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PIGMENT TRANSFORMATION AND ELECTRICAL RESPONSES IN RETINULA CELLS OF DRONE, *APIS MELLIFERA* ♂

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SUMMARY

1. Receptor potentials in honeybee drone retinula cells were recorded with intracellular micro-electrodes in the dorsal part of the superfused retina. The light stimuli were sufficiently weak that the response amplitude was proportional to the intensity.

2. Responses to stimuli of different wave-lengths, although of different amplitude, all had the same time course.

3. The maximal sensitivity in all the cells recorded from was to a wave-length between 450 and 460 nm.

4. Microspectrophotometry showed the presence of a pigment with two stable states, interconvertible by light, absorbing maximally at 445 nm (rhodopsin) and 505 nm (metarhodopsin).

5. There was a good match between the absorption spectrum of rhodopsin and the spectral sensitivity of retinula cells.

6. Transformation of a large fraction of rhodopsin to metarhodopsin by light reduced the sensitivity of the retinula cell but did not alter the shape of the relative spectral sensitivity curve or the time course of the responses.

7. It is concluded that for weak lights the receptor potential is determined only by the number of rhodopsin molecules that absorb photons: neither the presence of metarhodopsin nor its phototransformation to rhodopsin produces a detectable effect.

INTRODUCTION

Results of microspectrophotometry on the single rhabdome of the honeybee drone reveal the presence of a visual pigment with two stable states absorbing maximally at 445 and 505 nm (Muri, 1979). Absorption of light at an appropriate wave-length causes the transformation of the pigment molecules from one state to the other and this process is completely reversible.

Photopigments with similar properties have already been described in insects (Hamdorf, Schwemer & Täuber, 1968) and in *Balanus* (Hochstein, Minke & Hillman, 1973). These last authors, in order to explain the electrical responses evoked by stimuli at different wavelengths, proposed a model in which transformation from the 532 nm state into the 495 nm state of the pigment produces a substance which increases the membrane conductance, while transformation in the opposite direction produces a different substance which is an inhibitor neutralizing the excitor and

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being itself neutralized by it. This model of Hochstein *et al.* (1973) was designed to account for responses to light so intense that a considerable fraction of the photopigment was converted from one state to the other. In the present work we have examined whether a similar model appears to be necessary to account for the responses to much weaker lights in drone.

METHODS

Preparation and recording. Honeybee drones were kept together with worker bees, necessary to their survival, in heated transparent boxes and fed with water almost saturated with sugar. Under favourable conditions they survive 2 or 3 weeks. The head of the animal was cut off and a slice of chitinic tissue was cut from the posterior part of the head, with a cut parallel to the longitudinal axis of the ommatidia, to expose retinae of both eyes, as already described by Baumann (1968). Pl. 1 shows the preparation as it appears under the dissecting microscope. The head was pinned on the central pivot of the Plexiglass chamber with the exposed ommatidia facing upwards and superfused with oxygenated physiological solution. This solution, the same as that used by Bader, Baumann & Bertrand (1976), had the following composition (m-mole/l.): Na^+ 280, Cl^- 287, K^+ 3·2, Ca^{2+} 1·8, Tris 10, adjusted with HCl to a pH of 7·4.

