visual pigments (Menzel and Blakers, 1976; see also below) and their metarhodopsins. (*b*) Aqueous-extractable all-*trans* retinal declined precipitously (82% drop, $P \ll 0.01$), with a concomitant increase in retinol, primarily in the 11-*cis* configuration. Total retinol increased by 4.4 pmol/eye, whereas retinal decreased by 6.1 pmol/eye. Retinyl esters were present at 0.22 pmol/eye (74% all-*trans;* 26% 11-*cis*), but these levels did not change in response to illumination, either in absolute

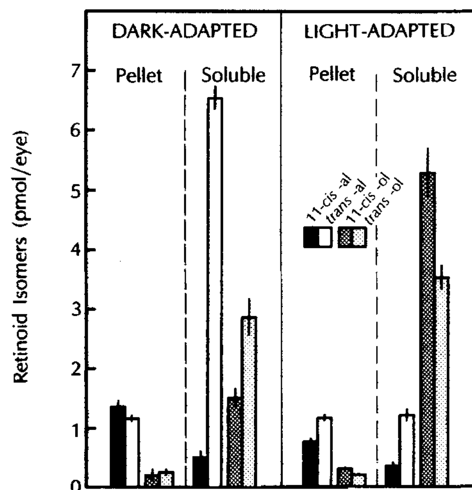

FIGURE 1. Retinoid isomers in pellet and aqueous-soluble fractions after 24 h dark and light adaptation. Error bars, SEM; $n = 6$.

quantities or in relative isomeric composition ($P \gg 0.1$). (*c*) There was thus a net loss of retinoids (1.7 pmol/eye in this experiment) in going from the dark- to the light-adapted state.

To investigate the possibility that during light adaptation the missing retinoid is transported out of the head, whole bodies of light-adapted and dark-adapted bees were lyophilized and extracted for retinoid analysis. No isomers of retinal, retinol, or retinyl esters were detected in the thorax or abdomen of either dark-adapted or

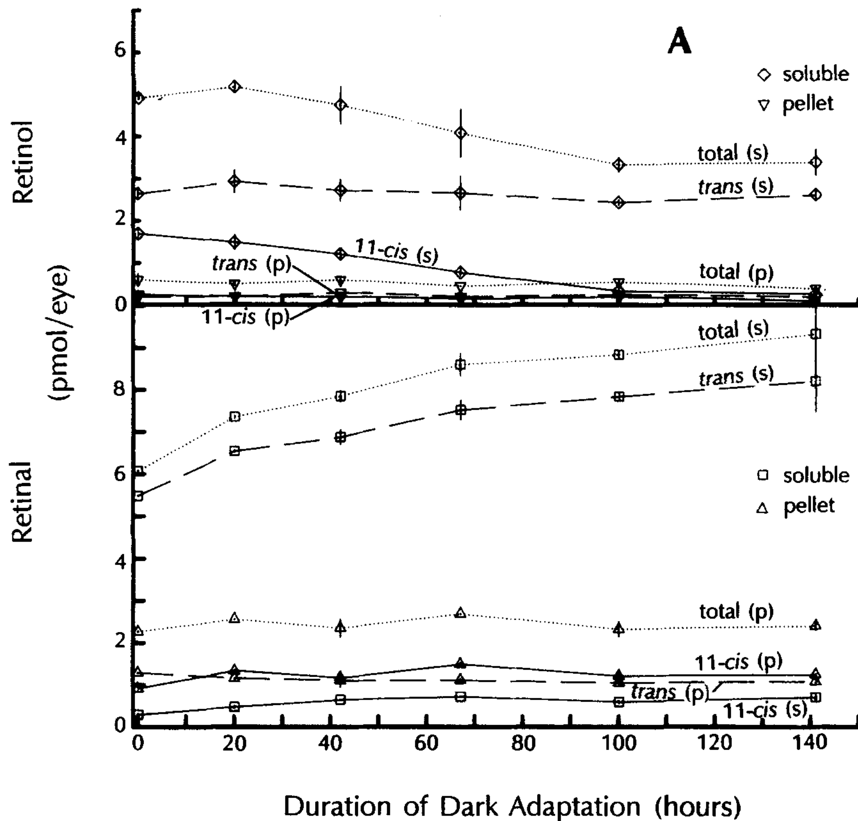

FIGURE 2. Changes in retinoid isomers with extended dark adaptation (*A*) and light adaptation (*B*). Before dark adaptation bees were adapted to room lighting. Before light adaptation bees were dark adapted overnight. Error bars, SEM; $n = 4$.

light-adapted bees. The fate of the balance of the retinoid (1.7 pmol/eye) therefore remains unresolved.

To examine the time course, bees were dark or light adapted for 5 d, and retinoids were assayed daily (Fig. 2). Several trends are readily apparent. First, during extended dark adaptation total retinal steadily increased, due almost exclusively to the increase of all-*trans* retinal in the aqueous fraction (Fig. 2 *A*, lower panel). Retinol changed significantly ($P < 0.05$) only in the 11-*cis* isomer, which declined throughout

dark adaptation (Fig. 2 *A*, upper panel). Extended dark adaptation resulted in a net gain of 1.7 pmol/eye of retinoid.

The effects of light adaptation after overnight dark adaptation are shown in Fig. 2 *B* for another group of bees. After the dramatic initial changes that were observed with 24-h light adaptation, (i.e., 6.3 pmol/eye decrease in soluble all-*trans* retinal [Fig. 2 *B*, lower panel] and 3.8 pmol/eye increase in soluble 11-*cis* retinol [Fig. 2 *B*, upper panel], extended light adaptation had the following effects. Soluble isomers of retinol

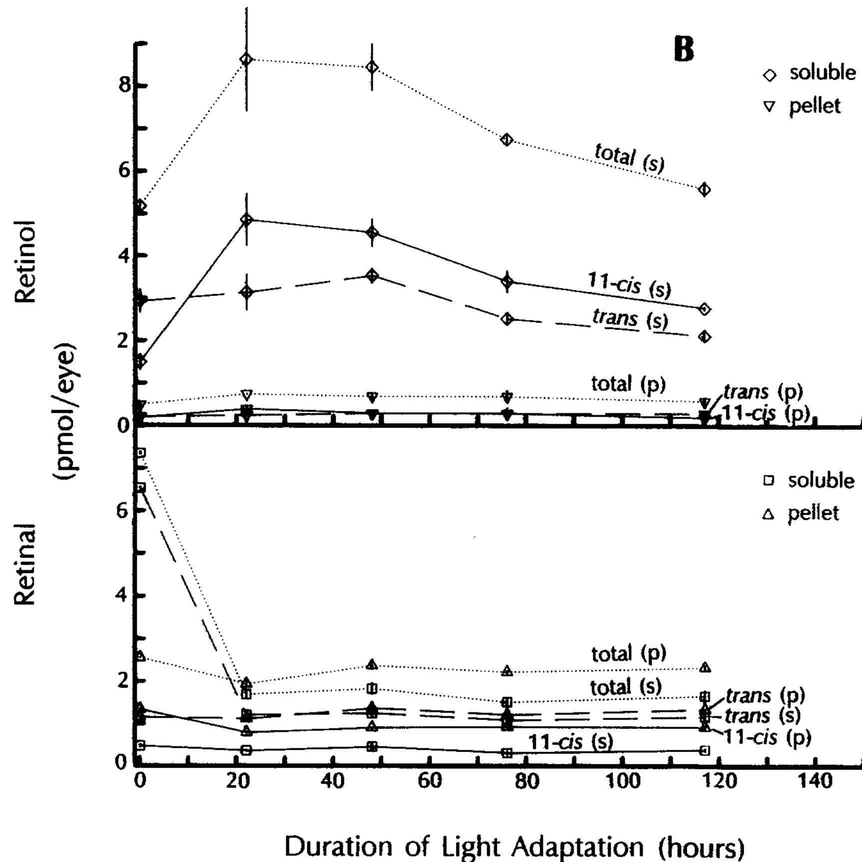

FIGURE 2 (continued)

(11-*cis* and all-*trans*) declined steadily (Fig. 2 *B*, upper panel). In contrast, all isomers of retinal remained essentially constant (Fig. 2 *B*, lower panel). After 5 d of light adaptation there was a net loss of 5.4 pmol/eye of retinoid ($P \ll 0.01$). When the remaining bees were subsequently returned to the dark, there was a recovery of retinoid to the original level ($t_{1/2}$ = 2 d, data not shown).

Because there are negligible amounts of retinol in the pellet fractions and the

isomeric composition did not change in response to light, these levels were not determined in subsequent experiments.

