19. The rC/rI was made by annealing equal amounts of polyribocytidylic acid and polyriboinosinic acid (Miles Laboratories, Elkhart, Indiana) at 25°C for 1 hour and observing a hypochromic effect at 260 mμ.
20. Pyran is a random copolymer of maleic acid and divinyl ether synthesized by Hercules Company, Wilmington, Delaware (NSC-46015-C).
21. *Salmonella enteritidis* endotoxin, Difco Laboratories, Detroit, Michigan.
22. Supported by PHS grant AI-05629.
* Dernham fellow of the American Cancer Society, California Division (J-116).
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Microspectrophotometry of Photoreceptor Organelles from Eyes of the Prawn Palaemonetes

Abstract. *Microspectrophotometric measurements of individual dark-adapted rhabdoms of the prawn* Palaemonetes vulgaris *reveal the presence of two light-sensitive pigments. A pigment with maximum absorbancy at 555 nanometers is converted by light to a long-lived intermediate with wavelength of maximum absorbancy at 496 nanometers. A second pigment with wavelength of maximum absorbancy at 496 nanometers bleaches in the light, seemingly without forming detectable products at wavelengths longer than 375 nanometers. Both pigments occur in each layer of microvilli.*

The direct demonstration by microspectrophotometry of three types of cone cells maximally sensitive in different spectral regions, coupled with the observation that the outer segments of individual cones contain only one visual pigment (*1*) has established the fact that the trichromacy of human color vision has its basis in the retinal receptors. In arthropod eyes the receptor cells associate in groups of usually seven or eight where they cooperate in the formation of the rhabdom. The rhabdom, which is a compound organelle formed by masses of microvilli from several sense cells (*2*), contains the visual pigment. Each cluster of sense cells together with its rhabdom is called a retinula and comprises the functional part of a single ommatidium. Because each sense cell has its own axon, yet is closely associated or even fused with the other cells via their shared rhabdom, it is important to determine the extent to which each receptor cell represents a separate input channel to the central nervous system. Not only is it important to establish whether cells in the same retinula are electrically connected, but one would also like to know whether arthropods with color vision have more than one visual pigment in each rhabdom (*3*). What, in brief, are the individual color receptors?

As part of a study of the microspectrophotometry of single arthropod rhabdoms we have made observations on the prawn *Palaemonetes vulgaris*. Animals were dark-adapted overnight, and the preparations were mounted under near infrared light with the aid of an infrared image converter. Rhabdoms were obtained by squashing the eyes with a glass rod in the bottom of a conical centrifuge tube under several milliliters of saline solution (*4*). Detached rhabdoms and tissue debris were separated from most of the fine particles and pigment granules by light centrifugation for several minutes. A drop of suspension containing rhabdoms was mounted between cover slips on the stage of a dual-beam recording microspectrophotometer (*5*). Rhabdoms were located under infrared light, and spectra were recorded with lateral illumination and spots of light that were small with respect to the approximately 10 μm width of the rhabdom.

The rhabdoms of *Palaemonetes* contain two photosensitive pigments. Figure 1 shows the kind of experiment on which this conclusion is based. Curve 1 is the initial spectrum, relative to the base line recorded when the sample beam passed through a clear region of the slide. Although it is not shown in Fig. 1, a back scan retraces the initial spectrum, indicating that the measuring beam is not significantly actinic under the conditions of measurement. If now the preparation is illuminated for 2 minutes with a bright red light from the field illuminator of the microscope (Corning filter 2-59, wavelengths longer than 620 nm), there is a fall in absorption between 540 and about 625 nm and a rise in absorption in the region 425 to 540 nm (Fig. 1, curve 2). This represents the conversion of a pigment absorbing maximally at 555 nm (P_{555}) to a product (or products) with peak absorption at 496 nm. Controls demonstrate that the exposure to red bleaching light was sufficient to remove all of the P_{555} initially present. A yellow irradiating light (Corning filter 3-71, wavelengths longer than 470 nm) then causes the absorption to drop

