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Photoregeneration of Visual Pigments in a Moth

A Microphotometric Study*

K. Hamdorf, G. Höglund, and H. Langer

Institut für Tierphysiologie der Ruhr-Universität Bochum
Department of Physiology, Karolinska Institutet, Stockholm

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Summary. The spectral absorbance by the visual pigments in the compound eye of the moth *Deilephila elpenor* was determined by microphotometry. Two visual pigments and their photoproducts were demonstrated. The photoproducts are thermostable and are reconverted to the visual pigments by light. The concentrations of the visual pigments and the photoproducts at each wavelength are determined by their absorbance coefficients at this wavelength.

P 525: The experimental recordings (difference spectra and spectral absorbance changes after exposure to monochromatic lights) were completely reproduced by calculations using nomograms for vertebrate rhodopsin. The identity between experimental recordings and calculations show: One visual pigment absorbs maximally at 525 nm (P 525). The resonance spectrum of the visual pigment is identical to that for a vertebrate rhodopsin (λ_{max} at 525 nm). The photoproduct of this pigment absorbs maximally at 480 nm (M 480). It is similar to the acid metarhodopsin in cephalopods. The relative absorbance of P 525 to that of M 480 is 1:1.75. The quantum efficiency for photoconversion of P 525 to M 480 is nearly equal to that for reconversion of M 480 to P 525. Wavelengths exceeding about 570 nm are absorbed only by P 525, i.e. P 525 is completely converted to M 480. Shorter wavelengths are absorbed both by P 525 and M 480. At these wavelengths a photoequilibrium between the two pigments is formed. Maximal concentration of P 525 is obtained at about 450 nm.

P 350: A second visual pigment absorbs maximally at about 350 nm (P 350), and its photoproduct at 450 to 460 nm. In the region of spectral overlap a photoequilibrium between the two pigments is formed.The visual pigment and the photoproduct are similar to those in the neuropteran insect *Ascalaphus*.

Introduction

The discrimination of wavelengths by a visual system requires at least two types of receptors with different spectral sensitivity maxima.

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Three groups of receptors have been identified in insects. One group is maximally sensitive in the ultra-violet region of the spectrum (about 350 nm), another group in the blue region (about 440 nm), and a third in the green region (about 520 nm). The number of receptor types can be determined by training experiments or electrophysiological recordings. Such measurements have shown that some compound eyes, e.g. the frontal eye of *Ascalaphus* (Gogala, 1967), have only one receptor type. At least three receptor types occur e.g. in the honey-bee (Daumer, 1956; Autrum and von Zwehl, 1964). A visual pigment with a spectral absorption corresponding to the ultraviolet spectral sensitivity has been identified in *Ascalaphus* (Gogala, Hamdorf, and Schwemer, 1970; Hamdorf, Schwemer, and Gogala, 1971; Schwemer, Gogala, and Hamdorf, 1971). The photoproduct of this pigment is thermostable, and it is reconverted to the visual pigment by blue light.

The present study concerns the question of whether similar pigments, maximally absorbing at other wavelengths, exist in insects; and particularly whether such pigments contribute to the discrimination of wavelengths. A sphingid moth (*Deilephila elpenor*) was chosen for the experiments because several facts suggest that the eye of this species is well suited for investigations on the visual pigments in insects. Firstly, the rhabdoms form a large part of the receptor layer in this "superposition" eye. This fact indicates that the eye contains a large amount of photopigment. Secondly, most of the screening pigment, which obstructs measurements on the visual pigment, can be dissected away from the receptors. Finally, electrophysiological mass recordings (Höglund and Struwe, 1970) indicate the presence of three types of receptors with different spectral sensitivity; one type maximally sensitive in the ultraviolet, a second in the green, and possibly a third receptor type sensitive in the blue region of the spectrum. From this result can be concluded that the eye contains at least two visual pigments.

Materials and Methods

Material. The moth *Deilephila elpenor* (Lepidoptera: Sphingidae) was used for the experiments. No variation between sexes in the experimental results was obtained. Pupae were kept at 23 to 28° C at 80% humidity until the imagines emerged. They were usually used for experiments 2 to 3 days after emergence. The moths were dark-adapted for at least one hour before the experiment. Measurements were also made on eyes that had been deep-frozen (−20° C). The results obtained on such eyes were similar to those obtained on fresh eyes.

Dissection and Mounting. The receptor layer of the compound eye was isolated by two parallel cuts with a razor blade. The major part of the dioptric apparatus, together with the distal screening pigments, was removed by a cut through the cornea. A second cut was made through the receptor layer close to the basal pigment. The receptor layer was then easily removed. Measurements parallel to the optical axis of the receptors were made on the intact receptor layer. Measurements at right

angle to this axis were best made on a small group of receptors cut from the central part of the retina. The preparation (intact retina, or a small group of receptors) was put in Ringer's solution (Ephrussi and Beadle, 1936) between quartz cover glasses. The chamber was sealed by silicone grease. The preparation gave reproducible results for more than 24 hours.

Measurements were made with a one-beam photometer (Zeiss MPM). Ultrafluar objectives (enlargement 10 or 32 times) and ultraviolet condensor were used. The diameter of the measuring area was usually 30 or 100 μm. Conversion and reconversion of the visual pigments were accomplished by a mercury lamp (Zeiss HBO, 150 W). Measurements were made using a tungsten lamp (12 V, 5 A). Monochromatic illumination was obtained by interference filters (Schott). The filters were inserted between aperture and condenser. A filter holder permitted rapid exchange of filters. Measurements were displayed on a recorder (Beckman).

Advantages of the Zeiss photometer are: good stability over time, high amplification of photomultiplier current, and possibility to calibrate the change in current at known intervals of intensity of monochromatic illumination.

