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Fly Visual Pigments Difference in Visual Pigments of Blowfly and Dronefly Peripheral Retinula Cells

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Summary. The visual pigments of peripheral retinula cells in fly eyes have been investigated by microspectrophotometry *in vivo*. Since flies have a pupil mechanism (Kirschfeld and Franceschini, 1969) which may invalidate the visual pigment measurements, the technique has been applied to the pupil-less mutant chalky of the blowfly *Calliphora erythrocephala*. It proves that the data acquired previously from wild type blowflies with the *in vivo* method (Stavenga et al., 1973) are indeed reliable.

Blowfly peripheral retinula cells contain a blue-green absorbing rhodopsin P495, which is photo-interconvertible with a yellow absorbing metarhodopsin M580. The transformation of rhodopsin into metarhodopsin occurs within milliseconds.

Peripheral retinula cells of wild type droneflies contain a rhodopsin P460 and a metarhodopsin M550. Both blowfly and dronefly belong to the suborder of Brachycera and both have a strongly bathochromic shifted metarhodopsin; yet, the characteristics of their visual pigments appear to be quite distinct.

Introduction

Visual pigments of insects are rhodopsins: the chromophore retinal is attached to a protein moiety, an opsin. In contrast with the rhodopsins of vertebrates, insect rhodopsins do not photolyse, but upon photoconversion they transform into a thermostable metarhodopsin state (review: Goldsmith, 1972). In view of the apparent simplicity the photochemistry of insect visual pigments might seem to be an obvious topic to study. However, the field has been little explored, probably due to the total amount of visual pigment in an insect eye being very small, the standard extraction methods then becoming laborious (see Goldsmith and Fernandez, 1966). Successful extractions have been recently obtained, notably by Hamdorf and co-workers, from eyes of the neuropteran *Ascalaphus macaronius* and the sphingid moth *Deilephila elpenor*, both eyes having the advantage of relatively high visual pigment content (Hamdorf et al., 1971; Schwemer and Paulsen, 1973).

Another approach, proven to be powerful in visual pigment research is that of microspectrophotometry in situ (review: Liebman, 1972). It was first applied to insects by Hamdorf and Langer (1965), and Langer and Thorell (1966) in measuring from rhabdomeres in eye-slices of *Calliphora erythrocephala*, mutant chalky. From these and subsequent investigations on this mutant blowfly (Hamdorf et al., 1973; Hamdorf and Rosner, 1973; Kirschfeld and Franceschini, 1975) as well as from the in vivo experiments performed on the wild type blowfly (Stavenga et al., 1973) it proves that blowfly eyes mainly harbour a blue-green absorbing rhodopsin which photo-interconverts with a bathochromic shifted yellow absorbing metarhodopsin. A visual pigment with very similar properties has been found in two other Diptera, namely the fruitfly *Drosophila melanogaster* (Ostroy et al., 1974; Kirschfeld and Franceschini, 1975) and the housefly *Musca domestica* (Kirschfeld and Franceschini, 1975).

On the other hand, in the larval mosquito *Aedes aegypti* the visual pigment found is a green absorbing rhodopsin which upon photoconversion transforms into a hypsochromic shifted, blue-absorbing metarhodopsin (Brown and White, 1972). Mosquitoes belong to the dipteran suborder Nematocera (thread-horns) whilst the flies mentioned above all are Brachycera (short-horns). So the main visual pigments of the two fly suborders have important differences. Still, as we will demonstrate in this paper, the picture shown so far is too simple, since, even within the suborder Brachycera the properties of the visual pigment can vary substantially.

The experiments reported here are performed on living flies. In wild type flies a major complication for photochemical visual pigment experiments is the activation, by the inevitable illumination, of the pupil mechanism, i.e. the system of migrating pigment granules acting within the visual sense cells (Franceschini, 1975; Franceschini and Kirschfeld, 1976). The influence of the pupil mechanism on the visual pigment measurements can be satisfactorily avoided by applying test-lights with intensity or duration beyond the sensitivity range of the pupil mechanism. Evidence for this claim can be elegantly produced by determining visual pigment spectra in the pupil-less blowfly mutant chalky with the same technique as used in the case of the wild type fly (see Stavenga et al., 1973). The agreement obtained between wild type and mutant spectra, reported below, reinforces the reliability of the measurements on another wild type fly, viz. the dronefly *Eristalis tenax* (Brachycera), the results of which are given subsequently.