Selective adaptation with different wavelengths: rationale. These experiments clearly show that in vivo there are prominent changes in retinoids under different light-adapting conditions. In an effort to distinguish between the effects of visual pigments and the RalPI in driving these changes, the visual cycle was dissected using selected wavelengths. The rationale is as follows. The ommatidia of worker bees have three visual pigments (P350, P440, P540; Menzel and Blakers, 1976; Bernard and Wehner,

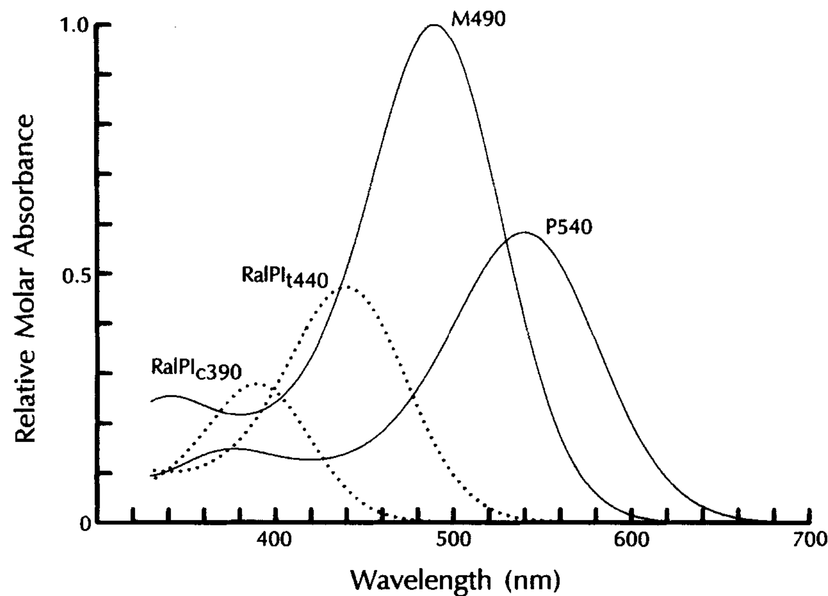

FIGURE 3. Spectral position and relative molar extinction of the two principal photopigments in the compound eye of worker honeybees. P540 and its metarhodopsin (M490) are modeled by a polynomial template developed by G. D. Bernard, Washington University, St. Louis, MO (unpublished). Dotted curves show the absorption spectra of the bee photoisomerase containing all-*trans* retinal ($RalPI_{t440}$) and 11-*cis* retinal ($RalPI_{c390}$), using the same polynomial template as a model. The extinction coefficients of the retinal photoisomerase are from Pepe et al. (1982), and those of rhodopsin/metarhodopsin are estimated from Bertrand et al. (1979) using $\epsilon = 43{,}000\ M^{-1}\ cm^{-1}$ for rhodopsin.

1980), but P540 accounts for half of the receptor classes over most of the compound eye. The absorbance spectra of P540 and its metarhodopsin (M490) are represented in Fig. 3 by a polynomial function (devised by G. D. Bernard, Washington University, St. Louis, MO; the heights of the curves indicate the relative molar absorbance). The RalPI is also a major photopigment, accounting for up to 80% of the total photopigments. The absorbance spectra of the photoisomerase with all-*trans* retinal ($RalPI_{t440}$) and 11-*cis* retinal ($RalPI_{c390}$) (Pepe et al., 1982) are also shown in Fig. 3.

Light with wavelengths longer than 565 nm will effectively drive more than

two-thirds of P540 to M490, with little or no effect on RalPI or the other visual pigments. Violet light (440 nm), on the other hand, will be very effective in converting the chromophore of RalPI to 11-*cis*, whereas the photosteady state between P540 and M490 will favor P540. Although some all-*trans* retinal should be formed by the conversion of the visual pigment P440 to its metarhodopsin at 490 nm (spectra not shown), the amount of this pigment is small by comparison with $RalPI_{t440}$. Conversely, some small amount of 11-*cis* retinal could be formed from the blue-sensitive metarhodopsin of P350. In summary, changes in retinoid levels driven by 565 nm and longer wavelengths should largely be due to conversion of P540 to M490, while changes driven by 440 nm should be dominated by conversion of $RalPI_{t440}$ to $RalPI_{c390}$.

Shifts in retinoids after selective conversion of visual pigment. For visual pigment conversion, dark-adapted bees were irradiated with orange light (wavelengths >565 nm) for 74 h, and samples for retinoid analysis were removed periodically during irradiation (Fig. 4). As expected, orange irradiation caused an initial shift in the aqueous-insoluble (pellet) retinal from 46% all-*trans*, 54% 11-*cis* to 78% all-*trans*, 22% 11-*cis* (Fig. 4, lower panel; $P \ll 0.01$). We interpret this as reflecting the conversion of rhodopsin to metarhodopsin, principally P540 to M490. As irradiation continued, insoluble 11-*cis* retinal (rhodopsin level) stabilized after 24 h and was maintained for the remaining 50 h of irradiation. Insoluble all-*trans* retinal (metarhodopsin level),

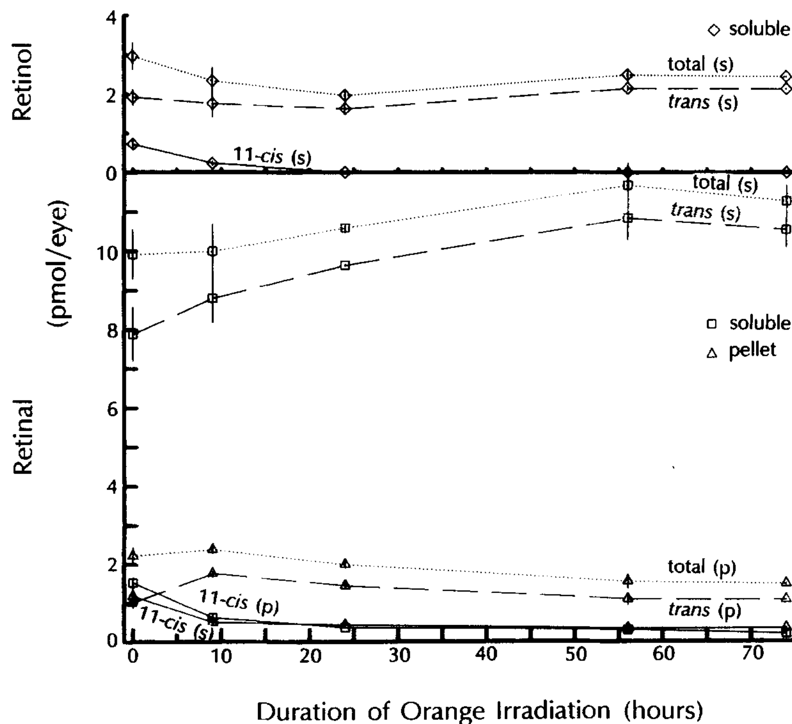

FIGURE 4. Changes in retinoid isomers with adaptation to orange light (wavelengths >565 nm). Before irradiation bees were dark adapted overnight. Error bars, SEM; $n = 4$.

however, showed a steady decline ($P \ll 0.01$) after the initial rise at light onset. In the soluble fraction retinal increased ($P \ll 0.01$) for up to 60 h of irradiation due to the increase in all-*trans* retinal ($RalPI_{t440}$). In contrast, soluble 11-*cis* retinal declined throughout orange irradiation, faster initially and then more slowly after 10 h ($P < 0.05$).

Analysis of aqueous-soluble isomers of retinol showed an interesting decline in the 11-*cis* isomer to 0 pmol/eye within 24 h of irradiation (Fig. 4, upper panel; $P \ll 0.01$). The all-*trans* isomer remained essentially constant throughout irradiation ($P > 0.1$). Retinoid totals tally closely, to within 0.2 pmol/eye (pellet retinal decreased 0.7 pmol/eye, supernatant retinal increased 1.4 pmol/eye, supernatant retinol decreased 0.5 pmol/eye).

Shifts in retinoids after photoactivation of the RalPI. For stimulation of the RalPI, dark-adapted bees were irradiated with violet light for 73 h, with samples taken periodically for retinoid analysis (Fig. 5). There was little change in the retinal isomers in the pellet; there was more 11-*cis* than all-*trans* throughout the experiment.

