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Dark-Processes Following Photoconversion of Butterfly Rhodopsins*

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Abstract. Photoconversion of rhodopsin to metarhodopsin by a short actinic flash creates photochemical changes in the absorbance spectrum of the butterfly rhabdom, which are measurable as changes in the reflectance spectrum of the intact eye. The difference spectrum relaxes in the dark, but changes considerably in shape when doing so. The positive peak caused by the accumulation of metarhodopsin relaxes to zero much faster than the negative peak caused by the loss of rhodopsin. The positive peak actually undershoots zero absorbance-difference before its final asymptotic approach to zero, whereas the negative peak approaches zero monotonically.

The entire temporal evolution of difference spectra can be quantitatively reproduced by only assuming different kinetics for the dark-processes of metarhodopsin's decay and of rhodopsin's recovery. A consequence of this analysis is that no long-lived, coloured intermediates can be detected in the rhabdom other than metarhodopsin.

Metarhodopsin's decay is well approximated by a first-order process, but has a time-constant that depends strongly on temperature. Examples are 71 min at 12.5° C, 18 min at 23° C, and 4 min at 26.5° C.

Rhodopsin's recovery is kinetically complex. The rate of recovery shortly after a small photoconversion is somewhat slower than the rate for metarhodopsin's decay. At later times, or for a large photoconversion, rhodopsin's recovery is very much slower than metarhodopsin's decay.

Key words: Insect photoreceptors – Visual pigments – Unstable metarhodopsin – Dark regeneration – Bleaching

Introduction

One of the cornerstones of the photochemistry of insect rhodopsins is that their metarhodopsins are stable. A considerable amount of work on the microspectrophotometry of insect visual pigments indicates that the rhodopsins of insects

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do not bleach but are changed by light to a stable metarhodopsin. Many of the techniques for photochemical measurement and analysis assume that the concentrations of rhodopsin and metarhodopsin in a rhabdom do not change if the eye is left in the dark. This assumption is adequately supported by a large body of work on some invertebrate species (Goldsmith 1972; Hamdorf 1979; Langer et al. 1982), but not for all species.

The concentrations of rhodopsin and metarhodopsin actually do change in the dark in some situations. Dark-regeneration with half-times of less than 30 min has been reported in four diverse groups of invertebrates [octopus – Schwemer (1969); fly – Stavenga et al. (1973); butterfly – Stavenga (1975b); lobster – Bruno et al. (1977)]. The thoroughly dark-adapted rhabdom contains no metarhodopsin in the lobster (Bruno et al. 1977) and crayfish (Cronin and Goldsmith 1982). The dark-processes can depend strongly on temperature. Bruno et al. (1977) showed that dark-regeneration of lobster rhodopsin has a half-time of 55 min at 15° C, but only 25 min at 22° C. They also showed that the dark-adapted lobster rhabdom contains no metarhodopsin, and that the chronically illuminated rhabdom contains much less metarhodopsin at 25° C than it does at 1° C.

Stavenga (1975a, b) studied the dark processes in a butterfly as its rhodopsin R535 was dark-regenerated from metarhodopsin M480, by following the relaxation process with dim monochromatic light of various wavelengths. The relaxation was monotonic at 567 nm, but was biphasic at 506 nm. He suggested two possibilities, *a*) M480 passes through a violet-absorbing intermediate as it is regenerated to R535, or *b*) more than one kind of rhodopsin was photoconverted by the yellow actinic light.

Bernard (1977) confirmed Stavenga's experimental results, but suggested that the metarhodopsin was unstable, and was decaying at a faster rate than the rate for dark-regeneration of rhodopsin, making it possible to bleach the rhabdoms. I recently presented evidence to support this suggestion (Bernard 1983). The purpose of the present paper is to quantitatively characterize the kinetics of both the decay of metarhodopsin and the regeneration of rhodopsin in the dark, to show how butterfly rhabdomeres can indeed be bleached in the living, intact animal, and to show that the kinetics of the dark-processes depend strongly on temperature.

Methods

An optical specialization of the butterfly eye allows in vivo measurements of photochemical difference spectra. In each ommatidium a tapetal reflector located at the proximal end of rhabdom creates coloured eyeshine (Miller and Bernard 1968; Ribi 1978; Miller 1979). By immobilizing an intact butterfly on the stage of an incident light microspectrophotometer (MSP) and focussing on the deep pseudopupil, it is possible to measure the light that has survived a double-pass through the rhabdom. Discrimination against stray light is superb (Franceschini and Kirschfeld 1971; Stavenga 1975a, b; Bernard 1977, 1979). Thus, the optical density (absorbance) of the rhabdom is proportional to one-half times the common logarithm of the measured reflectance.

