# FLUORESCENCE OF CRAYFISH METARHODOPSIN STUDIED IN SINGLE RHABDOMS

THOMAS W. CRONIN AND TIMOTHY H. GOLDSMITH, *Department of Biology, Yale University, New Haven, Connecticut 06511*

ABSTRACT Isolated photoreceptor organelles (rhabdoms) from eyes of crayfish (*Procambarus, Orconectes*) were examined on a microscope system designed for quantitative measurements of fluorescence. Although fully dark-adapted rhabdoms are nonfluorescent or very weakly fluorescent, an increasing emission appears on exposure to light. Over the 30-fold range of intensities studied, the rate of the appearance of this fluorescence is identical to the rate of formation of metarhodopsin from rhodopsin. Furthermore, the excitation spectra for the observed emission are similar to the absorption spectra of crayfish metarhodopsin at both neutral and acid pH. Finally, the amount of fluorescence observed in rhabdoms previously irradiated with selected wavelengths of light is proportional to the amount of metarhodopsin present in the photosteady state established by the prior irradiation. The emission therefore originates from crayfish metarhodopsin. Fluorescence emission peaks at 670 nm at neutral pH. The quantum efficiency is $1.6 \pm 0.4 \times 10^{-3}$. Although emission from other rhodopsin photoproducts has previously been noted, this is the first description of fluorescence from the metarhodopsin chromophore site.

## INTRODUCTION

The chromophore of all known visual pigments is retinal (or the closely related dehydroretinal), the aldehyde of retinol or vitamin A. The fluorescence properties of retinol are well known (Kahan, 1971), and some stereoisomers of retinal and its Schiff bases are also known to fluoresce (Balke and Becker, 1967; Waddell et al., 1973; Das et al., 1979). Although *N*-retinyl-opsin formed by reduction of the retinal-opsin Schiff base linkage of rhodopsin (Hall and Bok, 1976) has fluorescence properties similar to retinol itself (Ebrey, 1971), vertebrate rhodopsins emit only weakly if at all from their chromophore sites. Guzzo and Pool (1968) reported a 500-nm stimulated emission from cattle rhodopsin, peaking at 600 nm, but attempts to repeat this work have been unsuccessful (Busch et al., 1972).

Even if rhodopsin itself is not detectably fluorescent, some later intermediates in the visual cycle could fluoresce. A fluorescent photoproduct of fly rhodopsin discovered by Franceschini (1977, 1978) is formed under unusually intense irradiation (Stark et al., 1979). Although fluorescence emissions from some of the intermediates of the vertebrate visual cycle also have been reported (Guzzo and Pool, 1969), the published excitation spectra do not duplicate the absorption spectra of visual pigment intermediates, and more work is necessary to confirm these results.

Arthropod visual systems have some advantages for studies of rhodopsin and its photoproduct metarhodopsin. Metarhodopsin is thermally much more stable than it is in vertebrate photoreceptors, and rhodopsin and metarhodopsin are readily interconvertible by light. Consequently, the proportions of these two pigments can be varied in photosteady-state

mixtures by irradiation at appropriate wavelengths. Moreover, in crayfish the photoreceptor membranes (rhabdoms) are readily isolated, and single organelles can be studied spectrophotometrically. In this paper we describe fluorescence from crayfish rhabdoms and demonstrate that it arises from crayfish metarhodopsin.

## MATERIALS AND METHODS

Two species of crayfish, *Orconectes rusticus* (Connecticut Valley Biological Supply Co., Southamton, Mass.) and *Procambarus clarkii* (Carolina Biological Supply Co., Burlington, N.C.) were used for study. Previous work has found no differences in the absorption characteristics of their photoreceptors (Goldsmith, 1978), and in this study no differences in fluorescence were detected either. Animals were maintained in aerated aquaria on a 12:12 light:dark cycle and were usually used within 1 wk of arrival at the laboratory. Before use, animals were dark-adapted for at least several hours, but more often overnight. Suspensions of rhabdoms and other tissue fragments were obtained from dark-adapted crayfish by removing the compound eyes in dim red light and macerating them with a stout glass rod in 1 ml crayfish saline (van Harreveld, 1936) of desired pH (1.9 or 7.5) at 0°C. In most experiments rhabdom structure was stabilized by adding formaldehyde (prepared from paraformaldehyde) to a final concentration of 0.75%. After 20–30 min of fixation, the preparation was centrifuged and resuspended two times in crayfish saline and then kept at 0°C until use. Drops of the final suspension were placed on a slide in a ring of silicon grease and under a coverslip for examination at room temperature. For some experiments, rhabdoms were unfixed and were maintained in pH-7.5 crayfish saline. The use of glutaraldehyde fixation was avoided, since Collins and Goldsmith (1981) found that such treatment induces a photoenhanceable fluorescence with an excitation spectrum that peaks in the green.

