Microspectrophotometry of Rhodopsin and Metarhodopsin in the Moth Galleria

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ABSTRACT Fresh, frozen sections of the photoreceptor layer of the compound eye of the moth Galleria have been examined by microspectrophotometry, using 4 X 8 μm measuring beams that sampled from approximately two to four rhabdoms. The principal visual pigment absorbs maximally at 510 nm (P510), and on irradiation is converted to a thermally stable, pH-insensitive metarhodopsin with λmax at 484 nm (M484) and a 43% increase in molar extinction coefficient. Subsequently, short wavelength irradiation of the metarhodopsin photoregenerates some P510, but the absence of an isosbestic point in the cycle of spectral changes is consistent with the presence of smaller amounts of violet- or ultraviolet-sensitive visual pigment(s) that also are converted to a blue-absorbing metarhodopsin. Difference spectra for both P510 and M484 were measured, using hydroxylamine. The 484-nm metarhodopsin is reversibly converted to a form with λmax at 363 nm by high concentrations of glycerol. Dark regeneration of rhodopsin in vivo after several minutes exposure of thoroughly dark-adapted animals to full sunlight requires several days.

The insects are the last of the major groups of animals with conspicuous eyes to yield significant information on the biochemistry of their visual pigments. Although retinaldehyde has been implicated as the chromophore for some years (Goldsmith, 1958; Briggs, 1961; Goldsmith and Fernández, 1966), evidence for direct involvement of the 11-cis isomer in insect vision has been published only recently (Paulsen and Schwemer, 1972). Until about 1972 spectroscopic characterization of visual pigment extracts was meager and ambiguous (see Goldsmith, 1972 for review), but data on a neuropteran (Hamdorf et al., 1971; Schwemer et al., 1971) and a moth (Schwemer and Paulsen, 1973) are now available. Photometric studies on retinal slices or intact insects have also been executed (Brown and White, 1972; Carlson and Philipson, 1972; Hamdorf et al., 1973; Hamdorf and Rosner, 1973; Schwemer and Paulsen, 1973; Stavenga et al., 1973). This approach provides information on the spectral and thermal properties of the pigments and their intermediates of bleaching while they are still a part of the receptor membranes. Some interesting differ-
ences in absorption (Bruno and Goldsmith, 1974) and thermal stability (Schwemer and Paulsen, 1973) have emerged when these properties are compared with results obtained in detergent micelles. A second potential advantage of microphotometry is the capacity to resolve the cellular sites or origin of color vision pigments. In the eyes of most arthropods, however, the fusion of rhabdomeres from clusters of cells makes this goal much harder to realize than with rods and cones from vertebrate retinas.

This paper reports a microspectrophotometric study of the rhabdoms of a moth, *Galleria mellonella*. The retina is dominated by a green-sensitive rhodopsin which is converted by light to a stable metarhodopsin. Indirect evidence suggests that there are smaller amounts of one or more additional visual pigments maximally sensitive in the violet or ultraviolet (UV). A new finding is that the absorption spectrum of metarhodopsin can be reversibly shifted to the near ultraviolet by a high concentration of glycerol.

**METHODS**

Laboratory-reared adults of the moth *Galleria mellonella* were used throughout this study. Larvae were maintained in constant darkness at 28°C. Upon pupation the moths were moved to a room with a 12-h light-dark cycle. Experimental animals were placed in constant darkness approximately 18 h before their use.

All experiments were carried out under dim red light. Heads of dark-adapted moths were severed and dropped into 2-methyl-butane cooled in liquid nitrogen. The frozen heads were then quickly placed in Ames "O.C.T." freeze section mounting medium (Ames Co., Div. of Miles Lab., Inc., Elkhart, Ind.) and taken to −20°C in a cryostat. Frozen sections about 18 μm thick were cut and placed singly on microscope coverslips, to which they adhered. The sections were surrounded with a thin ring of silicone vacuum grease; one or two drops of bathing solution, either insect saline or glycerol, was introduced within the ring, and a second coverslip was placed over the section and pressed down lightly until a seal formed. The grease ring seal eliminated evaporation and served as a spacer which kept the upper coverslip from crushing the specimen. Moreover, the upper coverslip could be removed, the bathing solution changed, and the glass sandwich reassembled without damaging the section. Recordings were made at about 22°C.

The bathing solution used in some of the initial experiments was Grace's Insect Tissue Culture Medium (Grand Island Biological Company, Grand Island, N. Y.), but most of the experiments were performed in a saline consisting of 214 mM NaCl, 3.1 mM KCl, and 9 mM CaCl₂, buffered with TES (2.3 g/liter) and adjusted to pH 7.2 with NaOH. Identical results were obtained in the two solutions. In experiments in which the bathing medium was glycerol, concentrations other than 100% were obtained by mixing the glycerol with the physiological saline on a vol/vol basis. Where hydroxylamine was used, it was made up in the saline.

The dual beam microspectrophotometer used in these experiments is similar to that described by Liebman and Entine (1964), but the reference beam is external to the
microscope. For the measuring beam an image of an aperture was projected onto the specimen with a Zeiss X32 Ultrafluor glycerin immersion objective (Carl Zeiss, Inc., New York) (numerical aperture 0.4) mounted beneath the stage on the condenser carriage. An identical lens was used in the collecting optics. The light source for most experiments was a 6-V, 100-W tungsten ribbon filament lamp; for measurements in the range 300–400 nm a 500-W deuterium lamp (Kern Instruments, Inc., Port Chester N.Y.) was used. The monochromator was a Bausch and Lomb 250-mm focal length instrument (Bausch & Lomb, Inc., Rochester, N.Y.) with the grating ruled at 600 lines per millimeter, and the detector an EMI 9558 QA photomultiplier (EMI Electronics Ltd., Whittaker Corp., Gencom Division, Plainview, N.Y.).