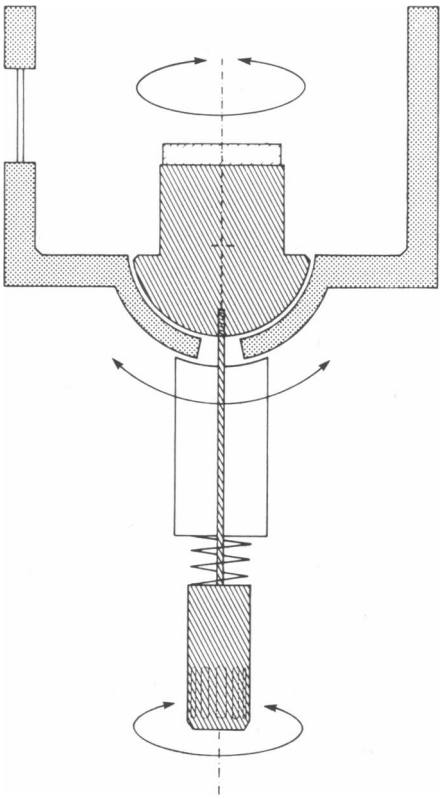
Perfusion chamber. In these experiments, in order to reproduce as faithfully as possible the physiological conditions, the stimulating light was applied through the cornea, rather than from above, as in previous work (Baumann, 1968). The technique of moving the stimulus with respect to the head by means of an optic fibre was discarded, due to the spectral properties of optic fibres, and a new technique was developed, based upon the principle of moving the preparation with respect to the stimulus. The device which allowed the movement is shown in Text-fig. 1. A central pivot, which can be tilted with respect to the horizontal plane and rotated around its axis, is mounted in a Plexiglass chamber with a quartz window on its side: the preparation, pinned on the top of the pivot, can be then oriented with the cornea perpendicular to the light which enters through the quartz window. When a spot of light is focused on the corneal surface, the ommatidia underlying the illuminated facette become bright up to the basal membrane. This visual criterion was used before penetrating a cell. The electrode was then advanced with a micromanipulator toward the illuminated ommatidia, perpendicularly to them, under visual control with a stereo microscope. When the tip of the electrode touches the tissue, a drop of potential of a few millivolts can be recorded. The continuous light was then substituted by flashes at a rate of one per second and penetration was usually achieved by applying to the electrode an oscillating voltage of $\pm$ 15 V at 2 kHz with a reed relay. Glass micropipettes were pulled with a Livingston puller from glass capillaries of 0·5 mm external diameter and when filled with 4 M-potassium acetate had a resistance ranging between 80 and 120 MΩ.

Illumination. The light source was a xenon lamp XBO 15C of 150 W, whose unattenuated flux on the preparation at 460 nm was $1{\cdot}7 \times 10^{14}$ photons sec^{-1} cm^{-2}. This intensity, which will be called intensity zero, was attenuated by means of neutral density filters (Balzers) in the visible range, or by narrowing the slit of the PMQ 2 Zeiss monochromator in the U.V. range. An electromagnetic shutter driven by a Tektronix pulse generator allowed pulses of light as short as 10 msec to be delivered. The light flux and filter transmission were measured with the same EMI photomultiplier used for spectrophotometry. The monochromator was calibrated by comparison with a pen-ray mercury lamp.

Data acquisition. All the experiments described here were performed with an on-line connexion with a Hewlett-Packard 21MX computer. The analogue signals were sampled and converted to digital at a rate of 1 kHz and then sent to the HP through a microcircuit interface. Stored on a magnetic disk, the data could be averaged on line and displayed on a Tektronix 4006 terminal; in this way it was easy to compare responses and to have controls throughout the whole experiment. An interactive program was specially developed for these experiments, allowing any response or average of responses to be displayed at any time. A second program was then employed for the display and tracing of the stored data on a digital plotter Tektronix 4662.

Controls. Only cells which attained saturated receptor potentials of over 55 mV with white

light, and had a resting membrane potential greater than 60 mV were accepted. At the beginning and at the end of each run of spectral sensitivity in the linear range, the shape and the amplitude of both linear and non linear responses were tested: units whose responses showed a significant difference in the two runs were discarded.



Text-fig. 1. Perfusion chamber. A cross-section drawing of the chamber employed in these experiments. The central pivot can rotate around its axis and tilt with respect to the vertical plane as indicated by the arrows. The preparation, mounted on the top of it, can be oriented with respect to the light, entering through a quartz window on the side of the chamber.

A chart recorder was used during all the experiments in order to check the stability of the resting membrane potential of the cell. Only units whose potential did not change more than 10% of its initial value during the whole experiment were accepted.

