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Ultra-violet sensitizing pigment in blowfly photoreceptors R1–6; probable nature and binding sites

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Summary. The spectral sensitivity of photoreceptors R1–6 in the blowfly *Calliphora erythrocephala* has a characteristic UV fine spectrum due to a sensitizing pigment complexed with the visual pigment, P490. The present study concerns the identity and steric configuration of the sensitizing pigment, and the number of its binding sites on P490.

1. Irradiation by "green" light demonstrated that "de novo" synthesis of visual pigment has priority over formation of UV sensitizing pigment, both using the same retinoid pool, and that in absence of sensitizing pigment an aberrant, more thermostable, visual pigment is formed.

2. The kinetics of increase in UV sensitivity after application of different retinoids to eyes with low content of P490, and the vibronic UV fine spectrum of the sensitizing pigment, make 13-cis-retinol the most probable candidate for the UV sensitizing pigment or its direct precursor.

3. β-carotene, lutein or zeaxanthin cannot be directly used by the eye as precursors for the UV sensitizing pigment. Vitamin A_2 probably is directly coupled to P490. At least two UV sensitizing pigment molecules can complex with P490.

4. Artificial UV sensitivity could be induced by the fluorescent thiol probe P–28, probably binding to cysteines 121 and 196 at the extracellular surface of P490.

Key words: UV sensitizing pigment – Spectral sensitivity – Retinoid incorporation – Photoreceptors R1–6 – Blowfly

Introduction

The spectral sensitivity of photoreceptors, $S(\lambda)$, is determined primarily by the spectral absorbance, $\alpha_p(\lambda)$, of the visual pigment (P) in the rhabdomere. Thus, in many photoreceptors an almost direct proportionality exists between the electrophysiologically recorded $S(\lambda)$ and the photometrically measured $\alpha_p(\lambda)$. As an example, the compound eye of insects that can discriminate colours, e.g. the worker bee, usually possesses three types of photoreceptors with different spectral sensitivity maxima. In the worker bee all 3 $S(\lambda)$-functions can be simply approximated by nomograms describing the spectral absorbance of retinal chromoproteids with λ_{max} at 345, 435 and 520 nm (Autrum and v. Zwehl 1964; Menzel and Backhaus 1989).

In many photoreceptors the direct relationship between $S(\lambda)$ and $\alpha_p(\lambda)$ is masked or secondarily modified by several different mechanisms: 1. "Self screening" by the light absorbing rhabdomere along its optical pathway. The extent of self screening varies with the densities of the visual pigment (P) and its photoproduct (metapigment, M) within the microvilli, and with the length of the rhabomere (Hamdorf 1979). 2. Absorption by photostable pigments (ommatins, pteridins or C_{40}-carotenes) located in front of, along or inside the rhabdomere. These pigments act as edge filters, or as spectral filters (Kirschfeld et al. 1988). 3. Wave guide properties of the rhabdomeric structure (Snyder 1979). Another mechanism is, 4. the action of "sensitizing" or "antenna" pigments, i.e. fluorophores associated with the P-molecules in the rhabdomeric membrane (Kirschfeld 1986; Vogt 1989). In accordance with Förster's (1951) theory of intermolecular energy transfer, energy absorbed by the fluorophore is transferred to the chromophore of the visual pigment thereby secondarily eliciting photoreceptor excitation.

The spectral sensitivity of all receptor types in the compound eye of flies is to some extent affected by the first 3 mechanisms (Kirschfeld 1986; Kirschfeld et al. 1988). The most prominent modification of the spectral sensitivity is, however, due to a sensitizing pigment, which causes the pronounced UV-sensitivity of receptors R1–6 (Burkhardt 1962; Stark et al. 1979; Gemperlein et al. 1980; see also Figs. 2 and 10). The sensitizing pigment is probably identical with the retinoid 3-hydroxy-retinol

Abbreviations: CRBP, Cellular Retinol Binding Protein; P490, Visual pigment in photoreceptors 1–6

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(Vogt and Kirschfeld 1984; Vogt 1989), as indicated mainly by two observations. One observation (Vogt 1983) is that the high UV-sensitivity is much reduced when the fly larvae are reared on a diet deprived of vitamin A, which also reduces the content of the highly fluorescent 3-hydroxy-retinol. The other observation is that the absorbance spectrum of cellular retinol binding protein (CRBP) from rat liver (Ong and Chytil 1978) has a "vibronic fine structure" with peaks within the UV region at 350 and 368 nm, which is very similar to that of the fly photoreceptors (as recorded photometrically and electrophysiologically by Gemperlein et al. 1980 and Vogt and Kirschfeld 1984; see also review by Vogt 1989).

The present study was made in an attempt to answer some still open questions concerning the identity and steric configuration of the sensitizing pigment in fly photoreceptors as well as the number and specificity of possible binding sites on the visual pigment and/or other membrane molecules. The measurements were based on electrophysiological recordings showing 1. that flies with a strongly reduced UV-sensitivity (due to vitamin A deprivation) rapidly gain a normal, or even supernormal, UV sensitivity when certain vitamin A derivatives are applied to the cornea or are injected into the retina, and 2. that the fine structure of the UV-sensitivity can be modified by application of fluorescent probes, e.g. N-(1-pyrene)-maleimide.

Material and methods

The experiments were made on the fly *Calliphora erythrocephala* white eyed mutant *chalky*. The flies were usually used 10 days after emergence. In order to modulate the content of visual pigment in the photoreceptors of the adult, the larvae were reared either on cattle heart (a diet deprived of vitamin A) or on cattle liver (a diet rich in vitamin A). In flies reared on cattle heart the content of P490 in photoreceptors R1–6 is maximally reduced to about 5% of the content in flies reared on cattle liver (Hamdorf 1979). In the present study some breeds had a higher content, up to 40%.

Preparation; recording of photoreceptor responses. The flies were fixed to a support by Krönig's mixture (bee's wax 1 part and colophony, 2 parts). The head was inclined about 30° to the left and fixed in this position. The chitinous plate on the right back of the head was removed by a fine needle and the air bags that surround the eye and its optic lobe were dissected away. A fine silver wire (diameter 50 µm), inserted through the cornea into the retina, served as indifferent electrode. Glass pipettes filled with KCl (3 *M*; impedance 30–50 MΩ) were used for the intracellular recordings. The pipette was inserted into the eye from the back of the head almost in parallel with the visual axis of the photoreceptors. The experiments were carried out at 23 °C in a small brass chamber with two opposite openings; one for the light stimulus and the other for the glass electrode. The chamber was permanently ventilated by humidified oxygen. Under these experimental conditions constant responses were recorded from single cells during up to 4 h.

The photoreceptor responses were recorded by a FET impedance converter (input impedance 400 MΩ) combined with a storage oscilloscope (Tektronix 5113 equipped with a differential amplifier 5A22N with band pass filter). The filtered analog signal of the oscilloscope (upper frequency = 1 kHz, lower frequency = 0.1 Hz) was digitized by the A/D converter (14 Bit, 25 kHz) of an AT-computer (Rival 286).

Stimulus equipment. A xenon arc lamp (DC, Osram XBO 2000 W) with a quartz condenser was used as light source. Wavelengths longer than 650 nm were attenuated by a solution of $CuSO_4$ in a quartz vessel. The plasma ball of the arc was imaged on the entrance slit of a computer controlled grating monochromator (Zeiss M20). Wavelengths were set by a step motor, whereby one step corresponded to a shift in wavelength by 0.42 nm. The intensity of the light leaving the monochromator was regulated by a variable slit. The monochromatic light was focused by quartz lenses onto the cornea of the eye. The energy of the light flux at the eye was determined by a thermopile (Dexter Research Center 2MQ). Figure 1a shows the relative number of photons (rel. $Q = Q_\lambda/Q_{520}$) at all stimulus wavelengths between 300 and 700 nm, normalized to the number of photons at 520 nm ($Q_{520} = 4 \times 10^{12} \times$ photons $\times$ cm^{-2} $\times$ s^{-1}). As seen in Fig. 1a, relative Q increased (between 300 and 450 m) or decreased (between 500 and 700 nm) almost continuously. Discontinuities were recorded only within the wavelength region between 450 and 500 nm, due to the spectral lines of the stimulus arc within this region. Light stimuli of constant duration were obtained by an electronic shutter (Compur) controlled by a pulse generator and pulse counter. The pulse opening the shutter also triggered the oscilloscope and the A/D-converter of the AT computer.

Measuring program and evaluation of spectral sensitivity. The eye was stimulated by 150 successive monochromatic light flashes of equal duration (50 ms) and increasing wavelength (in steps of 2.1 nm) from 300 to 630 nm. The time interval between the flashes was 2 s. The responses to all stimuli within the spectral range of the photoreceptor were thereby recorded (and analyzed by the computer) within only 5 min (with a spectral resolution of 2.1 nm). The time course of the responses (at each monochromatic wavelength) during 360 ms was described by 2500 data points. Maximal response amplitude within the 100th and 600th data point (between 14 and 86 ms after onset of the stimulus) was found by the computer. The mean amplitude of ±10 data points around this peak value was regarded as the response amplitude (V) and filed in the data bank. Spectral response amplitudes ($V(\lambda)$) were transformed into relative spectral sensitivity (rel. $S(\lambda)$) by using the amplitude/intensity function ($V/\log_{10} I$) of the photoreceptor (Hamdorf 1979). The critical low intensity part of this function was determined in each experiment using six stimulus intensities ($I_\lambda = 1.0, 0.52, 0.28, 0.15, 0.08$ and $0.04 \times Q_\lambda/Q_{520}$) at two wavelengths (488 and 370 nm). According to Matic and Laughlin (1981) the $V/\log_{10} I$ relationship can be approximated by the exponential equation, Eq. 1:

$$V/V_{max} = (I/I_{0.5})^n/(1+(I/I_{0.5})^n),$$

where V = response amplitude, V_{max} = maximal amplitude after a saturating stimulus, I = stimulus intensity, $I_{0.5}$ = stimulus intensity eliciting 50% maximal response and n = exponential slope of the function. V_{max} elicited by bright, saturating xenon flashes was found to be 46±3 mV. V_{max} was the same in P^+- and P^--flies. Introducing $V/V_{max} = Z$ into Eq. 1 leads to the double logarithmic relationship, Eq. 2:

$$Y = \log_{10}(Z/(1-Z)) = n(\log_{10} I - \log_{10} I_{0.5}).$$

If the response amplitudes in terms of Y against $\log_{10}$ stimulus intensity, I, are plotted in terms of $\log_{10}(Q_\lambda/Q_{520})$, the variables n and $I_{0.5}$ in Eq. 1 can be evaluated, whereby n corresponds to the slope of the straight line and $I_{0.5}$ corresponds to the value of $\log_{10}(Q_\lambda/Q_{520})$ at the point where the straight line intersects Y = 0 (see Fig. 1c).

