Photosensitivity Spectrum of Crayfish Rhodopsin Measured Using Fluorescence of Metarhodopsin

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ABSTRACT Discrepancies exist among spectral measurements of sensitivity of crayfish photoreceptors, their absorption *in situ*, and the number and absorption spectra of crayfish photopigments that are extracted by digitonin solutions. We have determined the photosensitivity spectrum of crayfish rhodopsin in isolated rhabdoms using long wavelength fluorescence emission from crayfish metarhodopsin as an intrinsic probe. There is no measurable metarhodopsin in the dark-adapted receptor, so changes in the emission level are directly proportional to metarhodopsin concentration. We therefore used changes in metarhodopsin fluorescence to construct relaxation and saturation ("photoequilibrium") spectra, from which the photosensitivity spectrum of crayfish rhodopsin was calculated. This spectrum peaks at ~530 nm and closely resembles the previously measured difference spectrum for total bleaches of dark-adapted rhabdoms. Measurements of the kinetics of changes in rhabdom fluorescence and in transmittance at 580 nm were compared with predictions derived from several model systems containing one or two photopigments. The comparison shows that only a single rhodopsin and its metarhodopsin are present in the main rhabdom of crayfish, and that other explanations must be sought for the multiple pigments seen in digitonin solution. The same analysis shows that there is no detectable formation of isorhodopsin in the rhabdom.

INTRODUCTION

The visual pigments and visual sensitivity of crayfish present perplexing anomalies. Spectral sensitivity of both the ERG (Kennedy and Bruno, 1961; Goldsmith and Fernandez, 1968; Wald, 1968) and of single retinular cells of the main rhabdom (Kong and Goldsmith, 1977) peak at ~560–565 nm, but the maxima can be at even longer wavelengths if care is not taken to prevent distortions by the migratory screening pigments (Nosaki, 1969; Waterman and Fernandez, 1970).

Microspectrophotometry (MSP) of single rhabdoms reveals an absorption with λ_{max} at 525–530 nm that is converted by light to a 515-nm metarhodopsin. (There is also a small, eighth retinular cell whose rhodopsin has λ_{max} at 450 nm [Cummins and Goldsmith, 1981], but it is not of concern in the present study.) The simplest interpretation of these MSP data is that the visual

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pigment absorbs maximally at 530 nm (Goldsmith 1978 *a*), and that the 565-nm maximum in the spectral sensitivity function is a result of screening of short wavelengths by the nonmigratory accessory pigments of the eye (Goldsmith, 1978 *b*). Studies of a white-eyed mutant of the crayfish *Procambarus* provide additional support for this interpretation (Kong and Goldsmith, 1977).

Digitonin extracts of crayfish photoreceptors, however, contain two light-sensitive pigments, with λ_{max} at 562 and 512 nm. The former bleaches through a metarhodopsin with λ_{max} at ~515 nm, but this intermediate is distinct from the 512-nm pigment originally present in the extract (Fernandez, 1965; Wald, 1967; Larrivee and Goldsmith, 1982).

What sense is one to make of the digitonin extracts? There are two possible explanations. The 562- and 512-nm pigments that are seen in digitonin solution might be artifacts of extraction and have no direct counterparts in the rhabdomal membranes. This explanation represents an interesting possibility because rhodopsins usually maintain their native absorption properties when recovered from the photoreceptor membranes by digitonin. Although shifts in absorption upon extraction of arthropod pigments are known (Bruno and Goldsmith, 1974), simultaneous hypsochromic and bathochromic shifts are unprecedented. There is no electrophysiological evidence for the 512-nm pigment in vivo, nor is it seen with MSP of isolated rhabdoms. As described above, the 565-nm spectral sensitivity function can be accounted for by screening effects on a single 530-nm rhodopsin (Goldsmith, 1978 *b*).

Alternatively, the MSP data might have been misinterpreted, and the 530-nm absorption band could represent a mixture of two pigments with λ_{max} at 562 and 512 nm. Although most of the evidence suggests that this is not the case (Goldsmith, 1978 *a*), it has been difficult to devise a microspectrophotometric experiment that eliminates this alternative. The recent discovery that crayfish metarhodopsin fluoresces (Cronin and Goldsmith, 1981) provides us with the opportunity.

In this paper we show that crayfish rhodopsin does not fluoresce measurably and that rhabdoms from healthy, dark-adapted animals contain no metarhodopsin. We then demonstrate that the photosensitivity spectrum for forming metarhodopsin (and thus the fluorescence signal) approximates the 530-nm absorption band in dark-adapted rhabdoms, rather than either of the pigments seen in digitonin extracts. Finally, we show by comparison with model pigment systems that the kinetics of the photoconversions occurring in the rhabdom indicate the presence of a single visual pigment.

MATERIALS AND METHODS

Experimental Preparation

The crayfish species *Orconectes rusticus* (Conn. Valley Biological Supply Co., Southhampton, Mass.) and, for some experiments, *Procambarus clarkii* (Carolina Biological Supply Co., Burlington, N. C.) were used for study. Both the maintenance of animals and the preparation of rhabdom suspensions for study were as previously described (Cronin and Goldsmith, 1981). Preparations were fixed for 20–40 min in 0.75%

formaldehyde and maintained in pH 7.5 crayfish saline (van Harreveld, 1936) unless otherwise noted. All fluorimetric work was performed on single rhabdoms devoid of screening pigment, using the system described in Cronin and Goldsmith (1981). The excitation source was a 12-V, 100-W quartz-halogen lamp run from a regulated DC power supply. Excitation wavelength was controlled by narrow-band interference filters (Ditric Optics, Inc., Hadson, Mass.; half bandwidth 5–12 nm), and intensity was regulated by neutral density filters (Ditric Optics). Emission was measured at wavelengths >600 nm (600-nm long-pass interference filter; Ditric Optics) unless otherwise noted. When changes in transmittance were monitored, the technique described in Cronin and Goldsmith (1981) was followed.