Major changes took place in the soluble fraction, however. All-*trans* retinal ($RalPI_{t440}$) declined to a level at 72 h that was 47% of the original, dark-adapted value ($P \ll 0.01$). Soluble 11-*cis* retinal, on the other hand, showed essentially no change ($P > 0.1$). Soluble retinol showed a change in the 11-*cis* isomer only, which more than doubled from its dark-adapted level (Fig. 5, upper panel; $P < 0.01$). The

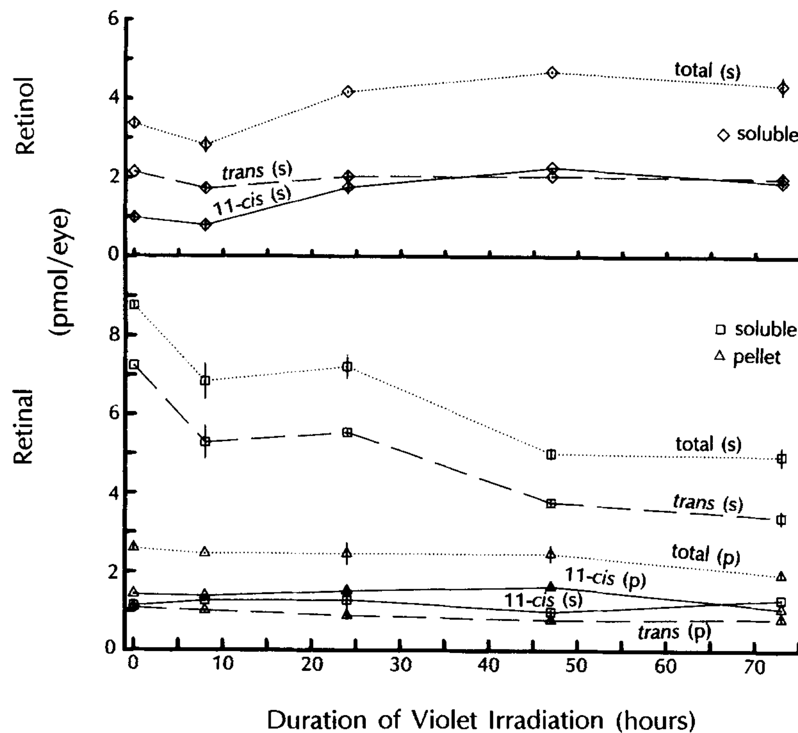

FIGURE 5. Changes in retinoid isomers with adaptation to violet light (440 nm). Before irradiation bees were dark adapted overnight. Error bars, SEM; $n = 4$.

increase reached a plateau after ~40 h of irradiation. The all-*trans* isomer of retinol showed no significant net change; a slight decrease at initial illumination was subsequently offset by a small but gradual increase.

In terms of total retinoids, retinal decreased 4.3 pmol/eye and retinol increased only 1.0 pmol/eye. As in the first series of experiments discussed, adaptation at wavelengths absorbed by $RalPI_{t440}$ not only leads to the formation of 11-*cis* retinol but also to a net loss of retinoid, in this experiment 3.3 pmol/eye. Analysis of retinyl esters in these samples showed only 0.25 pmol/eye, mostly in the all-*trans* configuration (95%). This level did not change significantly ($P > 0.1$) during violet illumination.

Absence of dark regeneration of visual pigment. Orange light should convert a substantial fraction of the visual pigment P540 to metarhodopsin. As expected, orange light caused the accumulation in the pellet of all-*trans* at the expense of 11-*cis* retinal (Fig. 4). If bees are able to regenerate visual pigment in the dark, prolonged dark adaptation should reverse this shift in the isomeric composition of the aqueous-insoluble retinal. Dark-adapted bees were irradiated for 24 h with orange light and subsequently kept in the dark for 4 d. Results are summarized in Fig. 6.

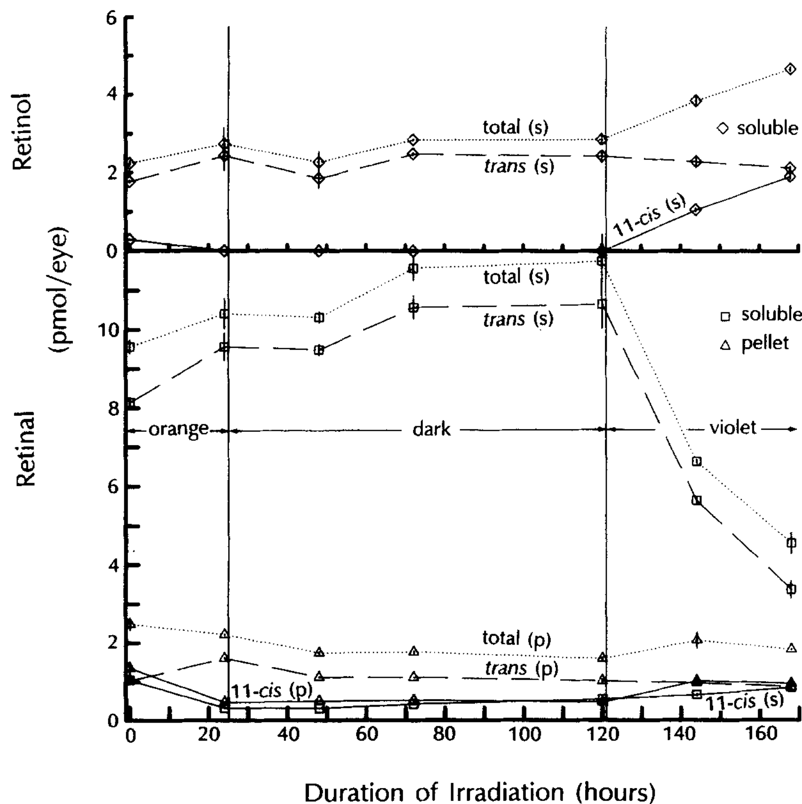

FIGURE 6. Changes in retinoid isomers in the dark after 24 h orange irradiation. After the dark period bees were irradiated for 48 h with violet light. Before orange irradiation bees were dark adapted overnight. Error bars, SEM; $n = 4$.

Note that the retinoid responses to orange irradiation are virtually identical to those presented in Fig. 4. Total retinoids increased 0.2 pmol/eye during orange irradiation.

During the course of dark adaptation, rhodopsin levels (pellet, 11-*cis* retinal) remained essentially constant ($P \gg 0.1$), whereas metarhodopsin levels (pellet, all-*trans* retinal) declined slowly ($P < 0.05$). Both the all-*trans* and 11-*cis* isomers of retinal in the soluble fraction increased, although the 11-*cis* increase was small (0.3 pmol/eye; $P < 0.05$) in comparison with that of all-*trans* (1.4 pmol/eye; $P < 0.05$). Retinol isomers showed no change during dark adaptation. Total retinoids increased 0.9 pmol/eye during dark adaptation. After 4 d of dark adaptation with no evidence for regeneration of visual pigment, the bees were illuminated with violet light for 2 d. Judging by the increase in 11-*cis* retinal in the pellet, violet light caused an increase in rhodopsin to 80% of the original dark-adapted level within 24 h. Metarhodopsin levels (all-*trans* retinal, pellet) were not significantly affected ($P > 0.1$). As before, violet irradiation caused a precipitous drop in soluble all-*trans* retinal (7.7 pmol/eye; $P \ll 0.01$). Soluble 11-*cis* retinal increased slightly (0.3 pmol/eye; $P < 0.05$). Retinol isomers followed the same trend seen previously, with no change in soluble all-*trans* retinol and a steady increase in soluble 11-*cis* retinol. With violet irradiation there was a net loss of 5.2 pmol/eye of total retinoid. Analysis of retinyl esters again showed no significant change ($P > 0.1$) in quantities in response to irradiation.

Properties of RalPI In Vitro

Formation of retinol. Aqueous extracts of bee eyes support the photoisomerization of all-*trans* retinal to 11-*cis* retinal (Schwemer et al., 1984). Our experiments, however, suggest that in vivo 11-*cis* retinol is the principal end-product of this reaction, and Goldsmith and Warner (1964) showed that aqueous extracts can generate retinol when given reduced cofactor. Experiments were therefore conducted to reconcile these observations. Table I summarizes the results of subjecting aqueous extracts of bee eyes to various light regimes and reduced cofactors. NADPH supports the formation of retinol, but only during irradiation. Furthermore, the threefold increase in retinol (1.1 pmol/eye) is stereospecific for the 11-*cis* isomer.

Although NADPH was added in great excess, other reactions appeared to be oxidizing it. Increasing the concentration of NADPH to 33 mM resulted in the formation of additional retinol (4.4 pmol/eye, 82% 11-*cis*), representing >80% conversion of the available (endogenous) retinal. The stereospecificity is possibly even greater if, as is likely, there is some nonspecific isomerization occurring in the crude extracts which secondarily converts 11-*cis* retinol to all-*trans* (see below).