The light intensity was adjusted for each unit in order to obtain linear responses, and the linear range was tested for each unit at different wave-lengths.

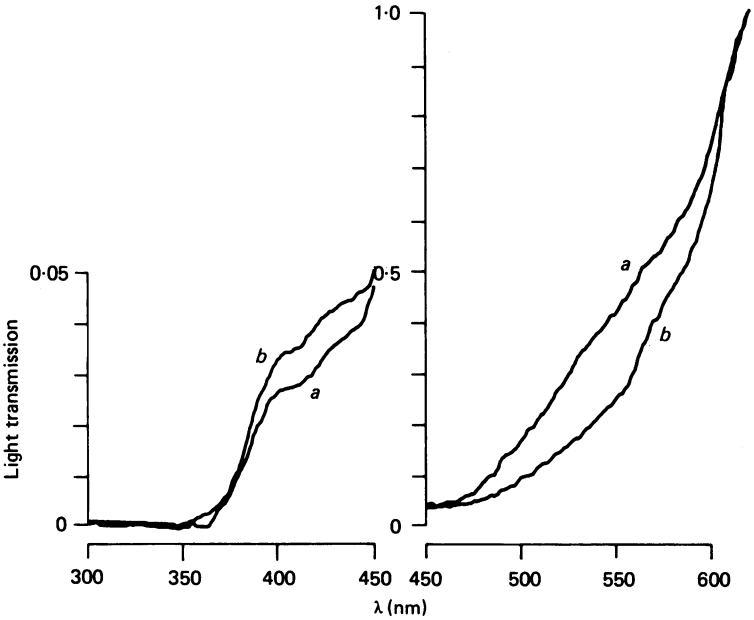
Before each run of spectral sensitivity the cell was allowed to dark-adapt to its maximal sensitivity: 2–7 min was sufficient.

The flashes necessary to evoke linear responses were so weak that they could be delivered one every second without affecting the condition of dark adaptation of the cell: the spectral sensitivity scans could be repeated in the two directions without any noticeable alteration either in shape or in amplitude of the responses.

Microspectrophotometry. The light source was a xenon arc type XBO 1600 whose beam was split in two: one bypassed the preparation and was taken as reference, while the other was focused on the rhabdome. The two beams were then chopped at a frequency of 13·5 Hz and collected on an S-20 EMI photomultiplier. A third beam was used to adapt the retina with

strong intensities. The dual pass Zeiss grating monochromator employed in these experiments had a spectral resolution of 0·1 nm in the range between 260 and 750 nm.

The preparation for microspectrophotometry was a section 100–200 μm thick perpendicular to the axes of the ommatidia. The section was cut with a razor blade and held between two quartz cover slips. The spot of the measuring beam, about 1 μm in diameter, was then focused on one rhabdome.



Text-fig. 2. Light transmitted through the rhabdome in two different conditions of pigment adaptation. Curve *a* is the transmission after conditioning for 3 min at a wave-length of 535 nm, curve *b* the transmission in the same cell after conditioning the pigment for 3 min at 405 nm. The gain is reduced of a factor of 10 at wave-lengths greater than 450 nm.

All the data of microspectrophotometry presented here were obtained from retinae fixed with glutaraldehyde. Results of a few experiments performed on fresh preparations did not show any significant difference from results obtained from fixed preparations. A detailed description of the microspectrophotometry is given in Muri, 1979.