Analytical Method

The kinetics of a photoreversible pigment system such as the one comprised of a rhodopsin (R) and its photoproduct metarhodopsin (M) have recently been analyzed (Hochstein et al., 1978; following the work of others such as Hamdorf et al., 1973; and Stavenga, 1975 *a*). At a given wavelength (λ) and photon flux (I), the rate of change of the fractional composition of metarhodopsin (f_M) in the pigment mixture is

$$df_M/dt = f_R K_R I - f_M K_M I \tag{1}$$

where $f_M + f_R = 1$, and K_R and K_M are the photosensitivies of the rhodopsin and metarhodopsin. Photosensitivity is defined as the product of the molecular absorbance at the wavelength of interest (α_λ) and the quantum efficiency (γ) for the transition to the alternate stable state (Dartnall, 1972). At the photosteady state, $df_M/dt = 0$, so that

$$f_M K_M = f_R K_R. \tag{2}$$

Defining F_M as the fractional composition of M in the photosteady state, it follows that

$$F_M = K_R/(K_R + K_M). \tag{3}$$

Thus, at each wavelength, the photosteady state concentrations of metarhodopsin and rhodopsin will depend only on their photosensitivities. A dependency of F_M or F_R on wavelength is called the *saturation spectrum* (Hochstein et al., 1978), photoequilibrium spectrum (Minke and Kirschfeld, 1979), Q-function (Stavenga, 1975 *a*), or R spectrum (Hamdorf et al., 1973).

The time-course by which this photosteady state is achieved can be obtained by integrating Eq. 1:

$$f_M(t) = F_M - [F_M - f_M(0)]\exp(-(K_R + K_M)It). \tag{4}$$

This exponential has a rate constant of $(K_R + K_M)I$. The sum of the photosensitivities of R and M is called the relaxation constant, and the wavelength dependency of $(K_R + K_M)$ is called the *relaxation spectrum* (Hochstein et al., 1978). Note that the exponential rate of attainment of the photostationary state is completely determined by wavelength and intensity, and the value of the relaxation constant is not affected by initial photopigment composition.

It can be seen from an inspection of Eq. 3 that the photosensitivity spectrum for R is simply the product of the saturation spectrum of M (Eq. 3) and the relaxation spectrum. Thus

$$K_R(\lambda) = F_M(\lambda)\,[K_R + K_M](\lambda). \tag{5}$$

To summarize, by measuring the saturation and relaxation spectra of a two-pigment system, the spectral sensitivity of photoconversion for each pigment of the pair can be determined.

Measurement of the Relaxation Spectrum

The relaxation spectrum represents the wavelength dependence of the summed photosensitivities of the two stable photopigments, which can be determined from the rate constants associated with the formation of the photosteady state (Eq. 4). The relaxation constant is simply the measured rate constant divided by the photon flux I.

In determining the rate constants, dark-adapted rhabdoms were exposed to wavelengths from 429 to 562 nm (center wavelengths of excitation interference filters: 429, 451, 469, 491, 510, 521, 531, 540, 548, and 562 nm, as determined with a Cary 14 spectrophotometer). Exposure and collection of emission were made through a 63-×, 1.4-n.a. Zeiss objective (Carl Zeiss, Inc., New York). The intensity of the excitation was adjusted to give a convenient total time for establishment of a photosteady state (10–30 s, requiring photon fluxes on the order of 10^{15} cm^{-2} s^{-1}). Excitation intensity was measured using a photodiode (PIN-10/UV; United Detector Technology Inc., Santa Monica, Calif.) placed in the position of the sample. The intensity was measured for the entire field of excitation; this was then corrected to give the actual intensity in the center 43 μm of the field, which was the region occupied by the individual rhabdoms. The correction factor was obtained for each excitation wavelength by comparing the intensity of the entire excitation field with that passing through a 43-μm electron microscope objective aperture, which was mounted on a slide in the same way as the experimental rhabdoms.

Because the rhodopsin chromophore has a restricted orientation in the microvillar membrane and because there is a slight shift in orientation upon photoisomerization (Goldsmith and Wehner, 1977), the measured relaxation constants will vary with the degree and polarization of the excitation source and the orientation of its e-vector with respect to the rhabdom. Rhabdoms were always placed in the field with their long axes parallel to the axis of tilt of the chromatic beamsplitter in the excitation beam, and unpolarized light was used for excitation. Although the chromatic beamsplitter that reflected the excitation beam to the microscope objective imparted a small degree of polarization, the measured degree of polarization was only 0.07 at all wavelengths tested, except at 562 nm, where it was 0.24. Since the entire volume of the rhabdom was used for measuring fluorescence and since the excitation beam was only weakly polarized, the effects of both polarization and chromophore orientation were small and were not considered further.

Excitation exposure and collection of emitted light from rhabdoms was automatically performed by computer, as described in Cronin and Goldsmith (1981). Individual rhabdoms were exposed to the excitation beam for at least five time constants for photoconversion, during which 200 measurements of emission intensity were obtained, and the excitation shutter remained open while another 80 measurements were made. These final 80 measurements were averaged to define the steady state level of fluorescence, and the exponential rate of approach to this stable fluorescence then could be determined by log-linear regression. Emission was usually measured with a 600-nm long-pass interference filter in front of the photomultiplier tube, although in some cases a 680-nm narrow-band filter was used.

Measurement of the Saturation Spectrum

Although in theory the saturation spectrum could be obtained from the same data used to calculate the relaxation spectrum, the amount of metarhodopsin fluorescence

at a given concentration would depend upon both the excitation intensity and the excitation spectrum of the metarhodopsin. To permit greater accuracy, the following procedure was used instead.

Fully dark-adapted rhabdoms were excited by light of 510 nm and the photopigments were allowed to attain a photosteady state. The time-course was fitted with a first-order exponential curve that was back-extrapolated to 0 s, thus giving a measure of the initial level of fluorescence in the dark-adapted photoreceptor. This was taken to be the stable background level of fluorescence. (For justification see Fig. 1 and Results). The rhabdom was then exposed to light of a second wavelength, using the excitation optics, to produce a new photosteady state. A second exposure to 510 nm was made, under identical conditions to the first, and this new time-course was also analyzed and back-extrapolated to 0 s, to give a measure of the amount of metarhodopsin found in the alternate wavelength's photosteady state. The final level of fluorescence was again proportional to the metarhodopsin in the 510-nm photosteady state. Thus, the ratio

$$(\text{initial fluorescence} - \text{background})/(\text{final fluorescence} - \text{background})$$

indicates the relative amounts of metarhodopsin at the two conditions of saturation. Each rhabdom was exposed to another equilibrating wavelength, tested at 510 nm a third time, and then discarded. Two ratios of fluorescence were therefore obtained for each experimental rhabdom. The equilibrating wavelengths used were the same as those used to determine the relaxation spectrum with the addition of 571, 581, 602, and 662 nm. The entire saturation spectrum from 429 to 662 nm was thus scaled relative to the amount of metarhodopsin in the 510-nm photosteady state.