Two methods were usually used for pigment analysis:

1. Difference Spectra. a) The receptors were exposed to a monochromatic light (e.g. 453 nm) of high intensity. During this "saturating" exposure, a photoequilibrium between visual pigment and photoproduct was established, as evidenced by a constant photomultiplier current during the later part of the light exposure.

b) They were then illuminated by a series of at least 10 wavelengths (395 to 636 nm). The photomultiplier current (J_1) was measured at each wavelength. The light intensity was kept so low that within the time (5 to 10 sec) for registration at each wavelength there was no change in current, showing that the absorbance did not change during the measurement.

c) The receptors were again exposed to a "saturating" monochromatic illumination of high intensity, different from that used in *a* (e.g. 608 nm). During the illumination, a new photoequilibirum between visual pigment and photoproduct was reached.

d) The photomultiplier current (J_2) during exposure to the series of wavelengths was again measured, as described in *b*. The relation between photomultiplier current (J_1/J_2) and change in light intensity (at each tested wavelength) was calibrated at the beginning and the end of each test series by inserting calibrated neutral density filters in the measuring beam. The change in absorbance (ΔE) at each wavelength (caused by the "saturating" illuminations in *a* and *c*) is given by:

$$\Delta E = \log (J_2/J_1).$$

2. Spectral Absorbance Change. a) The receptors were exposed to monochromatic light (λ_x). A constant photomultiplier current during the later part of the light exposure showed that a photoequilibrium between visual pigment and photoproduct was established by this "saturating" light exposure.

b) Exposure to another monochromatic light (λ_1, wavelengths between 414 and 636 nm) from the tungsten lamp. The change in photomultiplier current was measured during the light exposure. If the current change was not completed in a given time, usually 5 min, the receptors were exposed for 1 to 2 min to a high intensity "saturating" light from the mercury lamp.

c) Repeated "saturating" exposure to λ_x, as described in *a*.

d) Exposure to a second monochromatic light (λ_2, wavelengths between 414 and 636 nm) as described in *b*, and simultaneous measurement of change in photomultiplier current.

e) "Saturating" exposure to λ_x etc.

Variations in the measuring methods are described in connection with the results.

In the present paper ultra-violet refers to wavelengths about 350 nm, violet to wavelengths about 440 mn, green to wavelengths about 530 nm, and yellow to wavelengths about 570 nm.

The following symbols are used in this article: λ = wavelength, λ_{max} = wavelength maximally absorbed by pigment, $\alpha(\lambda)$ = absorbance coefficient at wavelength λ, $\gamma(\lambda)$ = quantum efficiency at wavelength λ, P = visual pigment (rhodopsin), M = photoproduct (metarhodopsin), E = absorbance, ΔE = absorbance change.

Results

Preliminary Microspectrophotometric Recordings

Attempts were made directly to record the spectral absorbance of the photopigments. A two-beam photometer (Chance, Perry, Åkerman, and Thorell, 1959) was used for the experiments. Recordings running from short to long wavelengths were different from those made in the opposite

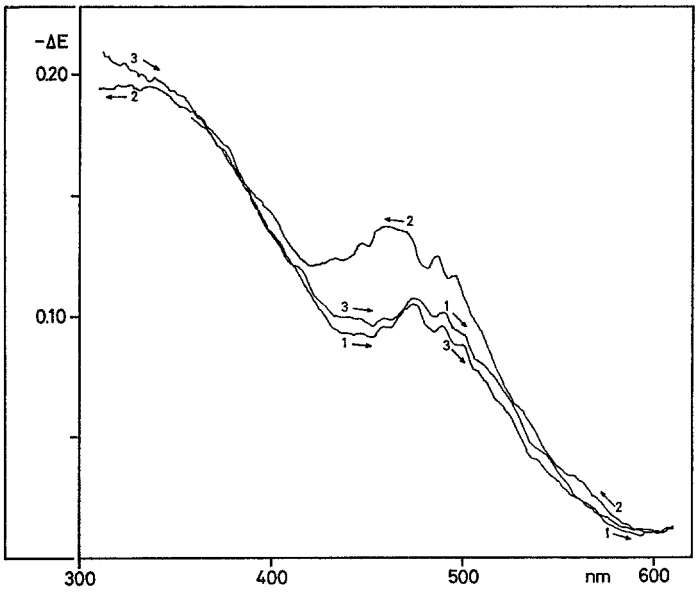

Fig. 1. Microphotometric recordings of absorbance spectrum from one rhabdom in *Deilephila elpenor*. Measurement made along optical axis. Diameter of measuring area 5 μm. Direction (arrows) and sequence (1, 2, and 3) of recordings indicated. Recordings 1 and 3 not significantly different. Absorbance of photostable screening pigment added to absorbance by photosensitive pigment. Absorbance change (mainly between 400 and 550 nm) due to changes in amounts of photosensitive pigments during recordings

direction (Fig. 1). This result shows the presence of photosensitive pigments the amount of which changed during the recording. The reason for the change was that the light intensity necessary for the measurements by the double-beam microspectrophotometer, caused a change in photoequilibrium, and thereby in absorbance, during the measurements.

Green Absorbing Pigment

1. Time Course and Amplitude of Absorbance Change

Onset of illumination along the optical axis of the receptors or at right angle to this axis, induced a gradual change in absorbance to a final value that then remained constant as long as the wavelength of the illumination was unchanged. The rate of change varied with the intensity of the adapting light, similarly to the absorbance change in the ultra-violet absorbing pigment in *Ascalaphus* (Schwemer, Gogala, and Hamdorf, 1971). The amplitude and direction (increase or decrease) of the absorbance change depended on the wavelength of test light and preceeding illumination. An example of the variation in amplitude with wavelength is seen in Fig. 2. The receptors were first illuminated by a long wavelength (571 nm), then by a short wavelength (453 nm), by a long wavelength (593 nm), and finally again by the short wavelength (453 nm). All light exposures caused a decrease in absorbance to a constant value. The absorbance change was equal during the second and fourth light exposure (453 nm). These exposures could be repeated indefinitely with the same result.

