Rhodopsin of the Larval Mosquito

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ABSTRACT   Larvae of the mosquito *Aedes aegypti* have a cluster of four ocelli
on each side of the head. The visual pigment of each ocellus of mosquitoes
reared in darkness was characterized by microspectrophotometry, and found to
be the same. Larval mosquito rhodopsin (λ_{max} = 515 nm) upon short irradia-
tion bleaches to a stable photoequilibrium with metarhodopsin (λ_{max} = 480 nm).
On long irradiation of glutaraldehyde-fixed tissues or in the presence of potas-
sium borohydride, bleaching goes further, and potassium borohydride reduces
the product, retinal, to retinol (vitamin A_1). In the presence of hydroxylamine,
the rhodopsin bleaches rapidly, with conversion of the chromophore to retinalde-
hyde oxime (λ_{max} about 365 nm).

INTRODUCTION

Most insects that undergo complete metamorphosis have, at various stages of
their life cycles, three different organs of photoreception. A simple visual sys-
tem, consisting of a few *lateral ocelli*, functions in the larva, whereas compound
eyes and *dorsal ocelli* differentiate at metamorphosis to the adult (Goldsmith,
1964). Most of our knowledge of the physiology of insect vision has come from
behavioral or electrophysiological experiments. Only a few attempts have
been made to measure insect visual pigments directly: in extracts of heads of
adult honeybees (Goldsmith, 1958 *a*, *b*) and cockroaches (Wolken and Sheer,
1963), in extracts of the compound eye of a neuropteran, *Ascalaphus macaronius*
(Gogala et al., 1970), and by microspectrophotometry of the compound eye
of the flesh fly *Calliphora* (Langer, 1966, 1967; Langer and Thorell, 1966 *a*, *b*).
The visual pigments of ocelli have never before been examined directly.

In this paper we describe the visual pigment in lateral ocelli of larvae of
the yellow fever mosquito *Aedes aegypti* L. Our particular interest in the bio-
chemistry of the mosquito ocellus springs from earlier morphological studies.
The configuration of photoreceptor membranes and other features of receptor
cell ultrastructure are altered by light (White and Sundeen, 1967; White,
1967, 1968). In order to begin making physiological sense of these morpho-
logical effects of light, we need to investigate the visual cycle and the nature of
the visual pigment.

The larva has a cluster of four ocelli on each side of the head (Figs. 1 and 2).

Each ocellus is a rosette of several photoreceptor cells, which in the light microscope are seen to bear a prominent lobed structure (Figs. 2 and 5 *c*) called the *rhabdom*. The dorsal and ventral ocelli contain 11–13 cells each and the central ocellus made up of two fused rosettes has approximately 26 cells. At the ultrastructural level, the rhabdom is shown to be a mass of microvilli which sprout from the distal surfaces of the receptor cells. The visual pigment is presumably localized in the microvillous membranes. In these features, the mosquito ocellus is organized as a typical arthropod photoreceptor.

We measured the visual pigment of the ocelli with a microspectrophotometer. Dense accessory pigmentation makes microspectrophotometry of normal ocelli difficult. However, animals of a white-eye mutant proved to be very suitable, since their ocelli, lacking all masking pigments, are transparent (Fig. 2 *a*). Indeed, the head is so transparent (Fig. 1) that the measurements can be made in living larvae. We anticipate that the entire visual cycle will be accessible to analysis in living animals. Moreover, we have been able to complement and extend our spectrophotometric experiments with measurements of the electrical activity (electroretinogram) of the ocelli (Seldin et al., 1972).

METHODS

The microspectrophotometer used in the following experiments is essentially that described by Brown (1961). A Zeiss WL microscope is mounted in the sample beam of a Cary 14 recording spectrophotometer (Cary Instruments, Monrovia, Calif.). The Zeiss Ultrafluor (ultraviolet transmitting) optics includes a × 100, 1.25 NA objective, a × 10 ocular, and a 0.8 NA condenser. The image of the measured field is delimited by an adjustable diaphragm at the entrance lens of the phototube housing. The reference beam is adjusted to match the size and energy of the sample beam by means of a diaphragm and neutral density screens. Only the sample beam passes through the specimen, and the base line is zeroed using the multipot system of the Cary spectrophotometer.

Ocelli for spectrophotometric measurement were mounted in an appropriate medium between quartz cover slips sealed with Vaseline. This microcell was mounted in the spectrophotometer, and a carefully selected 30 μm field within the specimen was aligned on the cross hair of a × 20 viewing ocular. Under these conditions, either a single ocellus or a group of ocelli could be scanned, depending on their orientation. Spectra were recorded from 650 to 300 nm at 25 A/sec at room temperature, with no measurable bleaching of the visual pigment.