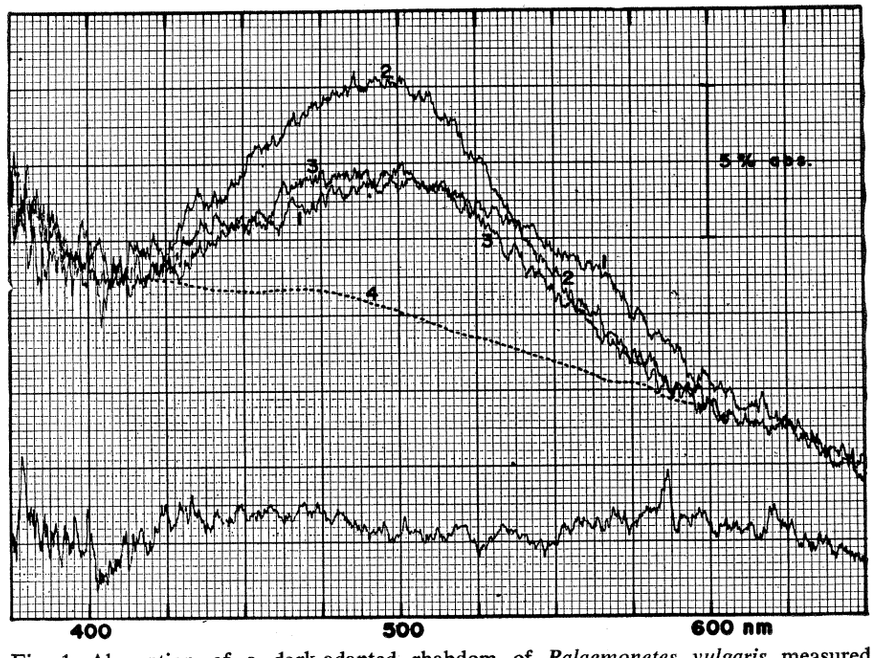

Fig. 1. Absorption of a dark-adapted rhabdom of *Palaemonetes vulgaris* measured with a 4-μm beam passing transversely through the organelle. Curve 1 is the initial spectrum; curve 2 was recorded after 2-minute exposure to bright red light (wavelengths greater than 620 nm); curve 3, after 2-minute exposure to bright yellow light (wavelengths greater than 470 nm); curve 4, 90 minutes later. Temperature, about 24°C; neutral *p*H; scanning speed, 2.1 nm/sec.

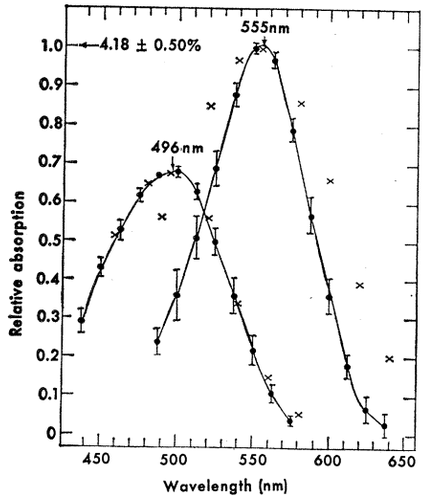

Fig. 2. Difference spectra for the two light-sensitive pigments of *Palaemonetes* rhabdoms. Points are averages of nine experiments with the standard errors; ×'s are hypothetical vertebrate rhodopsins with λ_{max} at 496 and 555 nm, taken from Dartnall's monogram (*8*).

to that shown in Fig. 1, curve 3, but no further. This represents the bleaching of a second pigment. The residual absorption slowly falls in the dark, usually with a time course of hours at 24°C. Curve 4, which was traced from the original record on another piece of chart paper, represents the final spectrum (*6*).

Bleaching experiments have been done in other ways as well. If the first exposure is to the yellow light, both photopigments are affected simultaneously, and the first spectrum recorded after irradiation is similar to curve 3 of Fig. 1. Alternatively, if a dark-adapted rhabdom is exposed to red

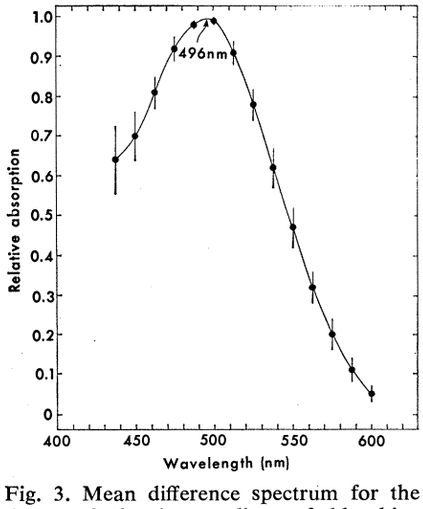

Fig. 3. Mean difference spectrum for the decay of the intermediate of bleaching formed from the 555-nm pigment. Points are averages of eight experiments (±S.E.).

