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The pigment system of the photoreceptor 7 yellow in the fly, a complex photoreceptor

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Summary. The 7y photoreceptor in the fly (*Musca, Calliphora*) retina harbours an unusually complex pigment system consisting of a bistable visual pigment (xanthopsin, X and metaxanthopsin, M), a blue-absorbing C_{40}-carotenoid (zeaxanthin and/or lutein) and a uv sensitizing pigment (3-OH retinol).

The difference spectrum and photoequilibrium spectrum in single 7y rhabdomeres were determined microspectrophotometrically (Fig. 2).

The extinction spectrum of the C_{40}-carotenoid has a pronounced vibrational structure, with peaks at 430, 450 and 480 nm (Fig. 3). The off-axis spectral sensitivity, determined electrophysiologically with 1 nm resolution shows no trace of this fine structure thus excluding the possibility that the C_{40}-carotenoid is a second sensitizing pigment (Fig. 4).

The absorption spectra of X and M are derived by fitting nomogram spectra (based on fly R1–6 xanthopsin) to the difference spectrum. λ max for X is 425 nm, and for M 510 nm (Fig. 5). It is shown that the photoequilibrium spectrum and the difference spectrum can be used to derive the relative photosensitivity spectra of X and M using the analytical method developed by Stavenga (1975). The result (Fig. 6) shows a pronounced uv sensitivity for both, X and M, indicating that the uv sensitizing pigment transfers energy to both X and M. A value of 0.7 for Φ, the relative efficiency of photoconversion for X and M, is obtained by fitting the analytically derived relative photosensitivity spectra to the absorption spectra at wavelengths beyond 420 nm.

Introduction

It is generally assumed that the spectral sensitivity of a photoreceptor is primarily determined by the absorption spectrum of the visual pigment with secondary modifications due, for example, to self-screening, waveguide effects and the presence of any coloured materials in the ray path acting as filters (e.g. cornea, oil droplets, overlying photoreceptors in a tiered rhabdom etc.; review Menzel 1979).

In many species of fly the situation is further complicated by the presence of photostable pigments which are located in the rhabdomeres themselves, and which can modify the properties of the receptors very considerably in several respects. E.g. both spectral and polarization sensitivity can be modified: 1) by sensitization of the visual pigment via a sensitizing pigment (Kirschfeld et al. 1977; Vogt and Kirschfeld 1983b), and 2) by the screening effect of a C_{40}-carotenoid (Kirschfeld et al. 1978; Hardie et al. 1979). Whereas electrophysiological data give important information on the functional properties of this kind of photoreceptor, in order to analyse the contribution of the different pigments in detail it is necessary in addition to measure the absorption properties of the pigments individually by microspectrophotometry.

With respect to its pigments, the most complicated photoreceptor yet described is probably the so-called 7 yellow (7y) photoreceptor found in several dipteran species (including *Musca* and *Calliphora*). It is characterized by a blue absorbing C_{40}-carotenoid which gives rise to the yellow appearance of its rhabdomere in transmitted light (Kirschfeld et al. 1978). Functionally it is an ultraviolet receptor, and it has been hypothesized that its spectral sensitivity is generated via a uv sensitizing pigment transferring the energy of absorbed quanta onto a blue absorbing visual pigment which is largely screened from direct excitation by the C_{40}-carotenoid (Hardie et al. 1979).

The absorption properties of the C_{40}-carotenoid have been investigated in some detail (Kirschfeld et al. 1978; McIntyre and Kirschfeld

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1981), and its chemical identity determined as a mixture of zeaxanthin and lutein (Vogt and Kirschfeld 1983a). Whilst the hypothesis that a sensitizing pigment is present has been strengthened by the demonstration of a fine structure in the uv spectral sensitivity of these cells (Hardie and Kirschfeld 1983) similar to that demonstrated in R 1–6 cells (Gemperlein et al. 1980; Kirschfeld et al. 1983), only preliminary data have been published on the properties of the visual pigment itself (Kirschfeld 1979).

It should be pointed out that, whilst there is no direct evidence, we assume that the chromophore of the visual pigment in 7y photoreceptors is the same as that in R 1–6 photoreceptors. This has recently been shown to be a novel chromophore, namely 3-OH retinal (Vogt 1983; Vogt and Kirschfeld 1984) and Vogt has proposed a common name for visual pigments with this chromophore, namely 'xanthopsin'.

The aim of the present work is a further characterization of this type of 'complex photoreceptor': in particular, to determine accurate absorption and photoequilibrium spectra of the visual pigment proper, to examine critically the question of whether the C_{40}-carotenoids might also play a role as a second sensitizing pigment, similar to their antenna function in photosynthesis, and to find out whether the uv sensitizing pigment transfers energy onto the xanthopsin (X) alone, or onto the metaxanthopsin (M) as well.

Methods

Microspectrophotometry. Experiments were performed on female specimens of *Musca domestica*; controls with white-eyed mutants gave similar results.

Tangential slices from the retina were cut with an oscillating razor blade (ca. 50 µm, 200 Hz, Kirschfeld 1967). The eye slices, which were only used if there was no noticeable distortion of the alignment of the rhabdomeres, were mounted in a drop of Ringer solution (Case 1957) in a chamber, so that the cut ends of the rhabdomeres could be observed in cross-section in the microscope. The chamber was cooled to 5 °C in order to stabilize the preparation and to prevent movement of pigment granules. The position of the preparation on the stage could be controlled with an accuracy of ca. 0.1 µm.

The microscope was a Leitz orthoplan equipped with ultrafluar optics (Zeiss). In the photometer head an adjustable diaphragm is incorporated into an ultrafluar eyepiece. The location of the image of the rhabdomere within this diaphragm could be inspected via a mirror which could be swung out of the ray path during measurements.

Electrophysiology. The electrophysiological experiments described in this paper were designed purely to answer the question of whether the C_{40}-carotenoid can function as a sensitizing pigment. To this end it was necessary to raise the flies on special diets with controlled carotenoid content (see Results), and for technical reasons it is easier to raise *Calliphora* on such a diet than *Musca*. The experiments were therefore performed on chalky mutants of *Calliphora erythrocephala*.

The flies were raised on horse meat (which has very low concentrations of carotenoids) to which either lutein or zeaxanthin was added as the sole C_{40}-carotenoid (Vogt and Kirschfeld 1983a).

The recording set-up has been described previously (Hardie et al. 1979). In brief, fine microelectrodes (tip resistances ca. 120 MΩ) were lowered vertically into the eye via a small hole in the cornea sealed with a drop of immersion oil. High resolution spectral sensitivity measurements were performed as described by Hardie and Kirschfeld (1983). Briefly, responses to repeated spectral scans in the range 400–520 nm were signal averaged and then converted to spectral sensitivity functions via the cell's response intensity function and the calibration of the lamp (quartz iodide) and monochromator (Zeiss MM 12). Calibrations were performed using an EG & G radiometer.

Results

The difference spectrum

Rhabdomeres R7 are identified by their position in the centre of the ommatidium (see Fig. 1). The majority of R7 cells in the *Musca* eye are 7y photoreceptors and can be positively distinguished from other classes of R7 by their yellow appearance in transmitted light and/or by their strong dichroism in the blue (Kirschfeld et al. 1978).

