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The Sensitizing Pigment in Fly Photoreceptors

Properties and Candidates

K. Kirschfeld*[1], R. Feiler[1], R. Hardie[1], K. Vogt[1], and N. Franceschini[2]

[1] Max-Planck-Institut für biologische Kybernetik,
Spemannstraße 38, D-7400 Tübingen, Federal Republic of Germany
[2] Institut de Neurophysiologie et de Psychophysiologie,
C.N.R.S., 31 Chemin J. Aiguier, Marseille, France

Abstract. Many lines of evidence suggest that the ultraviolet (uv) sensitivity found in the most common photoreceptor class in the fly is due to a sensitizing pigment which transmits the energy of absorbed light quanta to the visual pigment (Kirschfeld et al. 1977). It is shown that the uv extinction of the rhabdomeres has a vibrational fine structure corresponding to that found in the receptors' spectral sensitivity (Gemperlein et al. 1980). The uv extinction is greatly reduced when flies are reared on a carotenoid-deficient diet, in which case the vibrational fine structure in sensitivity is also lost. Properties (extinction, fluorescence) of several groups of substances that could represent the sensitizing pigment are illustrated.

Key words: Fly visual pigment, sensitizing pigment

Introduction

In the compound eye of the fly six of the eight receptor cells (called no 1–6, inset Fig. 1) in each ommatidium have a receptor potential action spectrum with two maxima of comparable height: one close to 500 nm, the other in the ultraviolet spectral range at 360 nm (Burkhardt 1962). The 500 nm peak is due to visual pigment (rhodopsin, *R*) absorption (Hamdorf et al. 1973, Stavenga et al. 1973), whereas evidence has been presented that the uv peak is due to a photostable pigment *(X)* that absorbs light quanta and transfers the energy to the visual pigment molecule ("sensitizing pigment") (Kirschfeld et al. 1977).

Flies grown on a low carotenoid diet have a reduced rhodopsin concentration in their microvilli (Boschek and Hamdorf 1976; Razmjoo and Hamdorf 1976; Harris et al. 1977). They also no longer exhibit the high uv sensitivity (Goldsmith et al. 1964; Stark and Zitzmann 1976; Kuo 1980), and the photosensitivity of their rhodopsin and metarhodopsin in the uv is also more reduced than in the

* To whom offprint requests should be sent

blue (Minke and Kirschfeld 1979). One explanation could be that the energy transfer from the sensitizing pigment to rhodopsin is reduced because of an increased distance from X to R. Another possible explanation would be that the concentration of the sensitizing pigment is also reduced in the microvilli, e.g., because its biosynthesis somehow depends upon carotenoids.

Electrophysiological analysis of the fine structure of the uv sensitivity gave an unexpected result: there is a vibrational fine structure with three maxima, near 330, 350, and 370 nm (Gemperlein et al. 1980), a result which, however, is not yet sufficient to chemically specify the sensitizing pigment.

In order to find out more about the sensitizing pigment in fly rhabdomeres we measured its extinction spectrum with improved resolution in normal flies and in flies grown on a low carotenoid diet. Also the spectral sensitivity of single cells was determined in such flies with high spectroscopic resolution in the ultraviolet. The results are compared with the spectral properties of known substances which could play the role of an ultraviolet sensitizing pigment.

Methods

Experiments were performed on specimens of both *Musca* and *Calliphora* reared in the laboratory. Normal diet for *Musca* was based on cottage cheese and yeast, that for *Calliphora* on bovine liver. Carotenoid-deprived flies were raised on lean horse skeletal muscle *(Calliphora),* or, in the case of *Musca,* Sang's medium with some added low fat cheese.

Eyecup-preparations were prepared as described earlier (Kirschfeld et al. 1978); extinction was measured either in individual rhabdomeres or, in order to improve the signal to noise ratio, by measuring several rhabdomeres type 1–6 together. Recently is has been shown that in the dorsal eye region of male *Musca* flies there are many ommatidia in which all rhabdomeres 1–7 have the same visual pigment so that seven rhabdomeres of one ommatidium can be measured all together (Franceschini et al. 1981a).