As operated, the instrument measures transmittance (\(T\), fraction of light transmitted). In an inhomogeneous medium the fraction of light lost in traversing the specimen (\(1 - T\)) is compounded of absorption and scattering, but in constructing difference spectra the latter is eliminated (or nearly so). In direct spectra such as Fig. 1 the vertical axis is \(1 - T\), but in the presence of scattering, \(1 - T\) is not, strictly speaking, absorptance. In labeling the ordinate, however, all transmission losses are indicated in the more familiar units of absorbance (log to the base 10 of the ratio of the incident to transmitted fluxes). Absorbance and absorptance (fraction of light absorbed) are directly proportional at the low densities of pigment encountered in these experiments.

In constructing difference spectra from raw records like Fig. 1, the higher frequency noise was first averaged out by penciling smooth curves over each of the traces on the original 10 X 15-inch chart recordings. These curves were then subtracted graphically at 12.5-nm intervals, using draftsman's dividers.

Deep red background illumination (712-nm interference filter, 22-nm half band width) was used to locate and align the section in the path of the scanning beam. The sections were cut approximately parallel to the ommatidial axes and the receptors were therefore irradiated from the side. The tracheal branches, which penetrate the basement membrane and extend into the retinulae for several micrometers, helped in locating rhabdoms, which cannot be visualized under these conditions. The measuring beam, which was 4 X 8 \(\mu\)m in the specimen plane and polarized parallel to its long axis unless otherwise noted, was placed in the region of the rhabdoms, just distal to the tracheoles, with its long dimension parallel to the ommatidial axes. These relationships are diagrammed in the inset in Fig. 9. The rhabdoms are about 6 \(\mu\)m thick. It is therefore probable that in most instances the measuring beam sampled from about three or four rhabdoms.

The energy of the measuring beam was sufficiently low as not to cause any detectable photoisomerization, for successive scans in the same location yielded identical spectra. Scans were recorded at 13 nm s\(^{-1}\).

The wavelength distribution of the light used to carry out photoisomerizations was limited with interference or Wratten cut-off filters; further details are provided with descriptions of specific experiments. The quantum fluxes of the isomerizing lights were determined with a calibrated photodiode (United Detector Technology, Inc., Santa Monica, Calif.).
RESULTS

Absorption of Rhodopsin and Metarhodopsin in Retinal Slices

Slices of the rhabdom layer from dark-adapted moths have a broad absorption band in the bluegreen region of the spectrum. Fig. 1 is an example of a recording from the microspectrophotometer. Curve 1 is the first spectrum recorded after removing the section from the cryostat, and curves 2–4 show the effect of successive exposures of 4, 8, 15, and 60 s to orange light from a 608-nm (15-nm half band width) interference filter. The hypochromic spectral shift indicates that rhodopsin is converted to a photoprodut, metarhodopsin. This metarhodopsin is stable and undergoes no further spectral changes in the dark. There is an isosbestic point at about 515 nm, which is in harmony with the interpretation that only two absorbing species are involved.

Photoregeneration

According to the current concepts of rhodopsin photochemistry, metarhodopsin contains the chromophore in a different stereoisomer (all-trans) from rhodopsin (11-cis). Consequently, light of wavelengths that are absorbed preferentially by metarhodopsin should cause isomerization of the chromophore.
from all-trans to 11-cis and photoregenerate some rhodopsin. The proportions of rhodopsin and metarhodopsin in the final photosteady state will depend on the relative molar absorbances at the wavelength of irradiation and on the relative quantum efficiencies for the forward and back reactions. (The possible formation of isorhodopsin is ignored here, for reasons detailed in the Discussion.)

Fig. 2 shows a typical result of efforts to photoregenerate visual pigment from *Galleria* metarhodopsin. These spectra were obtained from records like Fig. 1 by subtracting the instrumental base line. Curve 1 (filled circles) is the original spectrum. After an exposure to orange light sufficient to convert virtually all the visual pigment to its metarhodopsin, the absorption was as shown by curve 2 (open circles). The section was then irradiated for 1 minute

![Figure 2](image)

**Figure 2.** This, and Figs. 5-6, are difference spectra derived by subtracting the instrumental base line from original recordings such as seen in Fig. 1. Rhodopsin (curve 1) is converted to metarhodopsin (curve 2) by irradiation with orange light (\(\lambda\)'s > 550 nm). If the rhabdoms are then irradiated with violet light (412 nm, 18-nm half band width, 30 s), photoregeneration takes place; some of the metarhodopsin is reconverted to rhodopsin (curve 3). The lack of an isosbestic point for the three curves, as well as the greater-than-expected height of curve 3, indicates the probable involvement of more than two absorbing species. The violet isomerizing light had a flux at the specimen of \(8.7 \times 10^{14} \text{ h} \nu \text{ cm}^{-2} \text{s}^{-1}\) at the \(\lambda_{\text{max}}\).
with violet light (412 nm, 18-nm half band width); absorption rose at long wavelengths and fell at short wavelengths, a result consistent with a partial photoregeneration of visual pigment. Closer inspection, however, shows a quantitative discrepancy. The fall in absorption in the blue is too small relative to the increase in absorption in the green, and there is no isosbestic point. This result has been seen regularly, and on occasion the violet light caused an increase in absorption throughout the visible spectrum. These observations might be explained if the violet light, in addition to converting some metarhodopsin to rhodopsin, also produced additional absorption in the blue region of the spectrum. Such would be the case if, for example, a small quantity of visual pigment with absorption maximum in the violet or UV were converted to a blue-absorbing metarhodopsin.

Evidence for a second visual pigment

Fig. 3 is a further analysis of the experiment of Fig. 2. Curve 1 is the difference spectrum for the formation of metarhodopsin from rhodopsin, obtained by subtracting curve 2 from curve 1 in Fig. 2. Similarly curve 2 in Fig. 3 is the difference spectrum for the photoregeneration of rhodopsin by violet light, but for ease of comparison it is plotted as curve 3 (of Fig. 2) minus curve 2 (of Fig. 2). The absence of an isosbestic point is very clear.