Bleaching flashes were from two sequentially flashed M3 flashbulbs, each having a 20 msec flash duration. For yellow flashes, a Corning *3484* filter (cut-off 525 nm; Corning Glass Works, Corning, N.Y.) was used. For blue flashes, a Wratten *47* filter (maximal transmission 440 nm; Eastman Kodak Co., Rochester, N.Y.) was used. For ultraviolet flashes, a Wratten *18A* filter (maximal transmission 360 nm) was used. These filters were all coupled with a Jena *KG3* heat filter (Jenaer Glas Werke, Schott und Genoden, Jena, Germany). For long irradiations, a microscope lamp with a tungsten bulb was used, with appropriate filters.

The mosquitoes (*Aedes aegypti*) used in this study were grown from eggs kindly

supplied by Prof. George B. Craig, Jr., Department of Biology, University of Notre Dame. White-eye mutants (Bhalla, 1968) were generally used, but we also measured the visual pigment of wild-type animals of the Rockefeller strain. Larvae were hatched and reared in darkness to the fourth (final) instar. Larvae grown in darkness have much larger rhabdoms than those grown in light (White, 1967). Consequently we were able to use a relatively large scanning field. Growing animals in darkness also avoids the possible complication of having metarhodopsin present from a previous illumination. The metarhodopsin of these animals is very stable and possibly may persist even after a long period of dark adaptation.

Whole heads, or whole animals, were mounted in the microcell so that they were oriented as in Fig. 1. In this position, the ocelli are superimposed so that two or more are measured simultaneously. In most such preparations, the head was left intact, so that the medium bathing the ocelli was normal hemolymph. Alternatively, one side of the head capsule was cut away and mounted in the microcell with ocelli oriented as in Fig. 2 so that each ocellus could be measured individually. In such preparations, the ocelli were immersed in various media in the microcell.
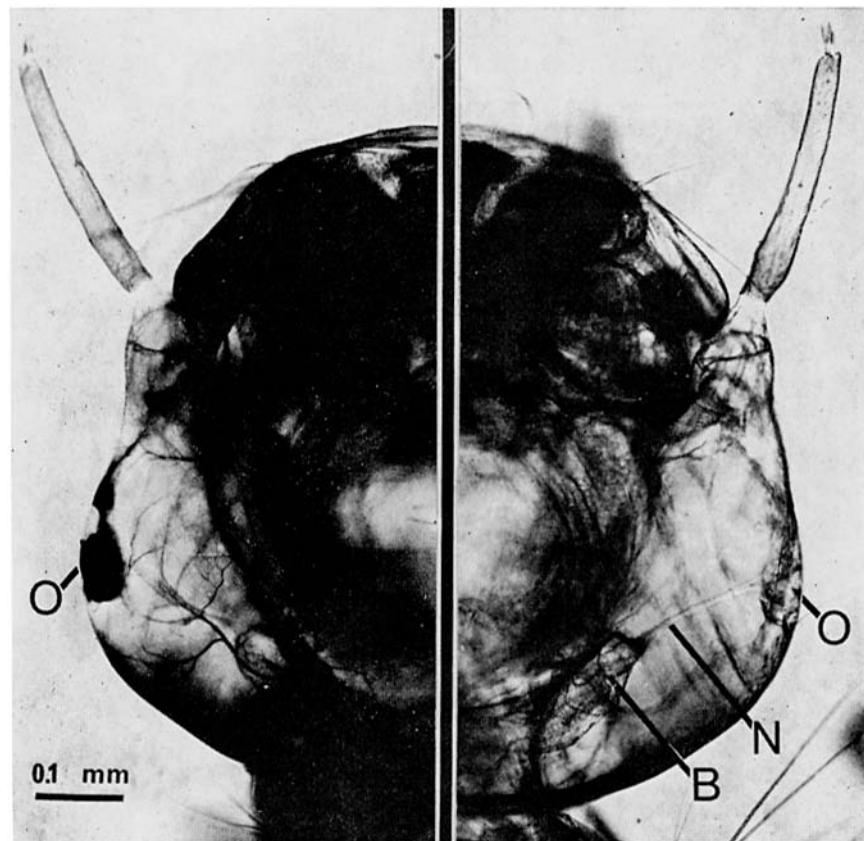

Figure 1.   Heads (dorsal aspect) of early fourth instar larvae: a wild-type animal at the left, a white-eye mutant at the right. Note the transparency of the head, and in the white-eye animal, of the ocelli. *O*, cluster of lateral ocelli; *B*, optic lobe of the brain; *N*, optic nerve.