In a few cases in which the steady state concentration of metarhodopsin at the test wavelength was very close to that of 510 nm, it was not possible to carry out an exponential analysis. In these cases, a linear regression was performed on the first 25 points of the fluorescence curve, and the fluorescence at 0 s was calculated from this. The use of a linear regression was acceptable because of the very slight slope and curvature of the (actual) exponential fluorescence curve.

RESULTS

Absence of Metarhodopsin in the Dark-adapted Rhabdom

At alkaline pH both rhodopsin and metarhodopsin can be photobleached, but the intensities required for rapid bleaching are substantially higher than those that simply convert rhodopsin to metarhodopsin (Goldsmith, 1978 *a*). We have taken advantage of this property of crayfish photopigment to measure the metarhodopsin content of dark-adapted rhabdoms. Development of fluorescence caused by irradiation of dark-adapted rhabdoms at 510 nm was measured at pH 9, and a typical experiment is illustrated in Fig. 1. The time-course of fluorescence development (Fig. 1, upper trace) was well described by a single-exponential function (Fig. 1, smooth curve). The rhabdom was then fully bleached by a 12-min exposure to a high-intensity light in the wavelength range 520–580 nm, using the excitation source (bleaching curve not shown). After the bleach, a second exposure at 510 nm and low intensity was made (Fig. 1, lower trace). The level of background fluorescence and scatter by the rhabdom after the total bleach of photopigment was close to the intercept of the smooth exponential curve at $t = 0$. In 13 experiments, the initial level of fluorescence from dark-adapted rhabdoms, calculated from the

back extrapolation of the best-fit exponential curve, averaged 99.8 ± 5.3% (SEM) of the level of the residual signal after bleaching. If metarhodopsin were present in the fully dark-adapted rhabdom, the fluorescence level after the bleach would have been consistently lower than that observed at 0 s in the original exposure; since this was not seen, we conclude that the rise in fluorescence is proportional to the total concentration of metarhodospin in the rhabdom.

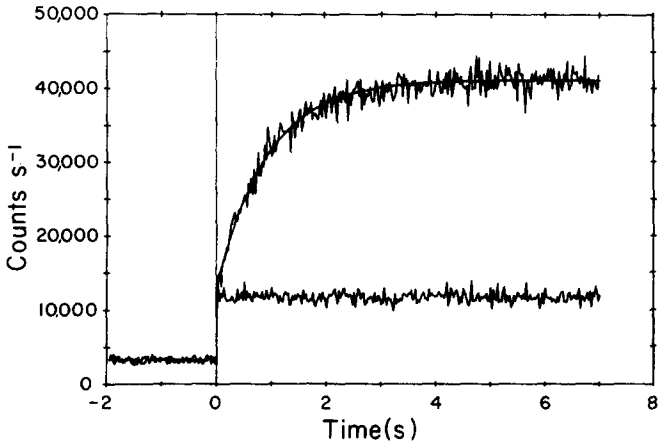


FIGURE 1. Metarhodopsin is absent in fully dark-adapted photoreceptors. The rhabdom was maintained at pH 9.2, a condition under which photobleaching of crayfish photopigment is possible (see text for details). The upper trace illustrates the appearance of fluorescence (photons counted per second) in a dark-adapted rhabdom (excitation at 510 nm, emission >600 nm), and is fit with a single-exponential function (smooth curve). The lower trace indicates the fluorescence after a total photobleach of metarhodopsin with long wavelength light, and illustrates the stability of the background. This stable background level is the same as the initial amount of fluorescence in the dark-adapted rhabdom (0 s intercept of upper trace or smooth curve), showing that there was little or no fluorescent photopigment initially present in the rhabdom.

Relaxation Spectrum

At all tested wavelengths, the curve of fluorescence development followed single-exponential kinetics. Fig. 2 shows, for four wavelengths, these time-courses of the increase in fluorescence and the exponential curves that were fitted to them. The variation in initial level of signal was probably due to spectral variations in reflection efficiency of the chromatic beamsplitter, scattering and reflection in the optics, and transmission of the barrier filter.

Relaxation constants were determined for 20–26 rhabdoms at each wavelength, and the average and normalized relaxation spectrum is plotted in Fig. 3. This curve has a maximum near 520 nm and declines smoothly on either side of this peak. In other words, the pigment system is driven most effectively to the photosteady state by light of wavelengths near 520 nm.

Saturation Spectrum

As described in Methods, the saturation spectrum was scaled relative to the amount of metarhodopsin fluorescence at 510 nm, defined as the fluorescence above the calculated level at 0 s. This was possible because (*a*) there is no metarhodopsin in the fully dark-adapted rhabdom, and (*b*) the background is stable.

We selected 510 nm as the reference wavelength for the saturation spectrum because the background signal at this wavelength was particularly low (see

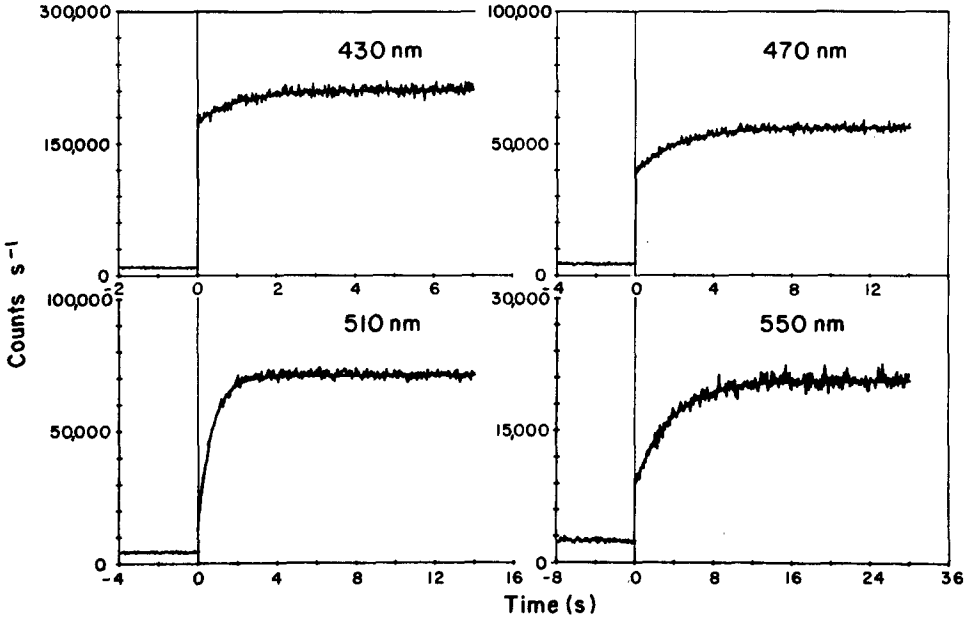


FIGURE 2. Appearance of fluorescence (photons counted per second) in fully dark-adapted rhabdoms with various excitation wavelengths. The excitation shutter was opened at 0 s, and emission was collected at $\lambda s > 600$ nm. Excitation wavelengths are indicated on the figure. The experimental fluorescence curves are well fit at each wavelength by single-exponential functions (smooth curves) whose time constants depend on the relaxation characteristics of the pigment system and the quantum flux at each wavelength. The variations in the initial heights of the fluorescence curves are probably due to spectral variations in the reflection efficiency of the chromatic beamsplitter, scattering in the optics, and transmission by the barrier filter.