Materials and Methods

Animals

Blowfly *Calliphora erythrocephala* chalky mutants have been supplied by Professor K. Hamdorf, Bochum. Droneflies *Eristalis tenax* were conveyed from Canberra and investigated in Groningen (in January 1975) upon a request by Professor G. A. Horridge.

Experimental Procedure

The experimental procedure was as described previously (Stavenga et al., 1973, 1975; Stavenga, 1975a). The living fly is immobilized with wax and mounted on a goniometer. The naked tip of a silvered and subsequently black painted quartz rod is inserted into the back of the fly's head through a negligibly small incision in the cuticle.

Monochromatic test-lights are applied antidromically via the quartz rod. The transmitted light is measured by a photomultiplier coupled to a microscope. Photochemical conversions of the visual pigment are induced by intense monochromatic orthodromic irradiations until a photoequilibrium is established. The intensity of the subsequent test-light is sufficiently low that during the measurement the photoequilibrium is not changed.

In the case of the wild type flies measurements are made sufficiently long (≥ 1 min) after irradiation to avoid contamination by the pupil mechanism. The experiments described here are only of peripheral retinula cells. Selective measurements of specific cell types is possible from the deep-pseudopupil (Franceschini and Kirschfeld, 1971; Franceschini, 1975) owing to its characteristic pattern. The most important advantage of the deep-pseudopupil is the enhancement of the signal-to-noise ratio (Franceschini and Kirschfeld, 1971) since one can measure the summed transmission of the same type of retinula cell from several ommatidia. In this way we have investigated the peripheral retinula cell type R_3 (Figs. 1-3) or, when a further signal increase was necessary, of cells R_2 , R_3 and R_4 together (Figs. 4-7).

Calculation of Spectra

The spectrophotometrical experiments are based on the theoretical analysis of photochromic pigment spectra given by Stavenga (1975b). Before we can go into the results a brief description of the theoretical basis is unavoidable.

We assume that a fly rhabdomere contains a photochromic visual pigment having two states, rhodopsin and metarhodopsin. Then the transmittance of the rhabdomere at a test wavelength λ_t is

$$T(\lambda_t) = \exp \left(- \int_0^L \eta(\lambda_t, x) [f_P \alpha_P(\lambda_t) + f_M \alpha_M(\lambda_t)] C(x) dx \right), \quad (1)$$

where η is a factor emerging from the fact that a fly rhabdomere acts as an optical waveguide; η equals the fraction of light power transmitted within the boundary of the waveguide (Snyder, 1975); x is the coordinate along the rhabdomere, L being its total length; $C(x)$ is the concentration of visual pigment molecules; f_P and f_M are the fractions of visual pigment molecules in the rhodopsin and metarhodopsin state respectively; α_P and α_M are the molecular absorption coefficients. If the two molecular states are photo-interconvertible and a photoequilibrium is established by stimulus wavelength λ_s , then the fraction of pigment in the metarhodopsin state is

$$f_{Me}(\lambda_s) = \left(1 + \Phi \frac{\alpha_M(\lambda_s)}{\alpha_P(\lambda_s)} \right)^{-1}, \quad (2)$$

where Φ is the relative quantum efficiency of the photoconversions and the suffix e indicates the photo-equilibrium. The relative quantum efficiency is the ratio of the quantum efficiencies of the conversions metarhodopsin to rhodopsin and rhodopsin to metarhodopsin respectively.

We further assume that a long wavelength λ_0 exists where $\alpha_P/\alpha_M = 0$. Then $f_{Me}(\lambda_0) = 0$. If photo-equilibria are created by stimulus wavelengths λ_0 and λ_s , then the difference in absorbance, defined by

$$D_e(\lambda_t, \lambda_s) = {}^{10} \log \frac{T_e(\lambda_t, \lambda_0)}{T_e(\lambda_t, \lambda_s)} \quad (3)$$

equals (from Eq. (1))

$$D_e(\lambda_t, \lambda_s) = f_{Me}(\lambda_s) N(\lambda_t) [\alpha_M(\lambda_t) - \alpha_P(\lambda_t)], \quad (4)$$