Had the effect of irradiation with violet light been simply a partial reversal
of the rhodopsin-metarhodopsin conversion, the difference spectrum would have been the same as curve 1, but lower by the fraction of unregenerated pigment, and with an isosbestic point at 515 nm. At long wavelengths, where metarhodopsin absorbs little, curve 2 is about 0.37 as high as curve 1, indicating 37% photoregeneration. Curve 3 shows a hypothetical difference spectrum for 37% photoregeneration, assuming the presence of only a long wavelength-sensitive rhodopsin and its metarhodopsin in stoichiometric relationship. The difference between curves 2 and 3 (open circles) is therefore the spectrum of those additional absorbers produced by the violet adapting light. This difference spectrum peaks in the blue.

On the hypothesis that this “extra” absorption represents metarhodopsin formed from a violet or ultraviolet absorbing visual pigment, we have sought direct spectroscopic evidence for such short wavelength receptors. These experiments have not been successful. They will be described in further detail below, in the section on glycerol.

**pH**

Certain invertebrate metarhodopsins (see Discussion) exhibit changes in absorption spectrum with pH. Retinal slices were therefore bathed in salines of different pH. Neither the rhodopsin nor metarhodopsin was altered in spectrum over a pH range of approximately 3.2–9.6.

**Glycerol**

Carlson and Philipson (1972) in their study of photoreceptors of the moth *Manduca* suspended their sections in whole or 25% glycerol in order to reduce light scatter. Because they did not observe the formation of metarhodopsin, it seemed to us desirable to make some of our measurements under similar conditions.

Fig. 4 compares the difference spectra for the photic conversion of rhodopsin to metarhodopsin, measured with the section in glycerol (open circles) and in saline (filled circles, average of several experiments). In glycerol the $\lambda_{max}$ of the photoproduct is in the near UV, at about 363 nm, rather than at 484 nm as in the experiments of Figs. 1 and 2. Light induces larger changes in absorption when the tissue is in glycerol because there is less overlap in the absorption spectra of the visual pigment and its photoproduct than there is in saline.

The most interesting feature of this result is that the spectral shift of photoproduct is reversible. Fig. 5 shows an experiment that illustrates this point. The first spectrum was recorded with the section in saline; the section was then irradiated with orange light, leading to the formation of metarhodopsin (spectrum 2). Using irregularities in the pattern of tracheoles around the bases of the rhabdoms as a landmark, careful note was taken of the position of the measuring beam. The preparation was then removed from the microscope, the coverslip lifted, and the saline replaced with glycerol. When the section was
Figure 4. Difference spectra for the bleaching of *Galleria* rhodopsin in saline and in glycerol (initial spectrum minus final). In saline the photoproduct (metarhodopsin) peaks in the visible (484 nm) whereas in glycerol the product of this conversion peaks in the UV (363 nm). Open circles describe a single experiment; closed circles are an average of several measurements.

covered and the measuring beam was once again positioned within a couple of micrometers of its original site, there was virtually no absorption in the visible region of the spectrum but rising absorption in the UV which could not be followed to the peak in this experiment (curve 3). On flushing out the glycerol and restoring saline, curve 4 was measured, which is similar to curve 2 except at short wavelengths where there appears to be additional scatter. Because of the reversibility of the glycerol effect, we consider the UV-absorbing photoproduct to be a form of metarhodopsin.

Although one might argue that the absence of absorption in curve 3 is due to unreliable replacement of the measuring beam after changing solutions, experiments can be done in such a way as to obviate this criticism. Fig. 4 (open circles) is an example; Fig. 6 is another. The preparation of Fig. 6 was originally mounted in glycerol and curve 1 recorded. Two minutes of orange light caused a fall in absorption throughout the visible region of the spectrum and a rise in the near ultraviolet that could not be followed in its entirety with the tungsten lamp. Replacing the glycerol with saline, as described above, shifted the metarhodopsin spectrum to longer wavelengths (curve 3). The hypsochromic shift of metarhodopsin in glycerol has been produced in over 30 experiments; its reversibility has been demonstrated six times (of six attempts) in variants of the experiments of Figs. 5 and 6.
The speed with which the metarhodopsin absorption is shifted into the UV is somewhat variable. The shoulder at 470–480 nm in curve 2 of Fig. 6 suggests that the reaction was not complete. In most experiments 2 min was sufficient, but in some preparations more than 15 min were required. This variability is very likely caused by different amounts of water trapped in the section, for high concentrations of glycerol are required to produce the UV shift. Seventy-five percent (vol/vol) glycerol is not adequate, and whole glycerol was used in the experiments just described. Allowing for residual water in the section, the actual concentration was probably in excess of 90%.

Average data for the absorption of metarhodopsin in glycerol are shown in Fig. 7, taken from difference spectra similar to that of Fig. 4 (open circles), measured with a deuterium lamp. The position of the $\lambda_{\text{max}}$ (363 nm) and the half band width are similar to the unprotonated Schiff's bases of alkaline indicator yellow (cf., for example, Bownds and Wald, 1965).

With a complete shift of the metarhodopsin to the UV, the use of glycerol provides a method for determining a relatively undistorted absorption spectrum of the visual pigment from the difference spectrum for a complete photo-bleach. Average results of 10 experiments are presented in Fig. 8 (open triangles). The curve, which peaks at 510 nm, describes the principal rhodopsin
Figure 6. Reversibility of the glycerol effect. An experiment similar to that of Fig. 5, except the initial bathing solution was glycerol. Curve 1, rhodopsin from a dark-adapted eye. Curve 2, after 2 min of orange light (wavelengths >550 nm) a rise in the UV is evident, and most of the absorption in the visible has decayed. Curve 3, after replacing the glycerol with saline the 484-nm absorption band of metarhodopsin is restored. (Use of a tungsten lamp in this experiment restricted data in the UV.)

of *Galleria*. Similar results were obtained when bleaching was done in the presence of hydroxylamine (see below).