Using the two monochromatic stimuli (488 and 370 nm), n was found to be almost constant, 0.66±0.03. Figure 1b shows the computer fit of the $V/\log_{10} I$ values at 488 and 370 nm, recorded in an individual experiment, to the theoretical function, Eq. 1: The distance between the parallel curves corresponds to the factor, f, by which the intensity of a 488 nm stimulus should be increased in order to elicit a response the amplitude of which is equal to that elicited by a 370 nm stimulus (see arrows in Fig. 1b). The relative spectral sensitivity is usually defined as the reciprocal of this factor, i.e. relative $S(\lambda) = 1/f(\lambda) \cong (Q^*_{\lambda max}/Q^*_\lambda)(\lambda)$, in which Q^*_λ is the

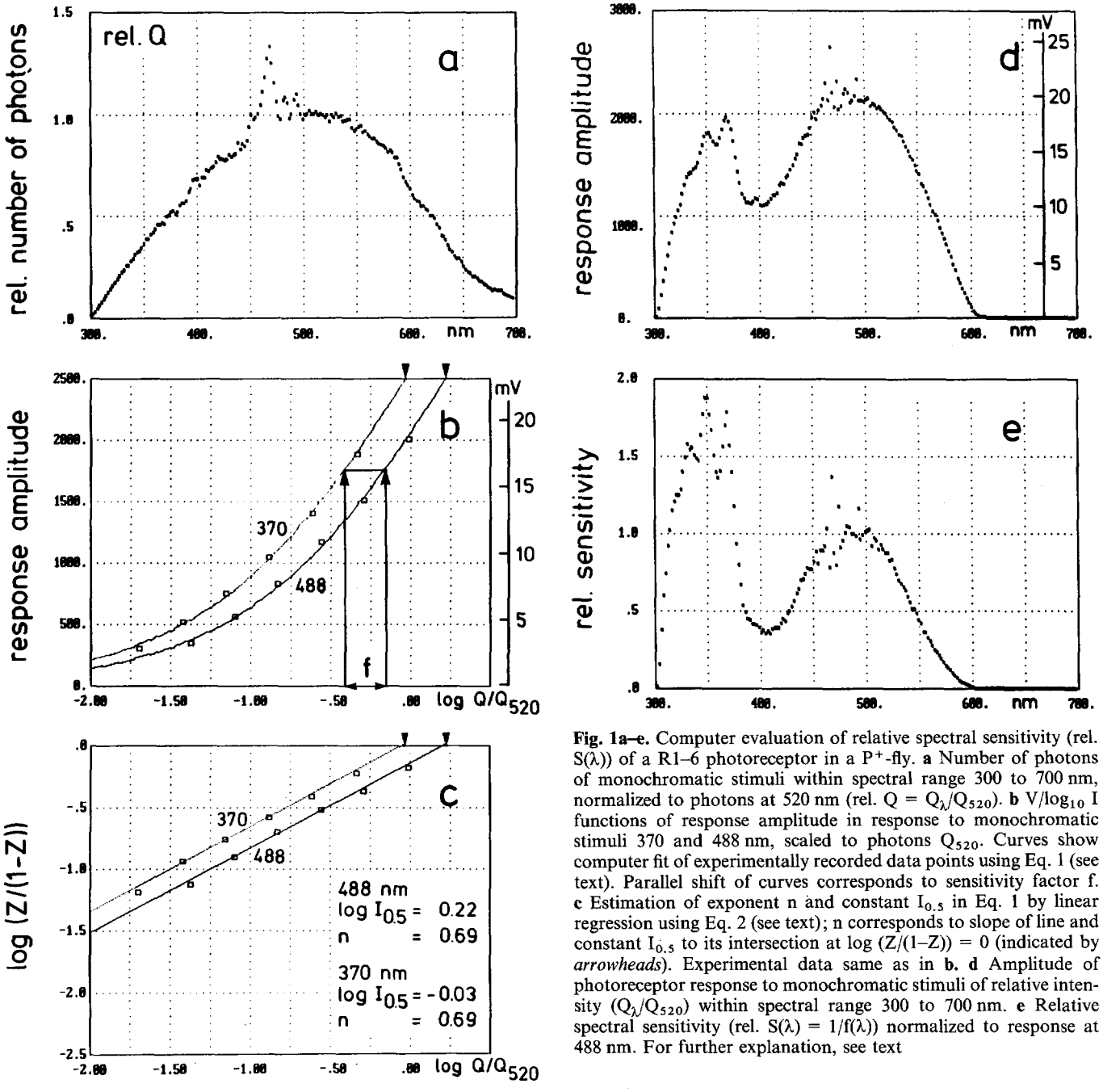

Fig. 1a–e. Computer evaluation of relative spectral sensitivity (rel. S(λ)) of a R1–6 photoreceptor in a P^+-fly. **a** Number of photons of monochromatic stimuli within spectral range 300 to 700 nm, normalized to photons at 520 nm (rel. $Q = Q_\lambda/Q_{520}$). **b** $V/\log_{10} I$ functions of response amplitude in response to monochromatic stimuli 370 and 488 nm, scaled to photons Q_{520}. Curves show computer fit of experimentally recorded data points using Eq. 1 (see text). Parallel shift of curves corresponds to sensitivity factor f. **c** Estimation of exponent n and constant $I_{0.5}$ in Eq. 1 by linear regression using Eq. 2 (see text); n corresponds to slope of line and constant $I_{0.5}$ to its intersection at $\log (Z/(1-Z)) = 0$ (indicated by *arrowheads*). Experimental data same as in **b**. **d** Amplitude of photoreceptor response to monochromatic stimuli of relative intensity (Q_λ/Q_{520}) within spectral range 300 to 700 nm. **e** Relative spectral sensitivity (rel. $S(\lambda) = 1/f(\lambda)$) normalized to response at 488 nm. For further explanation, see text

intensity of a stimulus at wavelength λ that evokes a response the amplitude of which is equal to that evoked by the stimulus intensity at the most effective wavelength ($Q^*_{\lambda max}$). In blowfly photoreceptors, λ_{max} alternates between the two wavelengths 350 nm and 490 nm, the position being strongly dependent on the amount of sensitizing pigment present in the receptor. To facilitate a comparison between functions, in the present article, 488 nm was chosen as reference wavelength, since it is close to the λ_{max} of the visual pigment, P490.

All relative sensitivity spectra were computed using the individual $V/\log_{10} I$ functions at the wavelengths 488 and 370 nm: Firstly, the intensity value corresponding to the response amplitude evoked by a monochromatic stimulus of intensity $I_\lambda = Q_\lambda/Q_{520}$ was read on the $\log_{10} Q/Q_{520}$ scale (Fig. 1b). Secondly, the difference between $\log_{10} Q_{488}/Q_{520}$ and $\log_{10} Q_\lambda/Q_{520}$ was calculated. This difference ($\log_{10} Q_{488}/Q_{520} - \log_{10} Q_\lambda/Q_{520}$ is equal to $\log_{10} 1/f_\lambda$. Thirdly, all $\log_{10} 1/f_\lambda$ values were transformed into numerical values, resulting in spectra normalized to 488 nm (Fig. 1e).

Modulation of the P490 and sensitizing pigment content in the R1–6 receptors by long lasting "green" or "blue" illumination. As demonstrated by Schwemer (1984, 1989), the content of P490 in the rhabdomeres can be drastically reduced within a few days by continuous "green" illumination. In contrast, the P490 content remains almost constant in continuous "blue" light. The present experiments (Fig. 3) were made using the same equipment as described in detail by Schwemer (1984). In brief, the flies were fed by sugar only and kept in cages (volume 51) covered either by a "green" or a "blue" acryl filter and illuminated by fluorescent light. Due to the acryl filter the bandwidth of the illumination was restricted to about 60 nm around 430 ("blue") or 530 nm ("green"). The change in visual pigment content in the treated eyes was continuously con-

trolled by recording difference spectra (spectrophotometer Perkin-Elmer 356) from isolated eyes (Hamdorf 1979).

Modulation of UV sensitivity by exogenous retinoids. The physiological effect of different retinal and retinol isomers (9-cis, 13-cis and all-trans, Sigma; 11-cis, gift from Schwemer) and of the retinal derivatives 13-ethyl-retinal and 13-propyl-retinal (synthesized by Bestmann) were tested. The retinoids were dissolved (concentration 10^{-2} *M*) in heptane, either directly or after having been previously dissolved in methanol or ethanol. A drop of the solution (usually 0.6–0.8 µl) was applied to the cornea of the right eye by a micro syringe. Vitamin A_2 (all-trans-3-dehydro-retinol; placed at our disposal by Hoffmann-LaRoche) was dissolved in methanol/heptane, or methanol, and then either applied to the cornea or injected into the retina. It was also tested whether carotenoids, known to be precursors of retinal and 3-hydroxy-retinal (β-carotene, lutein and zeaxanthin; Roth), can be taken up by the eye and transformed into antenna pigment.

Modulation of UV sensitivity by intraocular injection of fluorescent probes. The fluorescent analogs P–28 (N-(1-pyrene)maleimide) and A–485 (4-acetamido 4′-maleimidylstilbene 2,2′-disulfonic acid, disodium salt) of N-ethylmaleimide were used (Molecular Probes, Inc). These maleimides are thiol selective reagents. Their fluorescence drastically increases when the substance is covalently bound to protein (Haugland 1989). P–28 was dissolved in DMSO/methanol (1/5) and A–485 in NaCl (130 m*M*). The concentrations of the solutions (controlled photometrically) were 6–8 m*M*. Of the solution 0.2–0.4 µl was injected into the eye at its frontal rim. A small puncture had been made at the opposite rim to insure an almost equal distribution of the solution within the eye and to reduce the hydrostatic pressure.