The difference spectrum is the spectrum of the differences in absorbance between the two states of a bistable pigment system, and was determined by measuring the transmitted intensity, I, at a variety of test wavelengths in two different adaptation states. Typical raw data are shown in Fig. 1. The vertical lines are photomultiplier signals generated by flashes (0.3 s) of the measuring light. Between the test flashes (given at 2–10 s intervals) the rhabdomere is exposed to the adapting light. A shutter in front of the photomultiplier was closed during adaptation to protect it against this relatively strong light. Figure 1b shows that an adapting light of 500 nm leads to a high transmission at 520 nm, whereas adaptation to 400 nm reduces the transmission at 520 nm significantly. The measuring lights were too weak and short to shift measurable amounts of pigment themselves. The absorbance difference $\Delta E(\lambda)$ for each measuring wavelength λ used is calculated according to Eq. 1 (see Appendix 1).

Microspectrophotometric measurements of 7y rhabdomeres present particular technical difficulties. Any background light which is not transmitted by, or mode coupled (McIntyre and Snyder 1973; Wijngaard and Stavenga 1975) to the R7 rhabdomere introduces a systematic error into the difference spectrum. In general, background light is minimized in wild type flies by the strongly absorbing

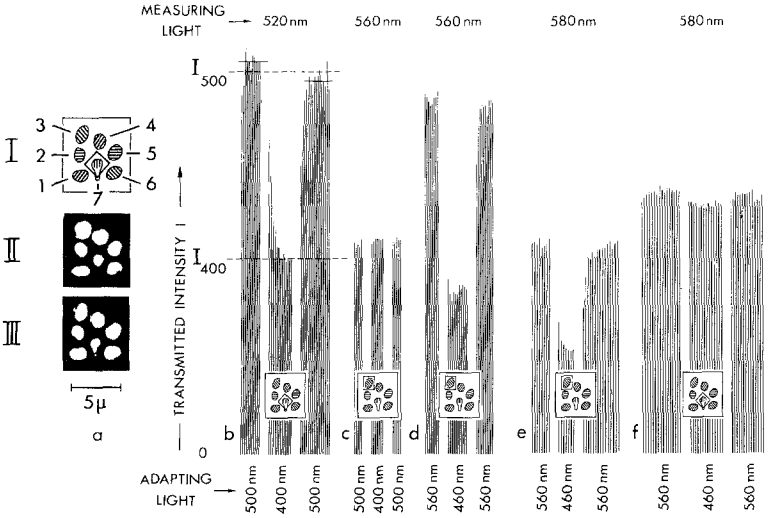

Fig. 1. a I: Arrangement of rhabdomeres no 1 to 7 in a single ommatidium of *Musca*. Position of diaphragm is indicated around rhabdomere no 7. II, III: Rhabdomeres of one ommatidium photographed on colour film (Kodak 5074) and reproduced in orange light (II) (6 mm Schottfilter OG 2) or in blue light (III) (4 mm Schottfilter BG 28). In blue light the light guiding neck of rhabdomere no 7 is clearly visible. **b–f** Vertical lines are photomultiplier signals evoked by 0.3 s flashes of measuring light of wavelengths given in the top line of the figure, transmitted through a single rhabdomere. Between the test flashes the adapting light of wavelength given in the bottom line was switched on. The inset indicates the position of the measuring diaphragm. Further explanation in the text

screening pigment. However, light is still transmitted by the surrounding rhabdomeres R 1–6, and the position of the R7 rhabdomere in the centre of the ommatidium makes it particularly susceptible to stray light signals. We attempted to minimize this by very careful positioning of the measuring diaphragm (we found that even a shift of 0.2 μm of the diaphragm was sufficient to modify the signal).

Another source of stray light comes from the R7 itself. The rhabdomere of the R7 cells is connected to the cell body by a slender 'neck' (see e.g. Trujillo-Cenóz and Melamed 1966). Surprisingly, this neck is itself capable of guiding light independently of the rhabdomere. This becomes particularly apparent with blue light, which is heavily absorbed in the rhabdomere by the C_{40}-carotenoid, allowing the light guided in the neck to be easily seen (Fig. 1a, III). With yellow light, by contrast, transmission in the rhabdomere is much higher and the neck is dark compared to the rhabdomere (Fig. 1a, II). The fact that in white light the 'neck' appears greenish compared to the yellow rhabdomere suggests that the light in the two structures is not mode-coupled. To minimize this effect we used preparations with shorter rhabdomeres, whereby the C_{40}-carotenoid absorption remains relatively small, and took special care in the placing of the measuring diaphragm.

Nevertheless, even with precise positioning of the diaphragm, signals from the R 1–6 cells may still be measured because of the waveguide properties of the rhabdomeres which may allow optical cross-talk (McIntyre and Snyder 1973). Such signals could affect the difference spectrum. In order to measure the difference spectrum in 7y rhabdomeres we chose an adapting wavelength pair which results in no net shift of the pigment in R 1–6, namely 400 nm and 500 nm. Figure 1c illustrates that this pair of adapting wavelengths indeed causes no measurable transmission change in a R3 rhabdomere, whereas in Fig. 1d it can be seen that a large change is induced by 560 nm vs. 460 nm. Figure 1e, f demonstrates, that even if there is any contribution of the R 1–6 transmission change measured in carefully selected rhabdomeres R 7, this signal is only small. The just measurable difference in Fig. 1f might be due to contamination from R 1–6 but could also be due to a pigment shift in R7 itself.

The difference spectrum presented in Fig. 2 is averaged from the results from 4 rhabdomeres 7y chosen for the stability of the measurements, using 400 and 500 nm as the adapting wavelengths to minimize any effect from R 1–6, and preparations with short rhabdomeres to minimize the effect of the neck. For comparison the dotted curve shows the mean from 5 preparations with longer rhabdomeres, measured before we were aware of the effect of the stray light from the neck, resulting in a systematic distortion in the blue.

Photoequilibrium spectrum

Further important information about a bistable pigment system is obtained from the photoequilibrium spectrum which describes the proportion of the visual pigment in one state as a function of the adapting (equilibrating) wavelength. We measured the relative proportion of metaxanthopsin at a test wavelength of $\lambda = 520$ nm, where there is virtually no absorption by xanthopsin. Further, this wavelength is close to the isosbestic point in R 1–6 rhabdomeres, and their transmission should

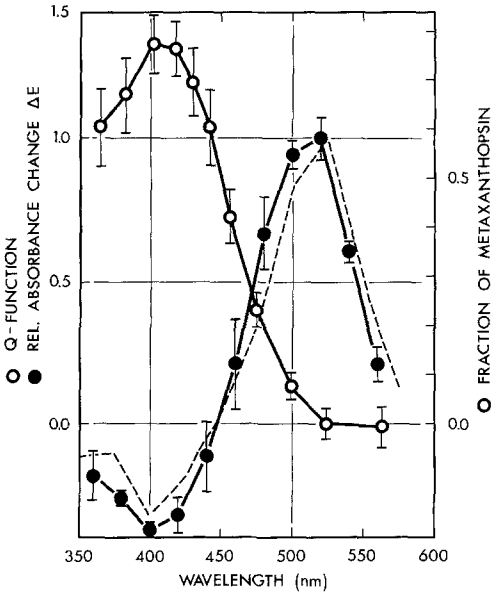

Fig. 2. Points: Difference spectrum ΔE measured after adapting the pigment first to light of 500 nm, then to light of 402 nm. Data from 4 rhabdomeres R7y with standard deviation. Circles: Q function measured at 520 nm. Pigment was shifted first with orange light ($\lambda > 520$ nm) into the xanthopsin state, then with the wavelength indicated at the bottom into the new equilibrium. Dashed curve: Difference spectrum (mean of 5 spectra) which shows systematic deviation in the spectral range of 400 to 500 nm because of the influence of the 'neck'-lightguide illustrated in Fig. 1a, III. Explanation in text. The ordinate at the right side indicates the fraction of metaxanthopsin created in photoequilibrium with wavelengths indicated on the abscissa, $\Phi = 0.7$