FIGURE 2 *a*.   Cluster of lateral ocelli of white-eye larvae (early fourth instar, grown in darkness) seen through the side of the head capsule. Montage of photomicrographs taken at different focal planes; anterior is to the left.
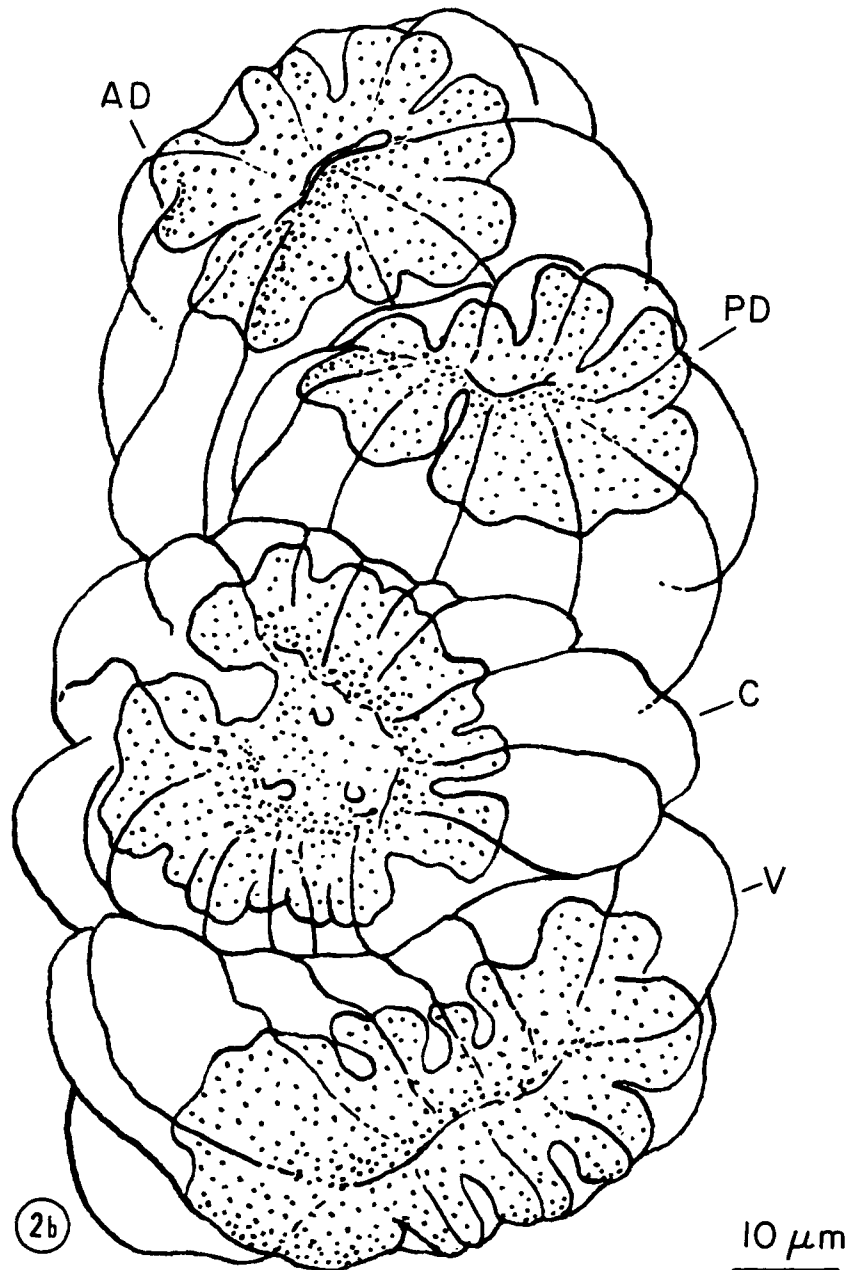
Figure 2 *b*. Diagrammatic interpretation of Fig. 2 *a*. The four ocelli making up the cluster have been designated anterior dorsal (*AD*), posterior dorsal (*PD*), central (*C*), and ventral (*V*). The rhabdoms (photoreceptor membranes) are stippled.

Unfixed ocelli were mounted in insect Ringer (NaCl 6.5, KCl 1.4, CaCl₂ 0.12, NaHCO₃ 0.1, and Na₂HPO₄ 0.01 g/liter) adjusted to an appropriate pH with phosphate buffer. In other experiments, the ocelli were fixed for 1 hr in 5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), and then washed in buffer. They were either used immediately or refrigerated in buffer for future use. All manipulations were carried out in dim red light of wavelengths longer than 690 nm.

RESULTS

Fig. 3 *a* illustrates an experiment in which a region of an ocellus that included the rhabdom (Field 1, curve 1), and then a more proximal region outside the rhabdom (Field 2, curve 2) were separately scanned. The difference between these spectra (Fig. 2 *b*) represents approximately the absorption spectrum of the rhabdom. It shows two major peaks, one with maximal absorbance (λ_{max}) at 515 nm, and the other with λ_{max} at 345 nm. The 515 nm band suggests the presence of a rhodopsin. There is also a minor band at 450 nm. The 345 nm substance, whose absorbance is several times greater than that of the rhodopsin, was always present in fresh preparations, but absent or greatly diminished in glutaraldehyde-fixed material. We do not know what it represents. However, we found no evidence that it changes in the light or is a visual pigment.