Fluorescence of stained rhabdomeres in P^+- and P^--flies. The methods used to isolate ommatidia and rhabdomeres, as well as the microspectrofluorometric measuring technique, were made as described in detail by Schlecht et al. (1987) and Juse et al. (1989). Isolated rhabdomeres were stained in NaCl solution (130 m*M*) by the pyrene fluorophores (Molecular Probes Inc.) P–28, P–223 (1-pyrenemethyl 3-β-hydroxy-22,23-bisnor-5-cholenate) and P–58 (N(1-pyrenesulfonyl)dipalmitoyl-L-α-phosphatidylethanolamine, triethylammonium salt). The fluorophores P–28 and P–223 were dissolved in methanol (0.45 m*M*) and P–58 in heptane (0.1 m*M*). Approximately 10 µl of the solution was added to the preparation. The increase of the rhabdomeric fluorescence was continuously measured by a microspectrofluorometer (based on a modified fluorescence microscope, Leitz Orthoplan; Schlecht et al. 1987) during 10 to 180 min after the staining. The wavelength 370 nm was used for excitation and the increase in emission was measured at 470 nm. Maximal emission was reached about 2 h after staining. In order to ascertain that all SH-groups were specifically stained by P–28 the cysteines in control experiments were reduced to cystine by dithiothreitol (DTT; 0.5 and 10 m*M*; 10 min pre-incubation before staining; Molecular Probes Inc.). Measurements were made both on P^+- and P^--flies in order to demonstrate that P–28 dominantly stains the rhabdomeric P490 molecules. Control experiments were made, using P–223 and P–58 as unspecific, neutral membrane probes.

Results

Sensitivity spectra of P^+ and P^--flies

The relative spectral sensitivities of the R1–6 receptors in flies reared on vitamin A deprived (P^-) or enriched (P^+) diet were characterised by the following distinctive features (Fig. 2): In P^--flies the relative S(λ) of the receptors well agreed with the absorbance spectrum of visual pigment P490 (Paulsen and Schwemer 1979) not only within the spectral range of the α-band (400 to 620 nm) but also within the range of the β-band (330 to 370 nm). Furthermore, the β-band displayed only a smooth peak around 350 nm and no "vibronic fine structure". In contrast, in P^+-flies the spectral sensitivity functions were somewhat broader than the α-band of the P490 absorbance spectrum. The deviation is most probably caused by the "self screening" effect of the visual pigment in rhabdomeres with high pigment content (causing an absorbance exceeding 0.3; Hamdorf 1979; Smakman and Stavenga 1986). A pronounced vibronic fine structure was seen within the range of the β-band due to the presence of sensitizing pigment. The fine structure was characterised by three sensitivity maxima, at 333, 350 and 369 nm, close to the wavelengths determined by FIS (Fourier Interferometric Stimulation; Gemperlein et al. 1980) and to electrophysiological data (Gribakin and Ukhanov 1990), and by two minima, at 339 and 362 nm. The relative sensitivities of the maxima and minima, normalized to 488 nm or 350 nm respectively, are summarised in Table 1. The content of P490 in P^+-flies was about 10 times higher than in P^--flies, as indicated by the intensity of a monochromatic stimulus (488 nm) necessary to elicit a criterion response of 20 mV (Hamdorf 1979). In P^+-flies a width of the monochromator slit of 0.02 ± 0.01 mm was sufficient to evoke the criterion response, whereas in P^--flies the slit had to be opened to 0.2 ± 0.10 mm, corresponding to 10 times higher intensity.

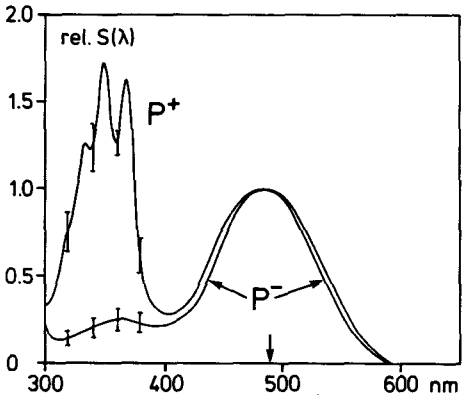

Fig. 2. Mean spectral sensitivity (rel. S(λ)) of R1–6 photoreceptors in P^+- and P^--flies normalized to sensitivity at 488 nm. *Bars* show SEM at 4 wavelengths within UV range. $n = 23$, 18. Sensitivity of P^--receptors about 0.1 that of P^+-receptors, due to corresponding reduction of visual pigment in rhabdomere as recorded photometrically. Note 1. that sensitivity spectrum of P^--receptors has no fine structure in UV region, indicating that relative $S_{360} = 0.25$ is exclusively due to β-band of P490, and 2. that band width of spectral sensitivity of P^--receptors is significantly smaller than that of P^+-receptors

Modulation of the spectral sensitivity induced by "blue" or "green" irradiation

The P490 content in receptors R1–6 of P^+-flies can be reduced to that of P^--flies by continuous irradiation by

Table 1. Fine structure of UV spectral sensitivity of photoreceptors R1–6 in P^+-flies before (control) and after application to cornea of 13-cis-retinol. Control, upper row: S_λ values normalized to sensitivity at 488 nm, S_{488}; lower row: S_λ values normalized to relative sensitivity at 350 nm, S_{350}. 13-cis-ol: S_λ values normalized to relative sensitivity at 350 nm, ($S_{350,488} = 1.93 \pm 0.12$). Mean and SD

Maxima / Minima		333 nm	339 nm	350 nm	362 nm	369 nm	*n*
P^+	control	1.28 ± 0.14	1.22 ± 0.12	1.73 ± 0.11	1.25 ± 0.10	1.64 ± 0.10	
		0.74 ± 0.08	0.71 ± 0.04	1.00	0.72 ± 0.06	0.95 ± 0.06	23
	13-cis-ol	0.73 ± 0.08	0.70 ± 0.08	1.00	0.66 ± 0.06	0.91 ± 0.06	14

green light (Schwemer 1984). Accordingly, in the present study, in parallel with a decrease in P490 content (determined photometrically) the absolute sensitivity of the photoreceptor (determined electrophysiologically) dropped to that in P^--flies during illumination by green light. In contrast to the receptors of P^--flies, the spectral sensitivity of the P^+-receptors within the UV region had the same fine structure as in the control animals. Furthermore, the amplitudes of the 3 sensitivity peaks in the UV region were equal to, or even higher, than in the controls. This finding indicates that the sensitizing pigment is incorporated into the rhabdomeric membrane as long as all-trans retinoids are available in sufficient quantity. As predicted by Schwemer's results (1986), irradiation of P^+-flies by continuous blue light affected neither the P490 content nor the spectral sensitivity of the photoreceptors.

In P^--flies, green light induced surprising results (Fig. 3): 1. The previously recorded smooth sensitivity spectrum in the UV region was transformed into an UV spectrum with a vibronic fine structure, and of a slightly higher amplitude than before the green irradiation (Fig. 3). As expected, in parallel the absolute sensitivity of the receptors dropped. This result can be explained by the same mechanism as that mentioned above, i.e. if free all-trans retinoid is made available by the destruction of visual pigment the retinoid can be used as sensitizing pigment. 2. In contrast to P^+-receptors, the spectral sensitivity of P^--receptors was modified not only within the UV range but also within the spectral range of the α-band of the visual pigment, as illustrated by the spectral sensitivity functions and the difference spectrum seen in Fig. 3. In comparison with the control, the spectral sensitivity was clearly shifted about 20 nm towards shorter wavelengths (arrowheads in Fig. 3). This hypsochromic shift, and the symmetry of the difference spectrum in the region between 400 nm and 600 nm, indicate that the absorption characteristic of the visual pigment is modified, probably due to a change in tertiary structure of the P490 molecule. As in P^+-receptors, blue light had no effect.

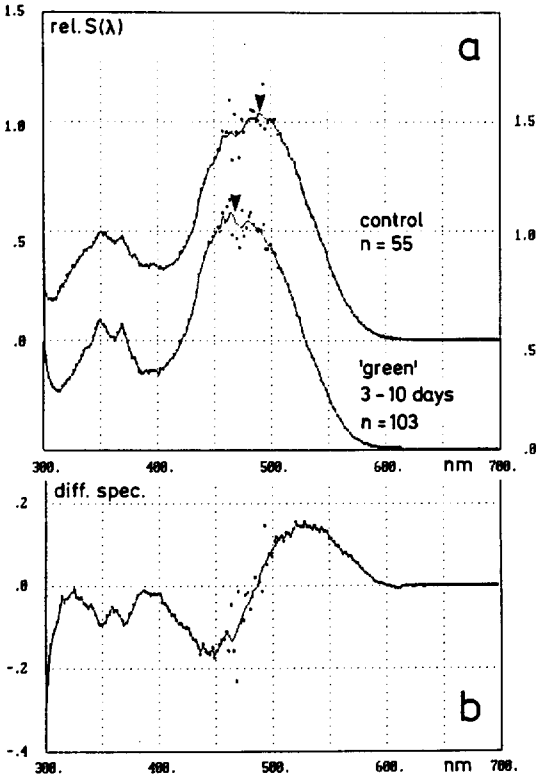

Fig. 3a, b. Modulation of spectral sensitivity of P^--receptors caused by continuous irradiation by "green" light for 3 to 10 days. **a** Sensitivity spectra of P^--flies kept in continuous "white" (control) or "green" light. Note 1. that UV sensitivity of control is higher (0.5) than that of untreated P^--receptors (0.25; Fig. 2) and that UV fine structure is just detectable, and 2. that "green" irradiation induces shift in λ_{max} from 490 to 470 nm (arrowheads). **b** Difference spectrum between curves in **a** (control minus "green"). Note symmetry of difference spectrum within range 400 to 600 nm and increase in UV fine structure induced by "green" irradiation

Induction of UV sensitivity in P^--flies by corneal application of retinoids

Application of carotenoids to the cornea of P^--flies led to a considerable increase in the UV sensitivity within a few hours. As an example, Fig. 4 shows the increase in UV sensitivity and the development of the vibronic fine structure in an individual photoreceptor following the application of all-trans retinal. Already 45 min after the application, the relative sensitivity at 350 nm was clearly higher (0.5, Fig. 4) than that of untreated receptors in P^--flies (0.25, Fig. 2), and after 80 min the fine structure with peaks at 350 and 369 nm was detected. The fine structure was more pronounced after 110 min, and after 160 min all 3 peaks, typical for the fine structure within the UV region, were clearly separated. Furthermore, the relative sensitivity at 350 nm was even somewhat higher