The experiment showed that long wavelength illumination causes the formation of a photoproduct, and that short wavelength light reconverts the photoproduct to the primary pigment.

2. Difference Spectra

A difference spectrum between short wavelength (453 nm) and long wavelength (608 nm) illumination was determined as described in Methods, *1*. The difference spectrum (Fig. 3) is characterized by a minimum at 550 nm and a maximum at 470 nm. Consecutive illumination by a short wavelength (453 nm) resulted in an inverted difference spectrum, i.e. a minimum at 470 nm, a maximum at 550 nm, and identical isosbestic point (wavelength at which $\Delta E = 0$). The difference spectra could be reproduced indefinitely by alternating short and long wavelength illumination. Wavelengths between 608 and 550 nm yielded similar difference spectra, the isosbestic point was the same while the amplitude of the maximum and minimum were smaller. Such difference spectra have been recorded from *Eledone* (Kropf, Brown and Hubbard, 1959;

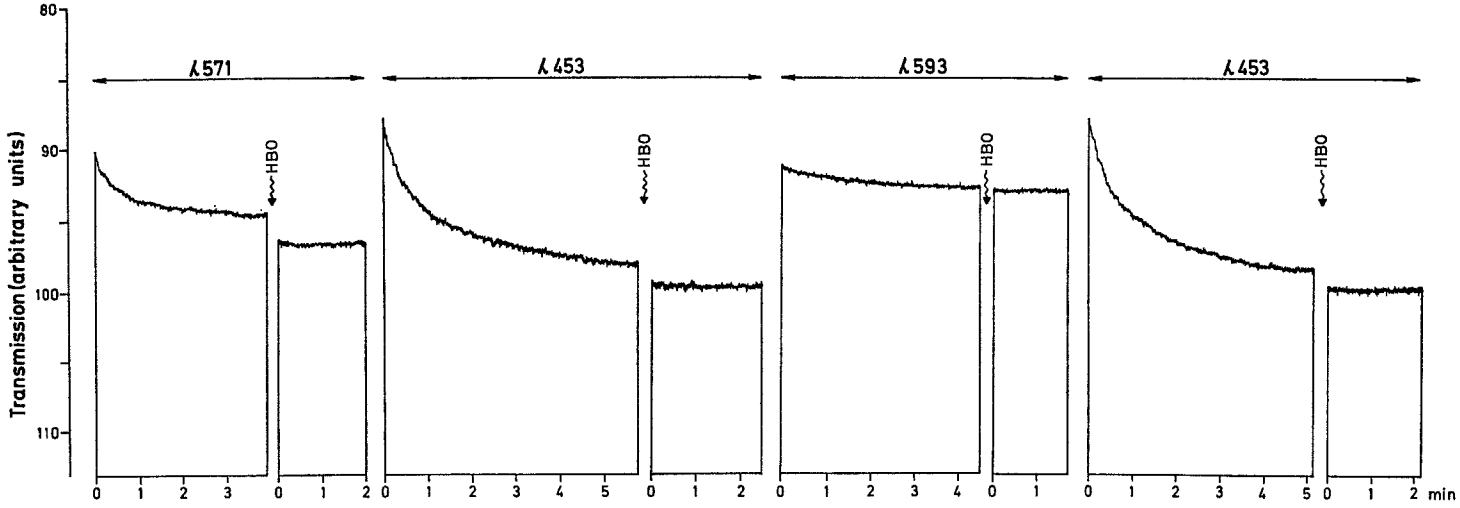

Fig. 2. Change in transmission (measured as change in photomultiplier current) caused by alternating monochromatic illumination (original recording). Receptors first illuminated by tungsten lamp, and then by high intensity mercury lamp (HBO). Note absence of transmission change after high intensity illumination, indicating that conversion of pigment (571 and 593 nm) or reconversion (453 nm) completed. Transmission change at 453 nm after exposure to 571 nm equal to change after exposure to 593 nm, indicating that equal amount of pigment reconverted

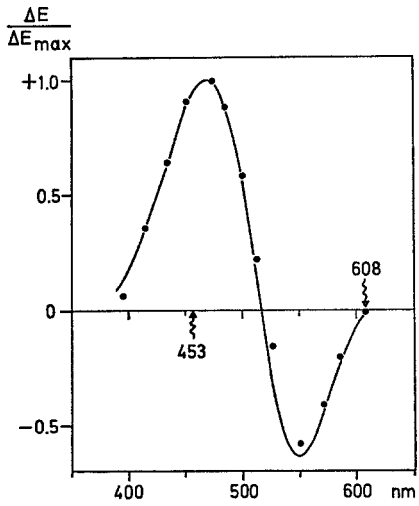

Fig. 3. Change in absorbance (dots) by receptors caused by alternating short (453 nm) and long (608 nm) wavelength illumination. Average of 2 recordings. Difference spectrum calculated (continuous line) from nomograms for vertebrate visual pigments with λ_{max} at 525 and 480 nm, with a ratio of absorbancies at $\lambda_{max} = 1:1.75$

Hamdorf, Schwemer and Täuber, 1968). In this species the visual pigment is converted by light to a thermostable photoproduct that is reconverted by light to the visual pigment. In *Eledone* the photoproduct absorbs maximally at a wavelength about 50 nm longer than the wavelength that is maximally absorbed by the visual pigment. In contrast, *Deilephila* is maximally sensitive to 525 nm (Höglund, Hamdorf and Rosner, 1973). The visual pigment thus absorbs maximally at this wavelength. Consequently, the considerable change in absorbance in the blue wavelength region (Fig. 3) shows that in *Deilephila* the photoproduct maximally absorbs at a shorter wavelength. As in *Eledone* the photoproduct is stable at room temperature and is reconverted to the visual pigment by light. The spectral absorbancies of the visual pigment and photoproduct partially overlap. The larger amplitude of the maximum at 470 nm (Fig. 3) in comparison with that of the minimum at 550 nm indicates that the molar absorbance of the photoproduct is larger than that of the visual pigment.