where

$$N(\lambda_t) = 0.4343 \int_0^L \eta(\lambda_t, x) C(x) dx. \quad (5)$$

From Equation (4) it follows that for a variety of λ_s difference spectra result with isosbestic wavelength(s) λ_{iso} defined by $\alpha_P(\lambda_{iso}) = \alpha_M(\lambda_{iso})$. It follows from Equation (2) that

$$f_{Me}(\lambda_{iso}) = \frac{1}{1 + \Phi}. \quad (6)$$

Irradiation with λ_{iso} results in a difference spectrum, according to Equation (4),

$$D_e(\lambda_t, \lambda_{\text{iso}}) \equiv D_{\text{iso}}(\lambda_t) = (1 + \Phi)^{-1} N(\lambda_t) [\alpha_M(\lambda_t) - \alpha_P(\lambda_t)]. \quad (7)$$

Relating the whole family of difference spectra to this particular one we obtain an important function (from Eqs. (4) and (6))

$$Q(\lambda_s) = \frac{D_e(\lambda_t, \lambda_s)}{D_e(\lambda_t, \lambda_{\text{iso}})} = \frac{f_{Me}(\lambda_s)}{f_{Me}(\lambda_{\text{iso}})} = (1 + \Phi) f_{Me}(\lambda_s) \quad (8a)$$

or (with Eq. (2))

$$Q(\lambda_s) = (1 + \Phi) \left/ \left(1 + \Phi \frac{\alpha_M(\lambda_s)}{\alpha_P(\lambda_s)} \right) \right. \quad (8b)$$

By dropping the suffixes in Equations (7) and (8b) the absorption spectra α_P and α_M can be derived

$$\alpha_P(\lambda) = \frac{D_{\text{iso}}(\lambda)}{N(\lambda)} \frac{Q(\lambda)}{1 - Q(\lambda)} \quad (9a)$$

$$\alpha_M(\lambda) = \frac{D_{\text{iso}}(\lambda)}{N(\lambda)} \frac{1 + \Phi - Q(\lambda)}{1 - Q(\lambda)}. \quad (9b)$$

It will be noted that before this analysis can be applied the first step is to provide evidence that the visual pigment under investigation is indeed a pigment with (only) two photo-interconvertible states.

Results

Spectrophotometry of the Retinula Cell Type R₃ in the Blowfly Calliphora erythrocephala, Mutant Chalky

In this section we shall first describe our spectrophotometrical experiments performed on the pupil-less blowfly mutant chalky. The antidromic transmission is measured from the retinula cell type R₃ at a series of test wavelengths λ_t , after photoequilibria are established by stimulus wavelengths λ_s being respectively 352, 398, 442, 470, 514 and 603 nm (Fig. 1). The photoequilibria were stable for at least several minutes. The latter, red, wavelength is taken as the reference wavelength λ_0 and the absorbance difference $D_e(\lambda_t, \lambda_s)$ is calculated (see Eq. (3), Methods). A family of difference spectra is thus obtained (Fig. 1). The proportionality of the curves and the common crossing point at the abscissa (an isosbestic point) are clear indications that the investigated type of retinula cell contains a photochromic visual pigment with two photo-interconvertible states, rhodopsin and metarhodopsin.

If we can take this view for granted then we can apply the tools described in the methods section to determine the characteristics of both the rhodopsin and the metarhodopsin state. Essential data are the shape of the difference spectra and the dependence of the relative magnitude on stimulus-wavelength. The shape is already given by the curves of Figure 1. The relative magnitude can only be obtained from Figure 1 for a few λ_s values. Hence a more detailed curve is determined by measuring the transmission at a fixed test-wavelength $\lambda_t = 583$ nm at a series of photoequilibria created by irradiation with a variety of stimulus wavelengths λ_s (Fig. 2). From the measurements the absorbance difference $D_e(\lambda_t, \lambda_s)$ is calculated (again with Eq. (3)). The longest stimulus wavelength, $\lambda_0 = 632$ nm, is taken as the reference wavelength. By normalizing the resulting spectrum (Fig. 2) to its value at the isosbestic wavelength the relative magnitude function

Fig. 1. Difference spectra from the peripheral retinula cells R_3 of the blowfly *Calliphora erythrocephala* mutant chalky. Photoequilibria are created by stimulus wavelengths $\lambda_s = 352, 398, 442, 470, 514$ and 603 nm. Absorbance differences have been calculated in respect of $\lambda_0 = 603$ nm with Equation (1). The inaccuracy is given by the bars at the abscissa

$Q(\lambda_s)$ is obtained (Eq. (8a)). The isosbestic wavelength proves, according to Figure 1, to be about $\lambda_{iso} = 510$ nm. Using this value, rescaling of the ordinate in Figure 2 yields $Q(\lambda_s)$.