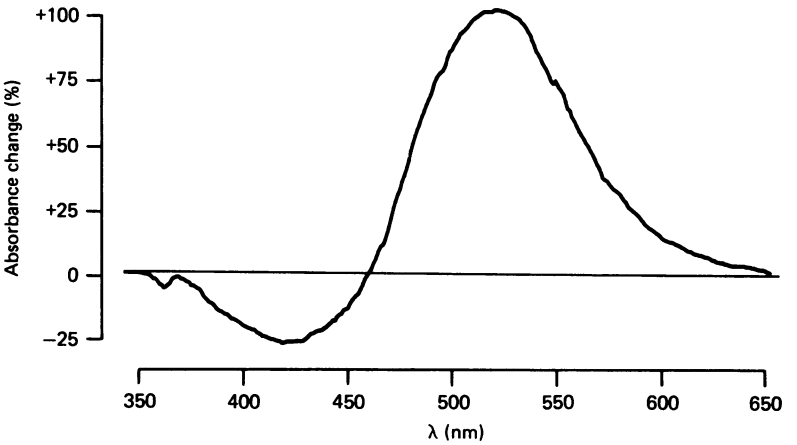
RESULTS

Microspectrophotometry

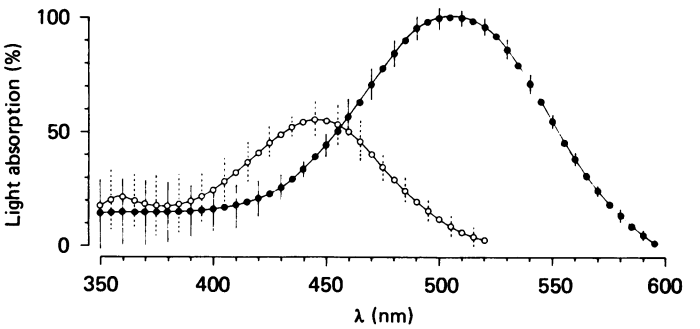
All data were obtained from measurements on single rhabdomes. Text-fig. 2 represents the transmission of light through the rhabdome in the range 300-600 nm, in two different conditions of pigment adaptation: curve *a* represents light transmission after conditioning at 535 nm for 3 min, curve *b* light transmission after 3 min of conditioning at 405 nm.

The difference spectrum obtained from curves of the kind of *a* and *b* (Text-fig. 3) shows two peaks, one negative at 420 nm and one positive at 518 nm. This result shows that conditioning with 405 nm light enhances absorption in the yellow-green, while depressing absorption in the blue-violet region, and that conditioning with 535 nm light depresses absorption in the green and enhances it in the blue-violet. This

demonstrates that the visual pigment of the drone can be converted by light from one state to another. From the difference spectrum and the equilibrium spectrum (Muri, 1979) the mean curves of absorption in the two states were computed and averaged for seven different rhabdomes; the result is shown in Text-fig. 4. In one state the maximal absorbance is at 445 nm; in the other it is at 505 nm.



Text-fig. 3. Difference spectrum. Plot of the relative absorbance change obtained from two curves of the kind of curves *a* and *b* of Text-fig. 2. Smoothed curve from one rhabdome.



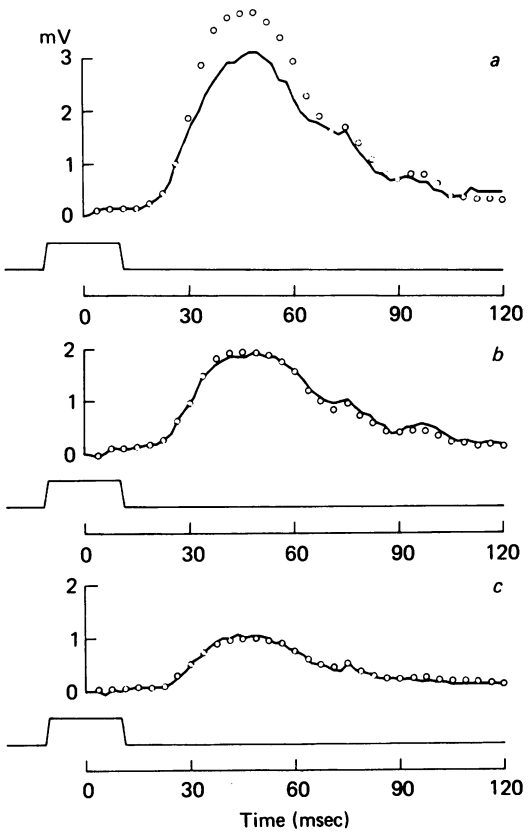
Text-fig. 4. Pigment absorption curves. The diagram represents the light absorbed by the pigment in the two states, i.e. rhodopsin and metarhodopsin, as a function of wave-length. Filled circles represent the absorption of metarhodopsin and are the mean of four different units normalized at their maxima. Open circles, which represent the absorption of rhodopsin, are the mean of seven units in % of the absorption of metarhodopsin. The bars represent standard deviations.