An attempt was made to test whether the visual pigment is a rhodopsin, i.e. retinal bound to a protein, that upon illumination converts to a compound similar to metarhodopsin. The experimentally recorded

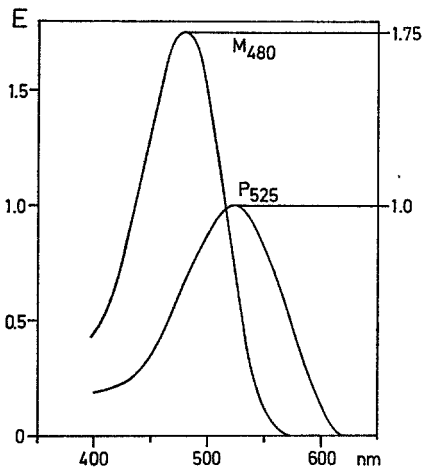

Fig. 4. Spectral absorbance by visual pigment (P 525) and photoproduct (M 480). Subtracting spectral absorbance by P 525 from absorbance by M 480 results in difference spectrum shown in Fig. 3

difference spectrum (Fig. 3) was reproduced by subtraction of two nomograms for vertebrate rhodopsins (Dartnall, 1953). The following characteristics of the experimentally recorded difference spectrum were used to optimize the reproduction. *1.* Wavelength at isobestic point, *2.* wavelength at ΔE_{max} and ΔE_{min}, *3.* the ratio between the amplitudes of the maximum and minimum ($\Delta E_{max}/\Delta E_{min}$). Fig. 3 (dots) shows that the recorded difference spectrum can be well reproduced by nomograms for a mixture of one rhodopsin with maximal absorption at 525 nm and another rhodopsin (here used as nomogram for a metarhodopsin) absorbing maximally at 480 nm, with a ratio of the molar absorbancies at λ_{max} $\alpha_{P\lambda_{max}} : \alpha_{M\lambda_{max}} = 1 : 1.75$. This ratio is the same as the ratio between the molar absorbancies of vertebrate 11-cis retinal and all-trans retinal (Rauen, 1964). The calculated absorbance spectra of the rhodopsin and corresponding metarhodopsin are seen in Fig. 4. The optimizing procedure has a high accuracy. The wavelengths that are maximally absorbed by the rhodopsin and the metarhodopsin can be determined on about ± 5 nm and the ratio of the molar absorbancies within about ± 0.1 to ± 0.2. The results therefore strongly suggest the presence in *Deilephila* of a rhodopsin absorbing maximally at 525 nm. Light converts this pigment to a thermostable photoproduct similar to a metarhodopsin absorbing maximally at about 480 nm.

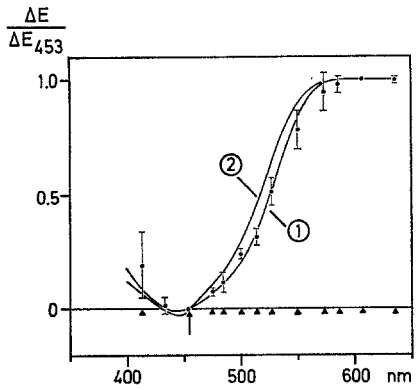

Fig. 5. Change in absorbance at 453 nm after exposure to wavelengths between 414 and 636 nm. Average of 3 recordings and standard deviation (dots with bars). Calculated change in absorbance (continuous lines) at 453 nm, assuming photoequilibrium between P 525 and M 480 (Fig. 3) with ratio of quantum efficiencies = 1.0 (curve 1) and 0.5 (curve 2)

3. Amplitude of Difference Spectra in Relation to Wavelength

The amplitudes of the maximum and minimum in the difference spectrum depend on the wavelengths of the "saturating" light exposures; but the ratio between the amplitudes of maximum to minimum, and the curve form, is constant. The relation between amplitude of the difference spectrum and wavelength of the "saturating" light exposure can be determined at any wavelength. Largest absorbance change, and thereby best accuracy of the measurements, is obtained by measuring at a wavelength close to the maximum or minimum of the difference spectrum. The wavelength 453 nm was therefore chosen for a determination (see Methods, *2.*) of the relation between amplitude of the difference spectrum and wavelength of the "saturating" light exposure.

The measurement resulted in the spectral absorbance change seen in Fig. 5. The amplitude was maximal after exposure to wavelengths longer than 570 nm It decreased considerably from 550 nm to a minimum at 450 nm.

The result shows that wavelengths longer than 570 nm completely convert the visual pigment to its photoproduct. In the region of spectral overlap between visual pigment and photoproduct (wavelengths shorter than about 560 nm, Fig. 4) the illumination converts part of the visual pigment to the photoproduct. The amount converted varies with the wavelength. It can thus be concluded that at these wavelengths a photoequilibrium is formed between visual pigment and photoproduct.

4. Test for Spectral Absorbance of Visual Pigment and Photoproduct. Pigment Concentration at Photoequilibrium, and Quantum Yield

The following procedure was used to test: *1.* The spectral absorbance of the visual pigment and the photoproduct, and *2.* the parameters that determine the concentration of the pigments at photoequilibrium.