The apparent stereospecificity of the alcohol dehydrogenase of the bee was investigated by comparison with frog ROS alcohol dehydrogenase as follows. It was noted during experiments to produce retinol in vitro that extracts filtered with 0.3-μm filters (Millipore) did not support the reduction of retinal. (Alcohol dehydrogenase activity was not retained on the filter, suggesting that contact with the filter leads to inactivation.) ROS extracts of frog alcohol dehydrogenase (with endogenous retinoids removed by hexane washes) were therefore added to crude bee eye extracts that had been filtered to remove bee alcohol dehydrogenase activity.

Results of this experiment are shown in Table II. No retinol was formed in the absence of light (rows 1 and 4), and only traces of retinol appeared when the endogenous alcohol dehydrogenase had been removed by filtering (row 3). With the

TABLE I

In vitro Reduction of Retinal in Aqueous Extracts of Bee Compound Eyes, in the Dark or with Green Irradiation, in the Presence or Absence of Reduced Cofactors (NADH or NADPH)

Condition	Retinal isomers			Retinol isomers		
	11-*cis*	all-*trans*	13-*cis*	11-*cis*	all-*trans*	13-*cis*
			pmol/eye			
Dark control	0.7	8.3	0.5	0.4	1.3	0.6
1 h green (no cofactor)	4.3	3.0	0.7	0.3	1.3	0.6
Dark + 2 mM NADH	0.7	8.2	0.4	0.4	1.2	0.5
1 h green + 2 mM NADH	4.4	3.2	0.6	0.5	1.4	0.6
Dark + 2 mM NADPH	0.7	7.4	0.3	0.4	1.2	0.5
1 h green + 2 mM NADPH	3.2	2.9	0.5	1.5	1.3	0.5

endogenous bee alcohol dehydrogenase present, light caused the formation of 1.33 pmol/eye of retinol (row 2), at least 84% of which was 11-*cis*. Frog alcohol dehydrogenase also supported the generation of retinol (row 5, 2.14 pmol/eye; at least 74% 11-*cis*). Because the alcohol dehydrogenase from frog ROS does not require 11-*cis* retinal for substrate (Wald, 1953), the stereospecificity of the coupled RalPI/alcohol dehydrogenase reaction probably has the following explanation. The all-*trans* retinal in $RalPI_{t440}$ is sequestered so that the alcohol dehydrogenase has no access to it. When light converts $RalPI_{t440}$ to $RalPI_{c390}$, however, the 11-*cis* retinal is not sheltered from the aqueous environment, and the alcohol dehydrogenase obtains access. The apparent stereospecificity of the alcohol dehydrogenase reaction is therefore governed by the availability of substrate rather than by the intrinsic properties of the alcohol dehydrogenase molecule. As further confirmation, when all-*trans* retinal in vesicles is added in excess to the RalPI extract, and therefore cannot be protected, there is reduction of all-*trans* retinal to retinol with no stereospecificity.

This explanation predicts that all of the retinol formed in the light using endogenous retinoids should be 11-*cis;* however, only 75–85% of the retinol recovered is actually the 11-*cis* isomer. The production of some 13-*cis* retinol indicates that

TABLE II

Comparison of Bee and Frog Alcohol Dehydrogenase (ADH) Activity on Bee Aqueous Extracts

Sample	Quantity of retinol made		
	11-*cis*	all-*trans*	13-*cis*
		pmol/eye	
RalPI + endogenous ADH activity (3 h dark)	0.00	0.00	0.00
RalPI + endogenous ADH activity (3 h green)	1.12	0.13	0.08
RalPI filtered to remove ADH activity (3 h green)	0.06	0.06	0.06
Filtered RalPI + frog ADH (3 h dark)	0.00	0.00	0.00
Filtered RalPI + frog ADH (3 h green)	1.59	0.46	0.09

All reaction mixtures contain 5 mM NADPH.

there is probably some nonspecific isomerization of retinoid occurring subsequent to the formation of $RalPI_{c390}$.

Selective use of retinal by the RalPI. To demonstrate that the substrate used by RalPI is retinal and not retinol, a 15× molar excess of all-*trans* retinol in lipid vesicles was added to crude RalPI extract. After irradiation on ice with green light (510 nm) for 1 h, retinoids were analyzed (Table III).

In the crude extract with endogenous retinoids and no added NADPH, blue-green light caused a change only in the retinal isomers, with an increase in 11-*cis* retinal. Even in extracts loaded with excess all-*trans* retinol there was no production of 11-*cis* retinol, nor was there an increase in 11-*cis* retinal above that which was produced without the excess retinol. The latter could have occurred if 11-*cis* retinol were being oxidized to 11-*cis* retinal as it was produced. To demonstrate that retinoid added in vesicles is accessible to the photoisomerase, all-*trans* retinal was also added in 15× molar excess to the endogenous retinal. Almost five times more 11-*cis* retinal was made than in the unsupplemented aliquot, totaling more than the available endogenous retinal. Continued irradiation resulted in still more production of 11-*cis* retinal,

TABLE III

Use of Retinoid Substrates by the RalPI

Sample	Retinal recovered		Retinol recovered	
	11-*cis*	all-*trans*	11-*cis*	all-*trans*
	pmol/eye			
RalPI (dark)	0.53	5.90	0.64	1.59
RalPI (1 h blue-green)	3.41	2.42	0.62	1.57
RalPI + 15× molar excess all-*trans* retinol (1 h blue-green)	3.75	2.11	0.68	22.60
RalPI + 15× molar excess all-*trans* retinal (1 h blue-green)	16.82	70.91	0.59	1.62

clearly indicating that RalPI can obtain retinoid from the lipid vesicles. This experiment demonstrates conclusively that the RalPI acts on retinal.

Transfer of 11-cis retinal to opsin. To investigate whether bee RalPI can donate chromophore directly to opsin, thereby regenerating rhodopsin, bleached frog ROSs were suspended in an aqueous extract of bee eyes that had been irradiated with green light to enrich it for 11-*cis* retinal. Opsin concentration was approximately equimolar to 11-*cis* retinal in the bee extract. ROSs were also presented with 11-*cis* retinal in lipid vesicles at the same molar concentration as the 11-*cis* in the bee extract. Fig. 7 summarizes the results.

Incubation of bleached ROSs with crude bee $RalPI_{c390}$ for 1 h results in some transfer of 11-*cis* retinal, reconstituting visual pigment (Fig. 7, curve *C*). Approximately 5% of the available opsin was successfully converted to rhodopsin with this highly artificial system. With 11-*cis* retinal in vesicles (Fig. 7, curve *D*) regeneration efficiency was ~25%. Bleached ROSs incubated for the same length of time with neither bee extract nor vesicles show no regeneration of rhodopsin (Fig. 7, curve *B*).

When the opsin is present in digitonin solution 100% regeneration was achieved with 11-*cis* retinal supplied in lipid vesicles, 100% regeneration when supplied attached to bovine serum albumin, and at least 80% regeneration when supplied as

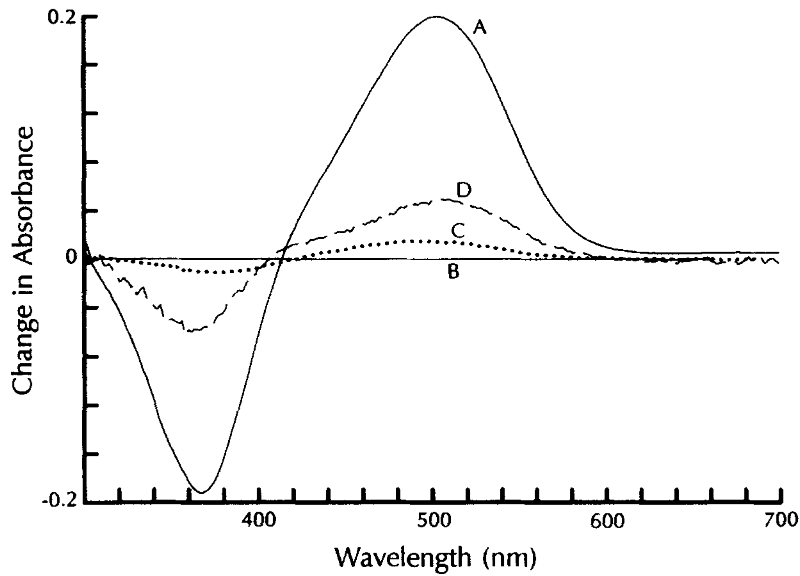

FIGURE 7. Difference spectra obtained from the bleaching of frog rhodopsin in 1% digitonin with yellow light (wavelengths >550 nm) in the presence of 1 mM hydroxylamine. Curve *A*, photobleach of native frog visual pigment. Curve *B*, photobleach of extract of an equal number of bleached ROS (control). Curve *C*, photobleach of extract of bleached ROS incubated with crude bee RalPI. Curve *D*, photobleach of extract of bleached ROS incubated with 11-*cis* retinal in lipid vesicles.