Fig. 2). Fluorescence changes during the initial 510-nm exposure of two dark-adapted rhabdoms, and to a second 510-nm exposure after formation of a new photosteady state at a second wavelength, are plotted in Fig. 4. The top part shows the results obtained by using a wavelength <510 nm, which led to a reduction in the amount of metarhodopsin in the photosteady state. In the lower part of the figure, a rhabdom was saturated with light at a somewhat longer wavelength; in this case the steady-state metarhodopsin concentration

was considerably higher than at 510 nm. In both cases, several percent of the pigment was destroyed during the test irradiation, but as it is the ratio of the initial/final metarhodopsin concentration that is measured in the second 510-nm reference exposure, the small prior loss of pigment is inconsequential.

To determine the saturation spectrum between 429 and 662 nm, 10–12 individual rhabdoms were photoequilibrated to light of each test wavelength,

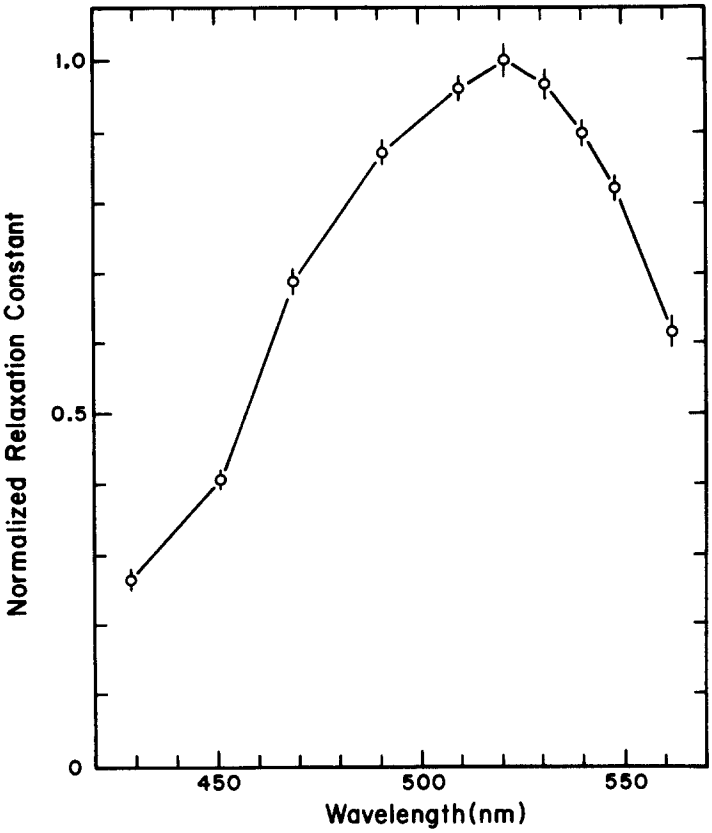


FIGURE 3. The relaxation spectrum of crayfish photopigment, determined from experiments such as those illustrated in Fig. 2. 20–26 rhabdoms were measured at each wavelength, and their individual relaxation constants were found by dividing the rate constant for the appearance of fluorescence (determined from best-fit single-exponential functions) by the photon flux at that wavelength. The plotted spectrum is normalized to the peak value; mean values and their standard errors are plotted for each test wavelength.

and the resulting spectrum is plotted in Fig. 5. There is a continuous increase in the steady-state proportion of metarhodopsin with exposure to wavelengths >450 nm. Very long exposure to wavelengths >662 nm leads to no further increases in metarhodopsin, so the point at 662 nm represents the maximum proportion of metarhodopsin attainable in any photosteady state.

In some other invertebrates with bistable visual pigments, it is possible

using red light to produce essentially 100% of one of the two states because their absorption coefficients are very different at long wavelengths. In such a situation, the saturation spectrum can be scaled to this 100% point, as has been done for the barnacle (Minke et al., 1978) or the fly (Minke and Kirschfeld, 1979; Stark and Johnson, 1980). Because the purpose of our study