Experimental Measurements. The spectral absorbance change was measured (Methods, *2.*) after "saturating" exposure to *1.* a long wavelength (608 nm) that converts all visual pigment to photoproduct, *2.* a wavelength (527 nm) close to the isosbestic point (about 520 nm), and *3.* a short wavelength (453 nm) that is maximally absorbed by the photoproduct (608, 527, and 453 nm correspond to λ_x in Methods, *2.*). The spectral absorbance change after exposure to 608 nm is seen in Fig. 6. There is a maximum at about 550 nm, and a minimum at about 460 nm. Two minima were recorded after exposure to 527 nm (Fig. 6). The ratio of the amplitudes of the minima after irradiation by 527 nm was about 1:2. Exposure to 453 nm caused a spectral absorbance change (Fig. 6) with a minimum at about 550 nm, and a small maximum at about 490 nm.

Calculation. The spectral absorbance changes after exposure to (λ_x) 608, 527, and 453 nm were calculated by the following method. The absorbance change ($\Delta E_{\lambda_x-\lambda_1,\lambda_1}$) at wavelength λ_1 after "saturating" exposure to λ_x is:

$$\begin{aligned}\Delta E_{\lambda_x-\lambda_1,\,\lambda_1} &= E_{\lambda_x,\,\lambda_1} - E_{\lambda_1,\,\lambda_1}\\ &= [\mathrm{P}]_{\lambda_x}\cdot\alpha_{\mathrm{P}\lambda_1} + [\mathrm{M}]_{\lambda_x}\cdot\alpha_{\mathrm{M}\lambda_1} - [\mathrm{P}]_{\lambda_1}\cdot\alpha_{\mathrm{P},\,\lambda_1} - [\mathrm{M}]_{\lambda_1}\cdot\alpha_{\mathrm{M},\,\lambda_1}\\ &= ([\mathrm{P}]_{\lambda_x} - [\mathrm{P}]_{\lambda_1})\,(\alpha_{\mathrm{P},\lambda_1} - \alpha_{\mathrm{M},\lambda_1})\end{aligned} \qquad (1)$$

in which $[\mathrm{P}]\lambda_x$ and $[\mathrm{M}]\lambda_x$ are the concentrations of visual pigment and photoproduct at photoequilibrium after exposure to λ_x, and $[\mathrm{P}]\lambda_1$ and $[\mathrm{M}]\lambda_1$ are the pigment concentrations after exposure to λ_1.

Assuming that the photoequilibrium (at wavelength λ) is determined by the absorbance coefficients ($\alpha_{\mathrm{P}}(\lambda)$; $\alpha_{\mathrm{M}}(\lambda)$) and the quantum efficiency ($\gamma_{\mathrm{P}}(\lambda)$; $\gamma_{\mathrm{M}}(\lambda)$) of the rhodopsin and metarhodopsin, the concentrations are determined by:

$$[\mathrm{P}]\,(\lambda) = \frac{\alpha_{\mathrm{M}}(\lambda)\cdot\gamma_{\mathrm{M}}(\lambda)}{\alpha_{\mathrm{M}}(\lambda)\cdot\gamma_{\mathrm{M}}(\lambda) + \alpha_{\mathrm{P}}(\lambda)\cdot\gamma_{\mathrm{P}}(\lambda)}\,,\quad [\mathrm{M}]\,(\lambda) = 1 - [\mathrm{P}]\,(\lambda). \qquad (2)$$

The spectral absorbance change after exposure to 608, 527, and 453 nm were calculated by Eqs. 1 and 2 (Fig. 6). Values for the absorbance coefficients of the rhodopsin and metarhodopsin were taken from the nomograms for P 525 and M 480 (Fig. 4). The concentrations of the pigments were calculated by Eq. 2. The ratio between the quantum efficiencies ($\gamma_{\mathrm{M}}/\gamma_{\mathrm{P}}$) was assumed to vary between 1.0 and 0.5. For comparison with

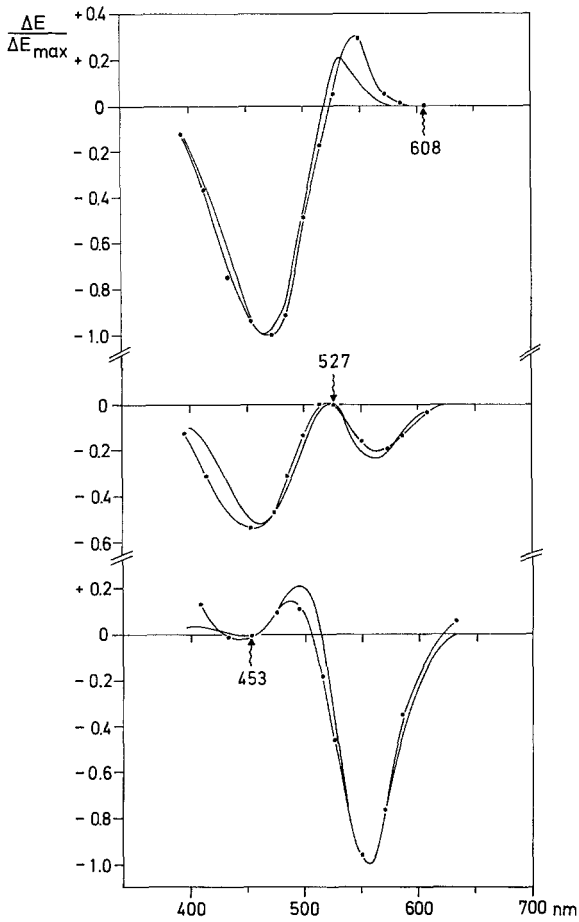

Fig. 6. Spectral absorbance change (Methods, 2) measured at wavelengths between 395 and 636 nm after exposure to 608 (upper curve with dots), 527 (middle curve with dots) and 453 nm (lower curve with dots). Upper and middle curves: one recording. Lower curve: average of 6 recordings. Calculated spectral absorbance change (continuous lines) at wavelengths between 395 and 636 nm after exposure to 608, 527, and 453 nm, assuming photoequilibrium between P 525 and M 480 (Fig. 4) with quantum efficiency = 1.0

the experimentally recorded spectral absorbance changes the theoretically calculated functions were normalized to 1.0 at maximal absorbance change.