Vanessa cardui butterflies were obtained as larvae from the Carolina Biological Supply, Burlington, NC. Twenty ommatidia in the medio-ventral region (azimuth = 20°, elevation = −15°) of the eye were illuminated with a 0.2 mm beam of N.A. = 0.07. The reflectance spectrum was measured with dim monochromatic flashes ($< 10^{10}$ photons/ommatidium/flash) that neither triggered a pupillary response (Franceschini and Kirschfeld 1971; Stavenga et al. 1977) nor altered the photochemical state. Unless stated otherwise, photochemical conversions were created by a 0.23 mm beam of N.A. = 0.07 that originated from a 45 W tungsten lamp covered by a 3 mm Schott KG3 heat filter plus a colour filter. The colour filter for "orange" flashes was a 3 mm Schott OG590, which created a beam of intensity 0.011 ft-candles at the eye. The colour filters for "wideband blue" flashes were a Hoya L42 cut-off filter and a Ditric 450 nm wideband (40 nm hbw) interference filter, and for "narrow-band blue" flashes were a Hoya Y44 cut-off filter and an Optics Technology 466 nm (12 nm hbw) interference filter. For a detailed description of the apparatus see Bernard and Stavenga (1979).

Results

Different Time-Courses for Metarhodopsin's Decay and Rhodopsin's Recovery

The following experiment with a two-month old *Vanessa cardui* was designed to determine how the difference spectrum changes with time in the dark, and to learn how the results depend on the photochemical state at the time of the actinic flash. The butterfly was dark-adapted in the MSP at 23° C for 12 h after which the reflectance spectrum (Fig. 1, curve *) was measured. This is the reference spectrum, $R_0(\lambda)$. An actinic orange flash was delivered that later analysis shows to have converted 38% of the rhodopsin R530 molecules into the metarhodopsin M490 state. The actinic flash activated the pupillary mechanism and, therefore,

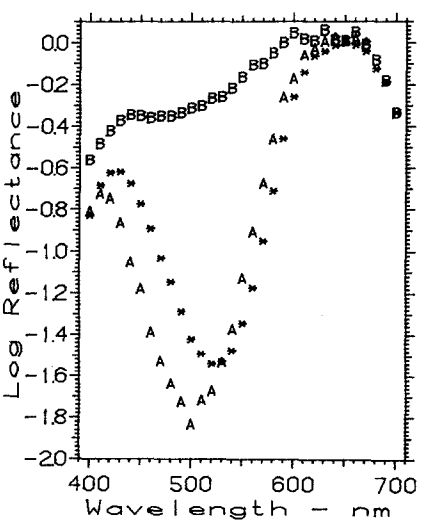

Fig. 1. Reflectance spectra from the deep pseudopupil of *Vanessa* in three photochemical states. Reference spectrum * was measured after 12 h of dark-adaptation at 23° C. Spectrum A was measured 3 min after a 3-s flash of bright orange light. Spectrum B was measured after bleaching the rhabdomeres of the green-receptors

3 min was allowed for complete pupillary recovery. Subsequently a series of reflectance spectra $R_t(\lambda)$ was measured during a dark-period of 2 h.

Absorbance-difference spectra were computed as

$$\tfrac{1}{2} \log_{10}[R_0(\lambda)/R_t(\lambda)] ,$$

which is the integrated, longitudinal absorbance difference for light that has propagated from the distal tip of the rhabdom to its proximal end (Stavenga et al. 1977). The first of such a difference spectrum is curve A of Fig. 2a which exhibits the familiar shape for photoconversion of a green-absorbing rhodopsin to a blue-absorbing metarhodopsin. Examples of the experimental difference spectra measured at later times are shown by curves B through E of Fig. 2a; for sake of clarity, only half of the measured spectra are plotted. The first feature of this family of spectra to catch the eye is the very rapid decay of the positive peak as compared to a relatively slow decay of the negative peak. Closer examination reveals that the relaxation of the positive peak is not monotonic. It actually undershoots zero for wavelengths less than the isosbestic point for the photoconversion.

After measuring curve E the 3-s orange flash was repeated. Half of the difference spectra measured throughout the subsequent hour in the dark are shown in Fig. 2b. The reference spectrum $R_0(\lambda)$ for this series and all others of Fig. 2 is the dark-spectrum * of Fig. 1. The initial spectrum A is about the same as before, but has a somewhat smaller positive peak. The temporal evolution is also about the same, but the negative peak after an hour in the dark is larger than before.