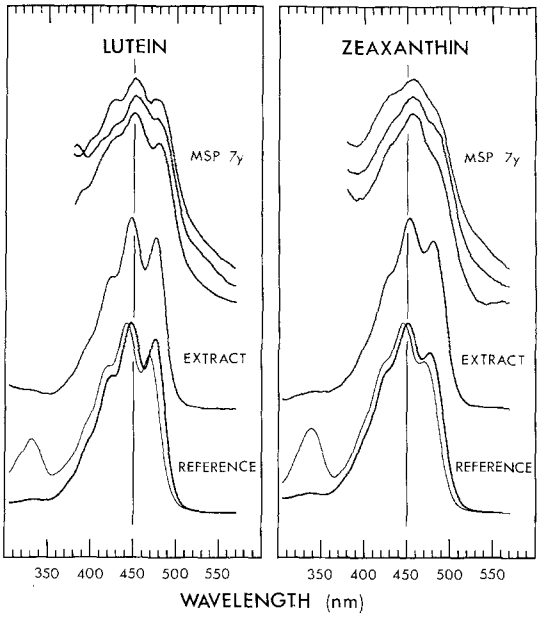

Fig. 3. C_{40}-carotenoids from the eyes of *Calliphora* reared on lutein and zeaxanthin rich diets, respectively. Extract: absorbance spectrum of the C_{40}-component isolated from whole eye acetone extracts, identifiable as lutein or zeaxanthin by HPLC and by comparison with reference spectra from lutein or zeaxanthin extracted from plant material (Vogt and Kirschfeld 1983a). (The second reference curve with the pronounced secondary peak at ca. 340 nm comes from a cis-isomer-presumably 15, 15'.) MSP curves show single extinction spectra of 7y rhabdomeres and reflect the λmax and the degree of modulation in the lutein/zeaxanthin. The bathochromic shift of 4–5 nm is due to the nature of the solvent (ethanol vs membrane lipids)

therefore be independent of the adapting wavelength with this measuring light. Any effect of straylight from R 1–6 is thus again minimized. The results give only the relative proportion of metaxanthopsin at any adapting wavelength, but by normalizing the data to the value at the isosbestic point in 7y, we obtain the so-called Q function (Fig. 2) which can be converted to the absolute photoequilibrium function with a knowledge of Φ, the relative quantum efficiency of photoconversion of xanthopsin and metaxanthopsin (right hand ordinate). The equations that have been used are given in Appendix 1.

Is the C_{40}-carotenoid a second sensitizing pigment?

Hardie et al. (1979) have already produced plausible arguments suggesting that the C_{40}-carotenoid is in fact only a screening pigment, however firm evidence for this hypothesis is lacking. We thus designed an experiment to test this hypothesis directly.

In order to appreciate the rationale behind the experiment it is first necessary to briefly recapitulate the results of the extraction of the C_{40}-carotenoid in 7y rhabdomeres and its identification as a mixture of zeaxanthin and lutein (Vogt and Kirschfeld 1983a).

In acetone extracts of freeze-dried *Calliphora* retinae the major C_{40}-carotenoid component (as identified by high performance thin layer chromatography and spectrophotometry of extracts) was all-trans zeaxanthin along with smaller amounts of all-trans lutein and cis-isomers of both. Further it was possible to raise flies on special diets containing either lutein or zeaxanthin as the sole C_{40}-carotenoid. In this case the extracts revealed only isomers of the one carotenoid. In addition MSP of the 7y rhabdomeres in such flies revealed characteristic extinction spectra allowing the identification of the C_{40}-carotenoid as lutein or zeaxanthin, respectively (Fig. 3). Of particular importance for the present experiment is that the spectrum of lutein shows a more pronounced vibrational fine structure than that of zeaxanthin and that this is clearly reproduced in the 7y extinction curve. (The enhanced vibrational fine structure is explained by the displacement of a double bond in the cyclohexene ring thus leading to a restriction of the torsional freedom of the C6–C7 bond.)

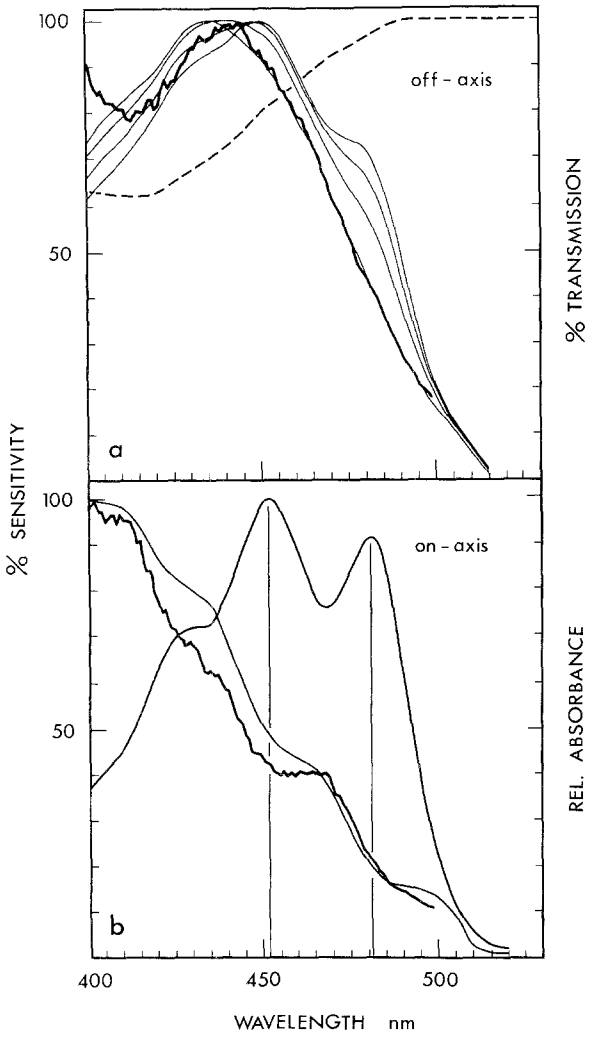

Fig. 4a, b. Spectral sensitivity of a 7y cell from *Calliphora* raised on a lutein-rich diet, measured in the range 400–520 nm with 1 nm resolution. **a** Off-axis, average of $n = 131$ scans. The family of smooth curves shows theoretical predictions based on the measured extinction spectra of the xanthopsin (shifted to λmax of 430 nm) and lutein. The transmission spectrum of the eye tissue (dashed line, Vogt, unpublished) has also been considered. The curves assume 0, 5, 10 and 15% efficiency of energy transfer. The best fit is with zero transfer efficiency. **b** On-axis data from same cell ($n = 76$ scans): data reveal an inverted fine structure, with the troughs corresponding to the peaks of the lutein absorbance spectrum (smooth curve). The data have been fitted with the X425 extinction spectrum assuming a purely screening effect of the lutein. Both on and off-axis predictions assume absorptivities (absorption/μm) of 0.008 and 0.024 for xanthopsin and lutein, respectively

It seemed to us that the clearest demonstration that the C_{40}-carotenoid could contribute actively to the cell's response (i.e. as a sensitizing pigment) would be the demonstration of this fine structure in the electrophysiologically determined spectral sensitivity function, and in order to maximize the chance of detecting this we used flies raised on a lutein diet.

The critical measurements were performed with non-axial light, since in the axial situation self-screening would necessarily reduce any modulation in $S(\lambda)$ and further, if the efficiency of energy transfer is not very high, then the screening effect of the carotenoid will predominate. By contrast, for light absorbed non-axially, the active contribution should be still just as high, whereas both self-screening and screening become negligible (Eq. 1, Appendix 2).