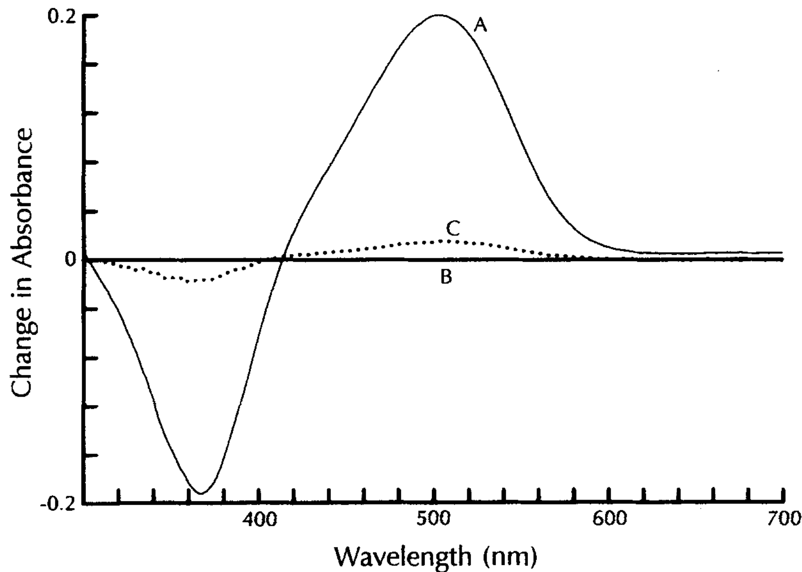

FIGURE 8. Difference spectra obtained from the bleaching of frog rhodopsin in 1% digitonin with yellow light (wavelengths >550 nm) in the presence of 1 mM hydroxylamine. Curve *A*, photobleach of extract of native frog visual pigment. Curve *B*, photobleach of extract of bleached ROS (control). Curve *C*, photobleach of extract of bleached ROS incubated with purified RalPI.

$RalPI_{c390}$. Regeneration in the latter case may have been > 80% because the presence of ommochrome pigments interfered with the direct spectrophotometric determination of rhodopsin. The rhodopsin was therefore separated from the ommochromes by ultrafiltration and the amount of rhodopsin was estimated by oxime extraction of the residue on the filter. This method probably entails some loss.

Since the RalPI extract used above is a crude preparation, it is possible that transfer of 11-*cis* retinal to membrane-bound opsin was not direct, but through some intermediate carrier. To eliminate this possibility, bleached ROSs were incubated for 1 h with purified RalPI (Fig. 8). As in the crude extract, there was 5% regeneration of rhodopsin in this preparation.

DISCUSSION

The results of this study suggest a model for the visual cycle of the honeybee (Fig. 9). The section enclosed within the heavy outline is addressed by the present experiments. Dotted arrows indicate likely reactions for which the evidence is less direct.

In the honeybee, as in other invertebrates, light produces a photosteady state between rhodopsin and metarhodopsin. This phenomenon is evident in the present experiments by the shift of 11-*cis* to all-*trans* retinal in the pellet fraction (Fig. 4) in response to irradiation. The fate of metarhodopsin in the dark is not clear in bees. In a variety of arthropods there is evidence from electron microscopy that metarhodopsin is stripped from the photoreceptor organelles and broken down in lysosomes (e.g., Blest, 1980; White et al., 1980; Waterman, 1982; Chamberlain and Barlow, 1984; reviewed by Schwemer, 1986), but little is known of the fate of the associated retinal. It is possible that at least some of the all-*trans* retinal from degraded metarhodopsin is sequestered by the RalPI and used again, although at this point we have no evidence for direct transfer. In flies the dark decay of metarhodopsin is much faster ($t_{1/2}$ = 20 h) than that of rhodopsin ($t_{1/2}$ = 1,200 h; Schwemer, 1984). The present experiments suggest that a similar catabolic removal of metarhodopsin occurs in the honeybee, as seen by the decline of all-*trans* retinal in the pellet during prolonged dark (Fig. 6) and orange adaptation (Fig. 4). The fate of opsin during the decay of metarhodopsin is not known. If bees are similar to flies, however, it is likely that the enzymatic digestion of opsin occurs subsequent to the removal of retinal, as Schwemer (1986) found in *Calliphora*.

Evidence from other sources also suggests that in arthropods rhodopsin is commonly either photoregenerated from metarhodopsin (e.g., Hamdorf et al., 1971; Hamdorf and Schwemer, 1975) or synthesized de novo (Stein et al., 1979; Paulsen and Schwemer, 1983). Chromophore exchange appears to be much less frequent than in the vertebrate eye. In flies visual pigment is not synthesized and incorporated into the membrane unless 11-*cis* retinal is available (Schwemer, 1988). In bees the role of RalPI is to supply this chromophore for the synthesis of all classes of visual pigments, which explains the requirement for blue light in order for bees, like flies, to recover from long-wavelength light adaptation.

In the absence of light absorbed by the RalPI, the total amount of $RalPI_{t440}$ increases (e.g., Figs. 2*A* and 4). After 24 h $RalPI_{t440}$ exceeds by severalfold the amount of visual pigment, and there is a net increase in retinoid in the eye. The

source of this new retinoid has not been identified, but probably involves C_{40} carotenoids that are present in honeybees (Goldsmith and Warner, 1964).

The RalPI clearly acts on all-*trans* retinal and not retinol since (*a*) the protein binds all-*trans* retinal covalently in vivo; (*b*) all-*trans* retinol does not change in response to wavelengths that drive the formation of 11-*cis* retinol in vivo (e.g., Fig. 5); and (*c*) in

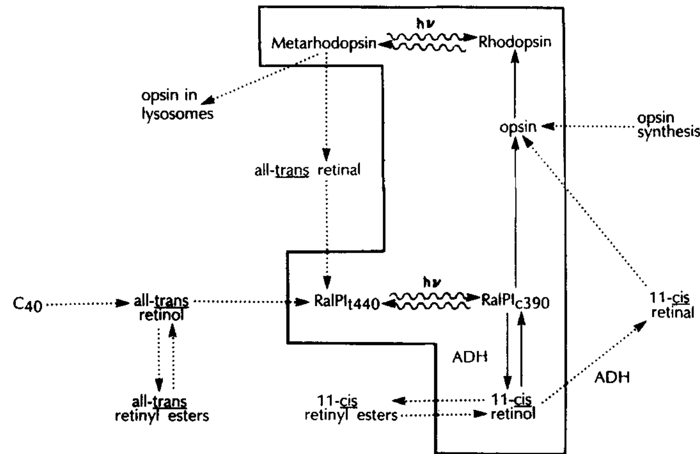

FIGURE 9. Model of the visual cycle of the honeybee. The elements enclosed within the "balloon" have an experimental basis described in Results. Dotted lines indicate likely pathways for which there is no direct evidence in this study. Much of the retinal of the bee's eye is associated with RalPI. RalPI forms 11-*cis* retinal in the presence of violet light, and most of the 11-*cis* retinal is immediately reduced to 11-*cis* retinol. Indirect evidence indicates that, as in other arthropods, formation of visual pigments is closely tied to opsin synthesis and membrane renewal, for which a supply of 11-*cis* retinal is required. The 11-*cis* retinal is supplied by RalPI, either directly from $RalPI_{c390}$ or indirectly via 11-*cis* retinol. Retinyl esters are present in small amounts and do not change significantly during the visual cycle. Further points require clarification. In the dark the stores of retinal (as $RalPI_{t440}$) build up, probably formed from C_{40} carotenoids and involving retinol as an intermediate (dotted arrows, lower left). When $RalPI_{t440}$ is converted to $RalPI_{c390}$ there is a net loss of retinoid from the retina by an unknown process (path therefore not shown). Use of 11-*cis* retinol to support formation of visual pigment could involve $RalPI_{c390}$ as an intermediate, but a second carrier protein is not precluded (dotted arrows, right). The mechanism by which opsin synthesis is controlled is not known (but see the text for discussion of possibilities). As in other arthropods, metarhodopsin is either photoconverted back to rhodopsin (photoregeneration) or shuttled to lysosomes during membrane turnover. We infer that some or all of the retinal from metarhodopsin may be recycled to RalPI, but there is no direct evidence to demonstrate this path. In flies retinoid is removed from metarhodopsin before loss of opsin from the photoreceptor membranes, and seemingly recycled. The path in bees may be similar.

vitro studies of RalPI activity show that there is selective conversion of only all-*trans* retinal and not retinol (Table III). The apparent stereospecificity of honeybee alcohol dehydrogenase is due to the fact that only $RalPI_{c390}$ (and not $RalPI_{t440}$) provides access to the retinal substrate.