Electrophysiology

Retinula cells respond to light with a membrane depolarization graded with light intensity; when the depolarization of the membrane attains the threshold, a single spike of constant amplitude superimposes on the leading edge of the receptor potential (Baumann, 1968). In the present investigation it was convenient to use weak test flashes so that they did not significantly alter the proportions of the two pigment states.

Linear range of responses. For very weak stimuli, the amplitude of responses is

linearly related to the number of photons absorbed. Text-fig. 5 is an illustration of that: for each trace the intensity of the stimulus is doubled and on the three responses the 'expected' depolarizations are superimposed. The expected value has been calculated, following the procedure already used by Baylor & Hodgkin (1973), by numerical summation of the first two curves, and then by multiplication of this

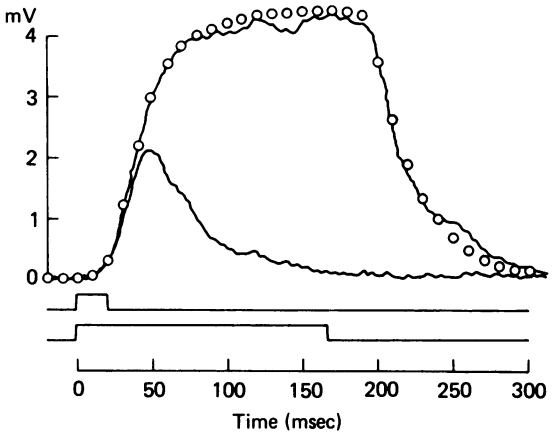


Text-fig. 5. Linear range of the responses. The continuous lines are the average of ten responses evoked by dim light whose intensity, from *c* to *a* was each time doubled. The predicted linear responses are represented by open circles. The value of these responses was computed taking the sum of traces *b* and *c* and multiplying this value by 1/3 for trace *c*, by 2/3 for trace *b* and by 4/3 for trace *a*. The actual response grows twofold from *c* to *b*, but less than that from *b* to *a*, thus indicating that responses exceeding 2 mV in amplitude are not any more linear with respect to the light. The limiting value of the linear range can vary from cell to cell. The light monitor is shown by the bottom trace.

value by 1/3 (trace *c*), 2/3 (trace *b*) and 4/3 (trace *a*). The linear relationship between response and stimulus in this cell did not hold for responses exceeding approximately 2 mV.

To see whether these weak stimuli produced detectable light adaptation we compared the response to a step of light with a hypothetical response calculated by assuming the principle of superposition (see Text-fig. 6 and its legend). The observed and calculated responses were undistinguishable showing that the sensitivity of the cell did not change during the step.

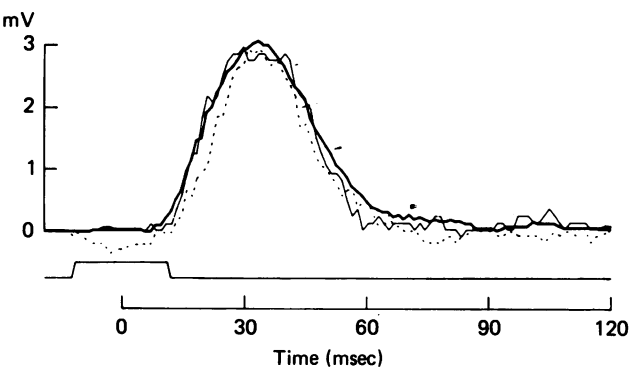
Spectral sensitivity in the linear range. The process leading to the electrical response in photoreceptors is initiated by absorption of photons by visual pigment. In the honeybee drone, since two thermostable states of the pigment had been demonstrated by microspectrophotometry, it was not possible with the available data to determine whether the two states of the pigment both contributed to the electrical