The spectrum from a central ocellus in pH 7.2 Ringer, recorded in the dark, is shown in Fig. 4 *a* (curves 1 and 2). After exposure to two flashes of yellow light the spectrum changed to curve 3. The difference spectrum of this photoconversion, plotted in Fig. 4 *c*, displays a maximal decrease in absorbance at about 550 nm, owing to the bleaching of rhodopsin, and a maximal increase at about 465 nm, owing to the formation of metarhodopsin. Similar results were obtained when ocelli were initially bleached with blue or ultraviolet flashes.

A second experiment of this kind is shown in Fig. 4 *b*. Curves 1 and 2 are dark scans from the ocelli of a living animal; curve 3 was recorded following two yellow flashes. The ocelli were then given two flashes of blue light and curve 4 was recorded. A large portion of the metarhodopsin produced by the yellow flashes was reconverted to rhodopsin by the blue flashes. We were able repeatedly to bleach and photoregenerate rhodopsin in these ocelli with either flashes or long irradiations. Hence, the product of each of our bleaches was a photoequilibrium, yellow light shifting it toward metarhodopsin, blue light toward rhodopsin.

Such photoequilibria were very stable. When the ocelli of living animals were flashed with sufficient yellow light to produce photoequilibrium and then left in darkness, the equilibrium initially established between rhodopsin and metarhodopsin remained stable for at least an hour, i.e., there was no measurable regeneration of rhodopsin in darkness during that period. Similar photoequilibria were established when glutaraldehyde-fixed ocelli were flashed

with yellow light, and some photoregeneration could be obtained following a subsequent flash with blue light. However, glutaraldehyde-fixed ocelli are irreversibly bleached by several minutes of irradiation with yellow light. We
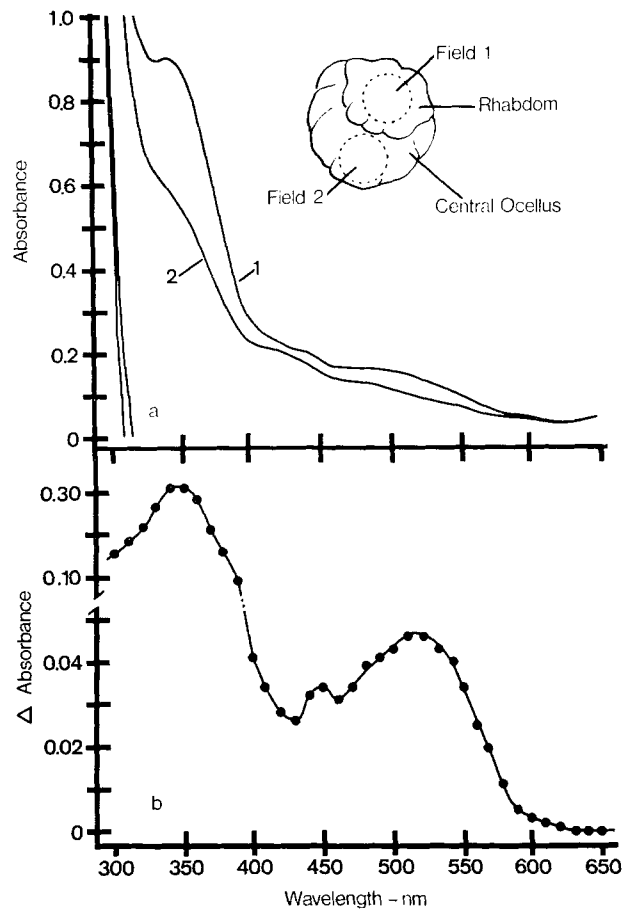

Figure 3.   (a). Recordings of absorption spectra of a central ocellus, mounted in insect Ringer at pH 7.2, measured in the dark. Spectrum curve *1* is that of a 30 μm field centered on the rhabdom, and spectrum curve *2* that of a field centered on a clear adjacent area. The *inset* diagram shows the position and relative size of the fields that were scanned. (b) Spectrum obtained by subtracting curve 2 from curve 1. It represents mainly the absorption spectrum of the rhabdom, including the rhodopsin at 515 nm, a small peak at 450 nm, and a large peak at 345 nm. The last two represent unknown substances, presumably not visual pigments.

have found that glutaraldehyde fixation increases the rate of photobleaching of mosquito rhodopsin, as was observed in the rhabdoms of the spider crab (Hays and Goldsmith, 1969), crayfish (Waterman et al., 1969), and lobster (Bruno, 1971).