Our microspectrophotometer (Leitz MPV II combined with an Oriel monochromator 7240) has been further developed into a dual beam instrument. The light intensity I transmitted through the rhabdomeres was recorded from one photomultiplier (cooled down to −30° C); the light intensity I_0, reflected from the first lens of the condenser (Zeiss, ultrafluar) was measured by a second photomultiplier. The extinction is

$$E = \log \frac{I_0}{I} + k ,$$

whereby k is an unknown constant. Before each actual measurement a scan of the spectrum was recorded for the measuring chamber with no preparation in the beam. This spectrum was corrected by means of a computer so that it gave, by definition, extinction equal to 0. The correction factors that take asymmetries, as e.g., in spectral sensitivity of the two multipliers etc., into account were stored and later applied to the data taken from rhabdomeres. k remains unknown since the measurements for preparations of rhabdomeres had the dioptric systems of

the ommatidia in place which introduce an unknown factor to *I;* the latter is assumed to be wavelength independent.

Electrophysiological measurements were made from R1–6 cells of white-eyed mutants (*Calliphora,* chalky) using standard intracellular recording techniques as previously described (Hardie 1979). High resolution spectral sensitivity measurements in the ultraviolet were achieved by recording the responses to continuous spectral scans in the range 310–400 nm using light from a 75 W Xenon arc lamp passed through a double prism monochromator (Zeiss MM 12). In order to obtain the necessary spectral resolution, the slit width was reduced such that the bandwidth of the monochromatic light did not exceed 1 nm. Further details are described elsewhere (Hardie and Kirschfeld, 1983). For the remaining spectral range (400–600 nm) sensitivity was monitored at discrete wavelengths only. In most cases light was delivered from a quartz fibre optics light guide focussed onto the eye with a quartz lens. Control experiments using axial and non-axial point sources revealed no significant difference with respect to the fine structure in the uv.

Results

The extinction spectra of rhabdomeres type 1–6 of dark adapted wild type flies typically show an extinction band with three peaks between 330 and 370 nm, and a pronounced maximum at 520 nm (Fig. 1, spectrum a). The peak at 520 nm is primarily due to absorption of a mixture of rhodopsin with metarhodopsin, which is created in photoequilibrium with the measuring light. That the 520 nm absorption is primarily due to a photointerconvertible pigment is shown by selective adaptation: blue light (λ = 460 nm) shifts a high percentage of the visual pigment into metarhodopsin (spectrum c), adaptation to orange light (λ = 560 nm) into the rhodopsin state (spectrum b) (see Hamdorf et al. 1973; Stavenga et al. 1973).

The conspicuous fine structure in the uv absorption band is likely to originate from the photostable sensitizing pigment. In our first analysis (Kirschfeld et al. 1977) we were not yet able to resolve this fine structure which clearly consists of one main peak at 350 nm, and two satellite peaks at 330 and 370 nm (Fig. 1).

Measurements of rhabdomere extinction carried out in flies grown on a carotenoid-deficient diet no longer show the conspicuous extinction at 520 nm, which is typical for the visual pigment, but they also lack the threepeaked extinction in the uv, indicative of the presence of the sensitizing pigment (Fig. 2). The difference between the extinction data of flies grown on normal and on β-carotene deficient media respectively (Fig. 2, spectrum d) illustrates the net effect of the diet on the extinction.

Electrophysiology

Gemperlein et al. (1980) used Fourier spectroscopy to measure the fine structure of the uv sensitivity spectrum in *Calliphora.* They found three peaks, identical in