Text-fig. 6. Principle of superposition. The average response to a 20 msec flash of very weak light is superimposed to the response to a step of 165 msec of the same intensity. Open circles represent the convolution of the flash response computed according to the equation

$$y(t) = \int_0^T h(t-t')\,dt',$$

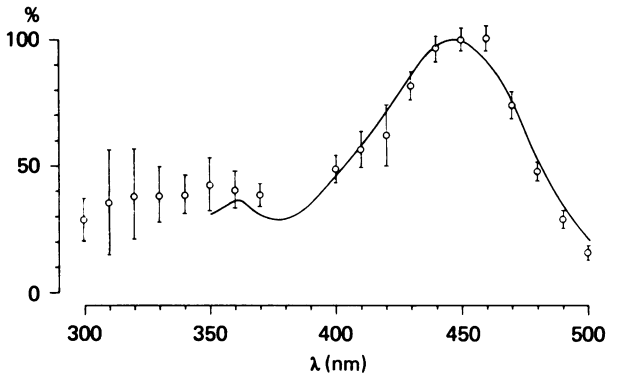
where $h(t)$ is the response to a Dirac pulse at t', T is the duration of the step and $y(t)$ is the voltage response.



Text-fig. 7. Univariance principle. Mean responses in the same cell at three different wave-lengths, matched in amplitude at the peak. The multiplication factor was 1 for the response at 460 nm, 2·45 for the response at 350 nm and 2·17 for the response at 480 nm. The three responses, even if of different amplitudes have the same time course. The bottom trace is the light monitor.

signal. To examine this point, linear responses at different wavelengths were matched in amplitude and superimposed, as shown in Text-fig. 7. The response is seen to be univariant with respect to wave-length, according to the definition given by Naka & Rushton (1966) who stated that 'each visual pigment can only signal the rate at

which it is effectively catching quanta; it cannot also signal the wave-length associated with the quanta caught'. Hence, from the similarity of the three curves of Text-fig. 7 over their whole time course, we can infer that whatever photosensitive substances lead to the electrical response of the cell, they produce similar responses.



Text-fig. 8. Comparison between spectral sensitivity and pigment absorption. Open circles and bars (S.D.) are the electrophysiological measurements in the linear range. The continuous line represents the absorption spectrum of rhodopsin, normalized at its maximum. The two curves superimpose quite satisfactorily, thus supporting the hypothesis that linear responses are proportional to the quantity of rhodopsin transformed by light into metarhodopsin.

The principle of univariance holds some way beyond the linear range, as it was possible to see by comparing responses evoked by intense but not saturating flashes of light at different wave-lengths and whose intensity was adjusted in order to match the peaks of the responses, and it was found to hold too when the stimulus was a step 100 msec long (two cells).

Having demonstrated univariance, we are justified in describing the spectral properties of these cells by plotting amplitudes of responses evoked by the same number of quanta as a function of the wave-length. The results obtained in twenty units, normalized at their maxima, are shown in Text-fig. 8.

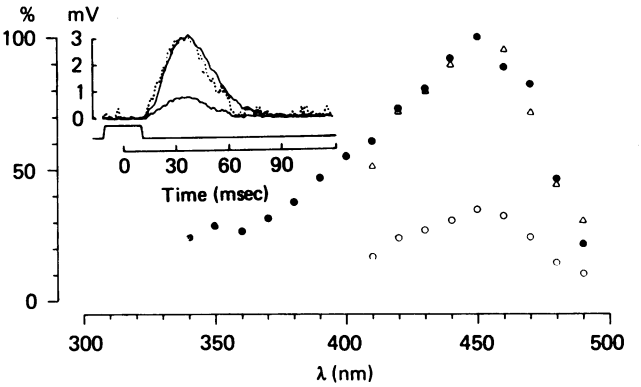
The retinula cells of drone in the dorsal part of the eye present a maximal sensitivity in the blue region (between 450 and 460 nm) and have a much steeper cutoff toward the red rather than in the U.V. region. Of all the cells examined so far, not a single one presented a peak in the U.V.