Such difference spectra as are shown in Fig. 4 *c* do not represent the true absorption spectra of either rhodopsin or metarhodopsin since the spectra of the two pigments overlap broadly. Bleaching ocelli in the presence of hydroxylamine, however, accurately determines the spectrum of the rhodopsin. Hydroxylamine speeds the hydrolysis of the metarhodopsin by irreversibly combining with its chromophore retinal, to form retinaldehyde oxime (Wald and Brown, 1950; 1953).

Such an experiment is presented in Fig. 5. A glutaraldehyde-fixed ocellus (shown in Fig. 5 *c*) was mounted in 0.1 M neutralized hydroxylamine. Dark spectra were recorded (curves 1 and 2). The rhodopsin was then bleached for 10 min with yellow light (curve 3). The difference spectrum (curves 1 and 2 minus 3; Fig. 5 *b*) shows rhodopsin with $\lambda_{max} = 515$ nm, and retinaldehyde oxime (upside down) with λ_{max} about 365 nm.[1] Since the oxime does not absorb light above 450 nm this difference spectrum accurately represents the true absorption spectrum of the rhodopsin above 450 nm. Glutaraldehyde fixation was helpful in these experiments, since it resulted in more stable base lines. However, it was not essential; similar spectra were obtained when fresh ocelli were bleached in the presence of hydroxylamine.

The true difference spectrum of metarhodopsin is more difficult to determine. For this we used an indirect method, illustrated in Fig. 6, to estimate its spectrum from such steady-state difference spectra as are shown in Fig. 4 *c*. Such a difference spectrum is the algebraic sum of absorbance changes due to both the bleaching of rhodopsin and the formation of metarhodopsin. Hence, subtracting the absorption spectrum of the rhodopsin bleached from the difference spectrum should yield the spectrum of metarhodopsin. In order to determine the amount of rhodopsin bleached in any particular experiment, we assumed that the absorbance change at 600 nm (asterisk in Fig. 6, curve 1) represents only the bleaching of rhodopsin. From the absorbance of rhodopsin at 600 nm (Fig. 7) we calculated the total rhodopsin bleached (Fig. 6, curve 2). Subtracting curve 2 from curve 1 yields curve 3, the spectrum of metarhodopsin ($\lambda_{max} = 480$ nm).

Difference spectra of the photoproduct were also obtained from experiments in which glutaraldehyde-fixed ocelli were flash bleached in the presence of hydroxylamine. In such experiments, the photoproduct initially produced gradually hydrolyzed in the dark. Difference spectra so obtained were similar to the calculated metarhodopsin spectra except that their λ_{max} lay at a slightly shorter wavelength (about 470 nm). This result is consistent with the unpublished observation of P. K. Brown, that a bleaching intermediate (glutaralde-