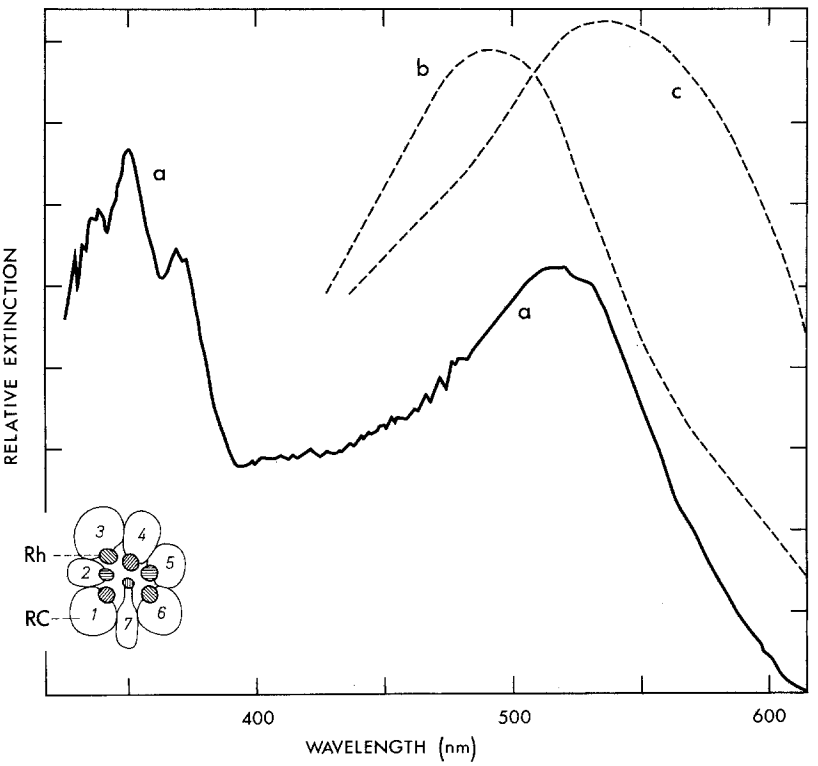

Fig. 1. Extinction spectra of rhabdomeres 1–7 measured all together from one ommatidium of Musca ♂ (wild type) in the dorsofrontal eye region, in which all rhabdomeres 1–7 contain the same visual pigment. **a** Spectrum measured in the condition in which the visual pigment is in photoequilibrium with the measuring light. **b** Extinction spectrum of rhabdomeres from a different eye as measured during adaptation to 560 nm. **c** Same rhabdomeres as in **b**, but during adaptation to 460 nm. The spectra **a** and **b**, **c** for clarity are shifted along the ordinate relatively to each other. Halfwidth of monochromator was 3 nm, zero of the spectra is not defined. Each division on the ordinate corresponds to $E = 0.1$. *Inset:* cross section through ommatidium indicating receptor cells RC and rhabdomeres Rh

position to those illustrated in Figs. 1 and 2. Their published data are from the mass response (electroretinogram) of the eye. Figure 3 (curve a) shows an intracellularly recorded spectral sensitivity curve which was measured with standard electrophysiological methods and which also shows the three peaks in the uv.

In addition we show that in carotenoid-deprived flies there remains a reduced uv sensitivity and, that the three uv peaks, if present at all, are reduced in amplitude (Fig. 3, curve b). This is interpreted as due to the fact that β-band absorption of the rhodopsin which has no threepeaked vibrational structure now gives a relatively higher contribution to the uv sensitivity compared to the sensitizing pigment molecules because the latter are reduced in concentration.

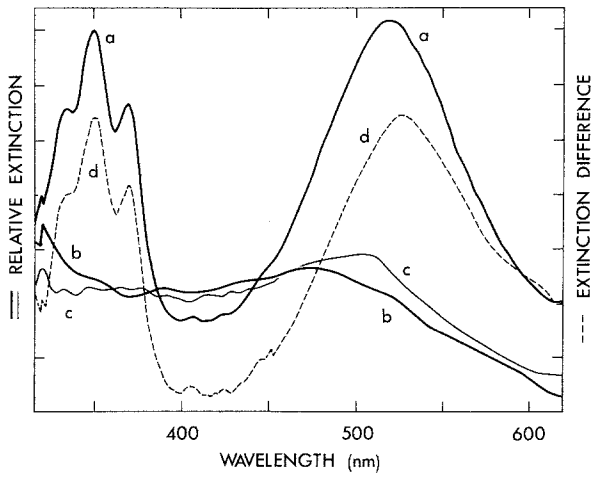

Fig. 2. Extinction spectra of rhabdomeres R 1, 2, and 3, from one ommatidium measured together in a condition where the visual pigment is in photoequilibrium with the measuring light (*Musca* ♂, wild type). **a** Fly grown on normal diet. This spectrum corresponds to the spectrum of Fig. 1a, but is from a different fly. **b, c** Two spectra from two different flies, the larvae of which have been grown on a carotenoid deprived medium. **d** Difference between spectra **a** and **c**. Halfwidth of monochromator ws 7 nm. Each division on the ordinate corresponds to $E = 0.1$ (left ordinate) and $\Delta E = 0.1$ (right ordinate)