light and then left in the dark, it requires the order of half an hour for spectrum 2 to fall to a position similar to spectrum 3. If the preparation is then irradiated with yellow light, photolysis of the second pigment is observed. These additional observations make it clear that most of the change between spectra 2 and 3 in Fig. 1 was the result of exposure to yellow light.

The difference spectrum formed by subtracting curve 4 from curve 1 gives the total bleach. This has two components, one of which can be measured by subtracting curve 3 from curve 2. This is the difference spectrum for the pigment bleached by the yellow light and has the wavelength of maximum absorbancy (λ_{max}) at 496 nm (P_{496}). The total bleach minus that of P_{496} yields the difference spectrum for the long wavelength-absorbing pigment and has λ_{max} at 555 nm (*7*).

These difference spectra are shown in Fig. 2 as averages of nine experiments, scaled in the proportions in which they absorb in the rhabdom. A number of years ago Dartnall (*8*) pointed out that the absorption spectra of vertebrate rhodopsins have the same shape if plotted on a frequency scale, a fact which enabled him to construct a nomogram for absorption spectra of visual pigments as yet undiscovered. The x's in Fig. 2 are nomogram pigments with λ_{max} at 496 and 555 nm; P_{496} follows closely the shape of a vertebrate rhodopsin, whereas P_{555} appears significantly narrower on both sides of the absorption maximum. However, because the absorption of P_{555} is obtained from a difference spectrum, the short wavelength side will be seriously depressed if even only relatively small amounts of colored intermediates fail to decay. Moreover, small movements of the rhabdom or alterations in its form between the times at which curves 1 and 4 (Fig. 1) were recorded introduce additional error. For these reasons the spectrum of P_{555} in Fig. 2 must be considered our best estimate to date.

The difference spectrum obtained by subtracting curve 4 from curve 3 in Fig. 1 is the "metarhodopsin" which forms from P_{555}. Figure 3 is the average of eight experiments. The λ_{max} is at 496 nm, but there is no proof that this curve represents the absorption of a single molecular species.

The microvilli of decapod crustacean rhabdoms are arranged with their long axes at right angles to the long axis of the rhabdom. In addition, they are organized in layers which are stacked along the axis of the rhabdom. Each layer is several micrometers thick, and the microvilli of alternate layers are oriented at right angles to each other (*2*). Phase-contrast photomicrographs of freshly prepared rhabdoms showed the layers of microvilli to be about 2.5 to 3.2 μm thick, as judged by the period of scalloping on their lateral edges.

To explore the distribution of pigments within the rhabdom, we used a measuring beam shaped as a slit whose image was 8.3 by 0.37 μm at the specimen. This slit was oriented at right angles to the long axis of the rhabdom, and measurements were made at successive positions along the length of the rhabdom. Precise movement of the measuring spot was achieved by mounting the aperture plate on a mechanical microscope stage equipped with a vernier on the rack and pinion. If alternate layers of microvilli contain only one of the pigments, this should be revealed in a series of measurements separated by half the band period (*9*). Twenty-one rhabdoms were examined at each of three positions separated by 1.5-μm intervals. At each position, the decrease in absorption following a red-light bleach was measured at 562 nm, and the decrease in absorption at 496 nm was measured after a yellow-light bleach. (The bleaching lights had the same spectral composition as in the experiment of Fig. 1.) Both pigments were invariably present, and in similar concentrations in adjacent spots. A single layer of microvilli therefore contains both pigments. Whether a single rhabdomere contains both pigments cannot be readily ascertained with *Palaemonetes*. Our results with crayfish confirm the observations on *Palaemonetes* that a single band of microvilli contains two pigments, for in *Orconectes* we have been able to measure, for both pigments, the difference in dichroism in alternate bands (*10*).