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[1] In this experiment, the ratio of retinaldehyde oxime to rhodopsin is low compared with that found in rhodopsin extracts. Moreover, the ratio varied from one experiment to the next. Possibly the oxime diffuses out of the rhabdom, or it may have a different orientation than the rhodopsin, as has been found in the vertebrate retina (Wald et al., 1963).
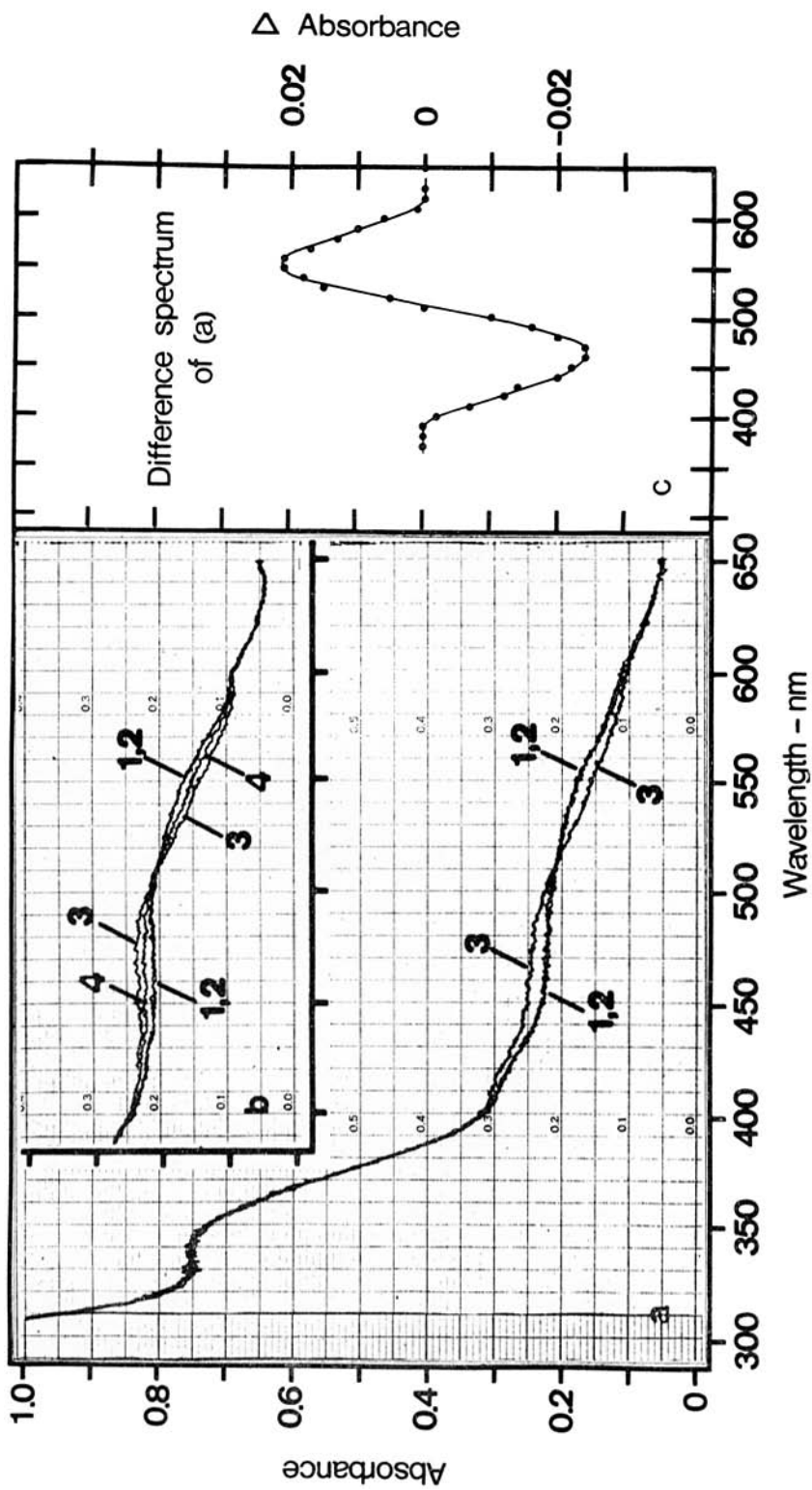
FIGURE 4. (*a*) Absorption spectra from a central ocellus mounted in insect Ringer at pH 7.2. Curves *1* and *2* were measured in the dark, curve *3* after the ocellus was flashed with yellow light. The difference spectrum (curves 1 and 2 minus curve 3) of the photoequilibrium between rhodopsin and its photoproduct, metarhodopsin, established by the yellow flash, is plotted in Fig. 4 *c*. (*b*) Spectra from the ocelli of a living animal. Curves *1* and *2* were measured in the dark, curve *3* was recorded after a yellow flash, and curve *4* was recorded after a subsequent blue flash. The yellow flash converted a portion of the rhodopsin to metarhodopsin; the blue flash reconverted some of the metarhodopsin to rhodopsin.