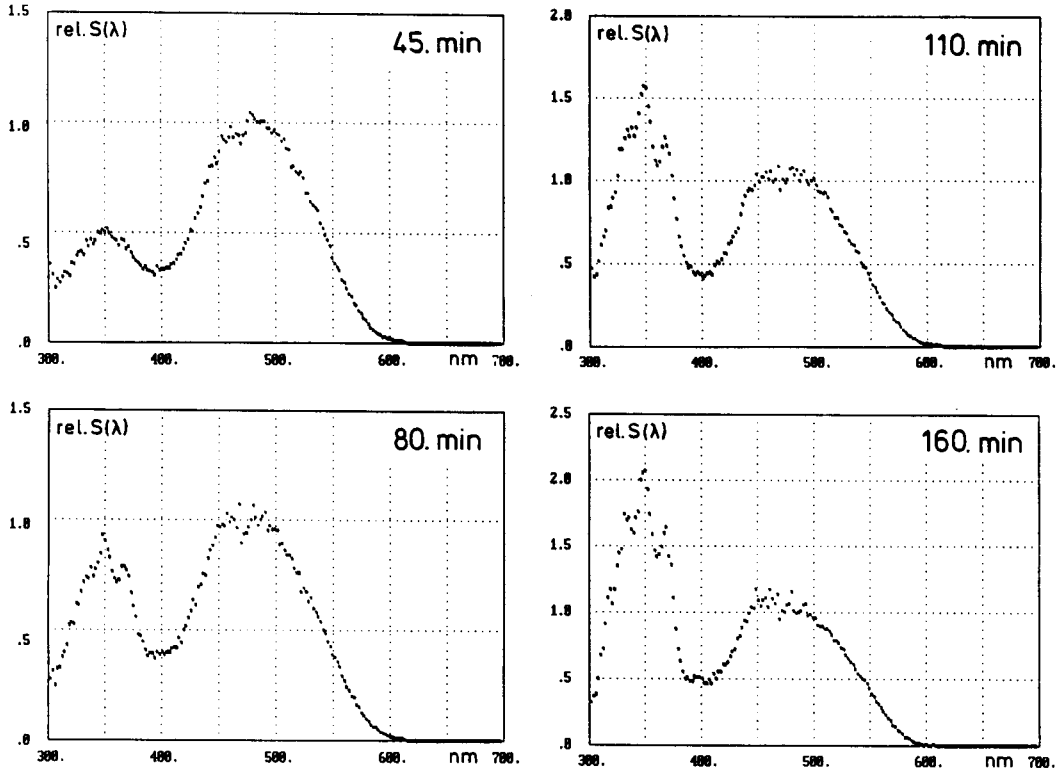

Fig. 4. Increase in UV sensitivity of individual P^--photoreceptor recorded 45, 80, 110 and 160 min after application of all-trans-retinal to cornea. Note that already 45 min after application UV sensitivity is clearly higher (0.5) than in control (0.25; compare spectra in Fig. 2 and in Fig. 5, 20–30 min)

(rel. $S_{350}=2.1$; Fig. 4) than in the P^+-receptors (rel. $S_{350}=1.7$; Fig. 2). In spite of the increase in UV sensitivity, the absolute sensitivity of the receptor within the range between 420 nm and 600 nm remained unchanged, as evidenced by the fact that the width of the slit necessary to evoke the criterion response of 20 mV at 488 nm remained unchanged (0.2 mm). A similar time course of increase in UV sensitivity after application of all-trans retinal was seen in all the receptors studied, as illustrated by the averaged spectra shown in Fig. 5.

Usually, immediately after application of the all-trans retinal the resting potential of the photoreceptors dropped and no response to light stimulation could be evoked, but within about 10 to 15 min the light sensitivity of the receptor was re-established completely. This "narcotic" effect was clearly due to the solvent heptane, since it was elicited also after application of heptane alone. Already about 30 min after the cessation of the "narcotic" effect the all-trans-retinal induced the first modulation in the UV sensitivity (Fig. 5, 40–50 min), and after about 3 h (Fig. 5, 150–270 min) UV spectra very similar to those recorded from P^+-flies were seen. The time course of increase at 350 nm could be approximated by a straight line within a large time interval (50 to 160 min; see Fig. 6). The slope of the line was about 0.8/h Δrel. $S_{350,488} \times h^{-1}$. Following this fast increase, the UV sensitivity remained constant for hours.

A very similar fast increase in UV sensitivity up to relative sensitivity 1.75 occurred after application of 13-cis retinal (Fig. 6). The recorded data points could be approximated by the same straight line as that describing the sensitivity increase after application of all-trans retinal. This result indicates that these two isomers of retinal can serve equally well as sensitizing pigment or its precursor.

The eye can use also the corresponding isomers of retinol as sensitizing pigment (or its precursor). Application of 13-cis-retinol led, however, to a much faster increase in UV sensitivity than application of the all-trans-isomere (1.0/h as compared to 0.3/h; Fig. 6). In addition, 13-cis-retinol was accepted by the receptor distinctly earlier and faster than both isomeres of the aldehyde. The effectiveness of a retinoid as sensitizing pigment (or precursor) thus clearly depends on whether it is a retinal or a retinol, as well as on its steric configuration.

Pilot experiments using 9-cis- and 11-cis-retinal suggested that the steric configuration is important also in aldehydes, since both isomers seemed to be accepted by the receptor somewhat slower than the all-trans and 13-cis forms. A similar effect, i.e. slow increase in UV sensitivity, was seen after application of synthetic derivatives such as 13-ethyl-retinal or 13-propyl-retinal. 13-ethyl-retinal (a mixture of all-trans and other isomeres) led to a somewhat slower increase in sensitivity (0.4/h) than naturally occurring retinals (all-trans, 13-cis; 0.8/h), but to a somewhat faster increase than all-trans retinol (0.3/h; Fig. 6). In spite of the slow increase, maximal UV sensitivity induced by 13-ethyl-retinal was much higher ($S_{350,488}=2.5$, recorded after 20 h) than that recorded after any other tested substance. An extremely slow increase was induced by 13-propyl-retinal. Nevertheless an

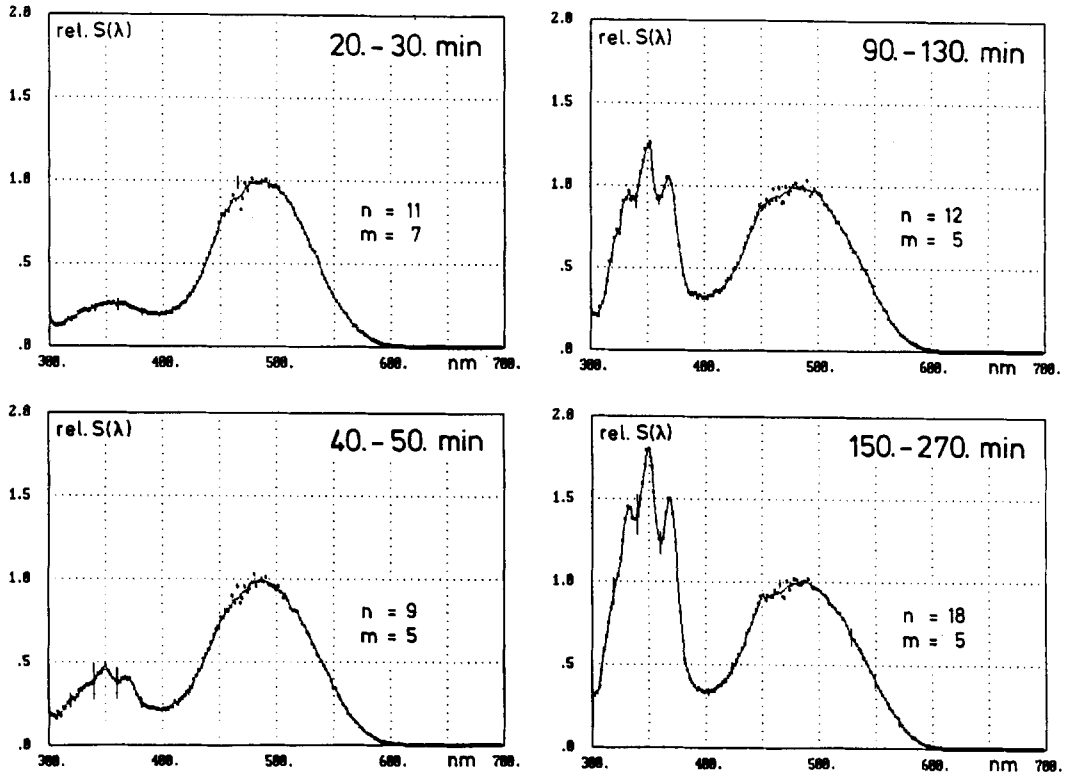

Fig. 5. Increase in relative UV sensitivity of P^--photoreceptors after application (20–30 min, 40–50 min, 90–130 min and 150–270 min) of all-trans-retinal to cornea. n = number of analyzed receptors; m = number of analyzed flies. Bars indicate SEM

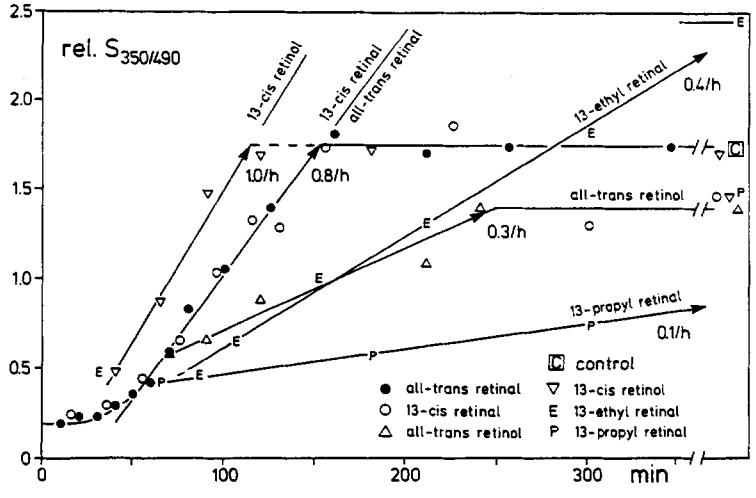

Fig. 6. Increase in relative UV sensitivity ($S_{350,488}$) of P^--photoreceptors following application of different vitamin A_1 derivatives to cornea. Values at right side of figure show maximal sensitivity recorded at least 20 h after application. Note 1. that fastest increase (Δrel. $S_{350,488} \times h^{-1} = 1.0 \equiv 1.0/h$) occurs after application of 13-cis-retinol, and 2. that 20–24 h after application of 13-ethyl-retinal relative $S_{350,488} = 2.5$ clearly exceeds value recorded from P^+-receptors ($\cong 1.75$, control). Corneal application of all-trans-vitamin A_2 (not shown in figure) leads to similar, slow increase in UV sensitivity (0.2/h) as application of all-trans-vitamin A_1 (all-trans-retinol)

UV sensitivity (1.5) similar to that of P^+-receptors was reached within one day. Both synthetic derivatives thus can replace the naturally occurring retinal or retinol, or its precursor(s), in the sensitizing pigment(s). In addition, the continuous increase in UV sensitivity up to the high value of 2.5 indicates that 13-ethyl-retinol leads to the formation of a sensitizing pigment, which is more effective than the naturally occurring sensitizing pigment.