All fluorimetric work was performed on a Zeiss epifluorescence microscope equipped with a 63X, 1.4 numerical aperture objective and an adjustable rectangular aperture in the upper image plane (Carl Zeiss, Inc., New York). Measurements of the fluorescence emission were obtained with a photomultiplier tube (Hamamatsu Corp., Middlesex, N.J., model R928) mounted in a cooled housing (Products for Research, Inc., Danvers, Mass., model TE177RF) and connected to a photon-counting system (Princeton Applied Research Corp., Princeton, N.J., model 1140). With the tube chilled, the dark count was $<2\ s^{-1}$. The results were corrected for the wavelength sensitivity of the photomultiplier tube, which was measured with a calibrated photodiode (United Detector Technology, Inc., Santa Monica, Calif.). The output of the photon counter was handled by a desktop computer (Hewlett Packard Co., Palo Alto, Calif., model 9825B), following analog-to-digital conversion. The computer also drove the monochromator and excitation shutter. The monochromator used for all spectral work consisted of a stepping motor, reduction gear, and semicircular continuously-variable interference filter (Optical Coating Laboratory, Inc., Santa Rosa, Calif., model VC 180-017; wavelength range 380–720 nm; half bandwidth 12–16 nm), contained in a light-tight housing that could be mounted either in the excitation path or as part of the collecting optics. Onset and duration of excitation was regulated by an electromagnetic shutter (Uniblitz model 225, Vincent Associates, Rochester, N.Y.) activated by the computer. The excitation source was either a 50-W high-pressure mercury arc or a 12-V, 100-W quartz-halogen lamp; both were powered by regulated DC power supplies. Excitation intensity was controlled by neutral density filters (Ditric Optics, Inc., Hadson, Mass.).

To obtain emission spectra, the monochromator was placed between the microscope and the photomultiplier tube and excitation characteristics were controlled by narrow-band interference filters (Ditric Optics, half bandwidth 5–8 nm) and a Zeiss 510-nm dichroic beamsplitter. For excitation spectra, the quartz-halogen excitation source was used, the monochromator was mounted between the excitation shutter and the microscope filter arm, and a Zeiss 580-nm dichroic beamsplitter was used. The spectral output of the excitation source was calibrated daily by means of a photodiode of known sensitivity (United Detector Technology Inc., Santa Monica, Calif.) placed in the position normally occupied by the rhabdom preparation. Emission was measured either at 670 ± 5 nm (half bandwidth) or at wavelengths >600 nm (600-nm long-pass interference filter, Ditric Optics). All fluorescence spectra

were corrected by subtracting any background light that could be detected in an empty field the same size as the area of rhabdom sampled. Background was typically <5% of the peak emission.

To measure the time-course of fluorescence, the mercury arc served as the excitation source, excitation wavelength was controlled by narrow-band filters, and emission was measured at wavelengths >600 nm. When transmittance changes in the rhabdom were monitored, the microscope substage illuminator was run from a regulated DC power supply and used as the monitoring light source. To monitor rhodopsin-metarhodopsin conversion, two 580-nm narrow-band filters were placed in the beam, one below the stage and the second in front of the photomultiplier tube. The filtered monitoring beam was kept at an intensity sufficiently low that it caused no measureable change in rhabdom transmittance over the experimental times used. Results of experimental runs, both spectra and time-courses, were stored on tape or magnetic disks for analysis by the computer.

The quantum efficiency of the fluorescence at pH 9.2 and room temperature was determined by a comparison of the integrated emission spectra of crayfish rhabdoms and a solution of Rhodamine B in ethanol (Parker and Rees, 1960). Rhodamine B was selected as the standard because its absorption resembles that of crayfish metarhodopsin and its emission spectrum falls entirely within the spectral range of our instrument, which allows an accurate determination of photon emission. Its quantum efficiency for fluorescence was taken to be 0.97 (Weber and Teale, 1957).

Excitation was at 490 nm, with the exciting beam polarized parallel to the long axis of the rhabdom. Emission was collected from identical areas of either crayfish rhabdom or Rhodamine B standard contained in a microcell with a 50-$\mu$m path length (Vitro Dynamics, Inc., Rockaway, N.J.). Rhodamine B absorptance was measured on the microscope at 546 nm, and the absorptance at 490 nm was calculated from the known absorption spectrum. The 490-nm absorptance of each rhabdom from which an emission spectrum was obtained was found by determining the difference spectrum before and after bleaching with long-wavelength light (520–590 nm). At high pH, crayfish photopigment can be completely bleached by such exposure (Goldsmith, 1978). These difference spectra were obtained with the same instrument used to measure fluorescence but with the substage illuminator used as a single-beam microspectrophotometer, the measuring beam polarized parallel to the long axis of the rhabdom. On the basis of the proportion of pigment present as metarhodopsin during the 490-nm excitation, we calculated that 50% of the absorptance was due to metarhodopsin. Absorptances of Rhodamine B and metarhodopsin were <0.025.

Quantum efficiency of fluorescence was calculated from the expression $\phi_m = \phi_s E_m A_s / E_s A_m$, where $\phi_m$ and $\phi_s$ are the quantum efficiencies of metarhodopsin and the Rhodamine B standard, $A_m$ and $A_s$ the absorptances at 490 nm (the exciting wavelength), and $E_m$ and $E_s$ the areas under the fluorescence emission spectra. $E_s$ was determined by summing the trapezoidal areas bounded by the corrected photon counts measured at 9-nm intervals over the full width of the emission spectrum of Rhodamine B. Because the rhabdom emission spectrum extended beyond our instrument's spectral range, its shape was assumed to resemble that of retinal with a band width at half maximum of 3,700 $cm^{-1}$. This required correction of the measured emission ($E_m$) by a factor of 1.78 to include emission at wavelengths >720 nm.