Intracellular recordings were made from 7y photoreceptors in two such lutein rich flies. The cells were identified by the overall shape of the spectral sensitivity function (Hardie et al. 1979; Hardie and Kirschfeld 1983). In the range 400–520 nm $S(\lambda)$ was measured with 1 nm resolution by using monochromatic scans (Hardie and Kirschfeld 1983). In 3 cells the recordings were stable enough to average the data from 50–100 scans using both axial and non-axial illumination.

The results (Fig. 4) show clearly that in the axial situation an 'inverted fine structure' is measured, where the troughs in the $S(\lambda)$ correspond to the peaks of the lutein extinction spectrum. This thus demonstrates directly a screening function but cannot exclude a moderate sensitizing function in addition. In the non-axial situation, however, there is no trace of any fine structure suggesting that the carotenoid has no sensitizing function. Of course an energy transfer of sufficiently low efficiency would go undetected. To get an idea of the upper limit for transfer efficiency which, theoretically, might still occur, predictions of $S(\lambda)$ were generated using different values of transfer efficiency. For the predictions we assumed the lutein spectrum measured in extracts, but with λmax shifted 5 nm to coincide with the MSP data (see Fig. 3). Initially we found a discrepancy in that a xanthopsin spectrum with λmax 440 nm was required to fit the data, whereas, as we shall see, the MSP data clearly show a λmax which is shorter (425 nm, Fig. 5). However, this discrepancy can be largely explained by taking the spectrally selective screening effect of the tissue with λmax at ca. 410 nm (Vogt, unpublished, see Fig. 4) into account[1]. The resulting predictions are presented in Fig. 4. With reasonable assumptions for the absolute extinctions of lutein and xanthopsin, the predictions indi-

[1] Actually, even with the tissue screen factor, the data of Fig. 4 were best fitted with a 430 nm xanthopsin spectrum. The data could also be well modelled with X425 if the tissue screening factor was assumed to be ca. 15% greater than that determined by Vogt (unpubl. and see Fig. 4). These differences, however, are insignificant and within the experimental error. What is important is that any model (425, 430 or 440 nm, with or without tissue screen) predicts a measurable fine structure when the energy transfer efficiency from the C_{40}-carotenoid is greater than 5–10%, and this was never observed.

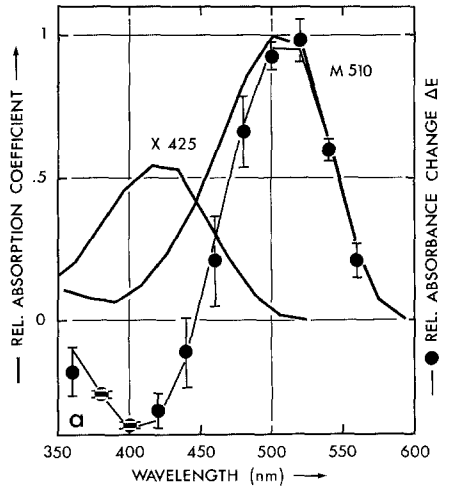

Fig. 5. Points: The difference spectrum from Fig. 2 (points) was fitted to a xanthopsin and metaxanthopsin spectrum. The shape of these spectra was taken from Schwemer and Henning (1984), the λmax-values and their ratios were shifted on a $\lambda^{\frac{1}{4}}$-scale (Barlow 1982), in order to fit the measured difference spectrum. The continuous difference spectrum is the difference between the *X* and *M* spectra

cate that even a transfer efficiency of 5–10% would have been sufficient to generate a measurable fine structure. By contrast the transfer efficiency of the uv sensitizing pigment in R1–6 is estimated as greater than 75% (Vogt and Kirschfeld 1983b).

We also modelled the axially measured spectral sensitivity, and in this case found that the results could be well modelled with the 425 nm xanthopsin spectrum, and again a purely screening effect of the lutein.

We can thus conclude that, with respect to xanthopsin, the C_{40}-carotenoid has little or no sensitizing function. However, it does have a significant effect on the axially measured spectral sensitivity function by virtue of its screening function.

Determination of the visual pigment absorption spectra

At least two methods are available for determining the visual pigment absorption spectra from the data collected in this study. In principle the most direct is an analytical method developed by Stavenga (1975) whereby the absorption spectra are derived from the difference spectrum and the *Q* function. However, this method is not generally applicable when there is a sensitizing pigment whose absorbance spectrum overlaps with that of the visual pigment. This is the case for 7y at wavelengths below ca. 420 nm. With certain assumptions, however, the analysis can then lead to the photosensitivity spectra of the visual pigments.

Approximation method

Initially, therefore we applied an approximation method (reviewed by Hamdorf 1979), whereby nomogram spectra are fitted to the difference spectrum by varying λmax and the ratio between X and M. This procedure, of course, requires a priori information on the shape of the absorption spectrum. Rather than using Dartnall (1953) or Ebrey-Honig (1977) nomograms, we used as templates the xanthopsin and metaxanthopsin spectra determined for *Calliphora* R1–6 photoreceptors (Schwemer and Henning 1984) and shifted these on a $\lambda^{\frac{1}{4}}$ scale introduced by Barlow (1982). This arbitrary scale has been found to give an excellent coincidence in shape for vertebrate visual pigments.

The result is shown in Fig. 5. λmax of the xanthopsin and metaxanthopsin are 425 nm and 510 nm, respectively. The maximum absorption of M is ca. 1.8× higher than that of X which is in the range of invertebrate bistable visual pigment systems (rev. Stavenga and Schwemer 1984).

In order to test whether the outcome of this procedure was critically dependent on the shape of the nomogram spectrum, we also fitted the data to Ebrey-Honig nomogram spectra (this fit was kindly performed by K. Hamdorf and P. Schlecht with their computer program). In this case λmax's of 426 nm and 506 nm were obtained but the quality of the fit was not quite so good.

Analytical derivation of absorption spectra

For wavelengths longer than 420 nm the analytical method of Stavenga (1975) should also produce the absorption spectra of the visual pigment without any assumptions as to the shape of the spectra. However, we must assume that the C_{40}-carotenoid, which absorbs in this range, has no sensitizing function. We have demonstrated that this is true with respect to the xanthopsin component (Fig. 4), and whilst this suggests that it is also 'silent' with respect to the metaxanthopsin, we have no direct evidence for this. In the range 350–420 nm, the action of the uv sensitizing pigment invalidates the analysis with respect to the absorption spectra, however, it is simple to show that the analysis should then provide the relative photosensitivity of the visual pigment (i.e. visual pigment absorption plus sensitizing pigment absorption × transfer efficiency) provided two conditions are met. The first is that the efficiency of energy transfer from the sensitizing pigment is the same onto both xanthopsin and metaxanthopsin, and the second that the difference spectrum does not equal zero (see Appendix). In the case of 7y, the latter condition

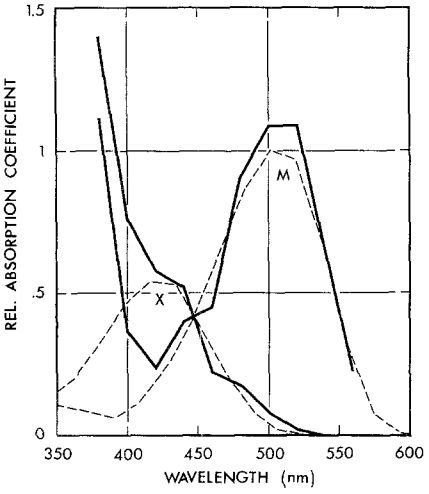

Fig. 6. Relative photosensitivity spectrum of *X* and *M* calculated from the difference spectrum and the *Q* function using the analytical method developed by Stavenga (1975), assuming a value of 0.7 for Φ, the relative quantum efficiency. The dashed curves are the spectra of *X* and *M* from Fig. 5 for comparison