The close fit between calculated and empirical functions demonstrates that: 1. The resonance spectra of the insect visual pigment and its

photoproduct are identical to those of vertebrate rhodopsins. 2. At each wavelength the concentration of the insect visual pigment and its photoproduct, i.e. the photoequilibrium, are determined by their molar absorbance coefficients.

The three functions shown in Fig. 6 vary only slightly with the size of the quantum efficiency. These functions are therefore not suitable to test the quantum efficiency for conversion and reconversion of the visual pigment. More accurate information about the quantum efficiency is given by the experiment illustrated in Fig. 5. As seen in this figure, the calculated functions vary with the quantum efficiency within a large wavelength range (460 to 570 nm). Within this range the experimentally recorded absorbance changes best agree with the function in which $\gamma_M = \gamma_P$. It can therefore be concluded that the quantum efficiency for conversion of the visual pigment is nearly equal to that for reconversion.

The experiment shown in Fig. 5 gives information also about the variation with wavelength in the concentration of the visual pigment in the receptors. In this experiment the spectral absorbance change measured at 453 nm after exposure to another wavelength is:

$$\Delta E_{\lambda x - \lambda 453, \lambda 453} = ([P]_{\lambda x} - [P]_{\lambda 453})\ (\alpha_{P, \lambda 453} - \alpha_{M, \lambda 453})$$

in which $(\alpha_{P, \lambda 453} - \alpha_{M, \lambda 453})$ is constant (c). The maximal spectral absorbance (ΔE_{max}) is:

$$\Delta E_{max} = \Delta E_{\lambda > 570 - \lambda 453, \lambda 453} = ([P]_{\lambda > 570} - [P]_{\lambda 453}) \cdot c.$$

The concentration of visual pigment after exposure to wavelengths exceeding 570 nm ($[P]\lambda > 570$) is 0. Therefore:

$$\frac{\Delta E}{\Delta E_{max}} = \frac{\Delta E_{\lambda x - \lambda 453, \lambda 453}}{\Delta E_{\lambda > 570 - \lambda 453, \lambda 453}} = \frac{[P](\lambda_x) - [P]_{\lambda 453}}{-[P]_{\lambda 453}} = 1 - \frac{[P](\lambda_x)}{[P]_{\lambda 453}}. \qquad (3)$$

Eq. 3 shows that the concentration of visual pigment in the receptors is directly proportional to the recorded values for $\Delta E/\Delta E_{max}$ (Fig. 5).

Ultraviolet Absorbing Pigment

The receptors were illuminated by a "saturating" exposure to monochromatic ultra-violet light (see Methods, *2.*). The absorbance change during a consecutive "saturating" exposure to 453 nm is seen in Fig. 7. A repeated exposure to ultra-violet light was followed by the same absorbance change during exposure to 453 nm. It can be concluded that an ultra-violet absorbing pigment is converted to another pigment, which absorbs blue light and is thereby reconverted to the ultra-violet absorbing pigment. *Deilephila* has a sensitivity maximum in the ultra-violet region (Höglund, Hamdorf, and Rosner, 1973). The ultra-violet absorbing

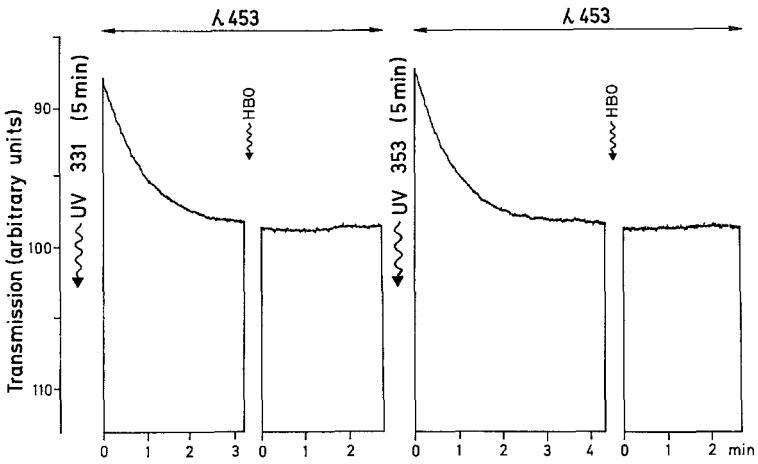

Fig. 7. Change in transmission caused by alternating exposure to ultra-violet (331 and 353 nm) and 453 nm (original recording), *cf.* Fig. 2. Measurements made at 453 nm

pigment ($\lambda_{max} = 350$ nm) is therefore the visual pigment, and the blue absorbing pigment is the photoproduct. The amplitude of the absorbance change at 453 nm varies with the wavelength of the preceeding "saturating" ultra-violet light exposure (Fig. 8a).

The result shows that both photoproduct and visual pigment absorb in the ultra-violet region and that in this region of spectral overlap, the amount of photoproduct formed varies with the wavelength, i.e. there is a photochemical equilibrium between visual pigment and photoproduct. The concentrations of visual pigment and photoproduct at photoequilibrium are determined by the molar absorbance coefficients of the two compounds, similarly to the green absorbing pigment.