## RESULTS

### *Development of Fluorescence in Fully Dark-adapted Rhabdoms*

Rhabdoms from fully dark-adapted crayfish (≥24 h in the dark) are initially weakly fluorescent or nonfluorescent. Upon exposure to the excitation beam, their fluorescence increases with time until a stable final level is achieved (Fig. 1, solid trace). Following the establishment of this stable level, a second light exposure of the same rhabdom immediately produces this same emission level (Fig. 1, dotted trace). The time-course of the initial appearance of fluorescence is well fit by a single exponential curve (Fig. 1, smooth curve superimposed on the solid trace). Fluorescence appears in unfixed material with the same

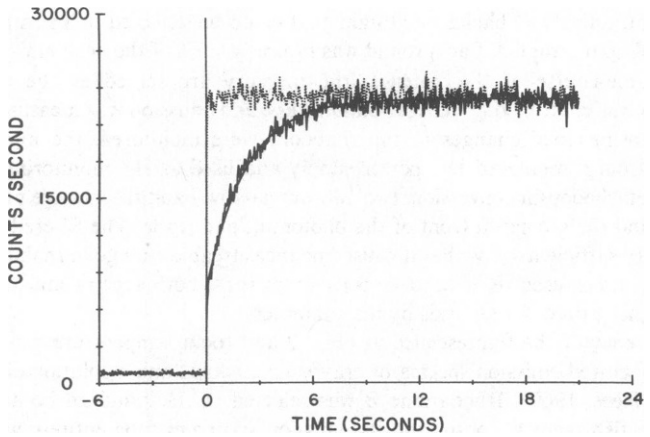


FIGURE 1 Time-course of the appearance of fluorescence in a previously dark-adapted rhabdom. Excitation at 450 nm, pH 7.5; the photon flux was ~7.3 × $10^{13}$ quanta $cm^{-2}$ $s^{-1}$. Excitation began at 0 s. Fluorescence was monitored at $\lambda$ >600 nm. The solid trace shows the measured photon count rate in a single experiment; the smooth curve is an exponential fit to these data. In this example, the time constant was 2.24 s. The dotted trace shows the time course of fluorescence after a second excitation of the same rhabdom. Note the immediate rise to the steady state level of the previous exposure, which indicates that the fluorescent substance formed during the first exposure persisted in the dark period between exposures.

kinetics as in the formaldehyde-fixed rhabdoms, which indicates that this fluorescence is not a fixation artifact (not shown). First-order kinetics are expected for the approach to a photosteady state mixture of rhodopsin and metarhodopsin (Hochstein et al., 1978). Since conversion of rhodopsin to metarhodopsin also proceeds from the onset of light, metarhodopsin is an obvious candidate for the source of the fluorescence.

### *Change in Transmittance upon Exposure to the Excitation Source Parallels the Change in Fluorescence*

If fluorescence originates from metarhodopsin, the rate of increase of fluorescence should reflect the rate of conversion of the original population of rhodopsin molecules in the rhabdom to the photosteady state mixture of rhodopsin and metarhodopsin. Since the absorption spectrum of crayfish metarhodopsin is shifted to wavelengths shorter than that of rhodopsin (see Fig. 2, inset), the appearance of metarhodopsin causes an increase in transmittance at long wavelengths. In order to measure the formation of metarhodopsin, the transmittance change at 580 nm was therefore monitored during exposure to the 450-nm excitation beam (Fig. 2). This change also is well described by a single exponential function. Although it is difficult to follow simultaneously the changes in fluorescence and transmittance in single rhabdoms, averaged curves obtained from large numbers of rhabdoms follow very similar time courses of fluorescence and transmittance change at a given intensity (Fig. 3).

### *Fluorescence and Transmittance Changes Show I-t Reciprocity*

A photochemical reaction should exhibit intensity-time reciprocity; the time constant of the process should be inversely proportional to the photon flux. The time constants of both transmittance and fluorescence change are not only equal over the range of intensities tested (1.5 log U), but they also follow closely the predicted inverse proportionality to intensity (Fig.

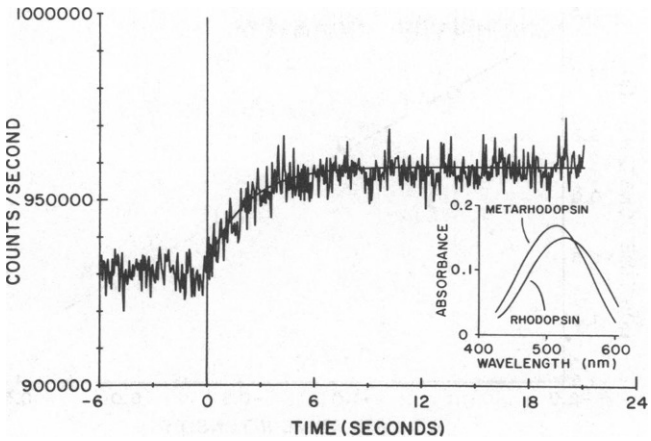


FIGURE 2 Time-course of increase in transmittance at 580 nm in a previously dark-adapted rhabdom, produced by the 450-nm light used to excite fluorescence. Other conditions were as in Fig. 1, but in this plot the ordinate scale has been expanded. At 0 s the excitation shutter was opened. The experimental trace shows the actual increase in transmittance (as measured count rate); the smooth curve is an exponential fit to these data (time constant, 2.22 s). Since only high-intensity light of 580 nm was monitored, the weak fluorescence emission at this wavelength does not contribute significantly to the measured change. *Inset:* Bleaching difference spectra of crayfish rhodopsin and metarhodopsin in rhabdoms exposed to 0.75% formaldehyde at pH 9, replotted from Goldsmith, 1978.