Next, a larger actinic dose of five orange flashes was delivered during a 4-min interval, yielding the difference spectra of Fig. 2c. Curve A is rather large, exhibiting a swing of 0.7 density units. Here, too, the positive peak relaxes rapidly while the negative peak relaxes slowly.

The larger actinic dose of five flashes was repeated, but caused relatively minor changes in absorbance, as shown in Fig. 2d. Because it seemed that the rhabdom was being depleted of rhodopsin R530, I delivered a series of 1-s orange flashes over a 21-min interval. The subsequent spectra shown in Fig. 2e indicate that R530 was essentially gone from the rhabdoms.

After measuring spectrum C of Fig. 2e, a series of five wideband blue flashes was delivered in order to see whether metarhodopsin, if present, could be converted back to rhodopsin. The bright blue light caused absorbance changes of less than 0.1 units, indicating that there was very little metarhodopsin present in the rhabdom. To confirm that the rhabdomeres of the green-receptors were essentially bleached, a 1-s UV-deficient, white flash (Hoya L42) was delivered. The subsequent absorbance spectrum was within 0.1 units of the spectrum before the white flash, which indicated that there could not have been much R530 or M490 left in the rhabdom.

The eye recovered from this total bleach. After 14 h about 60% of R530 had returned. The absorbance spectrum returned eventually to within 0.03 units of the initial dark spectrum.