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1. W. B. Marks, W. H. Dobelle, E. F. MacNichol, *Science* **143**, 1181 (1964); P. K. Brown and G. Wald, *ibid.* **144**, 45 (1964).
2. References to recent work on the anatomy of crustacean rhabdoms can be found in the chapter by E. Eguchi and T. H. Waterman

in *The Functional Organization of the Compound Eye*, C. G. Bernhard, Ed. (Pergamon Press, New York, 1966), pp. 105–124; for insects see T. H. Goldsmith in *The Physiology of Insecta*, M. Rockstein, Ed. (Academic Press, New York, 1964), vol. 1, pp. 397–462. The fine structure of the rhabdoms of *Palaemonetes* has been studied by E. Eguchi and T. H. Waterman and we thank them for making available to us their unpublished electron micrographs.

3. The fly *Calliphora* has been examined by H. Langer and B. Thorell, *Expl. Cell Res.* **41**, 673 (1965). There are two kinds of rhabdomeres in each retinula; two cells have a pigment with λ_{max} at 470 nm; the other six, at 510 nm. The dipteran retinula, however, is unique in having morphologically separate rhabdomeres.
4. The saline contained in final concentration the following: 524 m*M* Na^+; 612 m*M* Cl^-; 13.3 m*M* K^+; 12.4 m*M* Ca^{2+}; 24.8 m*M* Mg^{2+}; and sufficient $NaHCO_3$ to bring the solution to *p*H 7.
5. The design is but slightly modified from that of P. A. Liebman and G. Entine, *J. Opt. Soc. Amer.* **54**, 1451 (1964). The principal differences are that the photomultiplier tube was an EMI 9558QA, the collecting optics as well as the inverted microscope had Zeiss × 32 Ultrafluar objectives, and the programming circuit in the high voltage supply (Kepco, 2500 volts) was used in the feedback control of dynode voltage instead of a 2C53 regulator triode.
6. Usually, to effect a complete bleaching, an additional light exposure must be given after the preparation has sat many minutes in the dark. This suggests that some of the "metarhodopsin" present at the time curve 3 was recorded is converted—whether directly or indirectly is not known—into a photopigment perhaps identical to the pigment bleached by the initial exposure to yellow light.
7. This interpretation of the experiment can be represented as follows:

$$P_{555} \xrightarrow{h\nu} M_{496}$$
$$M_{496} \xrightarrow{h\nu} \text{colorless products}$$
$$P_{496} \xrightarrow{h\nu} \text{colorless products}$$

where P_{555} and P_{496} are the pigments initially present, and M_{496} is "metarhodopsin." On the basis of the evidence in Fig. 1, however, one might also argue that the rhabdom originally contained only one light-sensitive pigment with λ_{max} at about 540 nm. On irradiation, it formed a mixture of two photoproducts with similar absorption spectra, one of which was light-sensitive and bleached during the second, shorter-wavelength exposure. This alternative explanation can be summarized:

$$P_{540} \xrightarrow{h\nu} M_{496}$$
$$P_{540} \xrightarrow{h\nu} M'_{496}$$
$$M_{496} \xrightarrow{h\nu} \text{colorless products}$$
$$M'_{496} \xrightarrow{h\nu} \text{colorless products}$$