In principle from the data of Figure 1 and Figure 2 the absorption spectra of the rhodopsin and its metarhodopsin can be calculated using Equation (9). (We discuss the relatively unimportant function $N(\lambda)$ and the value of the quantum efficiency Φ below.) Clearly a blue absorbing rhodopsin and a yellow metarhodopsin are involved. Unfortunately, however, the accuracy of the experimental data is insufficient to yield exact spectra. Still, it can be estimated from the acquired data that the rhodopsin must have an absorption peak at approximately 495 nm and the metarhodopsin at 580 nm.

From a short examination of the figures we notice that at photoequilibria created by long stimulus-wavelengths the fraction of metarhodopsin $f_{Me}(\lambda_s) \approx 0$, since at long wavelengths $Q(\lambda_s) \approx 0$ (Eq. (8a) and Fig. 2). At shorter, blue, wavelengths $Q(\lambda_s)$, or $f_{Me}(\lambda_s)$, is large (Fig. 2), and $\alpha_M < \alpha_P$ (Fig. 1). Since we obtain a restricted value range for Q , $0 \leq Q \leq 1.6$, we can derive from Figure 2 a constraint

Fig. 2. Absorbance difference with respect to $\lambda_0 = 632$ nm resulting in photoequilibria at a variety of λ_s , measured at test wavelength $\lambda_t = 583$ nm. (Same preparation as Fig. 1.) By normalizing the curve to its value at the isosbestic wavelength $\lambda_{iso} = 510$ nm the function $Q(\lambda)$ results (see Eq. (8))

for Φ , the relative quantum efficiency. From $0 \leq f_{Me} \leq 1$ it follows, with Eq. (8a), that $\Phi \gtrsim 0.6$.

Returning to the concluded spectral characteristics of the visual pigment states, we describe Figure 3, which shows an alternative (and more common than that described hitherto) approach to the visual pigment spectra. After having finished the spectrophotometrical measurements which resulted in Figures 1 and 2, we measured the transmission of the blowfly eye outside the deep-pseudopupil, i.e. light that is transmitted outside the rhabdomeres.

Subsequently the retina (and, unavoidably, part of the visual ganglia) was removed by cutting it off with a razor blade, and the transmission of the remaining tissue was measured. In Figure 3 (upper curve) the difference in absorbance between the measurement outside the deep-pseudopupil and that with the retina removed (retina-off) is given. It follows, not unexpectedly, that this tissue has a higher absorbance in the blue compared to the yellow and red.

Furthermore, Figure 3b shows the difference in absorbance between peripheral retinula cell type R_3 , at a photoequilibrium created by $\lambda_s = 603$ nm, and the retina-off case. This absorbance difference, therefore, should represent the absorbance of the R_3 rhabdomeres alone, and, in fact, the absorbance of rhodopsin, since at $\lambda_s = 603$ nm, f_{Me} should be negligible. However, there is a large residual absorbance in the red, which we interpret as probably due to excess removal of scattering tissue proximal to the rhabdomeres by the cutting-off procedure. We have therefore corrected the R_3 retina-off spectrum with the absorbance spectrum of the eye-tissue (Fig. 3, upper curve) and the resulting corrected spectrum (Fig. 3) can then be taken to represent a rhodopsin spectrum. By adding the difference spectrum corresponding to $\lambda_s = 442$ nm (Fig. 1) we have an indication of the metarhodopsin spectrum, since, as we discussed above, after blue irradiation the rhodopsin content is small (large Q).