4). These results further support the origin of fluorescence from metarhodopsin and indicate the mainly photochemical nature of the rhodopsin-metarhodopsin transition under these conditions.

### *The Amount of Fluorescence Observed Can be Shifted in the Same Manner as the Metarhodopsin-Rhodopsin Composition of the Rhabdoms*

Because rhodopsin and metarhodopsin have different absorption spectra (see Fig. 2, inset), exposure to varying wavelengths of light will lead to varying photosteady-state mixtures of the

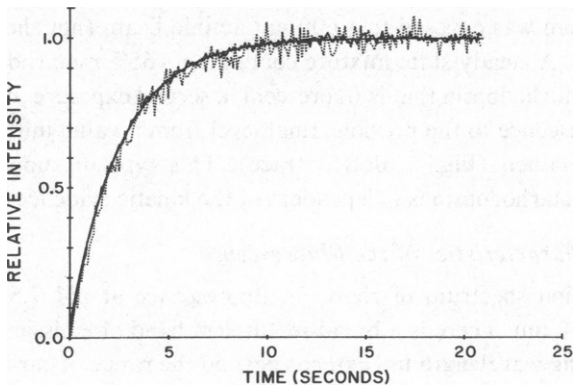


FIGURE 3 Average curves for change in fluorescence (solid trace, 19 rhabdoms) or transmittance (dotted trace, 24 rhabdoms). Conditions of exposure as in Fig. 1; all experimental curves were normalized before being averaged. The time-courses of both processes are very similar and are well described by the same exponential curve (smooth curve), with a time constant at this intensity of 2.60 s.

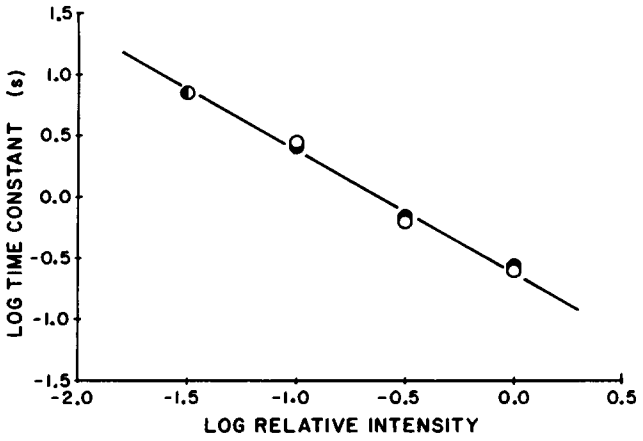


FIGURE 4 Time constants for averaged data for changes of fluorescence (●) and transmittance (○), plotted on a log scale, vs. log intensity. All data were collected at pH 7.5, and 16–24 individual measurements were averaged for each point. The intensity at $\log I = 0$ was $7.2 \times 10^{14}$ quanta $cm^{-2} s^{-1}$; the intensity at each point was measured with a calibrated photodiode placed in the position of the experimental preparation and was corrected for excitation spot size. The wavelength was 450 nm. That the line has slope = −1 indicates an inverse relationship between intensity and time constant. Higher photon fluxes were not measured because of limited instrument response times, and at lower fluxes the time courses were so long that the rhabdoms began to deteriorate.

two pigments. For example, at pH 7.5, and on the assumption of approximately equal quantum efficiencies for the reaction in both directions, photosteady states produced by short wavelength irradiation contain predominately rhodopsin, since metarhodopsin absorbance is greater than rhodopsin absorbance at wavelengths <530 nm. Conversely, with irradiation at long wavelengths, metarhodopsin predominates in the photosteady state. An experiment using these alterations in photosteady-state is illustrated in Fig. 5. The solid trace illustrates the development of fluorescence in a previously dark-adapted rhabdom exposed to 450-nm light. At this wavelength, the photosteady state mixture should contain ~40% metarhodopsin, as calculated from the relative absorbancies of the two pigments (Fig. 2, inset). After this initial exposure, the rhabdom was exposed to a 600-nm actinic beam (not shown) produced by the substage illuminator. A steady-state mixture containing ~65% metarhodopsin was produced. If it is chiefly the metarhodopsin that is fluorescent, a second exposure to 450 nm should cause a reduction of fluorescence to the previous final level from a value initially higher by ~50%. Such a result is obtained (Fig. 5, dotted trace). This type of support for the origin of fluorescence from metarhodopsin is independent of the kinetic evidence previously presented.