Because the analysis of experiments on photoregeneration suggested the presence of a violet- and/or UV-sensitive visual pigment present in small amounts, spectra were recorded in the near UV. No decreases in absorption that could be ascribed to the conversion of a UV rhodopsin to a ∼480-nm metarhodopsin were seen as a result of irradiation with short wavelength light. On the contrary, absorption appeared to increase due both to the formation of 484-nm metarhodopsin from 510-nm rhodopsin and to time-dependent increases in light scattering. The latter were decreased, but not eliminated, by the use of 60–70% glycerol, a concentration low enough to leave the absorption band of metarhodopsin well removed from any UV visual pigment that might have been present. In order to minimize the conversion of P510 to metarhodopsin by the UV irradiation, some sections were first exposed to green or blue lights (selected wavelengths longer than 480 nm) which should have spared a UV pigment while establishing a photosteady state.
between P510 and M484. Subsequent exposures to near UV light still failed to indicate the presence of a UV visual pigment, even when delivered with a Strobonar photographic flash (Strobonar, Honeywell Inc., Denver, Colo.) to further minimize the time, and thus the slowly increasing light scatter.

**Hydroxylamine**

In the presence of 0.1–0.2 M hydroxylamine, the metarhodopsin bleaches slowly over a time-course of 30–60 min. A constant light seemed to hasten somewhat the decay of metarhodopsin.

From these experiments it was possible to measure difference spectra for the bleaching of both rhodopsin and metarhodopsin, and average results are presented in Fig. 8 (open circles and X's, respectively). The difference spectrum for the rhodopsin is similar to that measured in glycerol, and a single

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**Figure 7.** The UV-absorbing form of metarhodopsin that is seen in glycerol. The $\lambda_{\text{max}}$ lies at 363 nm. Average of five experiments similar to the open circles in Fig. 4, measured with a deuterium lamp. The spectrum suggests an unprotonated Schiff's base.
Figure 8. Average difference spectra for the principal rhodopsin and metarhodopsin of Galleria. The triangles (Δ) represent the difference spectra for total bleaches in glycerol, and the open circles (○) for bleaches in the presence of hydroxylamine (initial spectrum minus final). The solid curve through these points is from the Dartnall (1953) nomogram for a vertebrate rhodopsin with a λ_{max} at 510 nm. The difference spectrum for metarhodopsin (X), obtainable in hydroxylamine because of the slow decay of the metarhodopsin, peaks at 484 nm. The solid curve was drawn through the points by eye. The relative scaling of the rhodopsin and metarhodopsin spectra reflects the 1.43 × greater molar extinction coefficient of the latter (standard error indicated by the heavy vertical bar through the peak of the metarhodopsin spectrum). See the text for further details.
curve with $\lambda_{\text{max}}$ at 510 nm, taken from Dartnall's (1953) nomogram of vertebrate rhodopsin, has been drawn for both sets of points. The curve for metarhodopsin ($\lambda_{\text{max}} = 484$ nm) was drawn through the points by eye.

Relative Molar Extinction of Rhodopsin and Metarhodopsin

With knowledge of the shapes of the absorbance spectra (Fig. 8) it is possible to determine the relative molar extinction at the $\lambda_{\text{max}}$ of metarhodopsin and rhodopsin ($\epsilon_M/\epsilon_R$) from absorbance changes measured in experiments like those of Figs. 1 and 2. The decrease in absorbance at 550 nm ($\Delta A_{550}$) is caused by the loss of rhodopsin, which is only partially compensated by the formation of metarhodopsin:

$$\Delta A_{550} = c \ell(0.62 \epsilon_R - 0.17 \epsilon_M),$$

where $c$ and $\ell$ are concentration and path length, and the coefficients describe the molar absorbances at 550 nm, relative to the peaks. Similarly, for the increase in absorbance at 475 nm,

$$\Delta A_{475} = c \ell(0.97 \epsilon_M - 0.79 \epsilon_R).$$

Combining, eliminating $c$ and $\ell$, and averaging data on absorbance changes from 20 experiments,

$$\epsilon_M/\epsilon_R = 1.43 \pm 0.05 \ SE.$$  

This figure provides the basis for the relative scaling of the spectra in Fig. 8, where the standard error ($\pm 0.05$) is represented as the heavy error bar through the $\lambda_{\text{max}}$ of metarhodopsin.

This analysis makes an important assumption: either the pigment molecules are randomly oriented in the measuring beam, or if they are not random, whatever dichroism exists is the same for both rhodopsin and metarhodopsin. The validity of this assumption must be demonstrated.

The measuring beam was laterally incident on the rhabdoms, with its long axis parallel to the ommatidial axes. When polarized parallel to the ommatidial axes, it should therefore be polarized at right angles to all the microvilli. When polarized in the orthogonal plane, however, there should be a distribution of relationships between the plane of polarization and the microvillar axes, from parallel to perpendicular. Thus some dichroism might be expected (cf. Moody and Parriss, 1961). The strength of the dichroism will depend on the degree of alignment of the chromophores with the microvillar axes (Goldsmith, 1975) as well as the obliquity of the section and the degree of disruption of the tissue during freezing and thawing. These geometrical factors are difficult to assess quantitatively by independent means, which makes the
preparation poorly suited for a study directed primarily at dichroic absorption in rhabdoms.

Fig. 9 shows a control experiment which indicates a small amount of dichroism. What is important, however, is that the ratios $\varepsilon_M: \varepsilon_R$ that are calculated from the absorption changes are not different for the two planes of polarization. In six experiments of this nature the calculated ratios of molar extinction were $1.38 \pm 0.056$ SE for $\varepsilon_1$ and $1.46 \pm 0.075$ SE for $\varepsilon_1$. These are neither significantly different from each other nor from the (independent) sample of 20 (measured with $\varepsilon_1$) that was cited above. There is thus no evidence that the dichroism of the metarhodopsin and rhodopsin are different, and the method of measuring the relative molar extinction is therefore appropriate.