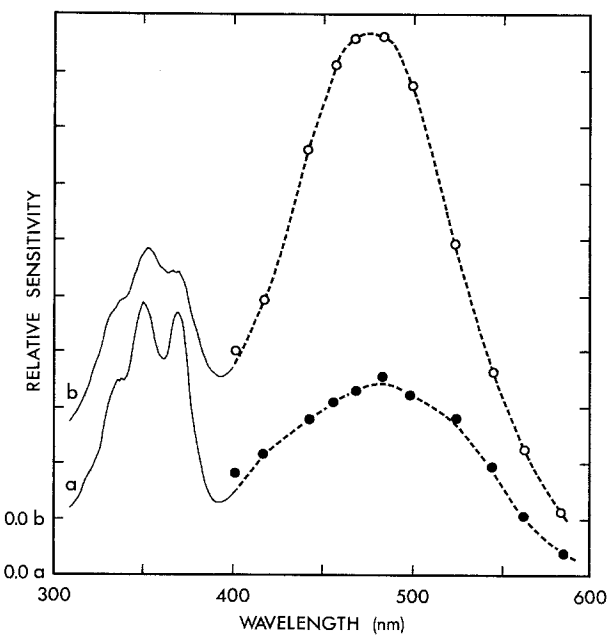

Fig. 3. Averaged spectral sensitivities measured intracellularly from single R1–6 photoreceptors in Calliphora (male, chalky). Curve a: Vitamin A rich flies (6 cells from 4 flies). Curve b: flies reared on vitamin A and carotene free medium (9 cells, 6 flies.) In the range 310–400 nm sensitivity was measured with 1 nm resolution using continuous spectral scans of narrow bandwidth monochromatic light. In the range 400–600 nm sensitivity was determined using flashes of monochromatic light at discrete wavelengths only. To demonstrate the difference in the fine structure in the uv the curves are normalised to the peak at 350 nm although this obscures the fact that vitamin A rich flies (curve a) are considerably more sensitive. The curves are shifted vertically with respect to each other for clarity, zero sensitivity for each curve being marked on the ordinate

Discussion

In flies grown on a low carotenoid diet, the relative sensitivity in the uv is reduced (Goldsmith et al. 1964; Stark and Zitzmann 1976 and Fig. 3). This might indicate that the presence of sensitizing pigment (or its incorporation into the membrane) is carotenoid dependent. However the result could also be simply explained by the known reduction in the rhodopsin concentration which might be expected to lead to an increase in the distance between rhodopsin molecules and sensitizing pigment molecules and thus a reduction in the efficiency of energy transfer (Förster 1951). The demonstration that sensitizing pigment is in fact dependent on carotenoid metabolism comes rather from the extinction data (Fig. 2) which show that in carotenoid-deprived flies, not only rhodopsin extinction but also extinction of the sensitizing pigment in the uv is virtually absent.

Obviously the biosynthesis of the sensitizing pigment molecules depends on the presence of carotenoids in the diet or at least their incorporation into the microvillar membrane is carotenoid dependent. Therefore one candidate for the sensitizing pigment could be vitamin A or a compound related to it, because these molecules absorb in the spectral range of 325–380 nm.

The microspectrophotometrical as well as the electrophysiological data show a clearcut vibrational fine structure which is not usually found in vitamin A or its derivatives. In the following we review the occurence of a fine structure in the uv absorption spectrum of vitamin A related substances, as observed under special circumstances (see also Franceschini 1982). The lack of a vibrational fine structure is considered as being primarily due to the fact that there are a large number of conformers differing in the torsional angle about the single bond connecting the β-ionylidene ring and the sidechain (between C6 and C7, Fig. 4). The absorptions of the various conformers are all shifted with respect to one another with the consequence that when they are superimposed the spectrum is broad and unresolved (Hemley and Kohler 1977).