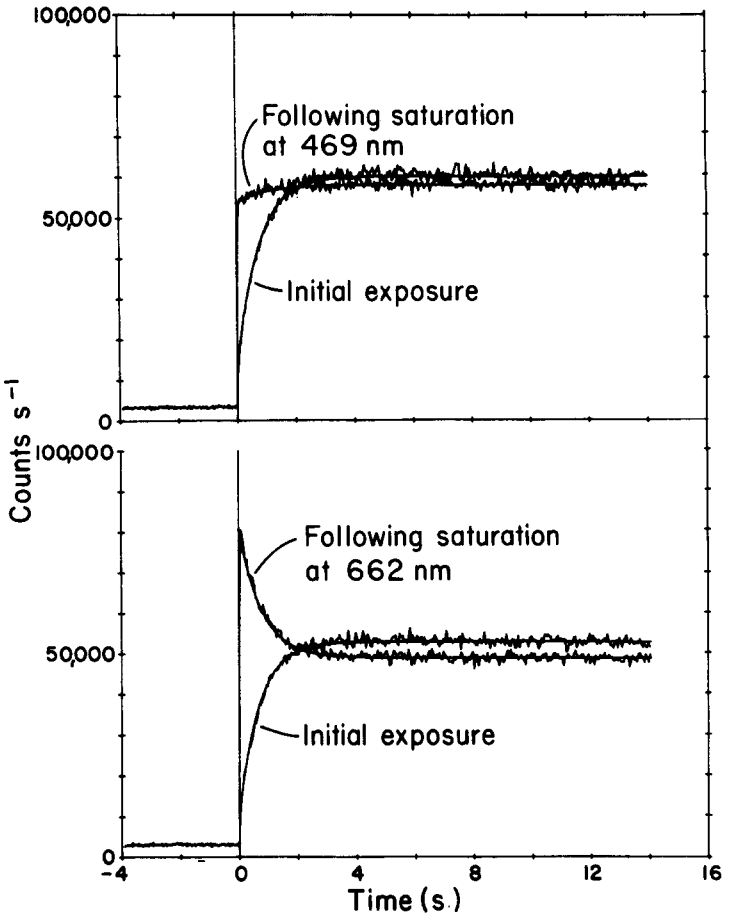


FIGURE 4. Changes in the steady-state metarhodopsin concentrations with various saturating wavelengths. Each of the two graphs shows the appearance of fluorescence upon excitation at 510 nm in an initially dark-adapted rhabdom and again after establishment of a photosteady state at a test wavelength, given on the figure. All traces are fit with computed single-exponential functions (smooth curves). In both cases, the second 510-nm saturation fluorescence level was lower than the first one, showing that a small amount of pigment was lost between the first and second measurements. The initial curve's intercept at 0 s gives the background signal level, used to determine the ratio of the initial:final fluorescence level during the second exposure (see Methods). In the upper part (λ_{test} = 469 nm), this ratio was 0.91; in the lower part (λ_{test} = 662 nm), it was 1.83.

is to determine the sensitivity characteristics of the visual pigment system, we make no assumptions about the amount of metarhodopsin created by long wavelength irradiation. However, the 0% point is established (by results of bleaching described above), so the relative heights of the spectral points are fixed. The absolute scale can only be determined with the aid of an independent measure of metarhodopsin concentration at one wavelength, but as we shall see, the absolute scale is not required for this study.

Photosensitivity Spectrum

As described by Eq. 5 in Methods, the product of the relaxation spectrum and the saturation spectrum is the photosensitivity spectrum. Because we do not know the actual units on the ordinate of the saturation spectrum we obtained,

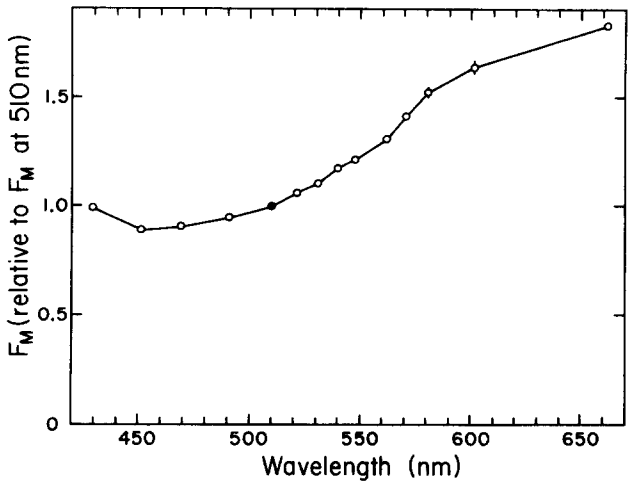


FIGURE 5. The saturation spectrum of crayfish metarhodopsin. The fraction of pigment as metarhodopsin in the photosteady state, F_M, relative to F_M at 510 nm, is plotted vs. wavelength. 10–12 rhabdoms were tested at each wavelength, as described in Methods and illustrated in Fig. 4. The standard errors are indicated when they are larger than the plotted symbols. Saturation at longer wavelengths than 662 nm produced no further increases in F_M.

we cannot calculate the absolute photosensitivity values for crayfish rhodopsin. However, since the saturation spectrum is plotted in relative units of metarhodopsin, its product with the normalized relaxation spectrum gives the actual shape of the photosensitivity spectrum in relative units. This spectrum is plotted in Fig. 6, and has a maximum near 530 nm. The difference spectrum derived from total photobleaches of crayfish rhodopsin in formaldehyde at pH 9.0 (Goldsmith, 1978 *a*) is also plotted in Fig. 6; it has a maximum at 526 nm and closely resembles the photosensitivity spectrum determined here using fluorescence measurements.

Because the ordinate scale of the saturation spectrum is unknown, it is not possible to determine by this analysis the photosensitivity spectrum of crayfish

metarhodopsin, even in relative units. However, the fluorescence excitation spectrum has been measured (Cronin and Goldsmith, 1981) and is similar to the MSP absorption measurements of crayfish metarhodopsin, having λ_{max} near 515 nm.

Photopigment Kinetics under Long Wavelength Irradiation

It has been demonstrated that the kinetics of both fluorescence and transmittance change are first order and have essentially identical rate constants when

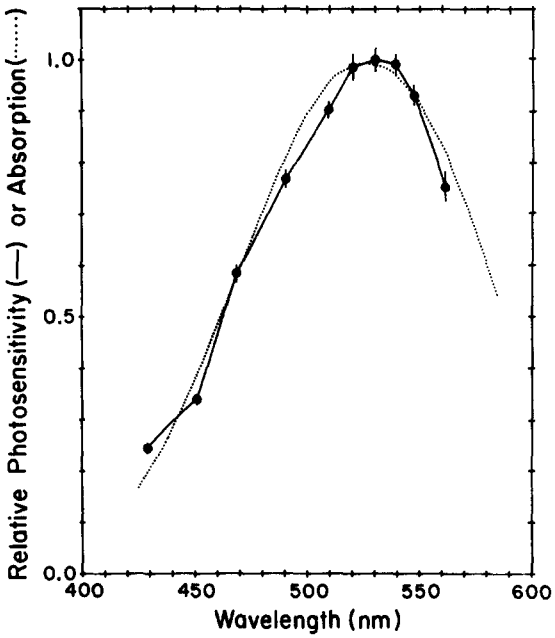


FIGURE 6. Photosensitivity spectrum of crayfish rhodopsin (solid curve), obtained by multiplying the relaxation spectrum (Fig. 3) by the metarhodopsin saturation spectrum (Fig. 5). The spectrum was obtained in relative units, because of the uncertainty associated with the ordinate scaling of the saturation spectrum, and is normalized to the peak at 531 nm. Standard errors, computed from the standard errors associated with the two source spectra, are included. The dotted curve represents the normalized absorption spectrum for crayfish rhodopsin, obtained from total bleaches of rhabdoms at pH 9.0 (Goldsmith, 1978 *a*).

rhabdoms are irradiated with 450-nm light (Cronin and Goldsmith, 1981; Fig. 7 A). To see whether this also holds with longer wavelength exposures, the previous work was repeated with 550 nm irradiation. Transmittance was measured at 582 nm, where the change is maximal. The results are given in Fig. 7 B; at this wavelength also, both time courses are again first order with virtually identical rate constants.