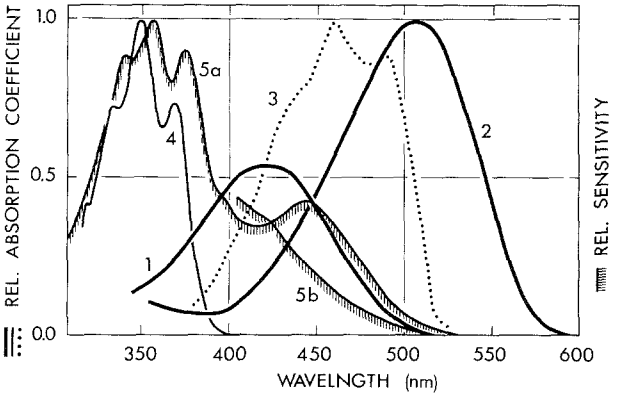

Fig. 7. Pigment absorptions and spectral sensitivity of photoreceptors R7y. 1, 2: *X* and *M* absorption spectra from Fig. 5. 3: Absorption spectrum of C_{40}-carotenoids from a rhabdomere R7y after waveguide correction (McIntyre and Kirschfeld 1981). 4: Absorption spectrum of the uv sensitizing pigment. The spectrum shown is from a receptor R1–6 (Kirschfeld et al. 1983), since only in these rhabdomeres are measurements possible with sufficient accuracy. 5a: Spectral sensitivity of a receptor 7y, measured off-axis, 5b: measured on-axis for wavelengths longer 400 nm. The small difference between the extinction- and sensitivity spectra in the uv seems to be characteristic for receptors 1–6 and 7y, respectively. It is not explained (Hardie and Kirschfeld 1983)

is met down to wavelengths of 350 nm (Fig. 2). The former condition is of course, a priori unknown, however, by analogy with R1–6 likely to be true (see Minke and Kirschfeld 1979).

Finally, for the calculation we need to assume a value of Φ, the relative quantum efficiency of photoconversion. Since we have already derived visual pigment absorption spectra by the approximation procedure above, we can vary Φ in the calculation to obtain the best fit with these data, and thus also derive an estimate for Φ.

The result of the analysis, using the equations presented in Appendix 3 and the data of Fig. 2, is shown in Fig. 6.

At wavelengths longer than 420 nm, and assuming a Φ value of 0.7 there is good agreement with the absorption spectra obtained semi-independently by the fitting procedure of nomograms to the difference spectrum. This agreement incidentally, is also evidence for our assumption that the C_{40}-carotenoid plays no sensitizing role onto metaxanthopsin. Below 420 nm both the spectra show a pronounced increase in the absorption coefficient in the uv, which represents the contribution of the sensitizing pigment provided our assumption of equal energy transfer is correct.

Finally, we use our estimate for Φ to convert the measured *Q* function into the absolute photoequilibrium function (see Appendix 1), and the result is shown in the right ordinate of Fig. 2.

Discussion

The 7y photoreceptor in flies has an exceptionally complicated pigment complex, and is in addition technically difficult to study due to its small size and position in the center of the ommatidium. Nevertheless, by a combination of microspectrophotometric, electrophysiological and biochemical techniques we believe now to have succeeded in providing a comprehensive description of the pigments and their functions.

The extinction spectra of the four components (X, M, C_{40}-carotenoid and uv sensitizing pigment) are summarized in Fig. 7 along with the resulting spectral sensitivity of the photoreceptor.

Spectral mechanisms

The visual pigment itself is a typical invertebrate bistable system with a xanthopsin absorbing maximally at 425 nm, that can be photointerconverted with a metaxanthopsin with λmax 510 nm. The spectral sensitivity function measured off-axis can be fitted with a 430 nm xanthopsin spectrum (derived from the MSP data) as long as the spectrally selective screening effect of the tissue (λmax 410 nm) is taken into account. The need to do so shows that caution must be exercised in deriving visual pigment spectra from electrophysiological data, since even white-eyed mutants (in this case *Calliphora* chalky) are not necessarily truly white.

With respect to Φ, the relative quantum efficiency, we estimated a value of 0.7 by a fitting procedure (Fig. 6) and found that this could be consid-

ered independent of wavelength beyond 420 nm where the uv sensitizing pigment no longer absorbs. We lay no great claim to accuracy, however. This value is significantly smaller than that found in R 1–6 photoreceptors (0.93, Hamdorf 1979), but well within the range of values in other invertebrates (review Stavenga and Schwemer 1984).

The C_{40}-carotenoid plays, most probably, a purely screening function and sensitizes neither xanthopsin nor metaxanthopsin. Whilst we have direct evidence for this assertion with repect to xanthopsin (Fig. 4), for the case of metaxanthopsin we point out that the absorption spectra determined analytically from the difference spectrum and the Q function fit closely to those determined from the difference spectrum alone assuming pigment nomograms (Fig. 6). If the carotenoid had sensitized metaxanthopsin we would have predicted a systematic discrepancy between the two methods.

As in R 1–6 photoreceptors (Minke and Kirschfeld 1979) a uv sensitizing pigment probably sensitizes metaxanthopsin as well as xanthopsin. The analytical method we used for determining the photosensitivity spectra assumes that sensitization of xanthopsin and metaxanthopsin is equal, if this assumption is false then an error is introduced into the spectra. However, as we show in the Appendix, this error would be the same for both the xanthopsin and metaxanthopsin spectra. Since the xanthopsin photosensitivity spectra show a similar uv sensitivity to the electrophysiologically determined spectral sensitivity this systematic error is presumably small or non-existent. Thus we can in fact be reasonably confident that the uv sensitivity of the metaxanthopsin photosensitivity spectrum is also genuine (Fig. 6).

A final logical possibility not yet considered is that the uv sensitizing pigment might also sensitize the C_{40}-carotenoid. However, for this to happen without the C_{40}-carotenoid being able to sensitize the visual pigment would demand a very specific spatial arrangement of the three absorbing dipoles. Further if such a sensitization was at all significant, the uv sensitizing pigment must also function as a screening pigment in which case it would be very unlikely that the uv sensitivity measured on axis could be so high (Hardie et al. 1979).

Chemical identity

The C_{40}-carotenoid has been identified as a mixture of zeaxanthin and lutein (Vogt and Kirschfeld 1983a, and see Fig. 3). Both these molecules have an OH group at the C3 position in the cyclohexene ring and would seem to be suitable as precursors for the visual pigment and sensitizing pigment chromophores. In flies raised on a normal diet (in our case liver), zeaxanthin is the dominant component. In diets with lutein or zeaxanthin as the sole carotenoid only the one or other pigment is found in the retina. If only α- or β-carotene are offered, eye extracts again reveal lutein and zeaxanthin, respectively, showing that the fly is capable of converting these simple carotenoids into the hydroxy-derivatives (Vogt and Kirschfeld 1983a).