If now the C6–C7 bond is stabilised not only is a vibrational fine structure in the extinction spectrum to be expected but also a redshift of some 30 nm if the β-ionylidene ring becomes coplanar with the side chain (Reppe 1970). There are several principle possibilities for such a stabilisation. Either by a sterical hindrance, e.g., if the vitamin A is incorporated into a membrane, or if a protein – vitamin A complex is formed, or if the conjugated chain of C = C double bonds is shifted by one C. In the latter case the C6–C7 bond now is a double bond and therefore no longer torsional. The latter derivatives of the molecule are called "retro"-derivatives (Fig. 4). In Fig. 5, examples of spectra of vitamin A derivatives are shown in comparison with the sensitizing pigment spectrum (curve a): The retro-vitamin A spectrum (curve b) is quite similar, as well as the spectrum of a protein (β-lactoglobulin)-retinol complex (curve c), which is only shifted slightly to longer wavelengths. How the all-trans retinol spectrum is modified by binding to another protein ("cellular retinol-binding protein") is further illustrated in Fig. 6, curves a and b, respectively (Ong and Chythil 1978): the absorption maximum is shifted from 325 nm to $\approx$ 350 nm, and the vibrational fine structure becomes obvious. Another example where the

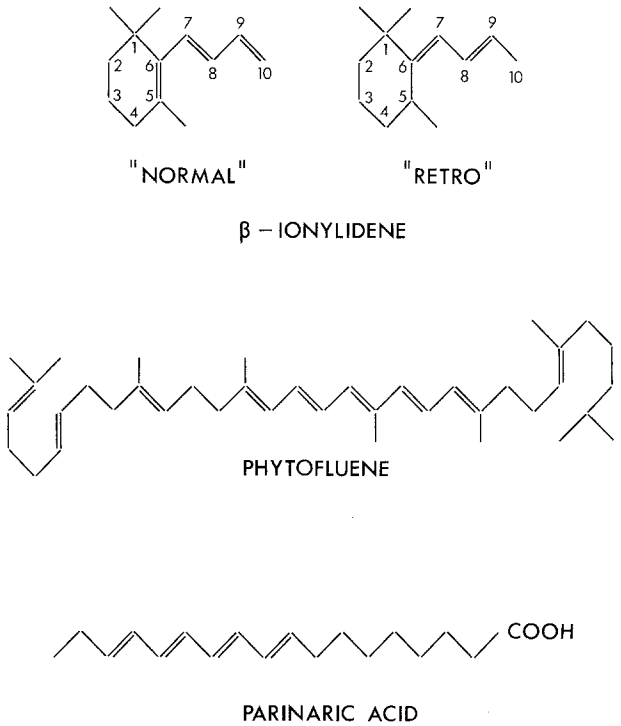

Fig. 4. Structural formulas of several compounds mentioned in the text

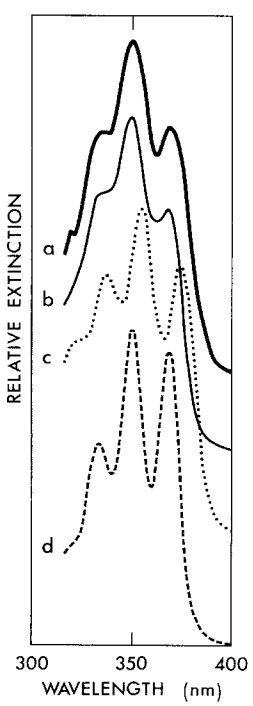

Fig. 5. uv-extinction spectra of sensitizing pigment **(a)** (from Fig. 1), retro-vitamin A **(b)** (Oroshnik et al. 1952), β-lactoglobulin – all trans retinol complex **(c)** (Fugate and Song 1980), and of phytofluene **(d)** (Koe and Zechmeister 1952)