DISCUSSION

Use of Fluorescence to Measure Photosensitivity Spectra

As described in detail in the Introduction, this study was prompted by the confusing inconsistencies among the MSP-derived spectra, in vivo spectral sensitivities, and absorption spectra of pigments in extracts. Following the changes in metarhodopsin fluorescence is similar in principle to the use of electrophysiologically obtained M potentials (Minke and Kirschfeld, 1979), since both quantities are proportional indicators of metarhodopsin concentration. Monitoring fluorescence has one methodological advantage, in that it allows a complete analysis of relaxation from a single exposure of a rhabdom, because the excitation beam also serves as the actinic source. Electrophysiological or MSP measurements of relaxation require a series of measurements interspersed with actinic exposures, with concomitant increases in effort if not in experimental error. Since fluorescence of invertebrate metarhodopsins may be quite common (Cronin and Goldsmith, 1981; Stavenga and Franceschini, 1981), it can potentially serve as a useful intrinsic probe for exploration of the properties of invertebrate photopigments. Furthermore, in some preparations fluorescence and electrophysiological measurements can be made on the same receptors, allowing the simultaneous examination of both photochemical and electrical changes in the cell.

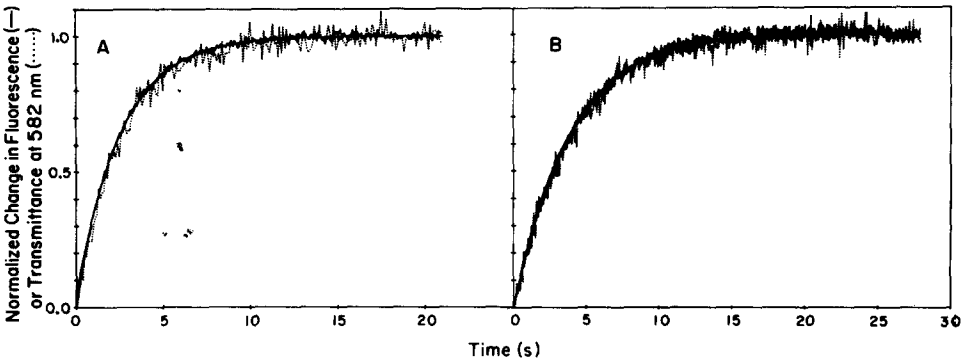


FIGURE 7. Averaged curves for change in fluorescence (solid traces) and transmittance at 582 nm (dotted traces) following exposure of dark-adapted rhabdoms to 450 or 548 nm light. A smooth single-exponential curve has been included in each case for comparison. (A) Exposure to 450 nm light, from Cronin and Goldsmith (1981). The fluorescence and transmittance changes are both well fit by one single-exponential function with a time constant at this intensity of 2.60 s. (B) Exposure to 548 nm light. Data were obtained in the present study, and 21 experiments were normalized and averaged for each curve. Again, one single-exponential fits both experimental curves (time constant = 3.85 s at this intensity).

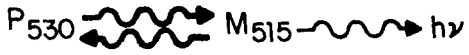
Absence of Metarhodopsin in the Dark-adapted Crayfish Photoreceptor

The absence of metarhodopsin in dark-adapted photoreceptors indicates that crayfish, like lobster (Goldsmith and Bruno, 1973; Bruno et al., 1977), can

physiologically regenerate rhodopsin in the dark. Although invertebrate, particularly arthropod, rhodopsin-metarhodopsin concentrations in the eye are sometimes thought to be primarily influenced by the wavelength distribution of incident light (see, for example, Hamdorf et al., 1973; Hamdorf, 1979), dark regeneration has also been demonstrated for some insects (Stavenga et al., 1973; Stavenga, 1975 *b*; Bernard, 1979). The mechanisms used for dark regeneration of arthropod photopigments are of considerable interest. The eyes of such animals are capable of producing massive turnover of photoreceptor membranes on time scales of hours or even minutes (White and Lord, 1975; Blest, 1978; Nässel and Waterman, 1979; Hafner and Bok, 1977; Chamberlain and Barlow, 1979; Stowe, 1980). Whether such synthetic activity is the sole means of replacing pigment molecules or whether there are supplementary enzymatic processes *in situ* remains to be determined.

The Single Rhodopsin of Crayfish Rhabdoms

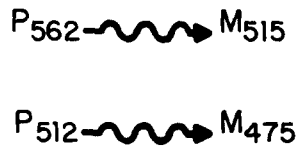
The experimental observations reported in this paper can be used to resolve some of the existing ambiguities that were outlined in the Introduction. For example, the photosensitivity spectrum (Fig. 6) shows that the precursor to the 515-nm metarhodopsin is maximally sensitive near 530 nm. Moreover, as shifts in photosteady state are described by first-order kinetics, there appears to be only a single pair of pigment forms participating. This pigment composition of the rhabdom can be described as



(I)

i.e., a 530-nm rhodopsin can be reversibly photoconverted to a 515-nm metarhodopsin, which in turn is fluorescent. In vivo, after a period of dark adaptation, the rhabdom contains only P_{530} with no detectable M_{515}.

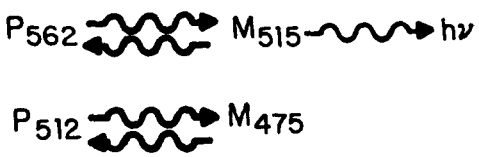
As described earlier, digitonin extracts of rhabdoms appear to contain a mixture of two pigments, each converted to a spectrally distinct metarhodopsin (Larrivee and Goldsmith, 1982):



(II)

The present study indicates that these pigments do not exist in the rhabdom and therefore must be generated in the process of extraction. To demonstrate that this is so, we shall consider several alternatives to model I.

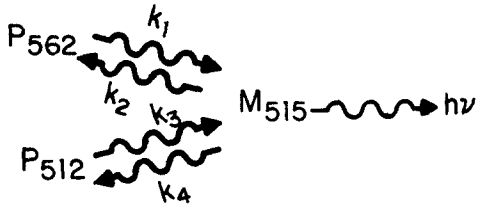
Suppose both pigments occurred in the rhabdom, and the 530-nm absorption band resulted from a mixture of P_{562} and P_{512}:



(III)

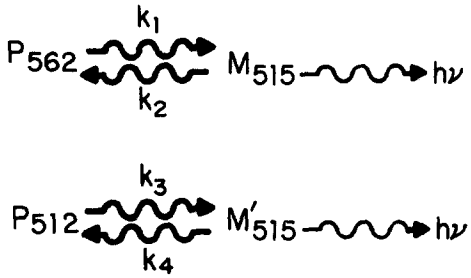
In order to keep model II consistent with the fluorescence excitation spectrum which has λ_{max} near 515 nm (Cronin and Goldsmith, 1981), we suppose that only M_{515} (and not M_{475}) fluoresces. There are two arguments against model II. First, there is no microspectrophotometric evidence for the occurrence *in situ* of M_{475}. More telling, however, is the rhodopsin photosensitivity spectrum (Fig. 6), which shows that the precursor of the fluorescent metarhodopsin is maximally sensitive at 530, not 562 nm. Model II can therefore be rejected.