In contrast to the retinoids so far tested, carotenes, like β-carotene, lutein or zeaxanthin cannot serve as precursors for the sensitizing pigment if applied to the cornea, as illustrated by the experiment seen in Fig. 7. Even 18 h after application of zeaxanthin no increase in UV sensitivity was recorded in P^--flies. The same negative result was obtained after application of β-carotene or lutein. This finding indicates that all cells in the compound eye of blowflies lack the enzyme necessary to split the carotenes into vitamin A molecules, i.e. to split β-carotene into two molecules of vitamin A_1 aldehyde (retinal) or zeaxanthin into two molecules of vitamin A_3 aldehyde (3-hydroxy-retinal).

Variations in fine structure of the UV spectrum with type of applied retinoid

All retinoids so far tested caused very similar sensitivity spectra in the UV region with maxima at 333, 350 and

Table. 2. Spectral sensitivity of photoreceptors R1–6 in P^+- and P^--flies within UV range after application to cornea of all-trans- or 13-cis-isomers of retinal and retinol. Before averaging individual sensitivity spectra were normalized to sensitivity maximum at 350 nm. *n* = number of analyzed photoreceptors; *hours* = time after application. Means and SD. Note: With one exception maxima and minima of fine spectra are very similar; exception is 13-cis-retinal which induced a maximum at 333 nm that was slightly larger, and maximum at 369 nm that was slightly smaller, than those of control

Maxima / Minima		333 nm	339 nm	350 nm	362 nm	369 nm	*n*	hours
P^+	control	0.79 ± 0.04	0.75 ± 0.03	1.00	0.75 ± 0.04	0.92 ± 0.04	23	
	13-cis-*ol*	0.78 ± 0.03	0.74 ± 0.04	1.00	0.68 ± 0.03	0.90 ± 0.06	14	17–26
P^-	all-trans-*al*	0.80 ± 0.06	0.75 ± 0.06	1.00	0.67 ± 0.05	0.84 ± 0.05	7	4– 4.5
	13-cis-*al*	0.85 ± 0.07	0.80 ± 0.08	1.00	0.72 ± 0.02	0.81 ± 0.08	5	2.5– 3
	all-trans-*ol*	0.77 ± 0.07	0.75 ± 0.10	1.00	0.73 ± 0.08	0.85 ± 0.05	4	4
	13-cis-*ol*	0.77 ± 0.06	0.72 ± 0.02	1.00	0.72 ± 0.06	0.89 ± 0.05	7	2.5– 3.5

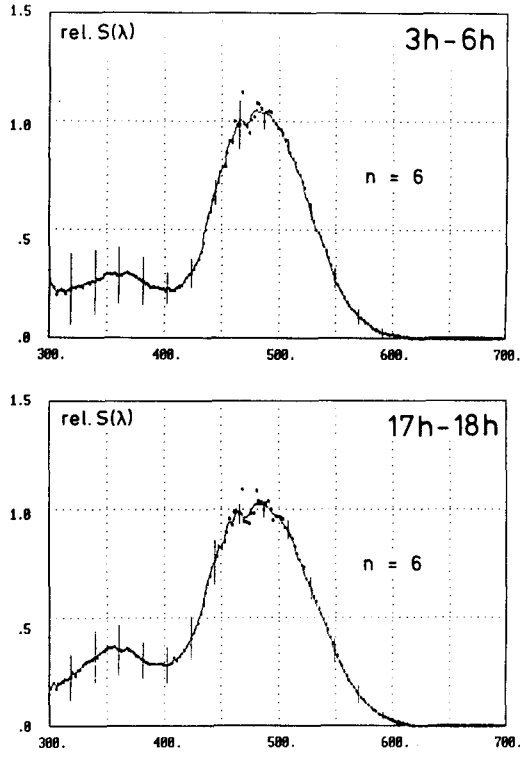

Fig. 7. Spectral sensitivity of P^--photoreceptors 3–6 h and 17–18 h after application to cornea of zeaxanthin (presumed optimal precursor for 3-hydroxy-retinol). Note that no increase in UV sensitivity occurs. Application of β-carotone or lutein also has no effect, indicating that all cells in eye lack enzyme to split carotenoids into two retinoids that can be used for visual excitation

369 nm and minima at 339 and 362 nm. In order to detect possible minor differences, the UV sensitivity spectra were normalized to the amplitude of the main maximum at 350 nm (Table 2). With one exception (13-cis-retinal) no spectrum was statistically different from the UV spectrum of P^+-flies. The spectrum recorded after application of 13-cis-retinal had a maximum at 333 nm that was slightly larger (0.85), and a maximum at 369 nm that was slightly smaller (0.81), than the corresponding maxima recorded from control eyes (0.79 and 0.92, respectively). The slight difference at 369 nm was verified by *t*-test ($P = 0.01$, Table 2). The same tendency (although not statistically significant) is revealed by a comparison of the sensitivity peaks seen after application of 13-cis-retinal with those recorded after application of 13-cis-retinol (Table 2). These results suggest that the fine structure of the spectral sensitivity in the UV region possibly is slightly modified on the one hand by the aldehyde- or alcohol group of the applied retinoid, and on the other hand by its steric configuration.

Supernormal UV sensitivity induced by application of retinoids

It was often observed that after application of retinoids to P^--eyes the UV sensitivity of the photoreceptors (rel. $S_{350,488}$) became very high (up to 3.3), clearly exceeding the sensitivity in P^+-eyes (1.73, Table 1). A similar increase was induced also in P^+-eyes after 13-cis-retinol had been applied to the cornea. About 20 h after the application the sensitivity had increased (slightly but highly significantly, $P = 0.0005$) from 1.73 to 1.93 (Table 1). The supernormal sensitivity increase in P^--receptors, as well as in P^+-receptors, after incorporation of different retinoids suggests a molar ratio exceeding one sensitizing pigment per visual pigment (see Discussion).

Modulation of the vibronic fine structure of UV sensitivity in P^--receptors induced by vitamin A_2

Application of 3-dehydro-retinol (vitamin A_2) to the cornea led to a slow (0.2/h) increase in UV sensitivity similar to that following application of all-trans-retinol (0.3/h, Fig. 6). The UV sensitivity spectrum recorded after the application had the same vibronic fine structure as that induced by vitamin A_1 derivatives. Contrastingly, if injected into the eye vitamin A_2 induced a very fast increase in UV sensitivity (> 1.0/h). The vibronic fine structure differed markedly from that caused by vitamin A_1 derivatives (Fig. 8, curve 120–180 min). The main maxima, at 350 and 369 nm, were shifted to 358 and 380 nm, respectively. This unusual spectral sensitivity converted into a normal one within a few hours (Fig. 8, curve 300–420 min). These observations indicate that

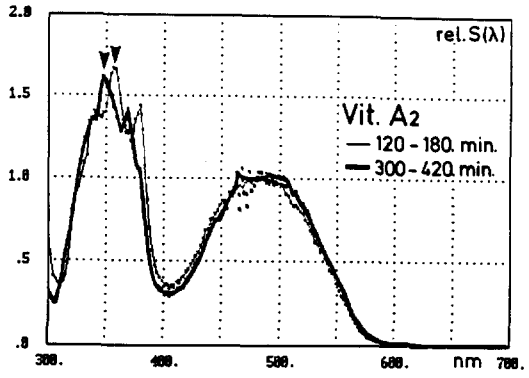

Fig. 8. Spectral sensitivity of P^--photoreceptors 120–180 min and 300–420 min after injection of vitamin A_2 into retina of P^--flies. Note that after injection, sensitivity peaks appear at 358 and 380 nm, and that spectrum changes to normal within a few hours only *(arrowheads)*

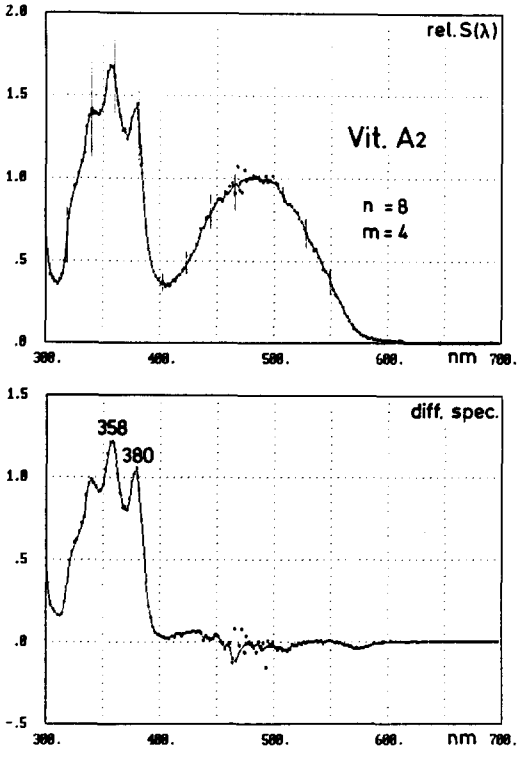

Fig. 9. Spectral sensitivity of P^--photoreceptors 120–180 min after injection of vitamin A_2 into retina and its difference spectrum against control. Difference spectrum (Vit. A_2, *upper graph*, minus P^--spectrum, Fig. 10) corresponds to absorption spectrum of transiently formed A_2 specific UV sensitizing pigment

vitamin A_2 can directly serve as sensitizing pigment, and that this vitamin very probably is replaced by naturally occurring antenna pigment formed enzymatically and using vitamin A_2 as precursor.