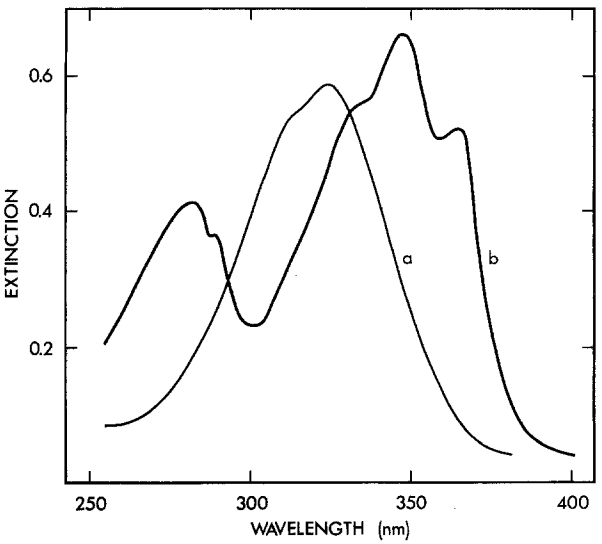

Fig. 6. Extinction spectra of all trans retinol in ethanol **(a)** and of cellular retinol-binding protein – retinol complex in buffer (pH 7.5) **(b)** (Ong and Chytil 1978)

same effects on noncovalently bound retinol is extensively discussed concerns intermediate states of bacteriorhodopsin (Schreckenbach et al. 1977, 1978a, b). The ability to bind to a protein, that is rhodopsin in the case of photoreceptors, might be of functional importance for the sensitizing pigment in order to maintain a close proximity to the rhodopsin chromophore which is important for Förster type of energy transfer (Förster 1951).

Actually, the distance $\Delta\lambda$ of some 20 nm between the uv peaks of the sensitizing pigment only indicates that the extinction should be due to a conjugated system as has been suggested already by Gemperlein et al. (1980), it need not be due to a vitamin A derivative. Since the presence of the sensitizing pigment depends upon carotenoid in the diet, it might be that the sensitizing molecule is a C40-carotenoid. However, carotenoids, like β-carotene, with a system of 11 conjugated double bonds absorb in the range of 400–500 nm, that is at much longer wavelengths than the sensitizing pigment. If, however, some of the double bonds in the carotene are hydrated, as e.g., in the phytofluene molecule (Fig. 4), the extinction would be shifted to shorter wavelengths (Fig. 5, curve d) (Zechmeister 1962). If the sensitizing pigment was derived from β-carotene then at least one double bond would have to be hydrated in the metabolism of the fly.

Recently a class of rather exotic naturally occuring substances has been described that cannot be completely excluded as a candidate for the sensitizing pigment: polyene fatty acids, or lipids containing these acids. These substances, like parinaric acid (Fig. 4) from the plant *Parinarium laurium*, exhibit the typical vibrational structure in their extinction spectrum (Fig. 7, spectrum a) (Sklar et al. 1975). Actually, it has already been shown that parinaric acid, artificially