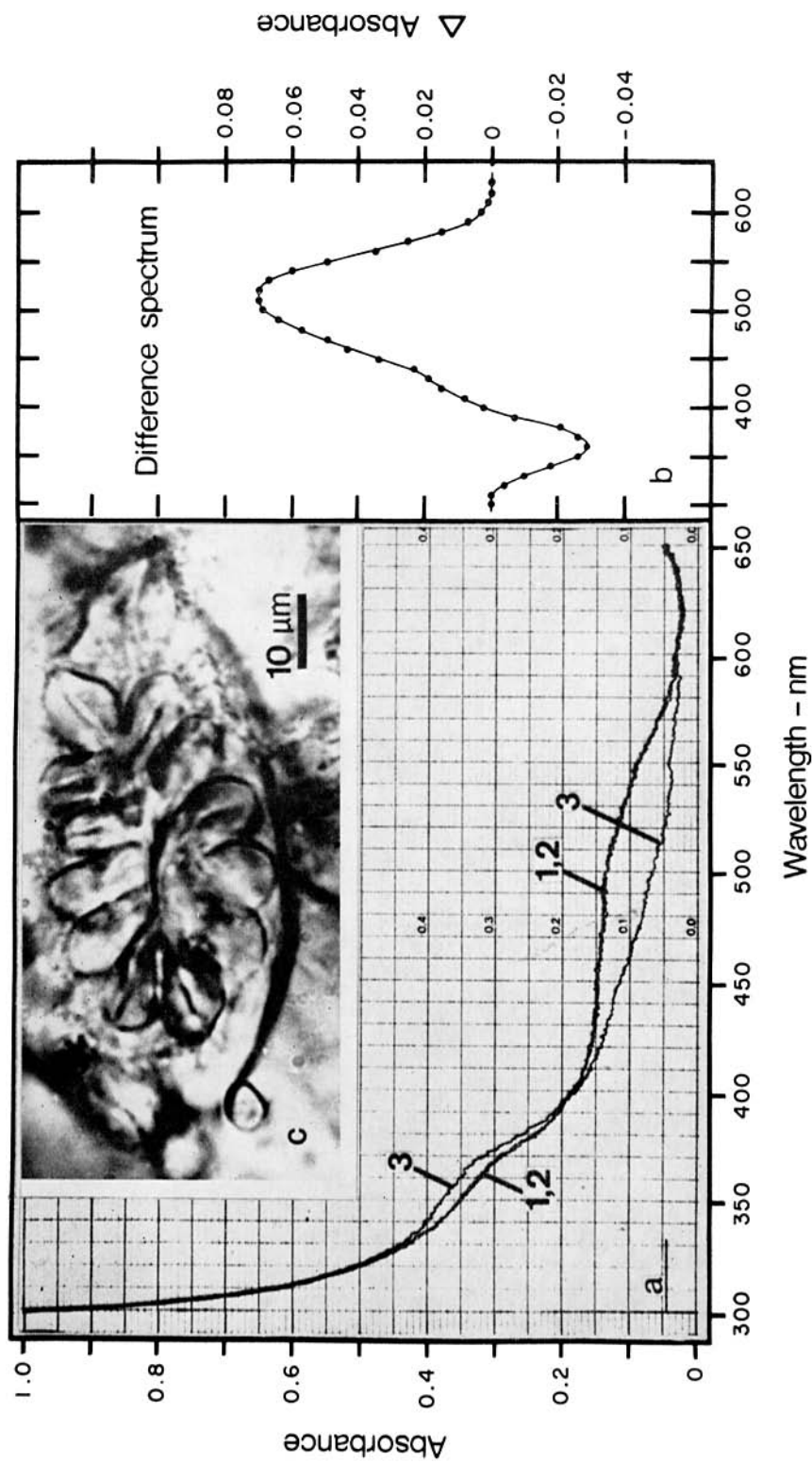
FIGURE 5. (a) Spectra from a ventral ocellus fixed in glutaraldehyde and mounted in neutralized hydroxylamine. Curves *1* and *2* were recorded in the dark, curve *3* after the ocellus had been bleached for 10 min with yellow light. The difference spectrum plotted in (b) represents the true absorption spectrum of the rhodopsin at wavelengths greater than 450 nm. The *inset* (c) is a photomicrograph of the ocellus that was measured as it appeared in the microspectrophotometer. A 30 μm central area of the rhabdom was measured.
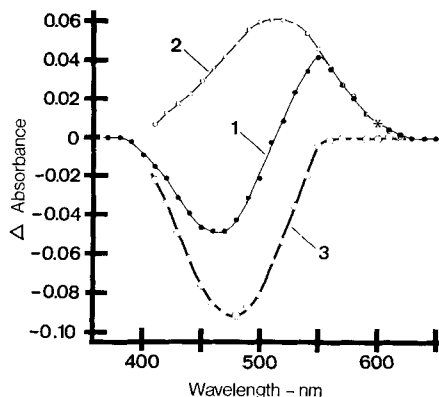
FIGURE 6. Estimation of the spectrum of metarhodopsin. Curve *1* is the difference spectrum from ocelli of a living larva flashed with yellow light. Curve *2* is the calculated spectrum of the rhodopsin bleached by the flash. Curve *3* is the spectrum of the meta-rhodopsin formed; it was obtained by subtracting curve 2 from curve 1. (See the text for further explanation.)
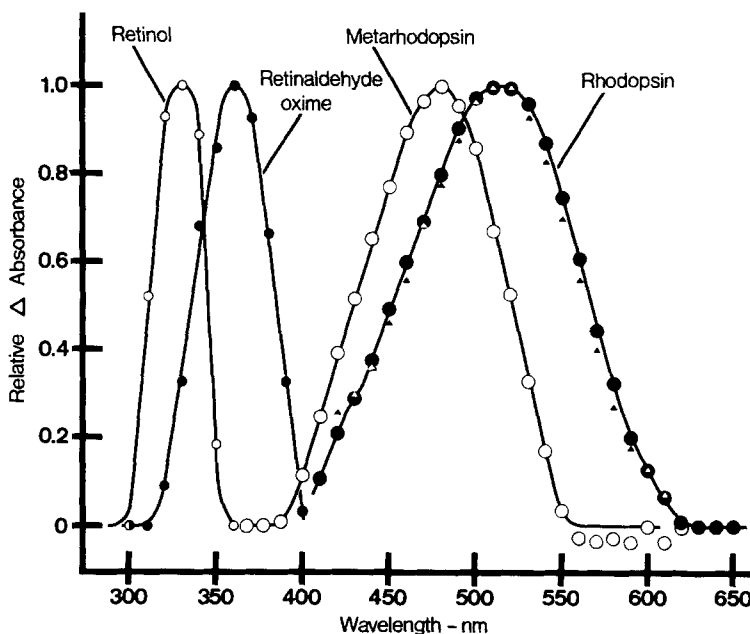