Spectral absorbance of UV sensitizing pigment

The spectral absorbance of a sensitizing pigment can be calculated as the difference between the sensitivity spectra recorded before and after application of a substance to the eye (Fig. 9). The sensitizing pigment induced by injection of vitamin A_2 is characterised by 3 vibronic peaks, at 340, 358 and 380 nm, and by a steep decrease in absorbance to zero at about 400 nm. In contrast, the spectral absorbance of the naturally occurring UV sensitizing pigment is characterised by vibronic peaks at 333, 350 and 369 nm and a steep decrease to zero already at about 390 nm (Fig. 10; function obtained by calculating the difference between the sensitivity spectra of P^+- and P^--receptors, which have an almost equal bandwidth within the range 430 to 600 nm).

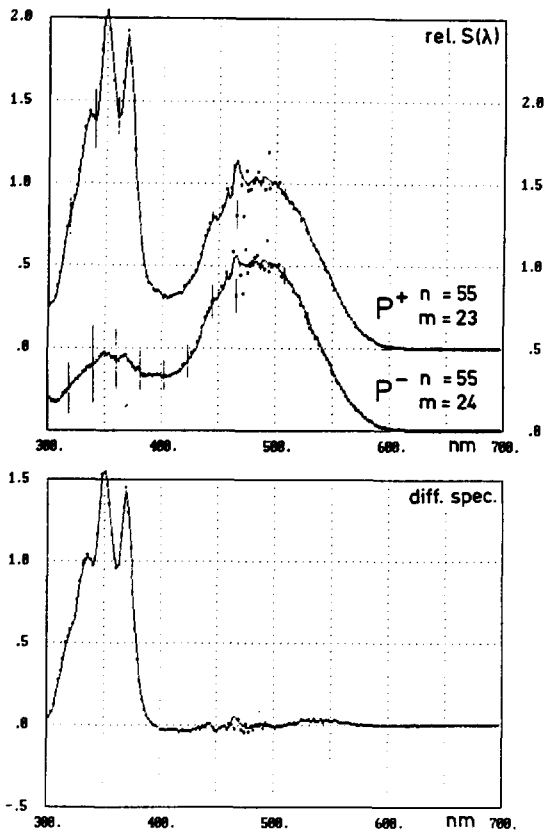

Fig. 10. Spectral absorbance of naturally occurring UV sensitizing pigment *(lower graph)* obtained by calculating difference between sensitivity spectra of P^+- and P^--photoreceptors of almost equal bandwidth within "visual" range *(upper graph)*

Induction of artificial UV sensitivity by the fluorophore P–28

Within 60 to 120 min after injection of the pyrene maleimide P–28 into P^--eyes an anomalous UV sensitivity appeared with peaks at 325 and 343 nm (Fig. 11). The difference between the artificial spectrum and that of the P^--control very well agreed with the spectral absorbance of the fluorophore pyrene. Pyrene maleimides are not appreciably fluorescent until after conjugation with thiols. It is therefore very likely that in order to act as an UV sensitizing pigment the probe has to be covalently bound to the visual pigment molecule.

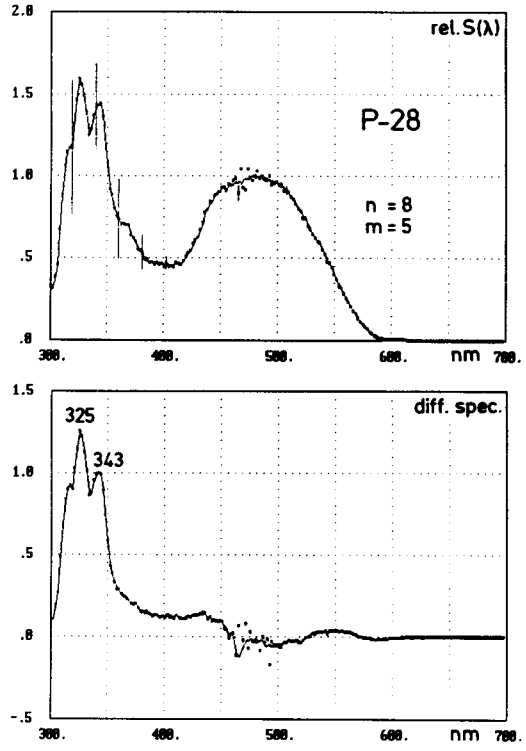

Fig. 11. Spectral sensitivity of P^--photoreceptors 60–120 min after injection of P–28 (N-(1-pyrene) maleimide; *upper graph*) and spectral difference against control (P^--receptors, Fig. 10, *lower graph*). Difference spectrum is very similar to excitation spectrum of pyrene (not shown)

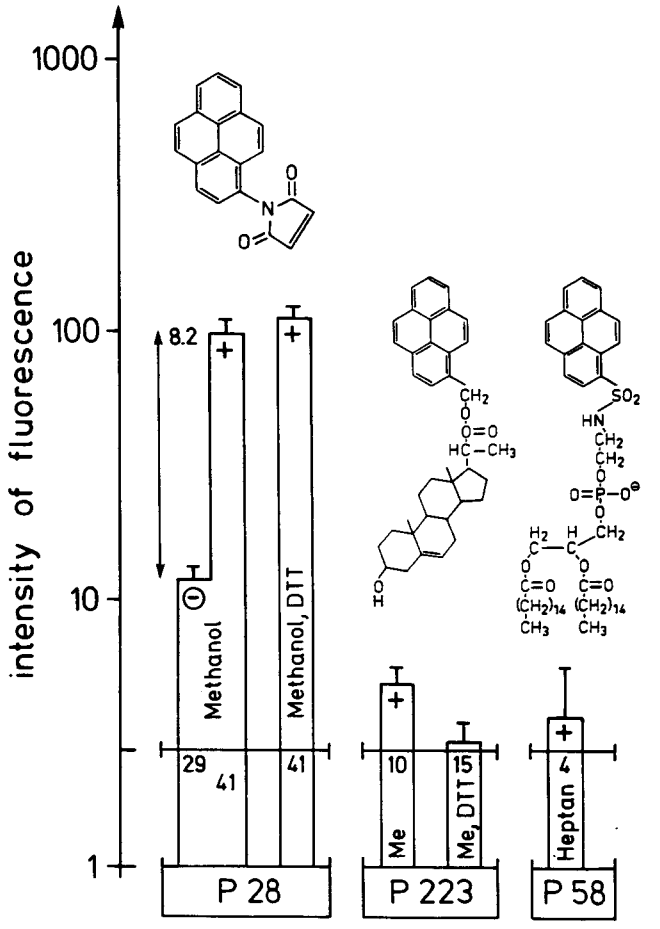

Fig. 12. Fluorescence of rhabdomeres in P^- (–) and P^+-flies (+) after staining by pyrene derivatives P–28, P–223 and P–58. Excitation of fluorescence by 370 nm and measurement at 470 nm. *n* of measurements on different rhabdomeres given below line indicating autofluorescence intensity. Note 1. that fluorescence from P^+-rhabdomeres was 8.2 times that from P^--rhabdomeres, 2. that reduction of cystines available for staining by DTT causes only slight increase in fluorescence, 3. that control experiments using neutral membrane probes cause only slight increase above autofluorescence

A covalent binding was supported by in vitro staining experiments using the -SH-probe P–28 and the neutral membrane probes P–223 and P–58. A comparison of the rhabdomeric fluorescence in P^--flies with that in P^+-flies (Fig. 12) clearly showed that the fluorescence was proportional to the content of P490 in the rhabdomere. The fluorescence in P^+-flies was 8.2 times higher than that in P^--flies, corresponding to the relative pigment content determined spectrophotometrically. Furthermore, pre-incubation using DTT, which specifically reduces cysteine to cystine, led only to a small further increase in fluorescence. In contrast to the staining by P–28, the neutral membrane probes P–223 and P–58 only caused a slight increase above the autofluorescence.

Reduction of the normal UV sensitivity by the fluorophore A–485

In contrast to P–28, injection of the maleimidyl stilbene A–485 did not enhance the UV sensitivity of P^--receptors. The opposite effect was obtained; a drastic reduction in UV sensitivity occurred. It is to be expected that also this water soluble probe binds to the visual pigment. Due to its hydrophilicity the probability for a parallel orientation of A–485 to the visual pigment is, however, smaller than for the hydrophobic pyrene P–28, and hence a transfer of absorbed photon energy to the chromophore of the visual pigment is also less probable. As a

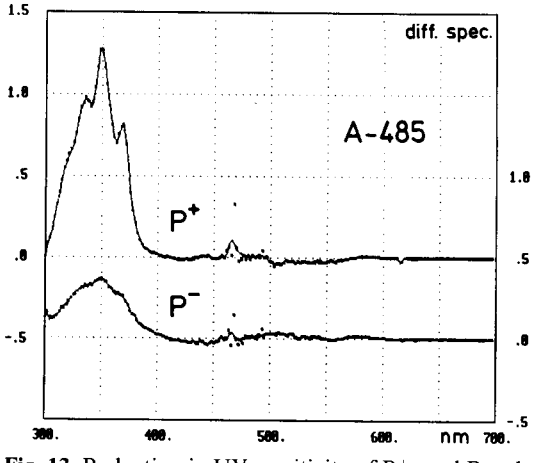

Fig. 13. Reduction in UV sensitivity of P^+- and P^--photoreceptors after injection of A–485. Difference spectra (control, Fig. 10, against A–485) show that A–485 causes a specific and drastic reduction in naturally occurring UV sensitivity of P^+-receptors as well as P^--receptors

result, A–485 probably acts only as a neutral screening pigment. Further support for this intepretation is the finding that also in P^+-receptors the normal high UV sensitivity was drastically reduced after injection of A–485 (Fig. 13, P^+-difference spectrum).

Discussion

Presence of sensitizing pigment

A variation in the UV sensitivity of fly photoreceptors with the carotene and vitamin A supply in the larval diet was first reported by Goldsmith et al. (1964) and Stark et al. (1977). The observation that the amplitude and "vibronic fine structure" of the sensitivity of photoreceptors R1–6 vary with the supply of retinoids during the larval stage (Kirschfeld et al. 1983; in the present study P^+, P^--flies; Figs. 2, 3, 10) strongly supports the concept generated by Vogt and Kirschfeld (1983) that the specific UV sensitivity of fly photoreceptors is due to a retinoid, probably 3-hydroxy-retinol, coupled to the visual pigment in the rhabdomere forming an UV sensitizing pigment. The photoreceptors in the P^--flies shown in Fig. 2 contained 0.1 the amount of P490 found in P^+-flies. The smooth UV sensitivity spectrum in these P^--flies very well agrees with the β-band of visual pigment P490, indicating that these flies completely lack the UV sensitizing pigment. In addition, the present results showed that also P^--receptors with a moderately reduced content of P490 (0.3 to 0.4 of maximal content; Fig. 10) have a disproportionally low UV sensitivity (rel. $S_{350} \cong 0.5$). These results strongly suggest that if retinoids are in scarce supply, the synthesis of P490 has priority over the incorporation of UV sensitizing pigment into the visual pigment molecule.