We have no direct evidence for the identity of the visual pigment and sensitizing pigment chromophores in 7y, but by analogy with R 1–6 photoreceptors we believe these to be 3-OH retinal and 3-OH retinol, respectively. The fine structure in the uv spectral sensitivity of 7y cells (Hardie and Kirschfeld 1983) is one argument for this; thus in a wide survey of other insect eyes, the presence of a fine structure in the uv spectral sensitivity (indicative of the sensitizing pigment) has been invariably correlated with the presence of 3-OH retinal, and particularly 3-OH retinol, in eye extracts (Vogt and Kirschfeld, in prep.). It has been suggested that the fine structure might be caused by a non-covalent bonding of 3-OH retinol to the opsin via hydrogen bonds at either end of the molecule, which results in a restriction of torsional freedom of the C6–C7 single bond (Vogt and Kirschfeld 1984, see Fig. 8).

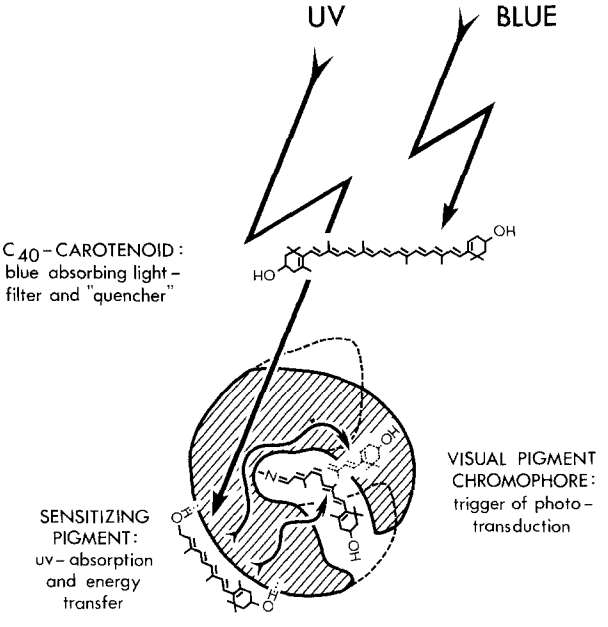

Fig. 8. Possible molecular arrangement of the three pigments present in rhabdomeres R7y. The Schiff-base-linked chromophore is shown in cis- and trans-configuration to illustrate that energy transfer from the sensitizing pigment happens to both (wavy arrows)

In R 1–6 photoreceptors estimates of the efficiency transfer (>75%) strongly suggest an attachment of the sensitizing chromophore to the opsin moiety (Vogt and Kirschfeld 1983), and whilst the critical measurements are lacking in 7y cells, a similarly high transfer efficiency is suggested by the high relative sensitivity in the uv.

Conversely, the lack of any demonstrable sensitization by the C_{40}-carotenoid would suggest that this molecule is not in such intimate association with the visual pigment, though this cannot be ruled out, since with a suitable angle between the dipole moments (90°), energy transfer can be minimized despite close physical contact.

Previously we quoted a stoichiometry of between 7 and 40 carotenoid molecules per visual pigment molecule (McIntyre and Kirschfeld 1981). However, we must now admit that an error of a factor of 10 was made for the extinction coefficient of the C_{40}-carotenoid. In addition, the numbers for the pigment density in the rhabdomeres no 7 used for the estimate were extrapolated from freeze fracture data of rhabdomeres 1–6 which are not necessarily relevant. Since we now know the photoequilibrium spectrum of the visual pigment we can use the extinction spectrum of the C_{40}-carotenoid and the extinction difference at 495 nm after preadaptation to 365 and 499 nm, respectively, that have been measured in the same rhabdomere (McIntyre and Kirschfeld 1981), in order to calculate the stochiometry between carotenoid and visual pigment. The calculation in Appendix 4 shows that the molecular concentration of both molecules is in the same order of magnitude.

Whilst a stoichiometry of 1:1 might suggest attachment of the carotenoid to the visual pigment, we tend to the view that the C_{40}-carotenoid is incorporated separately into the lipid membrane, in particular tending to align itself parallel to the lipid chains which would account for its preferred dichroism perpendicular to the microvillar orientation (McIntyre and Kirschfeld 1981). Our concept of the identity, function and incorporation of the various components of the complex pigment system is summarized in Fig. 8.

Functions

We have now a good understanding of the pigments and their contributions to the spectral sensitivity of this complex photoreceptor, but we have yet to address the question of why a uv-photoreceptor is created in such a complicated manner: by means of a blue absorbing visual pigment with two accessory pigments? The answer is not fully clear, but the following points should be considered.

1. As argued by Kirschfeld (1983), the advantage of a sensitizing pigment can be seen as increasing absolute sensitivity and extending the spectral range. Generally speaking, visual pigment molecules are packed very tightly into the photoreceptor membrane to maximize absorption. The addition of a second, sensitizing chromophore takes up practically no more space in the membrane, but can significantly increase the absolute sensitivity of the photoreceptor. This argument, valid for photoreceptors R 1–6 in the fly, is difficult to uphold for the 7y cell since the gain in sensitivity in the uv is largely lost by the screening effect of the C_{40}-carotenoid in the blue. Nevertheless, the bandwidth of the 7y photoreceptor is still greater than that of uv photoreceptors based on single rhodopsins.

2. With respect to the C_{40}-carotenoid, Kirschfeld (1982) has suggested that, as in plant cells, carotenoids in photoreceptors may serve a protective function against the damaging effects of photooxidation, either by simply absorbing potentially harmful rays or by quenching of singlet oxygen states. Indeed, with high levels of short wavelength illumination it has been shown that 7y rhabdomeres are less susceptible to damage than 7p or even R 1–6 rhabdomeres (Kirschfeld 1982; Zhu and Kirschfeld 1984). Of course most photoreceptors manage without any carotenoid pigments, however, there are other ways of dealing with the problem of photooxidation. Repair mechanisms are also most likely to be significant, exemplified, perhaps, by the massive 'shedding' described in many vertebrate and invertebrate photoreceptors; the metabolic cost, however, is presumably much higher.

3. For R 1–6 cells, the screening pigments of the retina help to maintain a high xanthopsin concentration (and hence sensitivity) since they are transparent to red light (Strother 1966; Langer 1975) which reconverts metaxanthopsin (M 580) to xanthopsin (X 490) (Stavenga et al. 1973). The metaxanthopsin in 7y absorbs in the green where the screening pigments also absorb highly, so that, for 7y, this mechanism is ineffective. The spectral filtering action of the C_{40}-carotenoid, however, does favour the conversion of 7y metaxanthopsin to xanthopsin and thus may have a similar effect as the screening pigments in R 1–6.

4. As already mentioned, both lutein and zeaxanthin are suitable precursors for the visual pigment chromophore – and also the sensitizing pigment. The possibility should thus also be consid-

ered that the carotenoid in 7y rhabdomeres is an intraretinal store for chromophore precursors (Vogt and Kirschfeld 1983a, b). If one takes into account that the volume of the rhabdomeres no. 7y is only 2–3% of that of all rhabdomeres (rhabdomeres no. 7 are shorter and thinner than no. 1–6 (Boschek 1971), and only 2/3 of them contain the C_{40}-carotenoid, Kirschfeld et al. 1978), it is clear that the absolute number of precursor molecules in this store is small compared to the number of sensitizing and Schiffbase-linked chromophores in the eye.

5. Finally, whilst the C_{40}-carotenoid does effect both spectral and polarization sensitivity in 7y, it has a more severe effect upon the spectral sensitivity of the underlying R8y cell (Kirschfeld et al. 1978; Hardie 1977; Hardie et al. 1979). The rhabdomere of R8 lies directly behind that of R7 and all axial light reaching R8 is first filtered by the 7y rhabdomere. As a result the R8y cell has a very sharply tuned spectral sensitivity function (λmax = 530 nm).