The wavelengths that are maximally absorbed by the photoproduct were determined as described in Methods, *2.* "Saturating" exposure to 372 nm was followed by "saturating" exposure to a test wavelength (414 to 628 nm). Fig. 8b shows that largest absorbance changes were recorded at 450 to 460 nm. It can therefore be concluded that the photoproduct absorbs maximally approximately at these wavelengths. For comparison, the corresponding recordings from *Ascalaphus* (Schwemer, Gogala, and Hamdorf, 1971) are also shown in Fig. 8. The recordings from *Ascalaphus* and *Deilephila* seen in Fig. 8a are almost identical, and those seen in Fig. 8b are very similar to each other. The ultra-violet absorbing visual pigment in *Deilephila*, and its photoproduct, are thus similar to

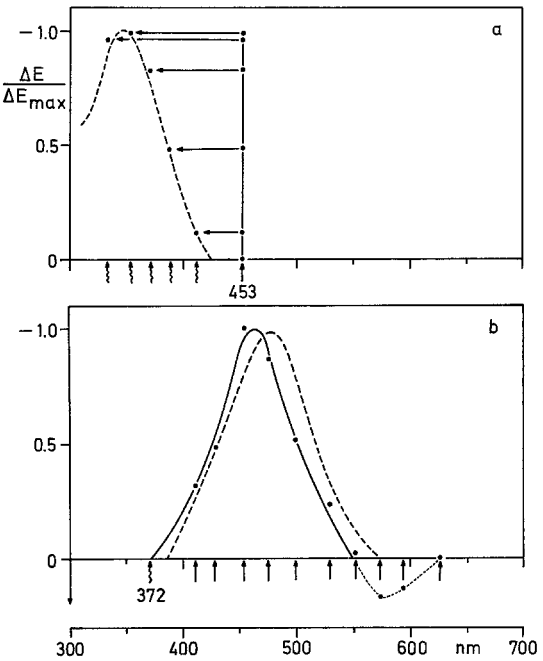

Fig. 8a and b. Spectral absorbance change caused by ultra-violet absorbing pigment and its photoproduct. a) Absorbance change (*Deilephila*) at 453 nm after exposure to 5 wavelengths in ultra-violet region (dots). b) Absorbance change (*Deilephila*) at wavelengths between 414 and 628 nm after exposure to ultra-violet light (372 nm). Dotted line indicates absorbance change caused by P 525 and M 480. Broken line in (a) and (b): corresponding absorbance change in *Ascalaphus*

those of *Ascalaphus*. The discrepancy between the recordings from *Ascalaphus* and *Deilephila* at wavelengths longer than about 500 nm can be explained by changes in the photoequilibrium between the green absorbing pigment and its photoproduct.

Discussion

Several methods have been developed for the study of visual pigments in situ (see e.g. Dartnall, 1972). Most investigations have been made on vertebrate receptors. The invertebrate visual pigments have attracted less attention.

The vertebrate visual pigments "bleach", i.e. retinal separates from the pigment molecule. For this reason the absorbance spectra of the visual pigments in the outer segments of rods and cones can be determined by

difference spectra between unilluminated and bleached receptors, and the spectral variation in the optical properties of the receptors can be measured after the visual pigments have been completely bleached. Measurements on the in situ thermostable and photoreversible invertebrate pigments are more difficult. The spectral variation in the optical properties of the receptors can not be separately determined. The absorbance spectra of the visual pigments and their photoproducts can only be deduced from difference spectra obtained by monochromatic illumination of varying wavelengths, as described by Hamdorf, Höglund, and Langer (1972) and in the present paper.

The results of microphotometric measurements can be influenced by the wave guide properties of the rhabdomeres (rhabdoms), and by the properties of the dioptric apparatus (Snyder and Miller, 1972; Snyder and Hamer, 1972; Snyder and Pask, 1972). Such influences are not to be expected in the present recordings. Snyder and Miller (1972, Figs. 3 and 4) showed that in small rhabdomeres, like in the 7. rhabdomere of the blow fly, the λ_{max} of spectral absorbance is shifted to shorter wavelengths, whereas in the six larger rhabdomeres the spectral absorbance would be nearly identical with the resonance spectrum of visual pigment. Assuming that the index of refraction of the much larger rhabdom of *Deilephila* is comparable to the values of the worker bee and of the fly (Varela and Wiitanen, 1970; Seitz, 1968, 1970) microphotometric measurements and resonance spectra would not differ. Furthermore, a change in recorded λ_{max} caused by the wave guide properties, would be accompanied by an increase in relative absorbance in the ultra-violet region. The recorded spectrum would thereby be considerably different from the resonance spectrum of a rhodopsin. Such difference was not observed. The spectrum of the green receptors calculated from the present measurements is nearly identical to the electrophysiologically recorded spectral sensitivity (Höglund, Hamdorf, and Rosner, 1973), and to the absorbance spectrum of the visual pigment in solution (Schwemer and Paulsen, 1973), and to the resonance spectra of vertebrate rhodopsin.

The microphotometric measurements on fresh eyes were identical to those obtained on deep-frozen eyes. This demonstrates that alterations in refractive indices due to retinomotor changes have not influenced the present results.

The electrophysiologically recorded sensitivity maximum at 525 nm (Höglund, Hamdorf, and Rosner, 1973) shows that the resonance spectrum with λ_{max} at 525 nm is that of the visual pigment (P 525), and the spectrum with λ_{max} at about 480 nm that of the photoproduct (M 480). The photoproduct is thermostable and is reconverted to the visual pigment by light. The concentrations of the visual pigment and the photoproduct at each wavelength are determined exclusively by the absorbance

coefficients of the pigments, and the quantum yield for conversion of the visual pigment to its photoproduct is nearly equal to that for reconversion.

The absorbance changes after exposure to wavelengths in the region about 400 to about 500 nm demonstrate another visual pigment that upon illumination converts to a thermostable photoproduct that is reconverted to the visual pigment by light. The absorbance maxima are in the ultra-violet (λ_{max} about 350 nm) and in the blue (λ_{max} about 450 to 460 nm) regions of the spectrum. From a comparison with the electrophysiologically recorded spectral sensitivity (Höglund, Hamdorf, and Rosner, 1973) can be concluded that the pigment with λ_{max} about 350 nm is the visual pigment, and that the photoproduct absorbs maximally at about 460 nm. A similar visual pigment and photoproduct occur in *Ascalaphus* (Gogala, Hamdorf and Schwemer, 1970).