Does the amount of antenna pigment depend exclusively on available retinoids?

The results of the experiments in which P^+- and P^--flies were irradiated by "green" (P^+) or "blue" (P^-) light indicate that the incorporation of sensitizing pigment does not exclusively depend on the total amount of available retinoids but also on the fraction of available all-trans retinoids: As demonstrated by Schwemer's pioneering work, continuous irradiation by green light leads to a drastic drop in the content of P490 in P^+-photoreceptors (for review, see Schwemer 1989). In contrast, the content of this pigment remains constant during irradiation by blue light for up to two weeks (Schwemer 1984). This result was the key to the understanding of the photochemical reactions and the molecular mechanisms of visual pigment turnover in an insect eye. Briefly, Schwemer (1986) describes the following pathway for pigment turnover. The rate of visual pigment disintegration mainly depends on the fraction of meta-molecules in the rhabdomere. Released all-trans chromophore is probably actively transported to cells near the dioptric apparatus, where it is complexed to a highly specific photoisomerase. Absorption of blue light by this complex isomerizes the all-trans chromophore predominantly into the 11-cis form, which is released from the protein and then transported back to the photoreceptor. Here the recycled 11-cis chromophore is used for "de novo" formation of visual pigment. The rate of pigment synthesis thus primarily depends on the amount of 11-cis retinoid freely available within the eye. In fact, Paulsen and Schwemer (1983) have shown that the 11-cis retinoid promotes the synthesis of visual pigment. Since recycling of the chromophore essentially depends on the blue absorbing photoisomerase, the continuous disintegration of meta-pigment can be compensated for by an adequate synthesis of P490, only if the receptors are irradiated by light containing blue wavelengths of sufficient intensity. During blue or violet irradiation the pigment content in the rhabdomere thus remains high, whereas during green irradiation the content of visual pigment drastically drops. In parallel, the content of "free" all-trans retinoids increases in the retina.

Presumably the increase in all-trans retinoid is used by the receptor to form additional UV sensitizing pigment. The effects on spectral sensitivity, experimentally induced in the present study by blue or green illumination of P^+- and P^--flies, are in accordance with Schwemer's model for retinoid turnover. Continuous blue (or white) illumination was seen to affect neither the absolute sensitivity nor the specific relative UV sensitivity of P^+- or P^--receptors. Contrastingly, green illumination was observed to cause qualitatively different effects. In both P^+- and P^--receptors the absolute sensitivity at 488 nm decreased nearly proportionally to the decrease in visual pigment content (determined spectrophotometrically), but while in P^+-receptors the relative UV sensitivity remained unaffected, in P^--receptors the UV sensitivity increased slightly and a vibronic fine spectrum became apparent (Fig. 3b) The slight sensitivity increase is possibly due to the surplus of retinoids in P^--receptors (derived from the disintegration of meta-pigment) being incorporated as UV sensitizing pigment. This interpretation is not unequivocal due to the following experimental observation: In P^--receptors green light not only causes a much smaller decrease in absolute sensitivity than that in P^+-receptors but also a pronounced shift in λ_{max} from 490 nm to 470 nm This shift is probably caused by the formation of an aberrant visual pigment, as indicated by the symmetry of the difference spectrum in this region (Fig. 3b). A possible explanation for such a formation is that meta-molecules (M) are more thermostable when sensitizing pigment is not coupled to the molecule. Due to such higher thermostability these molecules do not degrade. Instead they become modified by the permanent P↔M transitions during green irradiation and accumulate. In analogy with the 9-cis-rhodopsins in vertebrates (Dartnall 1972), the hypsochromic shift may be caused by a change in steric specificity of the photoregeneration, favouring the 9-cis configuration instead of the naturally occurring 11-cis form of the chromophore.

Which retinoids can serve as UV sensitizing pigment or its precursor?

The specific increase in UV sensitivity of P^--receptors after corneal application of different retinoids (Figs. 4, 5, 6) is a direct proof that the UV sensitizing pigment is a vitamin A derivative. It is, however, an open question which derivative(s) and steric conformation(s) are naturally used by the receptor since all substances so far tested can serve as sensitizing pigment (Fig. 6; compare maximal values of relative UV sensitivity reached about 20 h after application). Information about the substance naturally used by the receptor can possibly be obtained from the rate of incorporation, since the rate of sensitivity increase 1. depends on the steric configuration of the chromophore, as shown by comparing all-trans- with 13-cis-retinol, and 2. on the C_{15}-aldehyde or C_{15}-alcohol group, as shown by comparing all-trans-retinal and all-trans-retinol, and 3. is drastically retarded if the 13-methyl group of retinal is replaced by an ethyl or propyl group. Fastest increase was seen after application of 13-cis-retinol, suggesting that this substance is the most probable candidate to be the naturally occurring UV sensitizing pigment or its most suitable precursor. The vibronic fine structure also favours this hypothesis. Application of 13-cis-retinol leads to the best fit of maxima (333, 351 and 369 nm) and minima (339 and 362 nm) with those of the control (P^+; Table 2). Furthermore, exogenous 13-cis-retinol causes a supernormal increase in UV sensitivity in P^+-receptors, while the fine spectrum remains unchanged (Tables 1 and 2). Finally, very similar vibronic fine spectra are seen (MacDonald and Ong 1987) if different isomeres of retinol are complexed to the retinol binding proteins CRBP or CRBP(II). Figure 14 shows that the spectrum of the UV sensitizing pigment best fits the vibronic fine structure of the 13-cis-retinol CRBP(II) complex which has λ_{max} at 350 and 369 nm. The similarity of the vibronic spectra indicates that the ionone ring needs not necessarily be fixed to the protein by a 3-hydroxy-group, since in CRBP(II) the ionone ring is fixed in a protein pocket only (MacDonald and Ong 1987). From this fact alone it cannot be determined whether the UV sensitizing pigment in fly photoreceptors really is a 3-hydroxy-13-cis-retinol or simply a 13-cis-retinol. Fixation of the ionone ring to P490 by the 3-hydroxy group is, however, very probable, since only 3-hydroxy-retinal or 3-hydroxy-retinol was found by extraction from fly heads (Vogt 1983; Vogt and Kirschfeld 1984) or by analysis of retinoids in the blowfly eye (Schwemer 1988, 1989). An additional fixation in a protein pocket is not excluded.

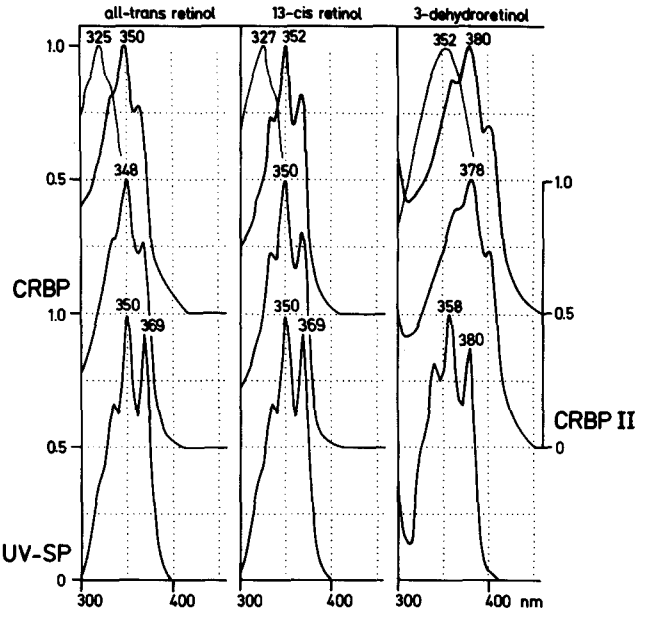

Fig. 14. Excitation spectra of CRBP – *upper spectra* – and CRBP(II) – *middle spectra* – compared with vibronic fine structure of sensitizing pigment – *lower spectra. Six upper spectra:* Excitation of CRBP when complexed with all-trans-retinol (350 nm), 13-cis-retinol (352 nm) or 3-dehydro-retinol (380 nm). Also shown are peaks of excitation spectra of all-trans-retinol (325 nm), 13-cis-retinol (327 nm) and 3-dehydro-retinol (352 nm). *Three middle spectra:* Excitation of CRBP(II) when complexed with all-trans-retinol (348 nm), 13-cis-retinol (350 nm) or 3-dehydro-retinol (378 nm). *Three lower spectra:* Vibronic fine structure of naturally occurring sensitizing pigment (350 and 369 nm; left and middle spectra are identical; data from Fig. 10, "diff. spec.") and of sensitizing pigment formed after application of 3-dehydro-retinol to eye (358 and 380 nm; data from Fig. 9). Upper and middle spectra after MacDonald and Ong (1987). All spectra normalized to 1.0. Note 1. that complexation of all retinoids with CRBP or CRBP(II) leads to similar shift of central peak in excitation spectra and that largest shift is caused by 3-dehydro-retinol, 2. that pronounced side peaks (369 nm) are seen only after complexation with 13-cis-retinol, 3. that complexation of 13-cis-retinol with CRBP(II) results in spectrum with closest similarity to that of naturally occurring sensitizing pigment, and 4. that peak of naturally occurring sensitizing pigment is at 350 nm, while addition of 3-dehydro-retinol to eye causes shift to 358 nm, and that this shift is somewhat smaller than changing complexation of CRBP(II) from 13-cis-retinol to 3-dehydro-retinol (from 350 to 378 nm)

Which biophysical and biochemical reactions are involved in the formation and incorporation of UV sensitizing pigment?