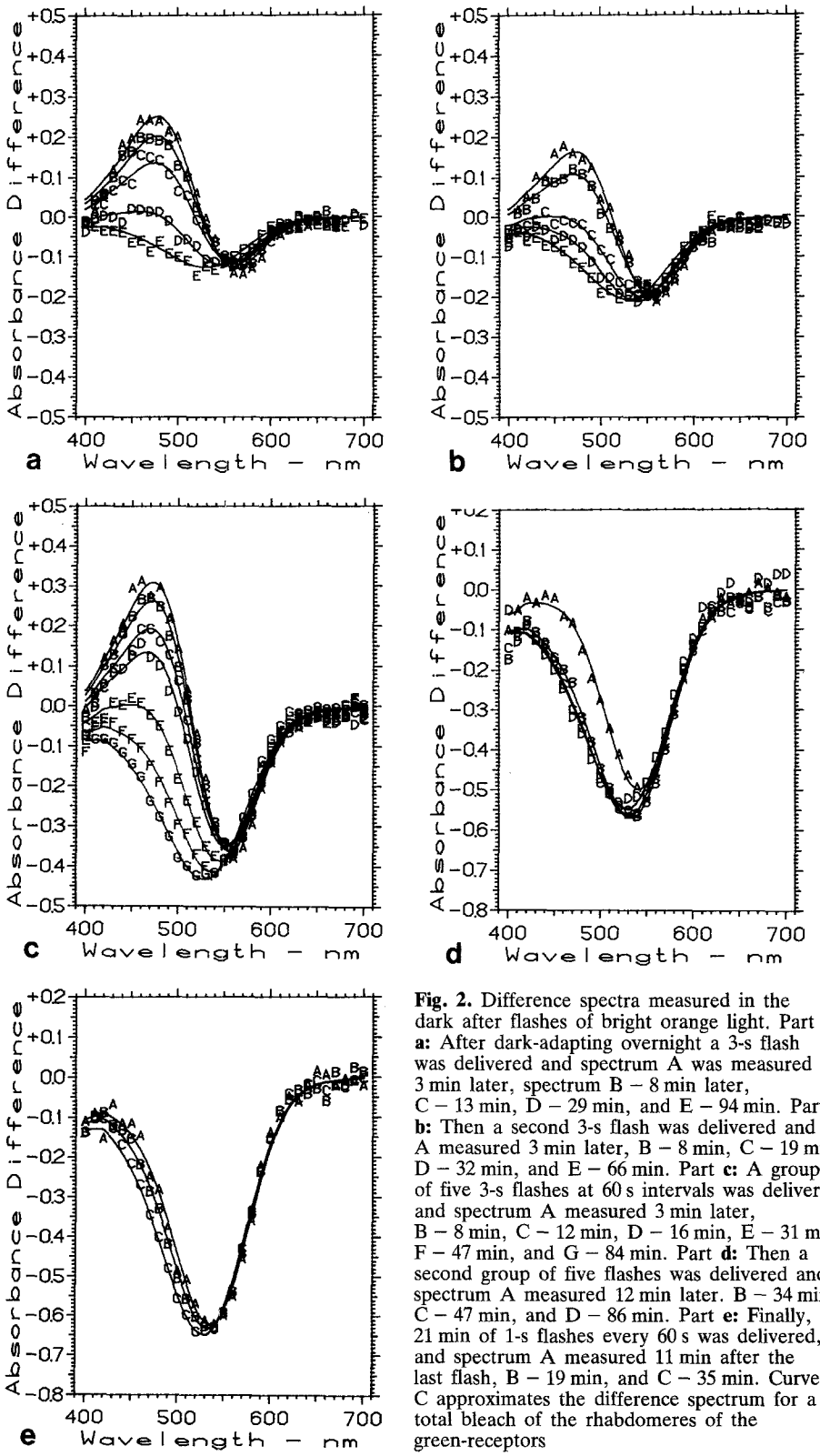

Fig. 2. Difference spectra measured in the dark after flashes of bright orange light. Part **a:** After dark-adapting overnight a 3-s flash was delivered and spectrum A was measured 3 min later, spectrum B – 8 min later, C – 13 min, D – 29 min, and E – 94 min. Part **b:** Then a second 3-s flash was delivered and A measured 3 min later, B – 8 min, C – 19 min, D – 32 min, and E – 66 min. Part **c:** A group of five 3-s flashes at 60 s intervals was delivered and spectrum A measured 3 min later, B – 8 min, C – 12 min, D – 16 min, E – 31 min, F – 47 min, and G – 84 min. Part **d:** Then a second group of five flashes was delivered and spectrum A measured 12 min later. B – 34 min, C – 47 min, and D – 86 min. Part **e:** Finally, 21 min of 1-s flashes every 60 s was delivered, and spectrum A measured 11 min after the last flash, B – 19 min, and C – 35 min. Curve C approximates the difference spectrum for a total bleach of the rhabdomeres of the green-receptors

The reader may have noticed that the final curve in each of Fig. 2a–e resembles very much the absorption spectrum of a green-absorbing rhodopsin. In fact, the solid lines passing through the experimental spectra E of Fig. 2a and b, spectrum G of Fig. 2c, D of Fig. 2d, and C of Fig. 2e are all least-squares fits (SE < 0.02) to the Dartnall nomogram (Dartnall 1953; Ebrey and Honig 1977) for R530. These curves form the partial bleach series which is shown in Bernard (1983).

Knowing the spectrum of the green-absorbing rhodopsin, the next step was to determine the spectrum of the blue-absorbing metarhodopsin from experimental difference spectra via an iterative procedure. The procedure is to take a large difference spectrum such as B of Fig. 2c and find the R530 absorbance spectrum that best fits the long-wavelength tail of the difference spectrum. Stripping that estimate from the difference spectrum yields an approximation to the absorbance spectrum of metarhodopsin. The iteration can be continued by taking the putative metarhodopsin spectrum and stripping it from the original difference spectrum, then finding the density and lambda-max for this new, putative rhodopsin spectrum. No matter which difference spectrum was chosen for analysis, the iterations converged to lambda-maxes of 490 nm and 530 nm. The fit of the metarhodopsin spectrum to the Dartnall nomogram is excellent.

These results led to the hypothesis that any spectrum of the entire series could be fit by

$$\{D_M(t)\alpha_M(\lambda) - [D_R(t_0) - D_R(t)]\alpha_R(\lambda)\},$$

where λ is the wavelength, $\alpha_M(\lambda)$ and $\alpha_R(\lambda)$ are normalized absorbance spectra taken from the Dartnall nomogram, where $D_M(t)$ is the optical density of metarhodopsin (at its λ_{max} of 490 nm) present at time t, $D_R(t_0)$ is the density of rhodopsin (at its λ_{max} of 530 nm) at time t_0 when the reference spectrum was measured, and $D_R(t)$ is the density of rhodopsin R530 present at time t.

A least-sqares procedure with only two free parameters,

$$D_M(t) \text{ and } \Delta D_R(t) = [D_R(t_0) - D_R(t)]$$

was used to fit each difference spectrum. In all cases the SE was less than 0.02. The results of this analysis are plotted semilogarithmically in Fig. 3a.