A problem for any interpretation of the complex 7y photoreceptor is the fact that the 7y cells are randomly distributed over the retina with so-called 7pale (7p) cells (Kirschfeld et al. 1978). These are also uv receptors, but use a completely different mechanism (namely a uv xanthopsin, Hardie et al. 1979; Kirschfeld 1979; Hardie and Kirschfeld 1983). For the strategy behind this, we may again look to the underlying R8 cells (8p) which are blue receptors with λmax at 460 nm (Smola and Meffert 1979; Hardie and Kirschfeld 1983). It is then obvious that such a photoreceptor would be effectively blind if it lay behind a 7y photoreceptor, the rhabdomere of which absorbs ca. 90% of the incident blue light (McIntyre and Kirschfeld 1981).

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Appendices

1. Definition of the difference- and the photoequilibrium spectrum

The difference spectrum $\Delta E(\lambda)$ was calculated from intensities transmitted through rhabdomere no. 7y according to

$$\Delta E(\lambda) = \log \frac{I_{500}(\lambda)}{I_{402}(\lambda)}. \quad (1)$$

The indices of I indicate adaptation wavelengths, in Fig. 2 $\Delta E(\lambda)$ is normalized to the maximum $\equiv 1$.

The photoequilibrium spectrum $Q(\lambda)$ is calculated as

$$Q(\lambda) = \log \frac{I_{\lambda}(520)}{I_{>520}(520)}. \quad (2)$$

In Fig. 2 $Q(\lambda)$ is normalized to 1 at the isosbestic wavelength. In addition the fraction of metaxanthopsin created in photoequilibrium f_M is calculated according to

$$f_M(\lambda_{iso}) = 1/(1+\Phi). \quad (3)$$

Φ is the relative quantum efficiency of photoconversion of xanthopsin and metaxanthopsin.

2. Modelling spectral sensitivities

In order to model spectral sensitivities on the basis of several pigments we used expressions derived from the theoretical absorption of a fused rhabdom (Snyder et al. 1973) and developed further by Hardie et al. (1979). In the present case we considered only wavelengths beyond 400 nm and ignored the contribution of the uv sensitizing pigment. The relative spectral sensitivity, $S(\lambda)$, is then given by:

$$S(\lambda) = T\left[\left(\frac{\gamma \cdot \alpha_L + \alpha_X}{\alpha_L + \alpha_X}\right) \cdot (1 - \exp -(\alpha_L l + \alpha_X l)\right] \quad (1)$$

α_L and α_X are the absorptivities (absorption/μm) of lutein and xanthopsin and were taken as 0.024 and 0.008, respectively. $T(\lambda)$, is the transmission spectrum of the (white) eye tissue (for the off-axis situation only). γ is the transfer efficiency (of sensitization) and l the rhabdomere length. $l = 120$ μm was assumed for the on-axis model, and $l = 1$ μm for the off-axis model in which case the exponential half of the equation becomes practically irrelevant.

3. Calculation of photosensitivity

The following formalism is based on Stavenga (1975). It will be extended to the case where besides the visual pigment a photostable sensitizing pigment is present in a rhabdomere.

First we consider a rhabdomere with a thermostable visual pigment system (without a sensitizing pigment), the photointerconvertible components (xanthopsin, metaxanthopsin) having the molar (decadic) extinction coefficients $\alpha_X(\lambda)$ and $\alpha_M(\lambda)$.

We determine the wavelength dependent extinction difference $\Delta E(\lambda)$ created by a pair of adapting wavelengths. One of them, λ, is chosen such that $\alpha_X/\alpha_M \approx 0$; i.e. all metaxanthopsin is converted to xanthopsin. The fraction of metaxanthopsin and

xanthopsin are 0 and 1, respectively. The other wavelength is λ_a.

This extinction difference $\Delta E(\lambda)$ is proportional to the concentration of total visual pigment ($C_V \equiv C_X + C_M$), the length (l) of the rhabdomere measured, the difference of the extinction coefficients of the two pigment states at the respective wavelength $\alpha_M - \alpha_X \equiv \alpha_X(\lambda) - \alpha_M(\lambda)$, and a term which represents the relative concentration of the metapigment created by the second adapting light (λ_a), i.e.

$$\Delta E(\lambda, \lambda_a) = C_V \cdot l \cdot (\alpha_M - \alpha_X) \cdot \frac{1}{1 + \frac{\alpha_M(\lambda_a)}{\alpha_X(\lambda_a)} \cdot \Phi} \tag{1}$$

with Φ the relative quantum efficiency of photoconversion from the metapigment to xanthopsin compared to the reverse, i.e. $\Phi = \Phi_{MX}/\Phi_{XM}$, regarded as constant over the spectrum.

If we use the isosbestic wavelength where $\alpha_X = \alpha_M$ as λ_a, Eq. 1 becomes:

$$\Delta E(\lambda, \lambda_{iso}) = C_V \cdot l \cdot (\alpha_M - \alpha_X) \cdot \frac{1}{1 + \Phi}. \tag{2}$$

Eq. 1 divided by Eq. 2 yields the so called Q-function (Stavenga 1975), that we have also determined experimentally (Eq. 1). That is:

$$Q(\lambda_a) = \frac{\Delta E(\lambda, \lambda_a)}{\Delta E(\lambda, \lambda_{iso})} = \frac{1 + \Phi}{1 + \frac{\alpha_M(\lambda_a)}{\alpha_X(\lambda_a)} \cdot \Phi} \tag{3}$$

which gives the concentration of the metapigment at all wavelengths normalized to its concentration if the rhabdomere is adapted to the isosbestic wavelength.

If now the Q-function and a difference spectrum $\Delta E(\lambda, \lambda_a)$ are measured as described in the text, the relative extinction coefficient α_X, is obtained by substituting α_M in Eq. 3 by Eq. 2 and dropping the suffixes ($\lambda \equiv \lambda_a$)

$$\alpha_X(\lambda) = \frac{\Phi}{C_V \cdot l} \cdot \Delta E(\lambda, \lambda_{iso}) \cdot \frac{Q(\lambda)}{1 - Q(\lambda)} \tag{4}$$

and provided that Φ is known the relative α_M

$$\alpha_M(\lambda) = \frac{1}{C_V \cdot l} \cdot \Delta E(\lambda, \lambda_{iso}) \cdot \frac{1 + \Phi - Q(\lambda)}{1 - Q(\lambda)}. \tag{5}$$

If there is an additional photostable sensitizing pigment with the molar extinction coefficient $\alpha_S(\lambda)$ and a relative concentration f compared to the visual pigment ($f = C_S/C_V$), Eq. 1 remains unchanged except for the term containing the relative concentration where both α_M and α_X have to be replaced by the 'combined relative photosensitivities' $\alpha_X^* = (\alpha_S \cdot f \cdot \Phi_{SX} + \alpha_X)$ and $\alpha_M^* = (\alpha_S \cdot f \cdot \Phi_{SM} + \alpha_M)$:

$$\Delta E(\lambda, \lambda_a) = C_V \cdot l \cdot (\alpha_M - \alpha_X) \frac{1}{1 + \frac{\alpha_S \cdot f \cdot \Phi_{SM} + \alpha_M}{\alpha_S \cdot f \cdot \Phi_{SX} + \alpha_X} \cdot \Phi} \tag{1*}$$

with Φ_{SM}, Φ_{SX} the quantum efficiencies of energy transfer from the sensitizing pigment to xanthopsin and metaxanthopsin, respectively.
If the adapting wavelength is the isosbestic:

$$\Delta E(\lambda, \lambda_{iso}) = C_V \cdot l \cdot (\alpha_M - \alpha_X) \cdot \frac{1}{1 + \frac{\alpha_S \cdot f \cdot \Phi_{SM} + \alpha_X}{\alpha_S \cdot f \cdot \Phi_{SX} + \alpha_X} \cdot \Phi} \tag{2*}$$

or if as in our case $\alpha_S = 0$ at the isosbestic wavelength

$$\Delta E(\lambda, \lambda_{iso}) = C_V \cdot l \cdot (\alpha_M - \alpha_X) \cdot \frac{1}{1 + \Phi} \tag{2*}$$

with the latter Eq. 2*

$$Q(\lambda_a) = \frac{\Delta E(\lambda, \lambda_a)}{\Delta E(\lambda, \lambda_{iso})} = \frac{1 + \Phi}{1 + \frac{\alpha_S \cdot f \cdot \Phi_{SM} + \alpha_M}{\alpha_S \cdot f \cdot \Phi_{SX} + \alpha_X} \cdot \Phi} \tag{3*}$$