![Figure 9](image)

**Figure 9.** Spectra recorded with the measuring beam polarized parallel and perpendicular to the ommatidial axes. Filled circles, rhodopsin; open circles, metarhodopsin produced by irradiation with orange light. From the absorbance changes at 550 and 475 nm the relative molar extinctions ($\varepsilon_M: \varepsilon_R$) are calculated (see the text). That the calculated values in this (and other) experiments are independent of the plane of polarization supports the validity of the method. Inset is a tracing of a light micrograph of a section of fresh-frozen *Galleria* eye as cut for recording. The approximate size of the measuring spot (MS) is also shown. Cor, cornea; PP, primary pigment; BP, basal pigment; Tr, tracheoles penetrating the proximal ends of the ommatidia; Rhb, region of rhabdoms; LG, lamina ganglionaris. In sections showing minimal disruption, reasonable spectra could be obtained anywhere in the band of rhabdoms.
**Dark Regeneration**

A series of experiments was performed to measure the recovery of rhodopsin in vivo after a complete photoconversion to metarhodopsin. Moths which have been dark adapted for 3–18 h were placed in clear plastic boxes and exposed for 2–4 min to full sunlight. Under these conditions the moths became agitated and flew about, which helped to expose all areas of the eye to the sun. The moths were then returned to the dark, and test animals were prepared for recording after various periods of recovery. The amount of rhodopsin present in a section from a sunlight-exposed moth, relative to the mean rhodopsin level in dark-adapted control insects, was estimated as follows. Absorption spectra were obtained before and after exposing the section to 10 s of an orange light sufficient to convert totally the rhodopsin to metarhodopsin. The absorption change at 550 nm is proportional to the amount of rhodopsin present, and the absorption at 515 nm (the isosbestic point) is a measure of the total pigment (rhodopsin plus metarhodopsin) in the section. The quotient, relative to dark-adapted controls, gives the fraction of the pigment present as rhodopsin. The normalizing of absorption changes at 550 nm to total absorption at 515 nm compensates for variations in thickness of section. Changes at 550 nm were measured directly, but total absorption at 515 nm was estimated by comparing records with other experiments in which total bleaches were achieved in the presence of hydroxylamine. By this means the relative contributions of absorption and scatter were estimated.

Several minutes exposure to sunlight converted virtually all of the rhodopsin to metarhodopsin, for the orange assay light caused no further change at 550 nm. There was, however, a small increase in absorption of around 2% in the neighborhood of 484 nm, but whether this represented the formation of additional traces of metarhodopsin or was due to an increase in light scatter could not be determined.

The time-course of recovery is shown in Fig. 10. The reappearance of rhodopsin is remarkably slow; by the fifth day the amount of rhodopsin present had risen to 75–80% of normal values.

**Discussion**

*Thermal and pH Stability of Metarhodopsin*

Thermally stable metarhodopsins have now been observed in the photoreceptors of several species of crustacea (Wald and Hubbard, 1957; Goldsmith et al., 1968; Hays and Goldsmith, 1969; Bruno et al., 1973; Goldsmith and Bruno, 1973; Bruno and Goldsmith, 1974) as well as in insects: the larval mosquito *Aedes* (Brown and White, 1972), the neuropteran *Ascalaphus* (Hamdorf et al., 1973; Schwemer and Paulsen, 1973), the fly *Calliphora* (Stavenga...
et al., 1973; Hamdorf and Rosner, 1973). In addition, electrophysiological evidence implicates stable metarhodopsins in the median eye of Limulus (Nolte and Brown, 1972), in Balanus (Minke et al., 1973; Hochstein et al., 1973), and in Drosophila (Cosens and Briscoe, 1972; Pak and Lidington, 1974).

In eight of the nine arthropods studied photometrically, the metarhodopsin absorbs maximally in the region 470-505 nm; the one exception being the higher dipteran Calliphora where the $\lambda_{max}$ is at much longer wavelengths, around 570 nm. The present results indicate that in this respect Galleria metarhodopsin, $\lambda_{max} = 484$ nm, is similar to most of the other arthropod metarhodopsins studied.

The stable metarhodopsin of cephalopod molluscs is pH sensitive, with an acid form ($\lambda_{max} = 497-516$ nm) and an alkaline form ($\lambda_{max} = 380$ nm) (Brown and Brown, 1958; Hubbard and St. George, 1958; Hamdorf et al., 1968). Similar observations have been made on the metarhodopsin formed in digitonin extracts of the UV-sensitive visual pigment of the insect Ascalaphus (Hamdorf et al., 1971; Schwemer et al., 1971).

In contrast, the metarhodopsin of Galleria does not change its spectrum with pH. In this respect it is similar to the lobster Homarus (Wald and Hubbard 1957), the spider crab Libinia (Hays and Goldsmith, 1969), and the mosquito Aedes (Brown and White, 1972). (Data are not available for Deilephila and Calliphora.) In general, this difference cannot be attributed to digitonin extraction. ERP (early receptor potential) data on squid retinas demonstrate that alkaline metarhodopsin can be formed in the photoreceptor membranes (Hagins and McGaughy, 1967); conversely, the spectrum of lobster metarhodopsin...
Rhodopsin is unaltered by pH in both digitonin extracts (Wald and Hubbard, 1957) and in isolated rhabdoms (Bruno and Goldsmith, unpublished).

The pK for the dissociation of acid to alkaline metarhodopsin varies for different species of cephalopod mollusc from 7.3 to 9.1 (Hara and Hara, 1973). Because in each case the reaction presumably involves deprotonation of the same Schiff's base linkage, retinaldehyde with the e-amino group of a lysyl residue, higher pK values indicate a relatively greater inaccessibility of the binding site. The metarhodopsin of Galleria, like that of Homarus, Libinia, and Aedes has such an inaccessible site that it cannot be titrated by changing the pH of the bulk solution. The slow reaction of hydroxylamine with Galleria metarhodopsin is consistent with this picture.

It seems a reasonable conclusion that in the metarhodopsins of higher arthropods the binding site of the chromophore is more tightly sequestered within the opsin than is the case for cephalopod molluscs. If further work should show this to be a general rule, the insect Ascalaphus is an apparent exception. It would nevertheless be interesting to see whether alkaline metarhodopsin forms in the photoreceptor membranes of this species, because examples are known in other arthropods of digitonin extraction altering the properties of the pigment. For example, in the moth Deilephila the rate of thermal bleaching of metarhodopsin is accelerated after extraction (Schwemer and Paulsen, 1973), and in the blue crab Callinectes the absorption spectrum of rhodopsin is shifted at least 20 nm by removal from the rhabdom (Bruno and Goldsmith, 1974).