Changes in Kinetics Caused by Cooling

Another *Vanessa* was dark-adapted overnight at 24° C. After cooling to 12.5° C, a 100-ms flash of orange light was delivered from a 150 W xenon arc. After measuring a spectum, a 100-ms flash of narrow-band blue light was delivered. Then the eye was illuminated with 5 s more of the blue light. After measuring two spectra, a 100-ms orange flash was delivered. Eleven spectra were measured over a dark-period of 3 h. All of these spectra were analyzed as described above. The results are plotted in Fig. 3b. The kinetics of the dark-processes are very much slower at 12.5° C than they are at 23° C.

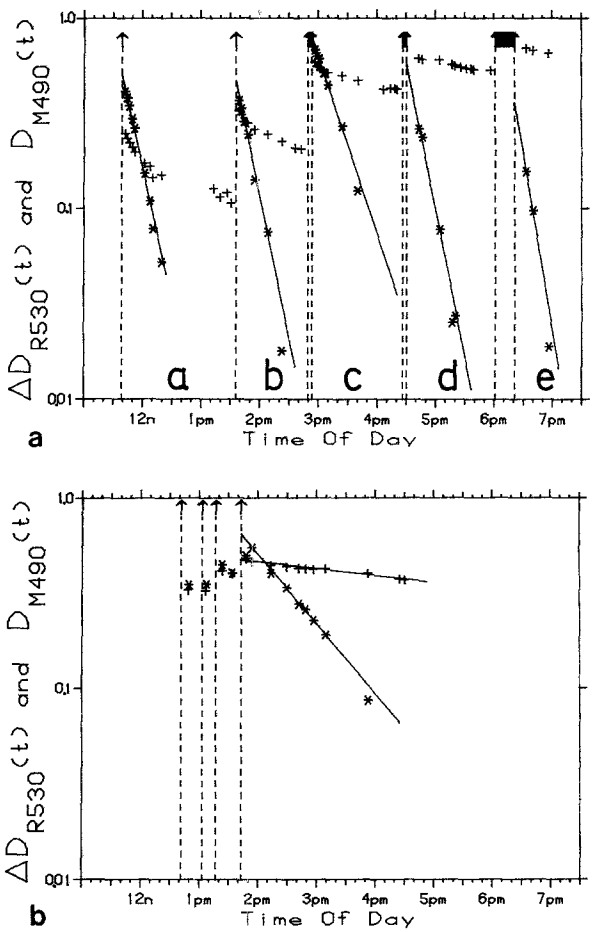

Fig. 3. a *Vanessa* at 23° C. Kinetics for decay of metarhodopsin M490, and for recovery of rhodopsin R530 for the experiment of Fig. 2. $D_M(t)$ is plotted with symbol *, and $\Delta D_R(t)$ is plotted with symbol +. The arrows and dashed lines show times of actinic illumination. The solid lines are least-square fits to simple exponential decay. Time constants for each line are 18.6 ± 0.7 min, 17.6 ± 1.0 min, 27.9 ± 1.0 min, 16.8 ± 1.8 min, and 14.0 ± 3.4 min, respectively; **b** *Vanessa* at 12.5° C. Kinetics for decay of M490 and recovery of R530. Symbols as in Fig. 3a but, in this case, least-square fits were made to both symbols. Time-constants are 71 min for M490 and 713 min for R530

Metarhodopsin's Decay and Rhodopsin's Recovery for the Viceroy at 26.5° C

The stock of *Vanessa* were commercially supplied as larvae in containers of undefined media. To address the question of whether the kinetics were different for freshly netted butteflies, I caught many species and did similar experiments. The results for *Vanessa* are qualitatively representative.

I have chosen the Viceroy (*Limenitis archippus*) as an example because it has a green-absorbing rhodopsin that is somewhat different from that of *Vanessa.*