The combined relative photosensitivities α_X^* and α_M^* are obtained from Eqs. 2* and 3* in analogy to the relative extinction coefficients (or rel. photosensitivities) in the case without sensitizing pigment

$$\alpha_X^* = \alpha_S(\lambda) \cdot f \cdot \Phi_{SX} + \alpha_X(\lambda) = \frac{\Phi \cdot Q(\lambda)}{1 - Q(\lambda)} \cdot \left[\frac{1}{C_V \cdot l} \cdot \Delta E(\lambda, \lambda_{iso}) + F \right], \tag{4*}$$

$$\alpha_M^* = \alpha_S(\lambda) \cdot f \cdot \Phi_{SM} + \alpha_M(\lambda) = \frac{1 + \Phi - Q(\lambda)}{1 - Q(\lambda)} \cdot \left[\frac{1}{C_V \cdot l} \cdot \Delta E(\lambda, \lambda_{iso}) + F \right] \tag{5*}$$

with

$$F = \alpha_S(\lambda) \cdot f \cdot (\Phi_{SM} - \Phi_{SX}) \cdot \frac{1}{1 + \Phi}.$$

For $F = 0$ the right hand side of Eq. 4*, 5* is identical to that of Eqs. 4, 5. This is obviously the case in the range ($\lambda \geqq 400$ nm) where $\alpha_S \approx 0$.

Calculating the α_X^* and α_M^* in Fig. 4 at shorter wavelengths we assumed that the sensitizing pigment transfers energy with the same probability to xanthopsin and the metapigment, i.e. $\Phi_{SX} = \Phi_{SM} = \Phi_S$, which means $F = 0$ too.

If however, $\Phi_{SX} \neq \Phi_{SM}$, then the values (α'_X, α'_M) that are presented in Fig. 4 represent:

$$\alpha'_X = \alpha_S(\lambda) \cdot f \cdot \Phi_{SX} + \alpha_X(\lambda) - \alpha_S(\lambda) \cdot f \cdot (\Phi_{SM} - \Phi_{SX}) \cdot \frac{\Phi \cdot Q(\lambda)}{1 + \Phi - Q(\lambda) - \Phi \cdot Q(\lambda)}$$

$$\alpha'_m = \alpha_S(\lambda) \cdot f \cdot \Phi_{SM} + \alpha_X(\lambda) - \alpha_S(\lambda) \cdot f \cdot (\Phi_{SM} - \Phi_{SX}) \cdot \frac{1 + \Phi - Q(\lambda)}{1 + \Phi - Q(\lambda) - \Phi \cdot Q(\lambda)}.$$

Then only the difference $\alpha'_M - \alpha'_X = \alpha_M - \alpha_X$ but not the absolute values stand for something reasonable;
alternatively:

$$\alpha'_X = \alpha_S(\lambda) \cdot f \cdot \Phi_{SX} + \alpha_X(\lambda) - F \cdot \frac{\Phi \cdot Q(\lambda)}{1 - Q(\lambda)}$$

$$\alpha'_M = \alpha_S(\lambda) \cdot f \cdot \Phi_{SM} + \alpha_M(\lambda) - F \cdot \frac{1 + \Phi - Q(\lambda)}{1 - Q(\lambda)}.$$

4. Calculation of stochiometry of carotenoid and visual pigment

a. Extinction of carotenoid, corrected for waveguide effects. From Fig. 9 (McIntyre and Kirschfeld 1981), the extinction maximum measured parallel ($E_{\parallel}$) and perpendicular ($E_{\perp}$) to the microvilli is

$$E_{\parallel} = 0.82, \quad E_{\perp} = 1.60.$$

From these numbers we calculate the extinction for unpolarized light

$$E_{up} = \log_{10}\{\tfrac{1}{2}(10^{-E_{\parallel}} + 10^{-E_{\perp}})\} = 1.05.$$

b. Extinction of visual pigment. From Fig. 1 (McIntyre and Kirschfeld 1981) the extinction differences after adapting the rhabdomere to light of 365 nm and 499 nm, are $\Delta E_{\parallel} = 0.17$ and $\Delta E_{\perp} = 0.10$, respectively. These numbers have to be corrected for waveguide effects, whereby the correction factor can be determined from Fig. 9 of the above quoted paper as 1.90 for *e*-vectors parallel to the microvillus and 1.64 for perpendicular *e*-vectors. This leads to the corrected $\Delta E_{\parallel,c} = 0.32$ and $\Delta E_{\perp,c} = 0.16$. For unpolarized light we arrive at

$$\Delta E_{up,c} = -\log_{10}\{\tfrac{1}{2}(10^{-\Delta E_{\parallel}} + 10^{-\Delta E_{\perp}})\} = 0.23.$$

From this difference we calculate the xanthopsin extinction by taking into account: 1) that the wavelengths 365 and 499 nm shift some 60% of the pigment from X to M and vice versa (Fig. 2), 2) that the difference at $\lambda = 495$ nm is close to the M-extinction maximum (Fig. 5), and 3) that the extinction maximum of X is 0.56 times that of M (Fig. 5.).

These factors leads to $E_{max} = 0.20$.

c. The relative concentrations of the carotenoid and xanthopsin. The concentration C of a substance with molar decadic extinction coefficient ε, measured in a vessel of length l is

$$C = E/\varepsilon \cdot l$$

where E is the measured extinction.

The relative concentration of the C_{40}-carotenoid and the visual pigment is

$$\frac{C_{C_{40}}}{C_X} = \frac{E_{C_{40}} \cdot \varepsilon_X}{E_X \cdot \varepsilon_{C_{40}}}.$$

If we assume that the extinction coefficient ε_X for xanthopsin is the same as that of rhodopsin we have $\varepsilon_X = 4 \cdot 10^4$ l/mol·cm. Lutein and zeaxanthin have an extinction coefficient $\varepsilon_{C_{40}} \approx 14 \cdot 10^4$ l/mol cm, and we arrive at

$$\frac{C_{C_{40}}}{C_X} \approx \frac{1.05}{0.2} \cdot \frac{4 \cdot 10^4}{14 \cdot 10^4} \approx 1.5.$$

This means that it is possible that there is a 1:1 relationship between visual pigment and C_{40}-carotenoid. The data, however, had to be corrected with many relatively large factors, including the waveguide parameter and Φ, neither of which is known accurately. In addition we used the extinction coefficients for the molecules in solution, which may also introduce an error, since the specific orientation in the membrane affects the extinction coefficient. Finally the estimate is based on a single measurement, which was not originally designed for this purpose. Therefore we think that it is justified only to conclude that both pigment molecules are present in the same order of magnitude.

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