One might argue that only M_{475} is an artifact of extraction, that both P_{562} and P_{512} exist in the rhabdom, and that they are related through a common metarhodopsin that fluoresces



(IV)

or that each gives rise to a spectrally similar and fluorescent metarhodopsin



(V)

In model III the fluorescence signal would depend on $[M_{515}]$, and the photosensitivity spectrum would be influenced by both P_{562} and P_{512}. In IV the fluorescence signal would be proportional to $[M_{515}] + [M'_{515}]$, and again both P_{562} and P_{512} would contribute to photosensitivity. Models III and IV therefore both appear to be consistent with the fluorescence excitation and the photosensitivity spectra. In terms of their kinetics, however, both III and IV are at variance with the data.

In model I the approach to the photosteady state is described by a single exponential term whose magnitude is a function of λ (Eq. 4, Methods). Experimentally, at each tested actinic wavelength, both fluorescence intensity and transmittance at 580 nm increase along the same single-exponential time-course (Fig. 7). In both III and IV, on the other hand, relaxation kinetics are described by the sum of two exponential terms whose relative magnitudes are dependent on wavelength, and in general, the development of fluorescence and the change in transmittance at 580 nm would have neither single-exponential increases nor the same time-course (Appendix). Some representative calculations are shown in Fig. 8. For Models III and IV, blue (450 nm) actinic exposures generate *decreases* in transmittance at 580 nm (broken curves in Fig. 8 A and B), opposite to what is observed. Moreover, even when

transmittance would increase, as with yellow (550 nm) actinic exposure in model II, it usually does not parallel the increase in fluorescence (Fig. 8 C; contrast with Fig. 7 B). Neither model III nor IV therefore is consistent with the data. We conclude that of the alternatives considered, only model I describes the photopigment system of crayfish rhabdoms.

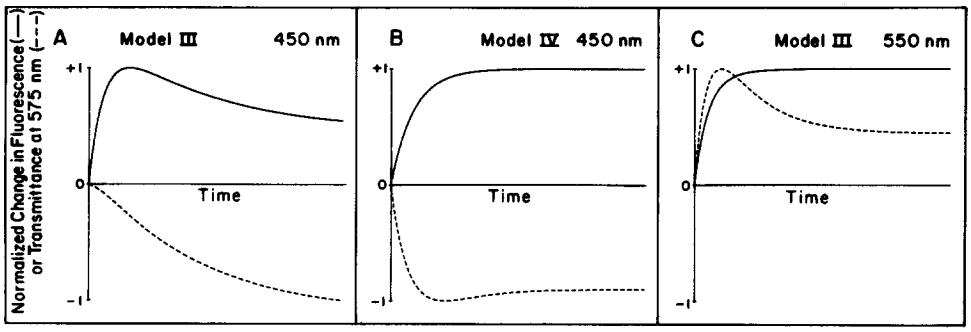


FIGURE 8. Predicted time-courses of changes in fluorescence (metarhodopsin concentration, solid curves) and 575 nm transmittance (broken curves), derived using models III and IV, which both assume the presence of two rhodopsins as observed in extracts (see Discussion and Appendix for the forms of the models). All curves are normalized to the maximum absolute value of each time-course and are plotted on an arbitrary time axis, the scale of which would vary with the intensity of actinic light. The quantum fluxes assumed in parts A and B are the same and equal to twice that of part C. The initial pigment mixture consisted of 0.65 P_{512} and 0.35 P_{562} (to match as closely as possible the 530-nm absorption of isolated rhabdoms) and no metarhodopsin (to remain consistent with the results of experiments; see Fig. 1). To construct all curves, the quantum efficiencies of conversions between both rhodopsins and their metarhodopsin(s) were assumed to be equal, and the metarhodopsin/rhodopsin extinction ratio(s) at their maxima were set at 1.2. These values are realistic and conservative; any reasonable variations in them do not affect the qualitative predictions of the models. Contrast this figure with the experimental results of Fig. 7. (A) Model III, actinic λ = 450 nm. The net increase in fluorescence is biphasic, with the early rise due to rapid net conversion of P_{512} to M_{515} and the later fall reflecting the further conversion of M_{515} to P_{562}. Transmittance *decreases* throughout, as there is a net gain in P_{562}, which makes up >70% of the photosteady state pigment mixture. (B) Model IV, actinic λ = 450 nm. The increase in fluorescence is monotonic, due to the net formation of metarhodopsins from both rhodopsins, but primarily from P_{512}. The transmittance at 575 nm again shows a net decrease, this time with two phases: an initial rapid fall due to formation of M'_{515} from P_{512} with a concomitant decrease in transmittance, and a subsequent partial recovery because of a slower gain in transmittance near 580 nm as P_{562} decreases. (C) Model III, actinic λ = 550 nm. Fluorescence rises monotonically, with both of the rhodopsins initially forming the common M_{515}. The loss of P_{562} causes a rapid initial rise in transmittance, but transmittance subsequently decreases as the large pool of metarhodopsin begins to regenerate P_{562}. Net conversion of P_{512} to M_{515} with a higher extinction maximum also contributes to the decreased transmittance.

Absence of Isorhodopsin

The 9-*cis* isomer of retinal binds to opsin to form isorhodopsin. Isorhodopsin is known principally from detergent solutions of rhodopsin, but in vertebrates it can also be produced in the photoreceptor. Exogenously applied 9-*cis* retinal will enter bleached skate rods to generate isorhodopsin (Pepperburg et al., 1978). In the eyes of living rats isorhodopsin is formed by sufficiently intense flashes of light that early intermediates absorb a second photon before they bleach; nevertheless, it is not normally found in the rat retina, and dark mechanisms for converting isorhodopsin to rhodopsin are not present (Huddleston and Williams, 1977).