Upon violet irradiation the RalPI catalyzes the stereospecific formation of 11-*cis*

retinal ($RalPI_{t440} \rightarrow RalPI_{c390}$). The 11-*cis* retinal has at least two known fates. The most obvious is reduction by an alcohol dehydrogenase to 11-*cis* retinol, which then associates with a retinol-binding protein (possibly RalPI). A second fate is transfer to newly synthesized opsin to form rhodopsin (Fig. 6). When honeybee RalPI is enriched with 11-*cis* retinal and mixed with bleached frog ROSs, there is a small but significant regeneration of frog rhodopsin (Figs. 7 and 8). Although inefficient in this system, more effective transfer of retinal might be expected in the confines of a cellular environment and in the presence of newly synthesized bee opsin, rather than with the enclosed membrane system of vertebrate outer segments. This result indicates only that a direct transfer between the RalPI and opsin is possible. In the squid, rhodopsin can be reconstituted using bleached rhabdomal membranes and metaretinochrome (Seki et al., 1980, 1982) even though in vivo a retinal-binding protein most probably shuttles 11-*cis* retinal from the myeloid bodies at the basal end of the receptor to the rhabdom (Ozaki et al., 1987; Hara, 1988).

Excess 11-*cis* retinal made by the RalPI is stored as the 11-*cis* isomer of retinol. This is evidenced by extended adaptation to white (Fig. 2) and violet lights (Fig. 5), which produce an increase in the 11-*cis* retinol, but essentially no change in aqueous-soluble 11-*cis* retinal. After 2 d of irradiation, the level of 11-*cis* retinol seems to reach an upper limit, possibly determined by the amount of protein to which 11-*cis* retinol binds. Retinyl esters play an inconsequential role in the visual system of the honeybee.

Aqueous-soluble 11-*cis* retinol clearly serves as a chromophore pool for the generation of visual pigment. This pool is readily depleted under conditions in which there is turnover of visual pigment without the generation of additional 11-*cis* retinol, as in the case of orange irradiation (Fig. 4). The involvement of 11-*cis* 3-hydroxyretinol has similarly been implicated in the visual cycle of the fly (Schwemer, 1988). The pathway from 11-*cis* retinol to visual pigment synthesis is unclear, but two possibilities are likely. First, 11-*cis* retinol may be oxidized in a reversal of the pathway that generated 11-*cis* retinol, leading to the formation of $RalPI_{c390}$ with subsequent transfer to opsin. Alternatively, 11-*cis* retinol may combine with a transport protein, and then be oxidized to retinal before transfer to opsin.

Although the proposed model accounts for a number of experimental observations, there are several results from this study that remain puzzling. First, what is the nature of the small pool of 11-*cis* retinal that is found in the aqueous extract? Levels remain constant during dark-, light-, and violet-adaptation (Figs. 2 and 5). However, with orange irradiation (light that depletes visual pigment), soluble 11-*cis* retinal declines to a basal level which is ~25% of that present before irradiation (Fig. 4), but does not decrease to zero even after 74 h of constant illumination. It appears that this pool is not completely available for visual pigment regeneration since rhodopsin levels can remain depleted without drawing further from this soluble 11-*cis* retinal. These results suggest that the soluble 11-*cis* retinal may be in two distinct populations, one of which (representing 75% of the total 11-*cis* retinal, 10.5% of the total soluble retinal) can contribute its 11-*cis* retinal for visual pigment renewal and is probably $RalPI_{c390}$; the other (5% of total soluble retinal) does not donate its chromophore. The latter pool may be small vesicles of shed microvilli known to be present in other insects (Williams and Blest, 1980; Schwemer, 1986). These small

vesicles would not be sedimented at the centrifugation speed and time used in this study.

Another puzzling result is that when $RalPI_{t440}$ has accumulated in the dark, violet light adaptation results in a net loss of retinoid. For example, in the experiment of Fig. 5 there was a net loss of 3.4 pmol/eye (23% of the total retinoid) compared with the level at the start of the irradiation. This loss requires photoactivation of RalPI since bees can be kept in orange light (wavelengths >565 nm) or in the dark with no loss of total retinoid. Further, retinyl esters are not implicated since they are present at only 0.2 pmol/eye and vary little with violet illumination. The retinoid is also not transported out of the head to some other storage site, since no retinoid (i.e., retinal, retinol, or retinyl ester) can be detected in either the thorax or abdomen. Another possibility is that the retinoid could be converted to 3-hydroxyretinal, a retinoid recently discovered to comprise 1% of the total eye chromophore in bees (Smith and Goldsmith, 1990). However, HPLC analysis of 3-hydroxyretinoids showed no elevation above the normal 1% level during violet illumination. Thus, it seems likely that the retinoid is destroyed (e.g., oxidative loss). Interestingly, in the fly, the only other arthropod in which the visual cycle has been studied, there is a similar net loss of as much as 2.4 pmol/eye (28% of total retinoid) when flies are irradiated with light that photoactivates the dipteran photoisomerase (Schwemer, 1988, 1989). Analysis of retinyl esters has not been reported for the fly.

The counterpart of retinoid loss in violet light is the net increase in retinoids during extended dark adaptation (Figs. 2 and 5). The increase occurs in $RalPI_{t440}$, with no associated change in retinyl esters. Retinoids are probably being generated from carotenoid precursors, but direct evidence is lacking.

The roles of all-*trans* retinol and all-*trans* retinyl esters in the visual cycle remain unresolved by these experiments. Although neither of these retinoids shows large changes in response to the various light regimes used here, there is a significant decline in all-*trans* retinol in response to prolonged light adaptation (Fig. 2 *B*). This decline suggests that all-*trans* retinol can be used in the visual cycle. All-*trans* retinol, for example, may be an intermediate in the formation of retinal from C_{40} carotenoids. However, we currently have no direct evidence to support this conjecture.

Comparison of Bee and Fly

The blowfly *Calliphora* is the only insect for which there is comparable information on the visual cycle, and a direct comparison of these two species is fruitful. Bees and flies are representative of the orders Hymenoptera and Diptera, groups that have been on separate evolutionary trajectories for at least 250 million years. The architecture of the photoreceptor layer is distinctly different in the two orders. The rhabdomeres of the photoreceptors in each dipteran ommatidium view slightly different points in space and converge on the same interneurons, providing the basis for the neural superposition optics that characterize the members of this order (Kirschfeld, 1967). Six of the cells (R1-R6) express the same visual pigment (Zuker et al., 1985); the remaining two share the same visual field but contain different visual pigments (Cowman et al., 1986; Hardie, 1986; Fryxell and Meyerowitz, 1987). In contrast, the rhabdomeres in a hymenopteran ommatidium are contiguous and view the same visual field. Three visual pigments are present in most of the ommatidia, providing

the basis for a color vision system that in bees appears to be substantially better developed than in flies.

Most Diptera employ 3-hydroxyretinal in their visual systems (an exception is Simuliidae, which use retinal [Vogt, 1987; Gleadall et al., 1989]); Hymenoptera, on the other hand, characteristically utilize retinal (exceptions are Sphecidae and Ichneumonidae, which have mostly 3-hydroxyretinal [Smith and Goldsmith, 1990]). In flies photoreceptors R1-R6 also contain an ultraviolet-absorbing sensitizing pigment, thought to be 3-hydroxyretinol, that passes energy to the visual pigment (Vogt and Kirschfeld, 1984). Such sensitizing pigments may be unique to Diptera. In flies the visual pigment of R1-R6 absorbs maximally at ~490 nm, but the metarhodopsin has λ_{max} at unusually long wavelengths, 570 nm (Stavenga et al., 1973). The metarhodopsins of the bee's visual pigments all absorb maximally in the blue, ~480 nm (Bertrand et al., 1979).