Fig. 3. The upper curve represents the difference in absorbance between a measurement outside the deep-pseudopupil and that with retina removed. The eye tissue shows a higher absorbance in the blue, most probably due to scattering. The absorbance difference between the measurement on R_3 with pure rhodopsin and the retina-off case, corrected by using the upper curve, yields a curve which represents approximately the rhodopsin absorption spectrum. By adding to the latter spectrum the difference spectrum of Figure 1, corresponding to $\lambda_s = 442$ nm, an indication of the metarhodopsin absorption spectrum is obtained

The two spectra of Figure 3 (lower part) support the conclusion that the peripheral retinula cell R_3 of the blowfly has a blue absorbing rhodopsin P495 and a yellow absorbing metarhodopsin M580 (approximate values). Since measurements from the other peripheral retinula cell types yield very similar difference spectra it seems that all these cells have one and the same visual pigment. Furthermore, the rhodopsin spectrum is similar to the sensitivity curve resulting from intracellular recordings (Dörrscheidt-Käfer, 1972). This notion is in line with the generally accepted view that rhodopsin conversion triggers the receptor potential. For a detailed understanding of the electrophysiological processes it is of interest to know whether or not intermediate visual pigment states exist (see Hamdorf and Rosner, 1973). We turn to this question in the next section.

Intermediate Visual Pigment States between Rhodopsin-Metarhodopsin Conversion

After photoconversion of rhodopsin the retinula cell is depolarized within a few milliseconds. In order to investigate whether or not the thermostable metarhodopsin state is reached within that short time the experiment of Figure 4 has been performed.

The antidromic transmission from the deep-pseudopupil of photoreceptors R_{2+3+4} is measured so as to increase the signal-to-noise ratio. The test-wavelength is chosen at $\lambda_t = 584$ nm since here $\alpha_M - \alpha_P$ is about maximal (Fig. 3). At the start of the irradiation the visual pigment is in photoequilibrium at 584 nm. Virtually all visual pigment molecules are then in the rhodopsin state (Fig. 2). The wavelength of irradiation $\lambda_s = 457$ nm is selected such that an extreme fraction of rhodopsin is transferred into the metarhodopsin state (see Fig. 2). Thus at the test-wavelength there will be an increase in absorbance (i.e. a decrease in transmission) during irradiation.

In Figure 4a and 4b the blue stimulus is shown in the lower trace. The transmission time course at $\lambda_t = 584$ nm during the blue irradiation is recorded in the upper traces of Figure 4a and 4b. In Figure 4a two experiments are superimposed, the stimulus durations being about 20 and 35 ms respectively. It appears that a new photoequilibrium is reached within approximately 30 ms. Since (some of) the created photoproduct(s) might be thermolabile the experiment has been repeated as demonstrated in Figure 4b. After the blue irradiation a dark time of 2 s has been intercalated in order to allow the intermediates, if any, to decay to the thermostable state. No such event occurs. Longer dark times give the same result. The reason for intercalating a dark time can be understood from the transmission increase later on in Figure 4b; the yellow test light induces a photochemical regeneration of rhodopsin. We conclude from Figure 4 that blowfly rhodopsin converts into its stable photoproduct metarhodopsin at least within a few milliseconds.

Before going into a discussion of the results obtained for blowfly visual pigment, first we shall proceed with some measurements performed on the visual pigment of the dronefly.

Spectrophotometry of the Visual Pigment in the Peripheral Retinula Cells of the Dronefly, Eristalis tenax

In this section we investigate the photochemistry of the visual pigment of wild type droneflies. The existing pupil mechanism appears to have properties very similar to those of the pupil mechanisms of other Brachycera (housefly, Kirschfeld and Franceschini, 1969; fruitfly, Franceschini, 1972a, b, 1975; blowfly, Stavenga, 1975a). To avoid the influence of the pupil a dark adaptation time ≥ 1 min has been given before the test measurements. In all other respects the procedure of investigation was as before.

In Figure 5 difference spectra are shown, obtained from transmission measurements of receptors R_{2+3+4} . Three photoequilibria established by stimulus wavelengths $\lambda_s = 470, 506$ and 632 nm have been analysed. As reference the red wavelength is taken ($\lambda_0 = 632$ nm). Hence two difference spectra result, which appear to be proportional in magnitude and intersect at the abscissa. In Figure 6 the difference spectrum between photoequilibria at 442 nm and 632 nm is given by the black squares (R_{2+3+4} in a different dronefly from that of Fig. 5). In order to compare the proportionality of the difference spectra, those of Figure 5 are magnified by a constant factor and plotted on Figure 6 (circles). A smooth curve