### *Spectral Characteristics of the Fluorescence*

The averaged emission spectrum of rhabdom fluorescence at pH 7.5 is plotted in Fig. 6. Excitation was at 450 nm. There is a broad featureless band of emission with a maximum at 660–670 nm. The long wavelength tail extends beyond the range of our instrument.

In obtaining the excitation spectrum, the emission was measured at 670 nm. By monitoring at the spectral peak, any possible contributions of other fluorophores or other extraneous light sources were minimized. The resulting excitation spectrum is also plotted in Fig. 6 (dotted curve), and has a $\lambda_{max}$ at ~525 nm. The excitation spectrum of unfixed rhabdoms is very

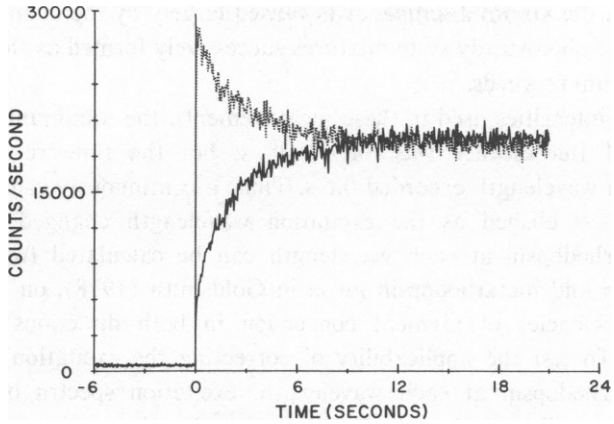


FIGURE 5 Alterations in fluorescence related to changes in the proportions of metarhodopsin in the photosteady state. The solid trace shows the development of fluorescence in a previously dark-adapted rhabdom (pH 7.5, excitation at 450 nm). After the establishment of a steady-state level of fluorescence, the rhabdom was exposed to 600-nm light for 2 min (not shown). The dotted trace was then obtained with a second exposure of the same rhabdom to 450 nm, and it reveals a decline in fluorescence to the same final level. This corresponds to the reduction in the proportion of metarhodopsin from ~65% in the 600-nm photosteady state to ~40% in the 450-nm photosteady state.

similar to this curve, which again indicates that the fluorescent component of the rhabdom is not significantly changed by paraformaldehyde. Metarhodopsin in aldehyde-fixed rhabdoms, however, has its maximum absorption at ~515 nm at pH 9, and has very similar absorption characteristics at pH 7.5 (Goldsmith, 1978), so that there is not an exact match between this excitation spectrum and the absorption of metarhodopsin. As is shown in the following

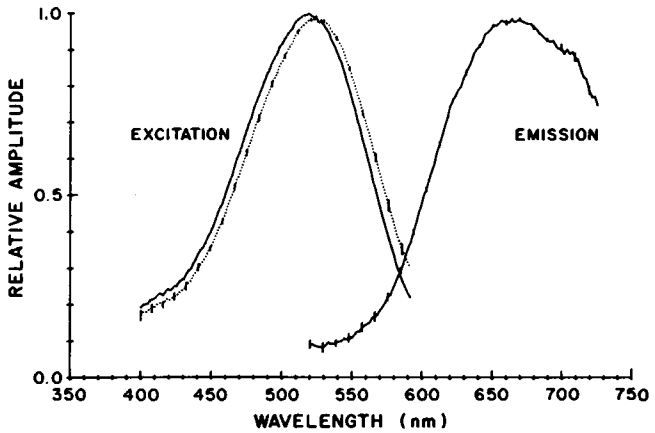


FIGURE 6 Fluorescence excitation and emission spectra at pH 7.5. The ordinate is a relative scale of photon flux per unit wavelength interval. The emission spectrum is an average (from six rhabdoms); vertical bars represent standard errors of the mean at ~9-nm intervals. The dotted excitation spectrum is an average from 21 rhabdoms measured under identical conditions, with emission at 670 nm, and is uncorrected for shifts in the photosteady state during measurement. The solid excitation spectrum is a replot of the dotted curve after correction at each wavelength for the proportion of metarhodopsin in the photosteady state mixture (see text for details).

paragraph, however, the 10-nm discrepancy is caused largely by the changing proportion of metarhodopsin in the photosteady state mixtures successively formed as the measurement of the excitation spectrum proceeds.

At the excitation intensities used in these measurements, the minimum time constant for the development of fluorescence was only 0.05 s, but the time required to measure fluorescence at each wavelength exceeded 0.6 s. Thus, a continuously changing photosteady state mixture was established as the excitation wavelength changed. The equilibrium proportion of metarhodopsin at each wavelength can be calculated from the absorption spectra of rhodopsin and metarhodopsin given in Goldsmith (1978), on the assumption of equal quantum efficiencies of pigment conversion in both directions (see Minke and Kirschfeld, 1979). To test the applicability of correcting the excitation spectrum for the proportion of metarhodopsin at each wavelength, excitation spectra of rhabdoms were measured under two conditions: (1) new steady state produced at each excitation wavelength (high excitation intensity, long waits at each wavelength before measuring the emission, and lengthy measurements) and (2) re-establishment of the photostationary state at a standard wavelength between each pair or measurements (low excitation intensity, minimal waits, and brief measurements, causing minimal departure from the reference photosteady state at each test wavelength). These excitation spectra are plotted in Fig. 7. The spectrum measured under condition 2 (curve 2) is hypsochromically displaced ~10 nm relative to the spectrum measured with a continuously changing photosteady state (condition 1) and corresponds more closely to the absorption spectrum of metarhodopsin. The spectrum measured with the changing photosteady state (curve 1) is similar, but not identical, to the dotted curve of Fig. 6.