The lack of an isosbestic point in photoregeneration (Figs. 2–3) has been interpreted as evidence for the formation of additional metarhodopsin from a short wavelength visual pigment. There is another possible explanation which cannot be eliminated with certainty but which we think rather less likely: formation of isorhodopsin.

When digitonin solutions of vertebrate rhodopsin are irradiated at temperatures low enough to arrest photodecomposition at the stage of metarhodopsin, a photosteady state of several components forms, one of which is isorhodopsin (Hubbard and Kropf, 1958). I sorhodopsin contains retinal in the 9-cis isomer. Mechanisms appear to exist, however, that severely restrict if not prevent the photoregeneration of isorhodopsin in eyes. In living flies (Hamdorf and Rosner, 1973; Stavenga et al., 1973) and isolated lobster rhabdoms (Goldsmith and Bruno, 1973) the isosbestic point in the photoconversion of rhodopsin and metarhodopsin indicates that little or no 9-cis isomer forms. Even in digitonin extracts the same result is obtained with cephalopod mollusc rhodopsin (Hubbard and St. George, 1958; Hamdorf et al., 1968). For steric reasons the 9-cis isomer may not be able to form without prior loss of intimacy between the chromophore and opsin. In addition, retinochrome from cephalopod molluscs very specifically directs the isomerization of all-trans to 11-cis
retinal, and when other cis isomers are provided as substrates 11-cis retinal is still the final product (Hara and Hara, 1973). Phosphatidylethanolamine plays a similar role in vertebrate rod outer segments. Photoisomerization of all-trans retinal performed in the presence of outer segments leads primarily to the formation of the 11-cis isomer; decreasing the ratio of all-trans retinal to outer segment membrane decreases the proportion of 9-cis retinal in the isomerate (Shichi and Somers, 1974). For these reasons we are inclined to interpret our results along the lines of Schwemer and Paulsen (1973) and Hamdorf et al., (1973) and to doubt the intrusion of isorhodopsin.

Effect of Glycerol on Galleria Metarhodopsin

The effect of high concentrations of glycerol on Galleria metarhodopsin can be viewed as a reversible denaturation. The spectral shift to 363 nm implies an opening up of the protein, exposing the Schiff's base linkage in its alkaline form with no measurable spectral enhancement through additional interaction with the opsin. This result is similar in direction to the effect of glycerol in shifting the equilibrium between bovine metarhodopsin I and metarhodopsin II toward the latter form, a reaction that also involves the addition of hydrogen ions (Matthews et al., 1963). In the latter system, however, whole glycerol produces irreversible denaturation even at 3.2°C.

The concentration of glycerol required to cause the spectral shift in Galleria metarhodopsin is higher than in the 2:1 glycerol-water mixture usually employed in low temperature studies of rhodopsin solutions. Whether other rhodopsin intermediates might be similarly affected at sufficiently low temperatures is therefore not known to us. Another interesting point which should be examined experimentally is the possibility that the glycerol effect is only reversible if the protein is stabilized by the presence of membrane lipids.

In their microspectrophotometric study of moth (Manduca) retinas, Carlson and Philipson (1972) reported several visual pigments, with λmax at 350, 450, 490, and 530 nm. The latter three bleached to a photoproduct with λmax at 370 nm which the authors interpreted as a mixture of free and bound retinaldehyde. Their procedures involved whole and 25% glycerol as immersion media, but they do not state in which experiments each was used. From our work it would appear that their 370-nm photoproduct might be reversibly denatured (glycerol-opened) metarhodopsin.

Comparison with Other Moths

Although Manduca is reported to have four visual pigments, with λmax at 350, 450, 490, and 530 nm, Carlson and Philipson (1972) state that “of the hundreds of . . . scans through transverse sections of the photoreceptor cells, relatively few experiments . . . provided well-defined absorption spectra.” Consequently the four visual pigments are characterized on the basis of
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two, four, six, and one experiment, respectively. In contrast we find that in Galleria, visual pigment can be detected in practically any section that includes rhabdoms. Whether this is a difference in technique or between species is not clear. Carlson and Philipson's failure to detect a blue-absorbing metarhodopsin may have been due to the presence of glycerol, as discussed above.

A more comprehensive analysis of Deilephila has recently been reported. In digitonin extracts, three visual pigments are observed, with \( \lambda_{\text{max}} \) at 520, 440, and 345 nm and present in relative concentrations of approximately 5:1:1. At room temperature all bleach directly to retinaldehyde and opsin, and only P520 and P450 can be detected spectrally. At \(-15^\circ\text{C}\), however, the 480 ± 10-nm metarhodopsins of all three pigments are stable (Schwemer and Paulsen, 1973). Isolated retinas have also been studied with 30–100-\( \mu \)m measuring beams, and difference spectra were measured for the photoconversion of P525 and P350 to M480 (Hamdorf et al., 1973). In the analysis of these experiments it was assumed that in vivo the spectra of both rhodopsins and metarhodopsins are described by the Dartnall (1953) nomogram. The measured difference spectra could then be accounted for by nomogram pigments P525 (or P350) and M480, with molar extinction coefficients \( \epsilon_p : \epsilon_m \) in the ratio of 1:1.75.

The work on Galleria which we report in the present paper is largely in agreement with the results on Deilephila. Galleria P510 and its metarhodopsin are similar to the major pigment of Deilephila. Moreover, difference spectra for total bleaches of Galleria rhodopsin and metarhodopsin measured in situ are, to a first approximation, fit by the Dartnall nomogram. This finding therefore justifies the assumptions made by Hamdorf et al. (1973) in their indirect analysis.