Microspectrophotometric measurements have recently been made on the moth *Manduca sexta* (Carlson and Philipson, 1972). The results gave evidence for four visual pigments with λ_{max} at 350, 450, 490, and 530 nm. The recordings were interpreted to demonstrate bleaching products with λ_{max} at about 370 nm, indicating a mixture of free and bound retinal; rarely a λ_{max} was recorded at 325 to 330 nm suggesting the formation of retinol. The ultra-violet sensitive pigment was reported to form a photo-product with λ_{max} at 290 to 300 nm. The present results on *Deilephila* agree with those obtained on *Manduca* in showing visual pigments with λ_{max} at about 350 and 525 nm. A receptor maximally sensitive at about 440 nm was demonstrated by electrophysiological recordings (Höglund, Hamdorf, and Rosner, 1973), and the corresponding visual pigment has been extracted (Paulsen and Schwemer, 1973). This visual pigment was not shown photometrically in the present study, because in *Deilephila* it absorbs in the same wavelength region as the (in situ) thermostable photoproducts of the visual pigments.

The functional significance of the thermostable, photoregenerated visual pigments for the discrimination of wavelengths is discussed in a succeeding article (Höglund, Hamdorf, and Rosner, 1973).

References

Autrum, H., Zwehl, V. v.: Die spektrale Empfindlichkeit einzelner Sehzellen des Bienenauges. Z. vergl. Physiol. **48**, 357–384 (1964)

Carlson, S. D., Philipson, B.:Microspectrophotometry of the dioptric apparatus and compound rhabdom of the moth (*Manduca sexta*) eye. J. Insect Physiol. **18**, 1721–1731 (1972)

Chance, B., Perry, R., Åkerman, L., Thorell, B.: Highly sensitive recording micro-spectrophotometer. Rev. Sci. Instr. **30**, 735–741 (1959)

Dartnall, H. J. A.: The interpretation of spectral sensitivity curves. Brit. med. Bull. **9**, 24–30 (1953)

Dartnall, H. J. A., ed.: Photochemistry of vision. In: Handbook of sensory physiology, VII/1, Berlin-Heidelberg-New York: Springer 1972
Daumer, K.: Reizmetrische Untersuchungen des Farbensehens der Bienen. Z. vergl. Physiol. **38**, 413–478 (1956)
Ephrussi, B., Beadle, G. W.: A technique of transplantation for *Drosophila*. Amer. Nat. **70**, 218–225 (1936)
Gogala, M.: Die spektrale Empfindlichkeit der Doppelaugen von *Ascalaphus macaronius* Scop. Z. vergl. Physiol. **57**, 232–243 (1967)
Gogala, M., Hamdorf, K., Schwemer, J.: UV-Sehfarbstoff bei Insekten. Z. vergl. Physiol. **70**, 410–413 (1970)
Hamdorf, K., Höglund, G., Langer, H.: Mikrophotometrische Untersuchungen an der Retinula des Nachtschmetterlings *Deilephila elpenor*. Verh. dtsch. zool. Ges. **65**, 276–280 (1972)
Hamdorf, K., Schwemer, J., Gogala, M.: Insect visual pigment sensitive to ultraviolet light. Nature (Lond.) **231**, 458–459 (1971)
Hamdorf, K., Schwemer, J., Täuber, U.: Der Sehfarbstoff, die Absorption der Rezeptoren und die spektrale Empfindlichkeit der Retina von *Eledone moschata*. Z. vergl. Physiol. **60**, 375–415 (1968)
Höglund, G., Hamdorf, K., Rosner, G.: Trichromatic visual system in an insect and its sensitivity control by blue light. J. comp. Physiol. **86**, 265–279 (1973)
Höglund, G., Struwe, G.: Pigment migration and spectral sensitivity in the compound eye of moths. Z. vergl. Physiol. **67**, 229–237 (1970)
Kropf, A., Brown, P. K., Hubbard, R.: Lumi- and meta-rhodopsin of squid and *Octopus*. Nature (Lond.) **183**, 446–448 (1959)
Rauen, H. M., ed.: Biochemisches Taschenbuch, S. 861. Berlin-Heidelberg-New York: Springer 1964
Schwemer, J., Gogala, M., Hamdorf, K.: Der UV-Sehfarbstoff der Insekten: Photochemie in vitro and in vivo. Z. vergl. Physiol. **75**, 174–188 (1971)
Schwemer, J., Paulsen, R.: Three visual pigments in *Deilephila elpenor*. J. comp. Physiol. **86**, 215–229 (1973)
Seitz, G.: Der Strahlengang im Appositionsauge von *Calliphora erythrocephala* (Meig.). Z. vergl. Physiol. **59**, 205–231 (1968)
Seitz, G.: Nachweis einer Pupillenreaktion im Auge der Schmeißfliege. Z. vergl. Physiol. **69**, 169–185 (1970)
Snyder, A. W., Hamer, M.: The light-capture area of a photoreceptor. Vision Res. **12**, 1749–1752 (1972)
Snyder, A. W., Miller, W. H.: Fly colour vision. Vision Res. **12**, 1389–1396 (1972)
Snyder, A. W., Pask, C.: How bees navigate. Nature (Lond.) **239**, 48–50 (1972)
Varela, F. G., Wiitanen, W.: The optics of the compound eye of the honeybee (*Apis mellifera*). J. gen. Physiol. **55**, 336–358 (1970)

K. Hamdorf
G. Höglund
H. Langer
Institut für Tierphysiologie
der Ruhr-Universität Bochum
D-4630 Bochum-Querenburg
Federal Republic of Germany