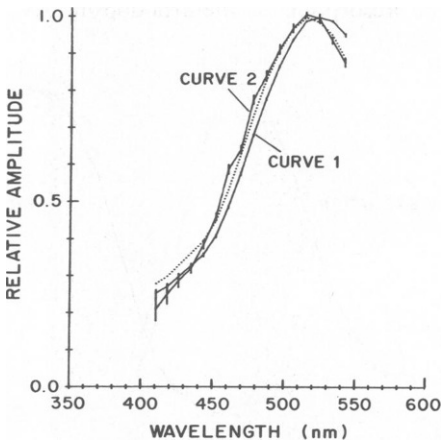


FIGURE 7 Excitation spectra of rhabdoms measured under conditions of continually changing photosteady-state amounts of metarhodopsin and with a constant metarhodopsin concentration. Excitation that allowed the continuous establishment of a new photosteady-state mixture of rhodopsin and metarhodopsin is plotted in curve 1 (average of 10 rhabdoms, 1 scan/rhabdom). Curve 2 shows the excitation spectrum obtained from the same 10 rhabdoms (2 scans/rhabdom) with a constant proportion of metarhodopsin maintained by repeated equilibrating exposures to light of a set wavelength (in this case, 520 nm) between each pair of exciting wavelengths. The error bars are longer for curve 2 than for curve 1 because of the lower quantum fluxes used for measurement. The dotted curve is a replot of curve 1 with correction for the calculated amounts of metarhodopsin in successive photosteady-state mixtures.

The small difference probably arises because emission at all wavelengths >600 nm was used to obtain these spectra (because of the very low photon fluxes required in condition 2), whereas in Fig. 6 only emission at 670 nm was measured. If the spectrum of rhabdoms with the varying photosteady state (condition 1) is corrected by dividing by the calculated steady-state proportion of metarhodopsin at each wavelength, the resulting curve (Fig. 7, dotted curve) corresponds well to the curve measured at a single metarhodopsin concentration (condition 2), particularly at wavelengths above 450 nm. This indicates both that the shift in the spectral position of the excitation spectrum is due to changing metarhodopsin concentration and that the calculated proportions of metarhodopsin are reasonably accurate. The entire excitation spectrum (Fig. 6, dotted curve) was therefore similarly corrected for the steady-state proportion of metarhodopsin at each wavelength. This corrected spectrum (Fig. 6, solid excitation spectrum) with a $\lambda_{max}$ at 518 nm is similar to the published absorption spectrum for metarhodopsin with $\lambda_{max}$ at 515 ± 2 nm (Goldsmith, 1978; see Fig. 2, inset).

*Dependence of the Fluorescence Spectra on pH*

The absorption spectra of crayfish rhodopsin and of metarhodopsin are both shifted hypsochromically at lowered pH (Goldsmith, 1978). Similarly, both the excitation and emission spectra for rhabdom fluorescence are shifted to shorter wavelengths at pH 1.9 (Fig. 8), with the excitation maximum at 460 nm and the emission maximum ~650 nm. Because the absorption spectra of crayfish rhodopsin and metarhodopsin are not accurately known at pH 1.9, the excitation curve cannot be corrected for changing proportions of metarhodopsin, but experiments similar to those of Fig. 7 suggest that the true excitation maximum is ~470 nm. In any case, the hypsochromic spectral shift and new location of the excitation maximum further implicate crayfish metarhodopsin as the source of fluorescence.

*The Quantum Yield of Fluorescence*

The quantum efficiency of fluorescence was measured for 10 individual rhabdoms at pH 9.2 and at room temperature, as described above in Methods. The calculated quantum yield was

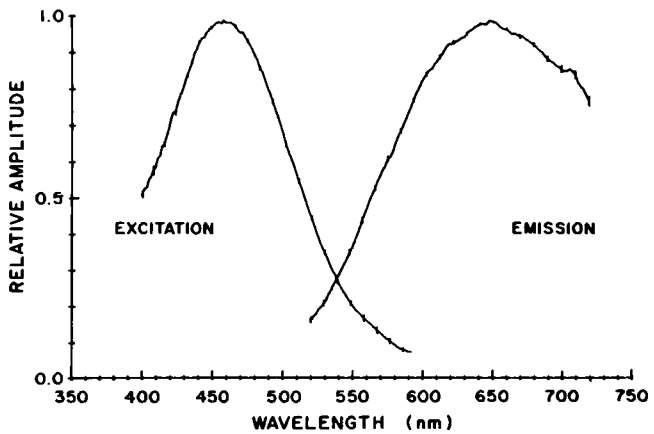


FIGURE 8 Average fluorescence excitation ($n$ = 25) and emission ($n$ = 25) spectra of crayfish rhabdoms at pH 1.9. Standard errors are indicated at 9-nm intervals.