Correlation between electrophysiology and microspectrophotometry. Since the first experiments on photoreceptors it has been observed that the response is not an immediate consequence of the absorption of light, but rather it is brought about by a process which develops slowly following illumination. In order to test whether there is a direct relationship between absorption by the pigment and amplitude of linear responses, we compared the electrophysiology and the spectrophotometry. Text-fig. 8 compares spectral sensitivity and the absorption curve of the 445 nm state of the pigment.

The two curves were matched in amplitude at the peak: circles represent the electrophysiological measurements and the bars the standard deviations. Light absorption is the continuous line. From the good agreement between the two, one

can conclude that the light response is correlated to the transformation of the pigment from 445 nm state to the 505 nm state. Hence we identify the 445 nm state as rhodopsin and the 505 nm state as metarhodopsin.

To examine whether absorption of light by metarhodopsin has an effect on the response, we remeasured the spectral sensitivity after converting much of the



Text-fig. 9. Spectral sensitivity in two different conditions of adaptation. The spectral sensitivity of a cell was determined after conditioning at 535 nm for 3 min (filled circles). In the inset the continuous line represents the linear response to a flash of 20 msec at 460 nm, in these conditions of adaptation. The spectral sensitivity determined in the same cell after a blue conditioning (404 nm) is represented by open circles, and the mean response to the same test flash at 460 nm is represented by the smallest response in the inset. A diminished amount of rhodopsin, caused by the blue illumination provokes then a diminished sensitivity on the whole spectrum. If now the curve of spectral sensitivity obtained after blue adaption is multiplied by a factor of 2·7, the two curves match quite satisfactorily (triangles). Multiplication of the single test response by the same factor gives the dotted response in the inset which superimposes to the control. The only effect of a diminished amount of rhodopsin is then a diminished sensitivity of the cell for any wave-length.

rhodopsin to metarhodopsin by illuminating the cell with an intense blue (404 nm) conditioning light. It can be computed from the absorption spectra (Text-fig. 4) that when a photoequilibrium is established about 60% of the pigment will be in the 505 nm state. After the conditioning light was turned off the cell's sensitivity was about the 37% of its initial value.

The spectral sensitivity curve of the cell still resembled that of rhodopsin (open circles in Text-fig. 9); when the curve was multiplied by a factor of 2·7 (triangles in Text-fig. 9) it coincided with the control spectral sensitivity curve obtained before the light conditioning (filled circles). At the longer wave-lengths many photons are absorbed by the metarhodopsin (more than by the rhodopsin) and yet no electrophysiological effect on the amplitude of the response was detected.

Moreover, a detailed analysis of the cell response to the same 460 nm flash in the two states of adaptation (inset of Text-fig. 9) shows that, even if the response after blue conditioning is smaller, it has the same time course as the control. This is even more evident after multiplication of the former by a factor of 2·7. The resemblance between the two curves indicates that the only difference between the two lies in a loss of sensitivity induced by blue conditioning. Hence one can conclude that the

only effect of a dimished number of rhodopsin molecules is a decreased sensitivity throughout the whole spectrum.