There are, however, some similarities that probably reflect the presence of shared molecular processes in the visual cycle. Neither the fly (*Calliphora erythrocephala*) nor the honeybee (*Apis mellifera*) seems able to form visual pigment without a supply of its 11-*cis* retinoid, and for this both species depend on a violet-absorbing photoisomerase. (*Drosophila* is reported to form visual pigment in the dark if supplied with C_{40} carotenoid, but not retinal [Seki et al., 1986].) In bees this photoisomerase is a soluble protein that binds the bulk of the retinal in the eye. Less is known about the chemistry of the photoisomerase in flies, where the evidence is all from in vivo experiments. For example, it is not known whether it is a soluble protein as in bees or a membrane protein as in cephalopod mollusks. The photoisomerase is presumed to work on 3-hydroxyretinal rather than 3-hydroxyretinol, but the evidence is indirect.

In flies, however, more is known about the relation between the rhodopsin cycle and opsin synthesis (Paulsen and Schwemer, 1983; Schwemer, 1983, 1984, 1986, 1988, 1989). In white (or blue) light 11-*cis* 3-hydroxyretinal is formed, and there is synthesis of opsin and turnover of rhabdomeric membrane. In darkness both rhodopsin and metarhodopsin are removed from the membrane (rhodopsin slowly [$t_{1/2}$ ~5 d], metarhodopsin ~60 times faster [$t_{1/2}$ ~20 h]) and there is no recovery of visual pigment. In green light, which does not activate the photoisomerase but which is absorbed by rhodopsin, total opsin is removed from the membrane faster than in darkness. This is because the photoisomerase is not activated in green light, with the result that rhodopsin renewal is blocked. At the same time, however, the residual rhodopsin in the membrane is in photosteady state with metarhodopsin, from which latter state opsin is removed from the membrane more rapidly.

A source of 11-*cis* retinal is thus required for rhodopsin replacement, and in fact for opsin synthesis (Paulsen and Schwemer, 1979). Whether 11-*cis* retinal regulates the expression of the opsin gene, or whether opsin synthesis is shut down in a negative feedback when opsin cannot be converted to rhodopsin (i.e., in the absence of 11-*cis* 3-hydroxyretinal) remains to be determined.

There is an additional twist to the removal of metarhodopsin from the membrane, in that it is light dependent. This effect of light is distinct from the interplay of opsin synthesis and degradation that can be regulated (indirectly) by blue light and photoisomerase activity. Metarhodopsin decay is inhibited by green light that does not activate the photoisomerase (Schwemer, 1984). This observation has led to the

hypothesis that metarhodopsin is converted thermally into a form susceptible to removal from the membrane (perhaps by phosphorylation, as observed by Paulsen and Bentrop, 1984); green light decreases the probability of this reaction by interconverting rhodopsin and metarhodopsin and decreasing the average time a pigment molecule spends in the initial metarhodopsin state.

Metarhodopsin (measured spectrally) disappears more rapidly than the membrane particles that appear to be opsin. This suggests that in flies the 3-hydroxyretinal is removed from the opsin before the opsin is stripped from the membrane.

The less extensive data on bees are consistent with the hypothesis that regeneration of visual pigment requires not only a source of 11-*cis* retinal but de novo synthesis of opsin as well. There are currently no measurements of opsin synthesis in the bee, however. Other evidence indicates that opsin synthesis and membrane renewal are probably closely associated in many other arthropods (moth [Goldman et al., 1975], mosquito [Stein et al., 1979], crayfish [Cronin and Goldsmith, 1984]; see also reviews by Waterman, 1982 and Schwemer, 1986).

In flies, under conditions where retinoids are removed from metarhodopsin and synthesis of new rhodopsin is blocked (e.g., green light), retinoids appear in the most peripheral part of the eye in cells close to the cornea. This is also where 11-*cis* retinoids first accumulate when the eye is irradiated with blue light. It is therefore hypothesized that the primary pigment cells are the location of the photoisomerase (Schwemer, 1988, 1989). In bees antibody staining and localization at the electron microscopic level shows the photoisomerase to be present in highest concentration in the primary pigment cells (Smith, W. C., and T. H. Goldsmith, manuscript in preparation), a further similarity between these two species.

In flies maintained in green light some of the retinoid removed from metarhodopsin may be converted to 3-hydroxyretinyl esters. We find no participation of retinyl esters in the honeybee, even under conditions where retinol is accumulating. The fly appears to recycle retinoid (Schwemer, 1989), although it has the capacity to generate 3-hydroxyretinoids from either carotenes or xanthophylls (Vogt, 1983; Stark et al., 1990). The bee appears to have a larger capacity for storing retinoids, and the present experiments demonstrate that the total retinoid pool ebbs and flows, depending on the ambient illumination, presumably drawing on carotenoids for replenishment.

For technical reasons involving the measurement of visual pigment in single retinas, most of the information on *Calliphora* was obtained from a white eye mutant lacking the ommochrome screening pigments. Schwemer has been careful to show, however, that none of his experiments are compromised by light damage to the photoreceptors. The interplay of different wavelength regions on the components of the visual cycle may nevertheless be expected to differ quantitatively in wild-type animals. In crayfish white eye mutants exhibit much smaller rhabdoms and a decrease in the areal density of visual pigment under ordinary room lighting, although the effect can be reversed by a period of dark adaptation (Kong and Goldsmith, 1977). On the other hand, the wide availability of eye mutants in *Drosophila* provides an untapped opportunity to dissect the visual cycle further.

Note added in proof: Pepe et al. (Pepe, I. M., C. Cugnoli, and J. Schwemer. 1990. *FEBS Letters.* 268:177–179) have recently observed a 40% regeneration of frog

rhodopsin when $RalPI_{C390}$ is mixed with membrane fragments of bleached frog rod outer segments.

This work was supported by grants EY00222 and EY00785 from the National Eye Institute.

Original version received 10 April 1990 and accepted version received 10 July 1990.

REFERENCES

Bernard, G. D., and R. Wehner. 1980. Intracellular optical physiology of the bee's eye. I. Spectral sensitivity. *Journal of Comparative Physiology.* 137:193–203.

Bertrand, D., G. Fuortes, and R. Muri. 1979. Pigment transformation and electrical responses in retinula cells of drone, *Apis mellifera*. *Journal of Physiology.* 296:431–441.

Blest, A. D. 1980. Photoreceptor membrane turnover in arthropods: comparative studies of breakdown processes and their implications. *In* The Effect of Constant Light on Visual Processes. T. P. Williams and B. N. Baker, editors. Plenum Publishing Corp., New York. 217–245.

Chamberlain, S. C., and R. B. Barlow, Jr. 1984. Transient membrane shedding in *Limulus* photoreceptors: control mechanisms under natural lighting. *Journal of Neuroscience.* 4:2792–2810.

Cowman, A. F., C. S. Zuker, and G. M. Rubin. 1986. An ospin gene expressed in only one photoreceptor cell type of the *Drosophila* eye. *Cell.* 44:705–710.

Cronin, T. W., and T. H. Goldsmith. 1984. Dark regeneration of rhodopsin in crayfish photoreceptors. *Journal of General Physiology.* 84:63–81.

Deigner, P. S., W. C. Law, F. J. Canada, and R. R. Rando. 1989. Membranes as the energy source in the endergonic transformation of vitamin A to 11-*cis* -retinol. *Science.* 244:968–971.

Fong, S.-L., A. T. C. Tsin, C. D. B. Bridges, and G. I. Liou. 1982. Detergents for extraction of visual pigments: types, solubilization, and stability. *Methods in Enzymology.* 81:133–140.

Fryxell, K. J., and E. M. Meyerowitz. 1987. An opsin gene that is expressed only in the R7 photoreceptor cell of *Drosophila*. *EMBO Journal.* 6:443–451.

Fulton, B. S., and R. R. Rando. 1987. Biosynthesis of 11-*cis*-retinoids and retinyl esters by bovine pigment epithelium membranes. *Biochemistry.* 26:7938–7945.

Gleadall, I. G., T. Hariyama, and Y. Tsukahara. 1989. The visual pigment chromophores in the retina of insect compound eyes, with special reference to the Coleoptera. *Journal of Insect Physiology.* 35:787–795.

Goldman, L. J., S. N. Barnes, and T. H. Goldsmith. 1975. Microspectrophotometry of rhodopsin and metarhodopsin in the moth *Galleria*. *Journal of General Physiology.* 66:383–404.

Goldsmith, T. H. 1958. The visual system of the honeybee. *Proceedings of the National Academy of Sciences, USA.* 44:123–126.