The time course of the sensitivity increase is of course the result of an interaction between several biophysical and biochemical processes. An important biophysical factor limiting the rate of pigment uptake is the diffusion of an applied substance across the dioptric apparatus and then up to 200 μm along the rhabdomere. It can be assumed that the diffusion constants of retinoids in lipid cell membranes are almost equal. Thus the observed large differences in increase of UV sensitivity after application of different retinoids are probably due to biochemical processes involved in the formation of the UV sensitizing pigment, e.g. 1. hydroxylation of the applied retinoids into 3-hydroxy derivatives, 2. reduction of the C_{15}-aldehyde group, 3. temporary binding of the retinoids to transport proteins, and 4. coupling of the sensitizing pigment to the molecular binding sites in the rhabdomere. Hydroxylation of retinal and retinol to 3-hydroxy derivatives is a very important reaction in the eye of the fly, since 3-hydroxy-retinal is needed as chromophore in

P490 and 3-hydroxy-retinol is probably complexed as sensitizing pigment (Kirschfeld 1986; Schwemer 1989). The substrate specificity of vitamin A hydroxylase is unknown. It can therefore not be excluded that derivatives, such as 13-ethyl- and 13-propyl-retinal are accepted by the enzyme less well than the naturally occurring precursor(s), whereby the incorporation of sensitizing pigment would be retarded. Other important factors in the retardation of UV sensitivity increase (from 1.0/h for 13-cis-retinol to 0.1/h for 13-propyl-retinal; Fig. 6) may be differences in affinity of the derivatives to the reductase, to the transport molecules, and not least, to the binding site on the rhabdomere.

The complexity of the chemical reactions presumably involved is not clarified by the present experimental data, but some new hypotheses can be made concerning the specificity of the binding sites at P490. After application of different retinoids, the UV spectra of P^--flies have a very similar vibronic fine structure. Hence it can be assumed (according the concept by Kirschfeld 1986 and Vogt 1989) that the UV sensitizing chromophore is complexed to the P490 molecule by hydrogen bonding at two specific binding sites located at a distance corresponding to the two hydroxy-groups (3-hydroxy- and 15-hydroxy-) of the chromophore. The binding sites are presumably located at the surface of P490 at different transmembranal α-helices, thereby fixing the sensitizing chromophore almost in parallel with the dipole of the 11-cis-3-hydroxy-retinal in the visual pigment (review by Vogt 1987). This hypothesis is to some extent supported by the present finding that of all substances so far tested 13-cis-retinol is most rapidly accepted by the receptor (compare time constants of sensitivity increase in Fig. 6). Provided that 13-cis-retinol is hydroxylated at C_3, both hydroxy groups of the 13-cis-3-hydroxy-retinol would point in the same direction, which would not be the case for the all-trans-3-hydroxy molecule. Thus, a sterically unhindered binding to the α-helices of the 13-cis isomere is more probable than a binding of the all-trans isomere.

The observation that 13-cis- and all-trans-retinal are accepted by the receptor (0.8/h) almost as fast as 13-cis-retinol (1.0/h) could possibly mean that not only the alcohols but also the aldehydes can bind to the visual pigment. The slight deviation of the UV spectrum following application of 13-cis-retinal in comparison with that recorded from P^+-receptors gives some support to such an interpretation. The distinct delay in incorporation by about 30 min favours, however, the alternative hypothesis that applied retinal has to be reduced to alcohol before the chromophore can complex to P490. Since the retinal tissue always contains 13-cis isomers (Schwemer 1988), it is possible that the enzymatic reduction preferentially leads to the formation of 13-cis-retinol. Such an "automatic" isomerization to 13-cis-retinol could also explain why application of all-trans-retinal causes a more rapid and larger increase in UV sensitivity than all-trans-retinol (which cannot be isomerized by the enzyme). It is also possible that the incorporation of the 13-ethyl and 13-propyl derivatives is preceded by the same enzyme reaction, although retarded, since the large and flexible side chains presumably drastically hinder the reduction of the 15-aldehyde group. That such enzyme reactions are involved in the production of an optimally fitting UV sensitizing pigment was demonstrated by the aberrant vibronic fine structure, which was transiently recorded after injection of vitamin A_2 (Figs. 8, 9). The transient bathochromic shift of the fine structure (350 to 358 nm, 369 to 380 nm) indicates, that vitamin A_2 is accepted by the visual pigment as sensitizing pigment. It is, however, accepted only as long as vitamin A_2 is available alone. The A_2 sensitizing pigment is replaced by the naturally occurring sensitizing pigment when, due to enzymatic activity, the naturally occurring pigment becomes available within a few hours, as indicated by the hypsochromic shift of the vibronic peaks (Fig. 8). The observation that vitamin A_2 complexed to CRBP or CRBP(II) causes a much larger bathochromic shift (380 or 378 nm; Fig. 14) than coupling of A_2 to the visual pigment (358 nm) suggests that the steric fit of A_2 to the visual pigment is much less perfect than that of the naturally occurring sensitizing chromophore.

How many binding sites for UV sensitizing pigment exist at the P490 molecule?

The following rough estimation of the number of binding sites can be made on the basis of the relative UV sensitivity $S_{350/488}$ and the molar extinction coefficients of P490 (about 40000 at $\lambda_{max} = 490$ nm, about 10000 at 350 nm) and of its UV sensitizing pigment (48000 at $\lambda_{max} = 350$ nm, corresponding to the value of 13-cis-retinol dissolved in ethanol at $\lambda_{max} = 328$ nm). Assuming a perfect energy transfer from the UV sensitizing pigment to P490 (i.e. an uppermost quantum yield of 1, instead of the lower value of 0.8 estimated by Vogt and Kirschfeld 1982; cf Smakman and Stavenga 1986) and a stoichiometry of 1 : 1, a relative $S_{350/490} = 1.45$ is calculated $(48000 + 10000)/40000)$. If the molar ratio is 2 : 1 the relative $S_{350/490}$ increases to 2.65. The experimentally determined relative $S_{350/488}$ values of P^+-receptors (1.73 in Tables 1 and 2, 1.85 in Fig. 5, 2.05 in Fig. 10) clearly exceed the uppermost theoretical limit (1.45) of a 1 : 1 molecular ratio, but are within the range from 1 : 1 to 2 : 1. A ratio of sensitizing pigment to P490 = 2 : 1 is thus much more probable than 1 : 1. This conclusion is further supported by the observations 1. that application of 13-cis-retinol to P^+-receptors leads to a further increase in UV sensitivity (up to 1.92 in Table 1), and 2. that after application of 13-cis-retinol or -*al* the relative UV sensitivity of P^--receptors very often almost reaches the uppermost theoretical ratio, i.e. 2 : 1. Such an effect is to be expected in receptors with low P490 content only, i.e. in receptors with negligible self screening along the rhabdomere and thus with almost direct proportionality between pigment content and receptor sensitivity (Hamdorf 1979). This conclusion is illustrated by the following calculation: The absorbance along the rhabdomere in P^+-receptors is assumed to be maximally about 0.5 at 490 nm (Schwemer, personal communication) and 1.33 (0.5×2.65) at 350 nm. The corresponding quantal absorption is 68.4% at 490 nm and 95.3% at 350 nm, result-

ing in $S_{350/490} = 1.39$. The absorbance in P^--receptors is about 0.05 only, and the absorption at 490 nm is 10.9% and at 350 nm is 26.3%, resulting in $S_{350/490} = 2.31$, i.e. almost twice the value in P^+-receptors. The experimentally induced large increase in relative $S_{350/488}$ of P^--receptors thus is probably caused by two pairs of binding sites, occupied by two UV sensitizing molecules. Also supporting this hypothesis is the result that application of 13-ethyl-retinal leads to a "supernormal" relative $S_{350/488}$ (about 2.5; symbol E at 20 h in Fig. 6). Doubtless, the 13-ethyl derivative can perfectly fill the place of the naturally occurring UV sensitizing pigment. It can, however, not replace the 3-hydroxy-11-cis-retinal in the "de novo" synthesis of P490 (Schwemer, unpublished). Hence one can assume that in the presence of 13-ethyl-retinal all P490 molecules have a chance to bind two UV sensitizing chromophores, while the content of P490 in the rhabdomere remains constant at the original low level. Both these conditions are necessary to approach the highest possible relative $S_{350/488}$ of 2.5.

How can UV sensitivity be artificially induced by non-retinoid chromophores?

The chromophore (3-hydroxy-retinal) of P490 is embedded between the 7 α-helices of the opsin (Huber et al. 1990), near the centre of, and almost parallel to, the phospholipid bilayer, the thickness of which is 50 Å. The loci of the binding sites of the UV sensitizing pigment are not known. They can be located either inside the membrane at the helices (as suggested by Kirchfeld 1986 and Vogt 1989) or at the intra- or extracellular loops of the visual pigment. At all these positions the distance between the antenna dipole and the P490 chromophore would never exceed the critical distance (25 Å; Förster 1951) for an optimal energy transfer between the chromophores.

The artificial UV sensitivity induced by injection of the pyrene-maleimide P–28 (Fig. 11) strongly supports the hypothesis that also extracellularly bound fluorophores can act as sensitizing pigments. Fluorescent maleimides like P–28 are specific thiol probes (Haugland 1989) and probably two probe molecules simultaneously bind to cysteines 121 and 196 at the extracellular surface of the opsin molecule (cf Huber et al. 1990). Support for the interpretation that two thiol groups are occupied simultaneously is the fact that single bound P–28 molecules fluoresce maximally in the UV region at 376 and 396 nm, whereas two juxtaposed P–28 molecules form excimers that fluoresce maximally in the visual range at 470 nm. That the pyrene-maleimide specifically couples to the P490 molecule was demonstrated by in vitro staining experiments (Fig. 12). The fluorescence was found to be directly proportional to the content of P490 in the rhabdomeric membrane. The binding of two molecules thus permits an optimal energy transfer to the visual pigment, P490. The recorded relative UV sensitivity at 325 nm, $\cong 1.6$, is also in line with this interpretation, since the molar absorbance of pyrene ($\varepsilon = 38000$) is somewhat lower than that of retinol ($\varepsilon = 48000$). The result that injection of A–485 did not induce an artificial sensitivity increase in the UV region can be correspondingly explained. Presumably two molecules of this probe also couple to cysteines 121 and 196. Nevertheless, it is to expect that this fluorescent probe does not markedly excite the visual pigment. The probe fluoresces maximally at 410 nm, i.e. almost at the absorption minimum of P490. In addition, it does not form excimers like P–28, and probably this hydrophilic fluorophore orients at right angle to the membrane surface, which does not allow an energy transfer to the 3-hydroxy-retinal of P490 oriented almost parallel to the membrane surface. A–485 therefore acts as an UV screening pigment along the rhabdomere, reducing the absorption by the UV sensitizing pigment. A screening effect was in fact observed after application of A–485 to P^+-receptors (Fig. 13).

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