Although we present evidence that in addition to P510, Galleria also possesses small amounts of one or more rhodopsins with \( \lambda_{\text{max}} \) at shorter wavelengths, we have been unable to measure such pigment(s) directly. In Deilephila P350, but not P450, could be detected in microphotometric experiments. The measuring beam was much larger (30–100-\( \mu \)m diameter) than the one employed in our work (4 × 8 \( \mu \)m), and it was usually incident along the axes of the rhabdoms. It therefore sampled a much larger population of cells. Furthermore, because of the density of tracheal branches near the basement membrane of Galleria, we found that light scatter made it impractical to sample effectively at the basal ends of the rhabdoms. These technical differences could account for the difference in results.

Relative Magnitudes of the Molar Extinction Coefficients of Rhodopsin and Metarhodopsin

It seems to be commonly true that the molar extinction coefficient of invertebrate metarhodopsin is larger than for rhodopsin. (See Goldsmith, 1972 for a summary of data on cephalopod molluscs.) The ratio of 1.75 which Hamdorf
et al. (1973) and Schwemer and Paulsen (1973) report for Deilephila is significantly larger than our finding of 1.43 ± 0.05 SE for Galleria. In crabs the ratio is close to unity (Hays and Goldsmith, 1969; Bruno et al., 1973; Bruno and Goldsmith, 1974) and for the lobster Homarus it is about 1.2, both in vitro (Wald and Hubbard, 1957) and in situ (Goldsmith and Bruno, 1973).

Regeneration of Rhodopsin from Metarhodopsin

Hamdorf and Rosner (1973) and Höglund et al., (1973) have drawn attention to the importance of photoregeneration of rhodopsin from metarhodopsin in the recovery process. Moreover, the failure of Brown and White (1972) to detect any dark regeneration of rhodopsin in the larval mosquito ocellus would seem to support the idea that in arthropods there may be little if any capacity for reconverting metarhodopsin to rhodopsin in the dark. On the other hand, in flies (Stavenga et al., 1973) and in lobster (Barnes, Bruno, and Goldsmith, unpublished data) dark regeneration of visual pigment has been measured over a time span of minutes. How does our observation that regeneration of Galleria rhodopsin requires several days (Fig. 10) fit this picture?

We suspect that the slow recovery of Galleria rhodopsin indicates a de novo synthesis of membrane protein. In other arthropod eyes there is fine-structural evidence for varying amounts of membrane turnover as a result of light adaptation (Eguchi and Waterman, 1967; White, 1967; White and Sundeen, 1967), but there is no direct evidence that this is occurring in Galleria. In any event, the conditions of our experiment may be somewhat special. In initial attempts to bring about a significant conversion of rhodopsin to metarhodopsin we used light-adapted moths and had little success. In the experiments of Fig. 10, however, we began with dark-adapted animals whose eye color and eyeshine indicated that the accessory screening pigment was in the fully retracted position (Höglund, 1966). The screening pigments offer 2–3 log units attenuation when they are in the fully light-adapted position (Höglund and Struwe, 1971; Goldsmith and Bernard, 1974), but in Galleria they require 30 min of light to reach this distributed state (Post and Goldsmith, 1965). This time is long relative to the 4-min light adaptation employed in the experiments of Fig. 10. Galleria in nature is rarely exposed to full daylight without time for a compensatory adjustment of the ommochrome granules, so the irradiation the photoreceptors received in this experiment fell near the extreme of natural conditions. More work will be required to establish whether under milder challenges there exist faster mechanisms for dark regeneration or whether Galleria, because it is short-lived as an adult, relies on a combination of pigment synthesis, largely during the pupal stage, and photoregeneration as an adult moth.

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REFERENCES

BOWND, D., and G. WALD. 1965. Reaction of the rhodopsin chromophore with sodium borohydride. Nature (Lond.). 205:254.

BRODS, M. H. 1961. Retinene, in insect tissues. Nature (Lond.). 192:874.

BROWN, P. K., and P. S. BROWN. 1958. Visual pigments of the octopus and cuttlefish. Nature (Lond.). 182:1288.

BROWN, P. K., and R. H. WHITE. 1972. Rhodopsin of the larval mosquito. J. Gen. Physiol. 59:401.

BRUNO, M. S., M. I. MOTE, and T. H. GOLDSMITH. 1973. Spectral absorption and sensitivity measurements in single ommatidia of the green crab, Carcinus. J. Comp. Physiol. 82:151.

BRUNO, M. S., and T. H. GOLDSMITH. 1974. Rhodopsin of the blue crab Callinectes: evidence for absorption differences in vitro and in vivo. Vis. Res. 14:653.

CARLSON, S. D., and B. PHILLIPSON. 1972. Microspectrophotometry of the dioptric apparatus and compound rhabdom of the moth (Manduca sexta) eye. J. Insect Physiol. 18:1721.

COENS, D., and D. BRISCOE. 1972. A switch phenomenon in the compound eye of the white-eyed mutant of Drosophila melanogaster. J. Insect Physiol. 18:527.

DARTNALL, H. J. A. 1953. The interpretation of spectral sensitivity curves. Br. Med. Bull. 9:24.

EUCHT, E., and T. H. WATERMAN. 1967. Changes in retinal fine structure induced in the crab Libinia by light and dark adaptation. Z. Zellforsch. 79:209.

GOLDSMITH, T. H. 1958. The visual system of the honeybee. Proc. Natl. Acad. Sci. U. S. A. 44:123.

GOLDSMITH, T. H. 1972. The natural history of invertebrate visual pigments. In Handbook of Sensory Physiology, vol. VII/I. H. J. A. Dartnall, editor. Springer-Verlag, Berlin. 685.

GOLDSMITH, T. H. 1975. The polarization sensitivity-dichroic absorption paradox in arthropod photoreceptors. In Photoreceptor Optics. R. Menzel and A. Snyder, editors. Springer-Verlag, Berlin. 392.

GOLDSMITH, T. H., and G. BERNARD. 1974. The visual system of insects. In The Physiology of Insecta. M. Rockstein, editor. Academic Press, Inc., New York. 2nd edition. 166.