Fig. 4a and b. Photoconversion of rhodopsin into metarhodopsin. The lower traces in Figure 4a and 4b represent an intense blue irradiation, $\lambda_s = 457$ nm. The upper traces show the antidromic transmission at test wavelength $\lambda_t = 584$ nm. At the start of the experiments in Figure 4a and 4b the photoequilibrium is at 584 nm. In Figure 4a two experiments are recorded; the duration of the two stimuli are about 20 and 35 ms respectively. As a consequence of the blue irradiation the transmission decreases (within approximately 30 ms, Fig. 4a) which does not change in the dark (Fig. 4b). This reveals that thermolabile intermediates between fly rhodopsin and metarhodopsin, if any exist, must have a lifetime shorter than a few milliseconds. Owing to the lack of screening pigments in the mutant chalky and the measurement being performed on receptor types R_{2+3+4} together the background contribution of stray light is substantial and may be valued to be 1.5 div

Fig. 5. Difference spectra of the peripheral retinula cells R_{2+3+4} of the dronefly *Eristalis tenax*, obtained by $\lambda_s=470, 506$ and 632 nm

Fig. 6. The difference spectra of Figure 5 are mapped to a difference spectrum corresponding to $\lambda_s=442$ nm while the reference wavelength $\lambda_0=632$ nm. The resulting isosbestic wavelength is about $\lambda_{iso}=485$ nm

Fig. 7. Absorbance differences measured in peripheral retinula cells of dronefly at test wavelengths $\lambda_t=442, 488$ and 570 nm. The measurements at $\lambda_t=488$ nm were performed to assure that no UV-pigment is involved

Fig. 8. The spectra of Figure 7 normalized to their value at the isosbestic point $\lambda_{iso}=485$ nm yield the function $Q(\lambda)$ of Equation (8)

Fig. 9. Absorption spectra of dronefly visual pigment states rhodopsin P460 and metarhodopsin M550, calculated with Equation (9) assuming that the relative quantum efficiency $\Phi=1$ and the waveguide function $N(\lambda)$ is constant

is drawn through the resulting set of points. From the experimental data we can assume that the visual pigment of the peripheral cells of the dronefly, as in the case of the blowfly, has two thermostable states, rhodopsin and metarhodopsin. Comparing Figures 6 and 1 we see that probably the dronefly pigment states are shifted towards the blue, since both the extremes and the isosbestic point are located at shorter wavelengths; the latter can be estimated to be approximately $\lambda_{\text{iso}} = 485 \text{ nm}$.

In Figure 7 the absorbance differences $D_e(\lambda_t, \lambda_s)$ at a few fixed test wavelengths are presented. Normalizing all these curves to their value at the isosbestic point should result in only one curve, assuming Equation (8) to hold. Figure 8 is the result of the normalization procedure. The drawn smooth curve seems to fit the experimental data reasonably well, so Figure 8 gives $Q(\lambda)$. We now assume that the difference spectrum of Figure 6 is proportional to $D_{\text{iso}}(\lambda)$ and that the relative quantum efficiency is about $\Phi = 1$ as has been shown for the similar visual pigment of octopus (Schwemer, 1969). Then from Equation (9) the absorption spectra of the two visual pigment states can be calculated, resulting in Figure 9. The spectra are approximate owing to the inaccuracy of the experimental values. It follows that dronefly rhodopsin P460 absorbs in the violet and its metarhodopsin M550 in the green. The inaccuracy in the wavelengths where absorption is maximum can be estimated at about 10 nm. The ratio of the maximal absorption coefficients $\alpha_{M\text{max}}/\alpha_{P\text{max}}$ is 1.4 ± 0.2 .

Discussion

We have investigated the visual pigments of the peripheral retinula cells of the blowfly mutant chalky as a comparison to the parallel experiments on wild type blowflies (Stavenga et al., 1973), using essentially the same technique. It proves that the measured absorbance difference spectra of both wild type and mutant are very similar. The isosbestic point, which is a critical measure, is in both cases about 510 nm. Also the extremes of the difference spectra are located at very similar wavelengths, 470 nm and 580 nm respectively. Furthermore, $Q(\lambda_s)$ in both cases is similar (compare Fig. 2 with Fig. 3 of Stavenga et al., 1973). The main difference to be noticed is the much higher magnitude of the spectra from the wild type fly. (Interestingly, this statement does not hold when the wild type flies are reared in the laboratory under the same conditions as the mutant. Evidently laboratory conditions are not healthy.) We conclude from our blowfly measurements that both wild type and mutant have the same visual pigment in the peripheral retinula cells characterized by rhodopsin P495 and its thermostable photoproduct metarhodopsin M580. The accurate absorption spectra are yet to be determined. Actually slightly different metarhodopsin peaks have been concluded for the mutant chalky by Hamdorf and co-workers (Hamdorf et al., 1973; Hamdorf and Rosner, 1973; see also Rosner, 1975) namely M550-560. (The rhodopsin P490-500 is in good agreement with our P495). The difference spectrum used for determining those spectra however was obtained from whole eye slices. Since the tissue in this case is far from homogeneous multiple scattering may then