Goldsmith, T. H., B. C. Marks, and G. D. Bernard. 1986. Separation and identification of geometric isomers of 3-hydroxyretinoids and occurrence in the eyes of insects. *Vision Research.* 26:1763–1769.

Goldsmith, T. H., and L. T. Warner. 1964. Vitamin A in the vision of insects. *Journal of General Physiology.* 47:433–441.

Groenendijk, G. W. T., W. J. DeGrip, and F. J. Daemen. 1979. Identification and characterization of *syn-* and *anti*-isomers of retinaloximes. *Analytical Biochemistry.* 99:304–310.

Groenendijk, G. W. T., W. J. DeGrip, and F. J. Daemen. 1980. Quantitative determination of retinals with complete retention of their geometric configuration. *Biochimica et Biophysica Acta.* 617:430–438.

Hamdorf, K., and J. Schwemer. 1975. Photoregeneration and the adaptation process in insect photoreceptors. *In* Photoreceptor Optics. A. W. Snyder and R. Menzel, editors. Springer-Verlag, Berlin, Heidelberg, New York. 263–289.

Hamdorf, K., J. Schwemer, and M. Gogala. 1971. Insect visual pigment sensitive to ultraviolet light. *Nature*. 231:458–459.

Hara, T. 1988. Visual photoreception of the squid: photopigment regeneration. *In* Molecular Physiology of Retinal Proteins. T. Hara, editor. Yamada Science Foundation, Yamada, Japan. 305–310.

Hara, T., and R. Hara. 1972. Cephalopod retinochrome. *In* Handbook of Sensory Physiology. Vol. VII. H. Dartnall, editor. Springer-Verlag, Berlin, Heidelberg, New York. 720–746.

Hardie, R. C. 1986. The photoreceptor array of the dipteran retina. *Trends in Neuroscience*. 9:419–423.

Isono, K., T. Tanimura, Y. Oda, and Y. Tsukahara. 1988. Dependency on light and vitamin A derivatives of the biogenesis of 3-hydroxyretinal and visual pigment in the compound eyes of *Drosophila melanogaster*. *Journal of General Physiology*. 92:587–600.

Kirschfeld, K. 1967. Die Projektion der optischen Umwelt auf das Raster der Rhabdomere im Komplexauge von *Musca*. *Experimental Brain Research*. 3:248–270.

Kong, K.-L., and T. H. Goldsmith. 1977. Photosensitivity of retinular cells in white-eyed crayfish (*Procambarus clarkii*). *Journal of Comparative Physiology*. 122:273–288.

Menzel, R., and M. Blakers. 1976. Colour receptors in the bee eye: morphology and spectral sensitivity. *Journal of Comparative Physiology*. 108:11–33.

Ozaki, K., A. Terakita, R. Hara, and T. Hara. 1987. Isolation and characterization of a retinal-binding protein from the squid retina. *Vision Research*. 27:1057–1070.

Paulsen, R., and J. Bentrop. 1984. Reversible phosphorylation of opsin induced by irradiation of blowfly retinae. *Journal of Comparative Physiology A*. 155:39–45.

Paulsen, R., and J. Schwemer. 1979. Vitamin A deficiency reduces the concentration of visual pigment protein within blowfly photoreceptor membranes. *Biochimica et Biophysica Acta*. 557:385–390.

Paulsen, R., and J. Schwemer. 1983. Biogenesis of blowfly photoreceptor membranes is regulated by 11-*cis*-retinal. *European Journal of Biochemistry*. 137:609–614.

Pepe, I. M., J. Schwemer, and R. Paulsen. 1982. Characteristics of retinal-binding proteins from the honeybee retina. *Vision Research*. 22:775–781.

Pringle, J. W. S. 1938. Proprioception in insects. I. A new type of mechanical receptor from the palps of the cockroach. *Journal of Experimental Biology*. 15:101–113.

Rando, R. R., J. Canada, P. S. Deigner, and W. C. Law. 1989. Is there a retinol isomerase? *Investigative Ophthalmology and Visual Science Supplement*. 30:331.

Schwemer, J. 1983. Pathways of visual pigment regeneration in fly photoreceptor cells. *Biophysics of Structure and Mechanism*. 9:287–298.

Schwemer, J. 1984. Renewal of visual pigment in photoreceptors of the blowfly. *Journal of Comparative Physiology A*. 154:535–547.

Schwemer, J. 1986. Turnover of photoreceptor membrane and visual pigment in invertebrates. *In* The Molecular Mechanism of Photoreception. H. Stieve, editor. Springer-Verlag, Berlin, Heidelberg, New York. 303–326.

Schwemer, J. 1988. Cycle of 3-hydroxy retinoids in an insect eye. *In* Molecular Physiology of Retinal Proteins. T. Hara, editor. Yamada Science Foundation, Yamada, Japan. 299–304.

Schwemer, J. 1989. Visual pigments of compound eyes: structure, photochemistry, and regeneration. *In* Facets of Vision. D. G. Stavenga and R. C. Hardie, editors. Springer-Verlag, Berlin, Heidelberg, New York. 112–133.

Schwemer, J., I. M. Pepe, R. Paulsen, and C. Cugnoli. 1984. Light-induced *trans-cis* isomerization of retinal by a protein from the honeybee retina. *Journal of Comparative Physiology A*. 154:549–554.

Seki, T., S. Fujishita, M. Ito, N. Matsuoka, C. Kobayashi, and K. Tsukida. 1986. A fly, *Drosophila melanogaster*, forms 11-*cis* 3-hydroxyretinal in the dark. *Vision Research*. 26:255–258.

Seki, T., R. Hara, and T. Hara. 1980. Reconstitution of squid rhodopsin in rhabdomal membranes. *Photochemistry and Photobiology*. 32:469–479.

Seki, T., R. Hara, and T. Hara. 1982. Reconstitution of squid and cattle rhodopsin by the use of metaretinochrome in their respective membranes. *Experimental Eye Research*. 34:609–621.

Smith, W. C., and T. H. Goldsmith. 1990. Phyletic aspects of the distribution of 3-hydroxyretinal in the class Insecta. *Journal of Molecular Evolution*. 30:72–84.

Stark, W. S., D. Schilly, J. S. Christianson, R. A. Bone, and J. T. Landrum. 1990. Photoreceptor-specific efficiencies of β-carotene, zeaxanthin and lutein for photopigment formation deduced from receptor mutant *Drosophila melanogaster*. *Journal of Comparative Physiology A*. 166:429–436.

Stavenga, D. G., D. G. A. Zantema, and J. W. Kuiper. 1973. Rhodopsin processes and the function of the pupil mechanism in flies. *In* Biochemistry and Physiology of Visual Pigments. H. Langer, editor. Springer-Verlag, Berlin, Heidelberg, New York. 175–180.

Stein, P. J., J. D. Brammer, and S. E. Ostroy. 1979. Renewal of opsin in the photoreceptor cells of the mosquito. *Journal of General Physiology*. 74:565–582.

Trehan, A., F. J. Canada, and R. R. Rando. 1990. Inhibitors of retinyl ester formation also prevent the biosynthesis of 11-*cis*-retinol. *Biochemistry*. 29:309–312.

Vogt, K. 1983. Is the fly visual pigment a rhodopsin? *Zeitschrift für Naturforschung*. 38C:329–333.

Vogt, K. 1987. Chromophores of insect visual pigments. *Photobiochemistry and Photobiophysics Supplement*. 1987:273–296.

Vogt, K., and K. Kirschfeld. 1984. The chemical identity of the chromophores of fly visual pigment. *Naturwissenschaften*. 71:211–213.

Wald, G. 1953. The biochemistry of vitamin A. *In* Symposium on Nutrition. R. M. Herriott, editor. Johns Hopkins Press, Baltimore. 73–107.

Waterman, T. H. 1982. Fine structure and turnover of photoreceptor membranes. *In* Visual Cells in Evolution. J. A. Westfall, editor. Raven Press, New York. 23–41.

White, R. H., D. Gifford, and N. A. Michaud. 1980. Turnover of photoreceptor membrane in the larval mosquito ocellus: rhabdomeric coated vesicles and organelles of the vacuolar system. *In* The Effect of Constant Light on Visual Processes. T. P. Williams and B. N. Baker, editors. Plenum Publishing Corp., New York. 271–296.

Williams, D. S., and A. D. Blest. 1980. Extracellular shedding of photoreceptor membrane in the open rhabdom of a tipulid fly. *Cell and Tissue Research*. 205:423–438.

Zuker, C. S., A. F. Cowman, and G. M. Rubin. 1985. Isolation and structure of a rhodopsin gene from *D. melanogaster*. *Cell*. 40:851–858.