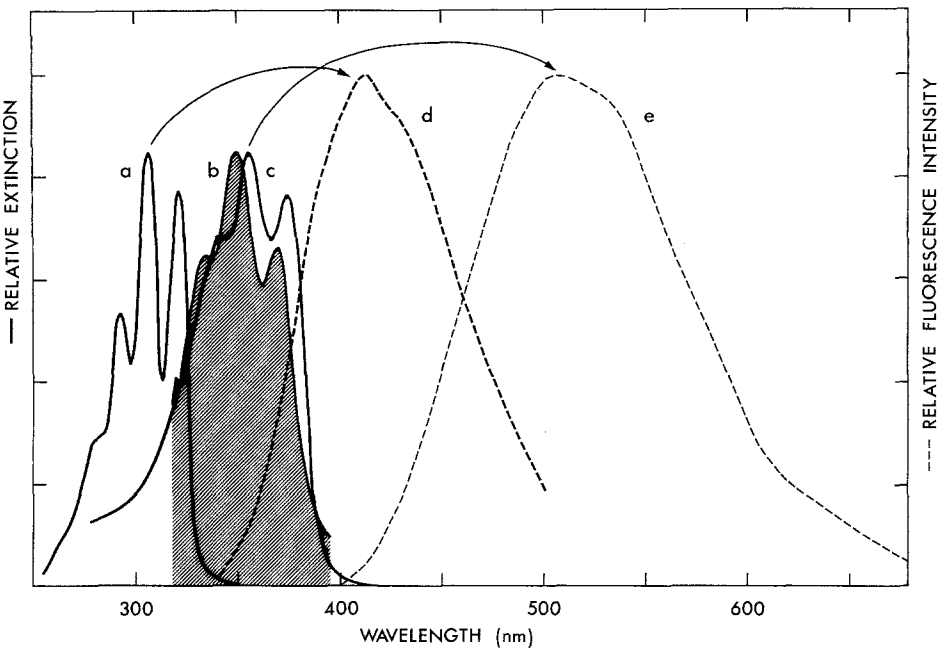

Fig. 7. a, d extinction and fluorescence emission spectrum of α-parinaric acid (Sklar et al. 1975). **c, e** excitation and fluorescence emission spectrum of β-lactoglobulin retinol complex (Fugate and Song 1980). **b** extinction spectrum of fly sensitizing pigment (from Fig. 1)

incorporated into rod outer segment membranes, is able to transfer energy to rhodopsin (Sklar et al. 1979). Of course, the sensitizing pigment need not be a fatty acid by itself, but the fatty acid could be incorporated into a lipid, similar to the parinaric acid labeled lecithins that are used as membrane probes.

The main difference between the properties of the parinaric acid and the fly sensitizing pigment is the fact that parinaric acid absorption (and fluorescence emission, see below) are shifted by 50 nm to shorter wavelengths (see spectra Fig. 7). However, it would for example be possible that the inclusion of one additional conjugated bond (leading to 5 conjugated double bonds instead of 4, as in the parinaric acid) into such a molecule could mediate a sufficient bathochromic shift. Alternatively by binding of a conjugated tetraene to a molecule like rhodopsin a sufficient bathochromic shift might be created, similar to the situation in the retinol-protein complex (Fig. 6).

If a polyunsaturated fatty acid like parinaric acid was the sensitizing pigment it should be metabolically derived from carotenoids, since the uv pigment is lacking in the carotenoid deprived flies. This seems not to be very likely and we therefore favour rather a protein retinol-complex or retro-retinal.

In addition to considerations of the extinction spectrum, we are able to predict another property of the sensitizing pigment: since the energy transfer is most likely of the Förster-type, the emission spectrum of the sensitizing pigment should overlap the absorption spectrum of the visual pigment. Under uv

excitation rhabdomeres R1–6 exhibit a broad band fluorescence, part of which possibly emanates from the sensitizing pigment (Stark et al. 1979; Franceschini et al. 1981b; Stavenga et al. 1982) Fluorescence spectra of two of the compounds mentioned (parinaric acid, β-lactoglobulinretinol complex) are shown in Fig. 7. The maxima are shifted by 100–150 nm to longer wavelengths compared to that of the extinction spectrum of the sensitizing pigment and the fluorescence of the β-lactoglobulin retinol complex nicely overlaps the absorption spectra of the visual pigment in the fly.

Paulsen and Schwemer (1979) discussed the possibility of a relatively enhanced β-band absorption of the rhodopsin due to dense packing of the molecules as a possible basis for the uv sensitivity peak in fly photoreceptors. Arguments against this possibility include the following facts: first, vibrational fine structure of the uv extinction is not expected for a rhodopsin β-band. Second, the relative loss in sensitivity of the uv-peak amplitude is coupled with a loss of the vibrational fine structure in carotenoid deprived flies. Third, the rhodopsin in flies shows a dichroic extinction, whereas the uv absorption band (Fig. 1) does not (Kirschfeld et al., in prep.). Correlated with this observation is the fact that these cells are polarisation sensitive in the visible, not in the uv (Hardie 1978; Kuo 1980). However, in vitamin A deprived flies there is again polarisation sensitivity in the uv, probably due to β-band absorption (Vogt and Kirschfeld 1983). Fourth, the photosensitivity spectrum of rhodopsin and of metarhodopsin coincide closely in the uv (Minke and Kirschfeld 1979). This would not be expected if the absorption in the uv were due e.g., to an increased β-peak.

All of these observations are either to be expected or at least easily interpreted according to the sensitizing pigment hypothesis. To interpret them on the basis of an increased β-band absorption needs assumptions about mechanisms which are so far unknown in other systems.

We are still unable to specify the sensitizing pigment molecule precisely. But several naturally occuring candidates with properties similar to those of the sensitizing pigment do exist. The aim of this paper was to prepare a basis for a biochemical approach which is needed for a final specification of the rhodopsin sensitizing molecules.

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