FIGURE 7. Summary of difference spectra of pigments of the larval mosquito ocellus. All spectra are arbitrarily brought to the same height. The rhodopsin spectrum is the average of seven difference spectra measured in the presence of hydroxylamine; the metarhodopsin spectrum is the average of three calculated spectra, the retinaldehyde oxime spectrum is the average of seven spectra, and the retinol spectrum is one difference spectrum. The triangles show the Dartnall nomogram of a hypothetical rhodopsin with λ_{max} = 515 nm.

hyde intermediate) at slightly shorter wavelengths than vertebrate metarho-dopsin I is found in glutaraldehyde-fixed vertebrate retinas.

Difference spectra from most of these experiments are summarized in Fig. 7. The rhodopsin and retinaldehyde oxime spectra are averages of seven difference spectra, and the metarhodopsin spectrum is the average of three calculated spectra. The spectrum of mosquito rhodopsin resembles a hypothetical rhodopsin with $\lambda_{max} = 515$ nm (triangles in Fig. 7) computed from Dartnall's nomogram (Dartnall, 1953). Though white-eye mutants were used for the bulk of our experiments, we also obtained similar results with wild-type animals, whose eyes have normal accessory pigments.

Fig. 7 also shows a difference spectrum of retinol (vitamin A_1) obtained when ocelli were illuminated for longer times in the presence of potassium borohydride. This speeds the hydrolysis of metarhodopsin, and reduces the product, retinal, to vitamin A (Wald and Brown, 1956). The potassium borohydride and hydroxylamine experiments, in which $retinol_1$ and $retinaldehyde_1$ oxime are formed, respectively, clearly demonstrate that the chromophore of mosquito rhodopsin and metarhodopsin is $retinal_1$.

Bleaching experiments in freshly dissected heads at pH 5, 6, 7, and 9, and intact larvae, gave results similar to those described above. At alkaline pH, however, base lines tended to be unstable, especially near 345 nm.

DISCUSSION

Analysis of insect visual pigments in extracts has proved difficult because of contamination with accessory pigments, and because the concentration of visual pigments is relatively low. Goldsmith (1958 a, b) extracted a pigment with λ_{max} about 440 nm from 20,000 honeybee heads with neutral buffer solution. This is as yet the only visual pigment that does not display the insolubility in water of typical membrane proteins. This pigment bleached to a 380 nm photoproduct, presumably retinal. Although Bowness and Wolken (1959) reported a similar buffer extraction of a pigment from housefly compound eyes, it is likely that the extract contained an accessory pigment, not a visual pigment (Goldsmith, 1964). Wolken and Sheer (1963) used digitonin to extract a 500 nm pigment from the cockroach (*Blatta orientalis*). It bleached like a rhodopsin, presumably to retinal, but its half-band width (67 nm) was unusually narrow, and did not fit the Dartnall nomogram. As both the honeybee and cockroach extracts were made from whole heads, the extracted pigments could have come from either the compound eyes or dorsal ocelli. Langer and Thorell (1966 a, b; Langer, 1966, 1967) measured with a microspectrophotometer the visual pigments in the compound eye of a white-eyed mutant of the blowfly (*Calliphora erythrocephala*). They found two pigments ($\lambda_{max} = 510$ and 470 nm) segregated in separate rhabdomeres.

Gogala et al. (1970) have recently reported the digitonin extraction, as well

as microspectrophotometric measurement, of a 345 nm visual pigment from the compound eye of a neuropteran, *Ascalaphus macaronius*. When irradiated with ultraviolet light, the pigment was converted to a 480 nm metarhodopsin. On long-wavelength irradiation the original 345 nm pigment was regenerated. Curiously, we also find a 345 nm substance associated with the mosquito rhabdom, but neither our spectrophotometric nor spectral sensitivity data (Seldin et al., 1972) indicate that it is a visual pigment.

Mosquito rhodopsin resembles the lobster rhodopsin measured in digitonin extracts by Wald and Hubbard (1957) and in isolated lobster rhabdoms (Bruno, 1971). Lobster rhodopsin has λ_{max} = 515 nm, and bleaches to a relatively stable metarhodopsin with λ_{max} = 490 nm. Both lobster and mosquito metarhodopsin appear to remain unchanged over a range of pH. Invertebrate metarhodopsins, unlike vertebrate, tend to be stable, at least in vitro (Brown and Brown, 1958; Hubbard and St. George, 1958; Mathews et al., 1963; Hays and Goldsmith, 1969). We have shown that mosquito metarhodopsin is stable in vivo as well. Again in contrast to vertebrate visual pigments, mosquito rhodopsin did not regenerate in darkness, suggesting that photoregeneration may be an important mechanism of rhodopsin renewal in the insect photoreceptor.

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