reduce the reliability of the measurements. Yet, to a first approximation the data referred to above and the data presented here are in agreement¹. Also the recent report by Kirschfeld and Franceschini (1975) on single rhabdomeres of the mutant chalky gives confirmatory results. All things considered, we conclude that our method of visual pigment measurements from living wild type animals, although being complicated by the pupil mechanism, is a reliable one.

As well as our blowfly experiments we have also investigated the visual pigment in the peripheral retinula cells of the dronefly. From our experimental results we have calculated the absorption spectra of the rhodopsin P460 and its metarhodopsin M550. The major uncertainty in the calculation is the value of the relative quantum efficiency Φ . We have assumed $\Phi=1$, as holds in the case of octopus visual pigment (Schwemer, 1969) and this may not be justified². Furthermore we have assumed that the function $N(\lambda)$ in Equation (9) is a constant. $N(\lambda)$ involves the parameter $\eta(\lambda)$, which is the fraction of light power transmitted inside the fly rhabdomere. We have shown that in the large peripheral rhabdomeres of flies η is only slightly dependent on wavelength (see Stavenga, 1974; Stavenga and van Barneveld, 1975). Since η decreases with wavelength its effect on the difference spectra is a suppression of the long wavelength side. So, the metarhodopsin peak is relatively too small. An exact calculation of the waveguide effects requires an accurate knowledge of the whole rhabdomere dimensions, which is not yet at our disposal. However, as argued above, it will only slightly influence the picture. All the same, the most important achievement of the dronefly data is the agreement and mutual reinforcement of the derived visual pigment spectra with the electrophysiological results of Tsukahara and Horridge (1976).

The results reported so far mainly concern the (for the human eye) visual wavelength range. Our set-up must be improved in order that reliable measurements can be performed in the UV also. Extension of the measurements towards the UV is of particular importance since this wavelength region is a still largely unknown area. Considerable arguments have been raised for and against the existence of a UV-visual pigment in fly peripheral retinula cells (pro: Rosner, 1975, and Horridge and Mimura, 1975; contra: Snyder and Pask, 1973; Stavenga et al., 1973; and Harris et al., 1976).

Since UV-pigments, investigated up to date, have a thermostable metarhodopsin absorbing maximally at about 475 nm (Hamdorf et al., 1973) the existence of UV-pigments can be investigated by measuring absorbance changes in the blue (metarhodopsin) region. If a UV-pigment should be located in the peripheral retinula cells in addition to the main blue or blue-green rhodopsin the isosbestic point of the latter visual pigment is a sensitive place for detecting absorbance changes after UV-irradiation. Neither in the blowfly nor in the dronefly were such events observed (Fig. 1 and Fig. 7; see Stavenga et al., 1973; and also Kirschfeld and Franceschini, 1975).

¹ The discrepancy seems to be solved since recently Razmjoo and Hamdorf (1976) take M580

² Recently obtained evidence (Tsukahara and Horridge, 1976) from electrophysiological retinula cell recordings in dronefly shows that the relative quantum efficiency $\Phi=0.8$ rather than $\Phi=1.0$. We have demonstrated (Stavenga, 1975b) that a change in Φ only affects the metarhodopsin spectrum (when this spectrum is estimated relative to the rhodopsin spectrum); see Equation (9). Taking $\Phi=0.8$ instead of 1.0 induces a slightly higher metarhodopsin peak and steeper slopes

On the other hand clear indications for a UV-rhodopsin in the central retinula cells were obtained in wild type blowflies (Stavenga et al., 1973), in blowfly chalky mutants and in dronefly (unpublished). Electrophysiological evidence for UV-sensitive retinula cells in flies has been provided by Burkhardt (1962, blowfly), Bishop (1974, dronefly), Horridge et al. (1975, dronefly) and Harris et al. (1976, fruitfly). Still, the suggestion that the superior central retinula cell R_7 is the UV-sensitive cell (Stavenga et al., 1973; Harris et al., 1976) is in general unjustified, according to the findings of Kirschfeld and Franceschini (1975).