$1.6 \pm 0.4 \times 10^{-3}$. The precision of ±25% was obtained by considering the errors associated with each of the several measurements on which the calculation is based.

## DISCUSSION

### *Fluorescence of Crayfish Metarhodopsin*

Several independent types of measurements made in this study all indicate that crayfish metarhodopsin is fluorescent. First, the fluorescence develops in fully dark-adapted receptors with first-order kinetics. The time constants of both fluorescence increase and metarhodopsin formation, measured as transmittance change, are equal to each other, and both are inversely proportional to the photon flux at the rhabdom over the range of intensities measured. Second, rhabdoms which have been previously irradiated with long wavelength light to produce maximal concentrations of metarhodopsin show an initial decline in fluorescence upon excitation with shorter wavelengths. Finally, when appropriately corrected, the excitation spectra at neutral and low pH are similar to the absorption spectra of metarhodopsin at the corresponding pH. Taken together, the experimental evidence shows that crayfish metarhodopsin is fluorescent, emitting most strongly at ~670 nm. The emission and excitation spectra for metarhodopsin are separated by a wider interval than is common to most fluorescent organic compounds, with a Stokes shift of 4,400 $cm^{-1}$ at neutral pH, and 6,350 $cm^{-1}$ at pH 1.9.

The time-course of appearance of fluorescence thus reflects the changing proportions of crayfish rhodopsin and metarhodopsin in the rhabdom, and eventually reaches a steady-state level when transitions in both directions become equal. The formation time of individual metarhodopsin molecules from their rhodopsin precursors is probably at least an order of magnitude more rapid than the fastest time constants measured here, which would be too rapid to influence our measurements of time constants for the photoconversion of the population of molecules to the photosteady state mixture.

### *Other Fluorescence Sources Located in Photoreceptors*

Although this paper is the first description of emission from the chromophore site of any rhodopsin-metarhodopsin system (see Busch et al., 1972; Lewis et al., 1976), Stavenga and Franceschini (1981) report that the metarhodopsin of flies also fluoresces in the red. Fluorescence has been observed from aromatic amino acids of vertebrate rhodopsins (e.g., Ebrey, 1972), and from retinol in photoreceptors (Hagins and Jennings, 1959; Eakin and Brandenburger, 1978), as well as from *N*-retinyl-opsin (Ebrey, 1971).

Bacteriorhodopsin from *Halobacterium halobium* has fluorescence characteristics similar to those of crayfish metarhodopsin (Lewis et al., 1976; Gillbro et al., 1977; Govindjee et al., 1978). In the bacterial photopigment, emission at room temperature occurs with a broad maximum in the spectral range 710–790 nm, the particular peak value differing in each report. Although the shape of the emission spectrum, the general spectral location, and the values reported for the Stokes shift (4,850 $cm^{-1}$, Lewis et al., 1976; >3,000 $cm^{-1}$, Govindjee et al., 1978) are similar to our findings for crayfish metarhodopsin, the quantum yield of fluorescence from crayfish metarhodopsin ($1.6 \times 10^{-3}$) is higher than that from bacteriorhodopsin at room temperature ($1.2–2.4 \times 10^{-4}$, Lewis et al., 1976; $2.4 \times 10^{-5}$, Govindjee et al., 1978). As in metarhodopsin, the chromophore of bacteriorhodopsin is in the all-*trans*

configuration. Interestingly, while all-*trans* retinal is weakly fluorescent, the 11-*cis* isomer is not (Balke and Becker, 1967); all-*trans* retinal fluorescence is only observed when the retinal is hydrogen bonded (Takemura et al., 1978). Visual proteins which have stable conformations bearing the all-*trans* chromophore thus seem promising candidates for further fluorescence studies.

In addition to the newly discovered fluorescence from fly metarhodopsin, several other fluorescent components have been observed in the rhabdoms of flies. In the peripheral rhabdomeres R1-6 of each ommatidium of the fly eye there is a UV-excited fluorescence independent of the photopigment state (Franceschini, 1977; Stark et al., 1979). The source of this fluorescence is most likely the accessory pigment postulated to confer UV sensitivity upon these rhabdomers (Kirschfeld et al., 1977; Minke and Kirschfeld, 1979). In some of the central rhabdomeres (R7) of flies there is located a photostable pigment (Kirschfeld et al., 1978) that is also fluorescent (Franceschini, 1978). Finally, there is a fluorescent pigment photoproduced from fly rhodopsin, but it requires greater intensities for its formation than does fly metarhodopsin (Franceschini, 1978; Franceschini et al., 1981; Stark et al., 1979). It is not yet clear how this compound is related to the metarhodopsin of flies, although it is spectrally similar to and probably derived from metarhodopsin (Stavenga and Franceschini, 1981).

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## REFERENCES

Balke, D. E., and R. S. Becker. 1967. Spectroscopy and photochemistry of all-*trans* retinal and 11-*cis*-retinal. *J. Am. Chem. Soc.* 89:5061–5062.

Busch, G. E., M. L. Applebury, A. A. Lamola, and P. M. Rentzepis. 1972. Formation and decay of prelumirhodopsin at room temperature. *Proc. Natl. Acad. Sci. U.S.A.* 69:2802–2806.