GOLDSMITH, T. H., and M. S. BRUNO. 1973. Behavior of rhodopsin and metarhodopsin in isolated rhabdoms of crabs and lobster. In Biochemistry and Physiology of Visual Pigments. H. Langer, editor. Springer-Verlag, Berlin. 147.

GOLDSMITH, T. H., A. E. DIZON, and H. R. FERNÁNDEZ. 1968. Microspectrophotometry of photoreceptor organelles from the eyes of the prawn Palaemonetes. Science (Wash. D. C.). 161:468.

GOLDSMITH, T. H., and H. R. FERNÁNDEZ. 1966. Some photochemical and physiological aspects of visual excitation in compound eyes. In The Functional Organization of the Compound Eye. C. G. Bernhard, editor. Pergamon Press, Inc., Elmsford, N. Y. 125.

HAOINS, W. A., and R. E. McGAUCHY. 1967. Molecular and thermal origins of fast photocative effects in the squid retina. Science (Wash. D. C.). 157:813.

HAMDORF, K., G. HÖGLUND, and H. LANGER. 1973. Photoregeneration of visual pigments in a moth. A microphotometric study. J. Comp. Physiol. 86:247.

HAMDORF, K., and G. ROSNER. 1973. Adaptation and photoregeneration in the eye of the blowfly. J. Comp. Physiol. 86:281.

HAMDORF, K., J. SCHWEBER, and M. GOGALA. 1971. Insect visual pigment sensitive to ultraviolet light. Nature (Lond.). 231:458.

HAMDORF, K., J. SCHWEBER, and U. TAUBER. 1968. Der Sehfarbstoff, die Absorption der Rezeptoren und die spektrale Empfindlichkeit der Retina von Eledone moschata. Z. Vgl. Physiol. 60:375.

HARA, T., and R. HARA. 1973. Isomerization of retinal catalysed by retinochrome in the light. Nat. New Biol. 242:39.
HAYS, D., and T. H. GOLDSMITH. 1969. Microspectrophotometry of the visual pigment of the spider crab Libinia emarginata. *Z. Vgl. Physiol.* 65:218.

HÖGLUND, G. 1966. Pigment migration, light screening and receptor sensitivity in the compound eye of nocturnal lepidoptera. *Acta. Physiol. Scand. Suppl.* 69:5.

HÖGLUND, G., K. HAMDOFF, and G. ROSNER. 1973. Trichromatic visual system in an insect and its sensitivity control by blue light. *J. Comp. Physiol.* 86:265.

HÖGLUND, G., and G. STRUWE. 1971. Pigment migration and illumination of single photoreceptors in a moth. *Z. Vgl. Physiol.* 74:336.

HUBBARD, R., and A. KROPF. 1958. The action of light on rhodopsin. *Proc. Natl. Acad. Sci. U. S. A.* 144:130.

HUBBARD, R., and R. C. C. ST. GEORGE. 1958. The rhodopsin system of the squid. *J. Gen. Physiol.* 41:501.

LIEBMAN, P. A., and G. ENTINE. 1964. Sensitive low-light-level microspectrophotometer: detection of photosensitive pigments of retinal cones. *J. Opt. Soc. Am.* 54:1451.

MATTHEWS, R., R. HUBBARD, P. K. BROWN, and G. WALD. 1963. Tautomeric forms of meta-rhodopsin. *J. Gen. Physiol.* 47:215.

MINKE, B., S. HOHSTEIN, and P. HILLMAN. 1973. Early receptor potential evidence for the existence of two thermally stable states in the barnacle visual pigment. *J. Gen. Physiol.* 62:87.

MOODY, M. F., and J. R. PARKISS. 1961. The discrimination of polarized light by Octopus. *Z. Vgl. Physiol.* 44:258.

NOLTZ, J., and J. E. BROWN. 1972. Ultraviolet-induced sensitivity to visible light in ultraviolet receptors of Limulus. *J. Gen. Physiol.* 59:186.

PAK, W. L., and K. L. LIDINGTON. 1974. Fast electrical potential from a long-lived, long-wavelength photoproduct of fly visual pigment. *J. Gen. Physiol.* 63:740.

PAULSEN, R., and J. SCHWEMER. 1972. Studies on the insect visual pigment sensitive to ultraviolet light: retinal as the chromophoric group. *Biochim. Biophys. Acta* 283:520.

POST, C. T., JR., and T. H. GOLDSMITH. 1965. Pigment migration and light adaptation in the eye of the moth, Galleria mellonella. *Biol. Bull.* 128:473.

SCHWEMER, J., M. GOGEZA, and K. HAMDOFF. 1971. Der UV-Schärfstoff der Insekten: Photokhemie in vitro und in vivo. *Z. Vgl. Physiol.* 75:174.

SCHWEMER, J., and R. PAULSEN. 1973. Three visual pigments in Deilephila elpenor (Lepidoptera, Sphingidae). *J. Comp. Physiol.* 86:215.

SCHWEMER, J., and R. L. SOMBORS. 1974. Possible involvement of retinylidine phospholipid in photoisomerization of all-trans-retinal to 11-cis-retinal. *J. Biol. Chem.* 249:6570.

STAVENOVA, D. G., A. ZANTEMA, and J. W. KUPEL. 1973. Rhodopsin processes and the function of the pupil mechanism in flies. In *Biochemistry and Physiology of Visual Pigments.* H. Langer, editor. Springer-Verlag, Berlin. 175.

WALD, G., and R. HUBBARD. 1957. Visual pigment of a decapod crustacean: The lobster. *Nature (Lond.)* 180:278.

WHITE, R. H. 1967. The effect of light and light deprivation upon the ultrastructure of the larval mosquito eye. II. The rhabdom. *J. Exp. Zool.* 166:405.

WHITE, R. H., and C. D. SUNDESEN. 1967. The effect of light and light deprivation upon the ultrastructure of the larval mosquito eye. I. Polyrribosomes and endoplasmic reticulum. *J. Exp. Zool.* 164:461.