All the same, central retinula cells can contribute relatively enormously to the ERG of flies (Koenig and Merriam, in preparation), so conclusions concerning visual pigment processes in the peripheral retinula cells derived from extracellular recordings must be considered critically. In fact, Hamdorf and Rosner (1973), from extracellular electrophysiological experiments, conclude that the blue-green absorbing rhodopsin of blowfly has, in addition to its yellow metarhodopsin, intermediate states absorbing in the blue-green and the UV, having lifetimes in the order of milliseconds or longer. This view has not been confirmed by our spectrophotometrical measurements. On the contrary, the photoconversion of blowfly rhodopsin into its metarhodopsin state occurs fairly quickly, to wit within a few milliseconds (Fig. 4). Probably the electrophysiological results referred to can be explained by contamination of the ERG by UV-sensitive cells and by the complicated processes arising at higher illumination intensities when both rhodopsin and metarhodopsin conversions affect the receptor membrane potential (see Tsukahara, Horridge and Stavenga, 1976).

A final remark may be made concerning the visual pigments of flies particularly, and those of insects in general. Both the blowfly and the dronefly visual pigment show a huge bathochromic shift upon photoconversion of the rhodopsin state. A similar and even larger shift has been found for the UV-pigment of *Ascalaphus*, namely P345 converts into M475 (Hamdorf et al., 1971, 1973). The advantage of this bathochromic shift for photoregeneration of the rhodopsin state has been recognized by Hamdorf et al. (1971, 1973) and Stavenga et al. (1973, 1975). Yet, more difficult is to understand the thermostability of the metarhodopsin state of insect visual pigments. In the case of the vertebrate visual pigments conversion of the rhodopsin results in a batho-pigment which has a stronger absorption farther into the red than the rhodopsin state (Yoshizawa, 1972). It has been supposed that these spectral properties are the consequence of a highly strained molecular state and that this accounts for the enormous instability of the batho-pigments (Wald, 1973). Apparently those insect visual pigments with thermostable bathochromic shifted metarhodopsins create a challenge for a molecular interpretation.

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Note Added in Proof

Recently Tsukahara and Horridge (1976) have estimated, by purely electrophysiological methods, that the absorbance of a dronefly rhabdomere at the wavelength of the rhodopsin peak is $1.17 \pm 7\%$. This compares well with the microspectrophotometrically obtained dronefly data reported here. From Figure 8 we have $Q(\lambda_s = 442 \text{ nm}) = 1.4$, hence the fraction of metarhodopsin in equilibrium at 442 nm, $f_{Me}(442) = 0.70$ or 0.77 depending on the value of the relative quantum efficiency, $\phi = 1.0$ or 0.82 (from Eq. (8a)). A rhabdomere with pure metarhodopsin hence has an absorbance at $\lambda_r = 550 \text{ nm}$ of $1.5 \pm 5\%$. Since $\alpha_{M \max}/\alpha_{P \max} = 1.4 \pm 0.2$, we obtain for the absorbance at the rhodopsin peak $1.1 \pm 20\%$, in agreement with the number above. We thus conclude that between 87 and 95% of the incident light can be absorbed.

These results can be further fortified by comparing them with previously estimated absorption coefficients. Hamdorf (1975), calculating the density of visual pigment molecules in a fly rhabdomere, concludes that with a length $L = 200 \mu\text{m}$ from 50% up to 90% of the light can be absorbed, which means that the rhabdomere absorption coefficient $\alpha_{R \max} C$ has a value between 0.003 and $0.012 \mu\text{m}^{-1}$. When we take for a dronefly rhabdomere $L = 250 \mu\text{m}$ we obtain $\alpha_{R \max} C = 0.009 \pm 0.002 \mu\text{m}^{-1}$ (unpolarised light). (Approximately the same value is obtained when going through the calculation procedure given above for the blowfly data of Stavenga et al., 1973). Our result is about the mean of previously estimated absorption coefficient values $0.005 \mu\text{m}^{-1}$ (housefly *Musca*; Kirschfeld, 1969) and $0.013 \mu\text{m}^{-1}$ (spider crab *Libinia*; Hays and Goldsmith, 1969).