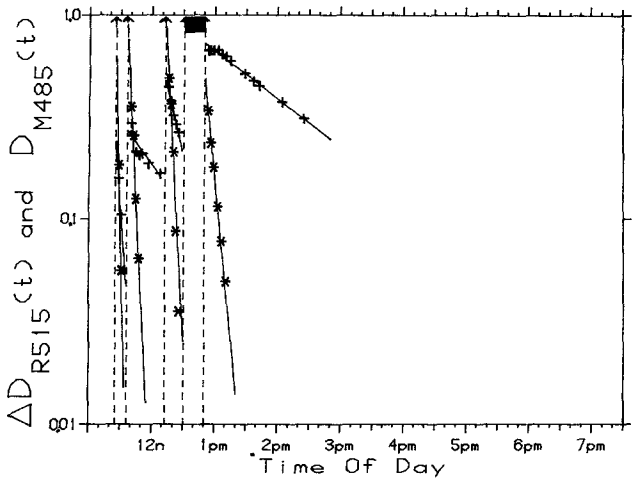

Fig. 4. The Viceroy at 26.5° C. Kinetics for decay of M485 and recovery of R515. Time constants for decay of M485 are 2.2 ± 0.4 min for the first segment, 4.4 ± 0.3 min for the second, 4.8 ± 0.3 min for the third, and 8.5 ± 0.6 min for the last segment. Recovery rates for R515 are 6.3 ± 1.9 min for the first segment, 60.4 ± 4.8 min for the second, 20.3 ± 2.4 min for the third, and 110.8 ± 2.4 min for the fourth segment

The rhodopsin of longest lambda-max in this eye is R515, which photoconverts to M485. After dark-adapting the Viceroy in the MSP overnight, a series of 3-s flashes was delivered, first using cut-off filter OG590, then OG570, then Hoya O56. Several spectra were measured after each flash. Then, an attempt was made at total conversion by delivering 3-s flashes through filter O56 every 30 s, for 20 min. The subsequent dark-processes were followed for 100 min.

The results of the analytical procedure are shown in Fig. 4. For the small, initial flashes the kinetics are very fast. For the larger actinic doses the decay rate for M485 slows moderately while the rate for recovery of R515 slows considerably.

Discussion

The fact that each of the difference spectra in Fig. 2 can be fit well by appropriate densities of R530 and M490 indicates that M490 did not pass through a long-lived coloured intermediate as it decayed from the rhabdom, nor did the orange light photoconvert another spectral type of rhodopsin. My measurements do not extend far enough into the UV to say anything about the presence of absence of hydrolyzed chromophore.

As shown in Fig. 3, the density of M490 decayed exponentially, with time-constant of about 18 min at 23° C. Thus the metarhodopsin content of the rhabdom is certainly not stable; more than 50% of M490 was lost within 13 min after the first flash.

The kinetics for recovery of R530 were not the same as for the disappearance of M490. Recovery of R530 was slower and was not first-order. The half-time for

recovery of R530 was 70 min for the first flash, 112 min for the second flash, and > 240 min for subsequent flashes. Recovery of rhodopsin was therefore considerably slower than decay of metarhodopsin. This result proves that dark-regeneration cannot consist of in situ reisomerization of the metarhodopsin chromophore.

I should state here that R530 of the butterfly can be photoregenerated from M490 by blue light just like that of any other invertebrate. After M490 has disappeared spectrally from the rhabdom, however, blue light only photoconverts R530 to M490, not the other way around.

Vanessa cardui and the Viceroy are not the only species that possess bleachable rhabdomeres. All of the thirty butterfly species I have studied exhibit this phenomenon.

Furthermore, green-absorbing rhodopsin is not the only spectral class of rhodopsin that can be bleached from rhabdoms. The rhabdomeres of UV-, blue-, and red-receptors can also be bleached. For example, if one takes a *Vanessa* that has been bleached of R530 and M490 (as in curve C of Fig. 2e) and delivers a bright UV flash, one measures an accumulation of about 0.3 units of the metarhodopsin (peak at about 475 nm) photoconverted from the UV-absorbing rhodopsin. Bright blue light causes photoregeneration, but repeated UV flashes over extended times can deplete M475, and reduce substantially the absorbance changes caused by blue flashes.

Conclusion

Photoregeneration is regarded as a much more important mechanism for invertebrates than dark-regeneration (Langer et al. 1982). Based on the results of this paper, the question of the physiological importance of dark-regeneration of invertebrate rhodopsins should be reconsidered. At the warmest temperature employed, 26.5° C, decay rates for M485 of the Viceroy were between 2 min and 9 min, depending on the magnitude of the photoconversion. Time constants for recovery of rhodopsin were 6 min for small conversions, and up to 111 min for large ones. It is interesting to compare 26.5° C to operating temperatures of butterflies in flight. According to Watt (1968), and Kingsolver and Moffat (1982), thorax temperatures fall in the range 32° C–37° C. Many species are not even able to fly if the thorax cools to 29° C. Because the thermal conductivity between head and thorax is high, the head temperature is likely to be at least 32° C. Although dark-processes in that temperature range have yet to be measured, it seems clear that metarhodopsin shall disappear very rapidly from the rhabdom, and regeneration rates shall be fast enough to firmly establish the dark-regeneration of insect rhodopsins as a physiologically important process.

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