Collins, J. S., and T. H. Goldsmith. 1981. Spectral properties of fluorescence induced by glutaraldehyde fixation. *J. Histochem. Cytochem.* 29:411–414.

Das, P. K., G. Kogan, and R. S. Becker. 1979. Spectroscopy of polyenes. III. Absorption and emission spectral investigation of polyene Schiff bases and protonated Schiff bases related to visual pigments. *Photochem. Photobiol.* 30:689–695.

Eakin, R. M., and J. L. Brandenburger. 1978. Autofluorescence in the retina of a snail, *Helix aspersa*. *Vision Res.* 18:1541–1544.

Ebrey, T. G. 1971. Energy transfer in rhodopsin, N-retinyl-opsin, and rod outer segments. *Proc. Natl. Acad. Sci. U.S.A.* 68:713–716.

Ebrey, T. G. 1972. The fluorescence from the tryptophans of rhodopsin. *Photochem. Photobiol.* 15:585–588.

Franceschini, N. 1977. *In vivo* fluorescence of the rhabdomeres in an insect eye. *Int. Congr. Physiol. Sci. Proc.* 13:237.

Franceschini, N. 1978. Bi-stable and photostable pigments in fly photoreceptor cells: evidence from 'ommatidial fundus fluoroscopy.' *Neurosci. Lett.* 1s(Suppl.):405.

Franceschini, N., K. Kirschfeld, and B. Minke. 1981. Fluorescence of photoreceptor cells observed *in vivo*. *Science (Wash. D.C.)*. In press.

Gillbro, T., A. N. Kriebel, and U. P. Wild. 1977. On the origin of the red emission of light adapted purple membrane of *Halobacterium halobium*. *Febs (Fed. Eur. Biochem. Soc.) Lett.* 78:57–60.

Goldsmith, T. H. 1978. The spectral absorption of crayfish rhabdoms: pigment, photoproduct, and pH sensitivity. *Vision Res.* 18:463–473.

Govindjee, R., B. Becher, and T. G. Ebrey. 1978. The fluorescence from the chromophore of the purple membrane protein. *Biophys. J.* 22:67–77.

Guzzo, A. V., and G. L. Pool. 1968. Visual pigment fluorescence. *Science (Wash. D.C.).* 159:312–314.

Guzzo, A. V., and G. L. Pool. 1969. Fluorescence spectra of the intermediates of rhodopsin bleaching. *Photochem. Photobiol.* 9:565–570.

Hagins, W. A., and W. H. Jennings. 1959. Radiationless migration of electronic excitation of retinal rods. *Discuss. Faraday Soc.* 27:180–190.

Hall, M. O., and D. Bok. 1976. Reduction of the retinal-opsin linkage in isolated frog retinas. *Exp. Eye Res.* 22:595–609.

Harreveld, A. D. van. 1936. A physiological solution for freshwater crustaceans. *Proc. Soc. Exp. Biol. Med.* 34:428–432.

Hochstein, S., B. Minke, P. Hillman, and B. W. Knight. 1978. The kinetics of visual pigment systems. I. Mathematical analysis. *Biol. Cybern.* 30:23–32.

Kahan, J. 1971. The fluorescence properties of vitamin A. *Methods Enzymol.* 18:574–591.

Kirschfeld, K., R. Feiler, and N. Franceschini. 1978. A photostable pigment within the rhabdomere of fly photoreceptors no. 7. *J. Comp. Physiol.* 125:275–284.

Kirschfeld, K., N. Franceschini, and B. Minke. 1977. Evidence for a sensitizing pigment in fly photoreceptors. *Nature (Lond.).* 269:386–390.

Lewis, A., J. P. Spoonhower, and G. J. Perreault. 1976. Observation of light emission from a rhodopsin. *Nature (Lond.).* 260:675–678.

Minke, B. and K. Kirschfeld. 1979. The contribution of a sensitizing pigment to the photosensitivity spectra of fly rhodopsin and metarhodopsin. *J. Gen. Physiol.* 73:517–540.

Parker, C. A., and W. T. Rees. 1960. Correction of fluorescence spectra and measurement of fluorescence quantum efficiency. *Analyst (London).* 85:587–600.

Stark, W. S., D. G. Stavenga, and B. Kruizinga. 1979. Fly photoreceptor fluorescence is related to UV sensitivity. *Nature (Lond.).* 280:581–583.

Stavenga, D., and N. Franceschini. 1981. Fly visual pigment states, rhodopsin 490, metarhodopsins M and M1, studied by transmission and fluorescence microspectrophotometry *in vivo. Invest. Ophthalmol. Visual Sci.* 20 (Suppl.):111.

Takemura, T., P. K. Oas, G. Hug, and R. S. Becker. 1978. Visual pigments. 8. Hydrogen bonding effects on fluorescence properties of retinals. *J. Am. Chem. Soc.* 100:2626–2630.

Waddell, W. H., A. M. Schaffer, and R. S. Becker. 1973. Visual pigments. III. Determination and interpretation of the fluorescence quantum yields of retinals, Schiff bases, and protonated Schiff bases. *J. Am. Chem. Soc.* 95:8223–8227.

Weber, G., and F. W. J. Teal. 1957. Determination of the absolute quantum yield of fluorescent solutions. *Trans. Faraday Soc.* 53:646–655.