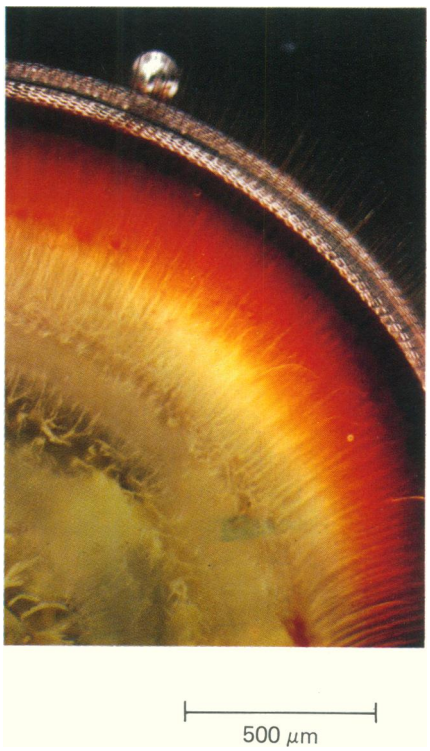
As a last test, the cell was exposed to a bright green (510 nm) conditioning light for 1 min and the response to a 460 nm test flash after 2 min of darkness was almost identical to the response to the same flash before the blue conditioning.

DISCUSSION

The most outstanding difference between vertebrate and most of the invertebrate photopigments is that the latter, following illumination, produce thermostable substances, instead of hydrolizing to opsin and retinal, as the vertebrate photopigments do (Hubbard, Bownds & Yoshizawa, 1965). This feature has been demonstrated in arthropods (Wald & Hubbard, 1957; Brown & Brown, 1958; Hubbard & St George, 1958) and in insects (Hamdorf *et al.* 1968; Gogala, Hamdorf & Schwemer, 1970; Razmjoo & Hamdorf, 1976), and some attempts have been made to correlate spectral properties of the different states of the pigments with electrical responses to light (Nolte & Brown, 1972*a*, *b*; Minke, Hochstein & Hillman, 1973; Hochstein *et al.* 1973; Tsukahara & Horridge, 1977). Nolte & Brown (1972*b*) proposed, for the *Limulus* median eye, that photoconversion of the U.V. pigment from its primary state (VP 360) into a stable photoproduct (M 480) induces a prolonged depolarizing afterpotential (PDA), while the reverse conversion in darkness is responsible for the slow return of the membrane potential to its resting level. Minke *et al.* (1973) presented evidence that no transformation takes place during the dark and therefore that there is no correlation between the electrical phenomenon of the slow recovery of the PDA and pigment conversion.

Working on another preparation, the barnacle, Hochstein *et al.* (1973) observed a closely parallel mechanism. The pigment has two thermostable states, interconvertible by light and absorbing maximally at 532 and 495 nm. They proposed that transformation of pigment molecules into the 495 state releases an excitor which increases the membrane conductance, while an inhibitor, released in the 495 → 532 transition, tends to neutralize the excitor. If this were true, responses elicited by light at a wave-length which is only absorbed by the pigment in its primary state would probably show a different time course from responses where the contribution of the inhibitor is significant. In the drone we have demonstrated (see Text-fig. 7) that no difference in time course can be detected between linear responses evoked by light even at wave-lengths as far apart as 350 and 480 nm.

In order to exclude the possibility that production of the excitor and of the inhibitor might require a time of several milliseconds, experiments were performed with steps of light instead of flashes. The principle of univariance holds as well for the step responses, and the spectral sensitivity curves obtained with steps are the same as those obtained previously with flashes of light, i.e., in the linear range we can find no electrophysiological sign of an effect of photon absorption by metarhodopsin. At very high intensities the drone, like the barnacle, has a PDA (Baumann & Hadjilazaro, 1972), and this can be supressed by stimulation at wave-lengths absorbed by metarhodopsin (F. Baumann, personal communication). These findings, although similar to those observed by Hochstein *et al.* (1973) in the barnacle, cannot be explained on the basis of the results presented here.


500 μm

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EXPLANATION OF PLATE

Section of a drone head showing the retina. The ommatidia extend from the corneal surface to the basal membrane for a length of approximately 500 μm. Their brown-red colour is due to the accessory pigment. The transparent layer surrounding the retina is the cornea. The darker zone immediately below is constituted by the crystalline cones which are surrounded by glial cells. The lamina and optic lobes are visible under the basal membrane.