where P_{540} is described by the difference spectrum for the total bleach (curve 1 — curve 4). There is a reason for favoring the first alternative at this juncture; Wald [G. Wald, *Nature* **215**, 1131 (1967)] has reported a pair of pigments in digitonin extracts of crayfish eyes which show a striking parallel to P_{555} and P_{496}. The pigment absorbing at longer wavelengths (λ_{max} at 556 nm in *Procambarus* and 562 nm in *Orconectes*) is converted by light into a long-lived intermediate with maximum absorbancy near 515 nm. The second pigment (maximum absorbancy at 525 nm in *Procambarus* and 510 nm in *Orconectes*) bleaches directly to retinal with no discernable intermediates at room temperature. Moreover, in solution the 562 nm pigment is selectively destroyed at *p*H 9, whereas the 510 nm pigment is broken down by 0.06*M* hydroxylamine. Although we have not been able to show corresponding chemical differences when the pigments are incorporated into the structure of the rhabdom (*Orconectes* or *Palaemonetes*), the fact that the differences exist in solution demonstrates the reality of two pigments.
8. H. J. A. Dartnall, *Brit. Med. Bull.* **9**, 24 (1953).
9. Although the width of the slit in the plane of focus was smaller than a band of microvilli, the rhabdom is sufficiently thick that the slit may be significantly out of focus at the upper and lower edges of the rhabdom, and therefore spread into adjacent bands. Even allowing for this complication, on the hypothesis that one of the pigments is missing from alternate bands of microvilli, one can show that if three spots are separated by intervals of one-half band period, the ratio of the pigment in one of the adjacent pairs of spots will be at least 10:1, regardless of the phase relation of the three spots to the banding system.
10. T. H. Waterman, H. F. Fernandez, T. H. Goldsmith, in preparation.
11. This work was supported by PHS grant NB-03333 to Yale University and postdoctoral fellowship NB 22,547 to H.R.F. We thank Dr. P. Liebman for his advice in the construction of the instrument, and N. Mandell for building it. Most of the experiments reported here were performed at the Marine Biological Laboratories, Woods Hole, Massachusetts.
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31 May 1968

Cytodifferentiation during Insect Metamorphosis: The Galea of Silkmoths

Abstract. *In the galea of silkmoths undergoing metamorphosis, generalized epidermal cells, which had previously secreted pupal cuticle, transform into highly specialized cells producing a new protein, the enzyme cocoonase. These cells first segregate by mitosis and displacement, then grow rapidly through endomitosis and accumulation of RNA-rich cytoplasm, and finally begin rapid synthesis of cocoonase. Replication of DNA continues in fully differentiated cells synthesizing cocoonase.*

The maxillary galea of certain saturniid moths (*1*) is a particularly favorable organ for the study of development. During the transformation from pupa to adult, highly specialized zymogen cells develop in the galea from the generalized epidermis in a predictable and swift sequence of events. When fully differentiated, these cells rapidly synthesize a characteristic protein, the cocoon-softening proteolytic enzyme cocoonase (*2*). In each galea, 20,000 cells with an aggregate dry weight of about 0.04 mg produce nearly 0.1 mg of enzyme in about 6 days. Cocoonase synthesis starts abruptly at a predictable stage. Glands at all developmental stages can be studied either in vivo or in short-term organ culture. One or more of these favorable characteristics are found in other metazoan cells that have proved useful for developmental studies in recent years (*3*). We describe here the normal course of development in the galea.

Pupae of *Antheraea pernyi* (diapausing first brood from Japan) and *A. polyphemus* (from North Dakota) were placed at 25° ± 0.5°C, after storage at 4°C for several months. Under these conditions, *A. pernyi* develops into an adult moth in 21 days (*A. polyphemus* in 18 days) measured from retraction of the wing epidermis (the first visible manifestation of development; day 0) to emergence of the mature moth (day 20 or day 17, respectively). Development proceeds according to a regular timetable (*4*). Galeae were excised from animals at the desired stage, fixed in 10 percent aqueous acrolein at 0°C overnight (*5*) or in 6 percent glutaraldehyde in half-strength Weevers' saline (*6*), and embedded in glycol methacrylate (*5*). Sections 1 to 1.5 μm thick were used. Autoradiograms were prepared with Kodak liquid emulsion NTB-2. Unless otherwise indicated, observations refer to *A. pernyi*.

In the diapausing pupa the galeae are two hollow, blood-filled, cuticle-covered projections of the epidermis in the head region, roughly conical in shape (*7*). The epidermal cells are arranged in a single squamous layer, separating the cuticle from the blood. The cells are uniform in appearance, each with a flat nucleus and very sparse cytoplasm, only 2 μm thick (Fig. 1a); they seem indistinguishable from epidermal cells elsewhere in the body.

With the onset of adult development, the cells of the galea, like all epidermal cells, retract from the cuticle, leaving behind them the molting gel, a protein-rich material that will eventually digest the endocuticle (*8*). They enlarge and become cuboidal. On days 2 and 3, administration of H^3-thymidine followed by autoradiography reveals DNA synthesis in most of the cells of the lateral half of the galea. Shortly thereafter, on day 4 and early day 5, mitotic figures appear in the same region, and the epithelium grows thicker as the cells proliferate (Fig. 1b).