In invertebrates with thermally stable metarhodopsins, on the other hand, there could be ample opportunity for the photoconversion of metarhodopsin to isorhodopsin, even at moderate quantum fluxes. Squid isorhodopsin can be photogenerated in digitonin solution (Yoshizawa and Wald, 1964), but the presence of an isosbestic point in the photoconversion of rhodopsin to metarhodopsin in the eyes of living flies (Stavenga, 1976; Hamdorf, 1979) or isolated rhabdoms of lobster (Bruno et al., 1977) suggests that isorhodopsin does not form in measurable quantities in these photoreceptors.

The reversible interconversions of rhodopsin, metarhodopsin, and isorhodopsin are formally described in model III. We have therefore done a computer simulation of the increase in transmittance at 575 nm and the increase in $[M_{515}]$ (and thus fluorescence signal) for a system in which P_{530} is reversibly photoconverted to M_{515}, and M_{515} is in turn reversibly photoconverted to isorhodopsin. The spectrum of crayfish isorhodopsin is not known; however, other isorhodopsins are hypsochromically displaced with respect to their rhodopsin but have λ_{max} at both longer and shorter wavelengths than the metarhodopsins (Yoshizawa, 1972, Table 2). We have therefore arbitrarily used spectra with λ_{max} at 505, 513, and 520 nm to approximate crayfish isorhodopsin. With a variety of values of quantum efficiency for the several reactions similar to those reported for other isorhodopsin systems (Kropf and Hubbard, 1958; Suzuki and Callender, 1981), the transmittance increase at 575 nm is always slower than the rise in metarhodopsin concentration by an amount that should have been detectable in our experiments (cf. Fig. 7). This conclusion is not altered by varying the ratio of extinction coefficients $\epsilon_{Iso}/\epsilon_{Rhod}$ at the λ_{max} between 1.0 and 1.2. We therefore conclude that the quantum efficiency for the formation of isorhodopsin in isolated rhabdoms is very small, possibly because there is a constraint on the conformations that invertebrate opsins can assume *in situ*.

APPENDIX

We set forth here the methods for calculating the theoretical curves in Fig. 8, which underpin the rejection of models III and IV on kinetic grounds.

The rate constants k_1–k_4 in models III and IV are the products of quantum flux and photosensitivity, the latter defined in Eq. 1. Knowledge of the absorption spectra and the relative molar extinction at the λ_{max} for each pigment, coupled with reasonable assumptions about the relative values of γ,

permit calculation of all photosensitivities and thus fix the rate constants for any photoisomerizing wavelength

In IV the kinetics of each pigment transition take the form of Eq. 4. Therefore the fluorescence signal, being proportional to M + M′, will depend on the sum of two expressions, each of the form of Eq. 4.

In III the kinetics of formation of M are mathematically somewhat more complex and their analysis involves finding a solution for the system of equations

$$df_R/dt = k_2 f_M - k_1 f_R$$

$$df_P/dt = k_4 f_M - k_3 f_P$$

$$df_M/dt = k_1 f_R + k_3 f_P - (k_2 + k_4) f_M$$

where f_R, f_P, and f_M are the fractional concentrations of P_{562}, P_{512}, and M_{515}, respectively, and $f_R + f_P + f_M = 1$. The solution to problems of this form is well known (e.g. see Haaser et al., 1964, pp. 522–527), and in this case can be written

$$f_P(t) = -k_4 c_1 \exp(\xi_1 t) - k_4 c_2 \exp(\xi_2 t) + k_1 k_4 / D \quad (6)$$

$$f_R(t) = (\xi_1 + k_3 + k_4) c_1 \exp(\xi_1 t) + (\xi_2 + k_3 + k_4) c_2 \exp(\xi_2 t) + k_2 k_4 / D \quad (7)$$

where $D = (k_1 k_3 + k_2 k_3 + k_1 k_4)$ and ξ_1 and ξ_2 are the roots (real and negative) of

$$\xi^2 + (k_1 + k_2 + k_3 + k_4)\,\xi + D = 0,$$

and the constants c_1 and c_2 can be evaluated from the initial condition $f_R(0) = 0.35$, $f_P(0) = 0.65$, and $f_M(0) = 0$ (see below for explanation of these proportions). Thus f_M can be obtained from

$$f_M(t) = 1 - f_P(t) - f_R(t), \quad (8)$$

which like Eqs. 6 and 7 includes the sum of two exponential terms in ξ_1 and ξ_2.

The absorbance at wavelength λ depends on the concentration of each of the several pigments, weighted by its molar absorption coefficient (ϵ_λ). Thus (for model III)

$$A(\lambda) = \epsilon_{P_{562}}(\lambda)[P_{562}] + \epsilon_{P_{512}}(\lambda)[P_{512}] + \epsilon_{M_{515}}(\lambda)[M_{515}]. \quad (9)$$

because the absorption coefficient of P_{562} at long wavelengths is greater than for either P_{512} or M_{515}, absorbance near 580 nm is disproportionately influenced by changes in P_{562}.

For any actinic exposure, the relative concentrations of the three pigments of model III can be calculated from Eqs. 6–8 and those of the four pigments of model IV from a pair of equations of the form of Eq. 4 (Methods). To use Eq. 9 and examine the absorbance as a function of time, we need only know in addition the absorption spectrum for each pigment, and the relative values of $\epsilon(\lambda_{max})$ and γ. The absorption spectra used for P_{512}, P_{562}, and M_{515} were

obtained by averaging their respective difference spectra measured in pigment extracts (Larrivee and Goldsmith, 1982). The molar extinction ratios for the extracted pigments (at their respective λ_{max}), ϵ_M/ϵ_R, range from 1.3 to 1.4 in pigment extracts (Larrivee and Goldsmith, 1982). We chose the more conservative value of 1.2 observed in intact rhabdoms (Goldsmith and Wehner, 1977). Selection of the relative values of γ to apply is somewhat more difficult. However, the quantum efficiency ratios $\gamma_{R\to M}/\gamma_{M\to R}$ have been measured for several arthropod visual pigments and are usually slightly above 1.0 (1.32: Goldsmith and Bruno, 1973; 1.20: Tsukahara and Horridge, 1977; 1.08: Hamdorf, 1979; 1.41: Stark and Johnson, 1980). We therefore used the conservative value of 1.0 for this ratio. Reasonable increases in the ratios of either extinction or γ have minimal effects on the qualitative predictions of the models and do not alter our conclusions.

As there is no metarhodopsin detectable in rhabdoms from dark-adapted eyes, in models III and IV the 530-nm absorption would have to arise from a mixture of ~35% P_{562}, 65% P_{512}, and no metarhodopsin. The proportion of 35:65 was determined by a computer-generated best fit of P_{562} and P_{512} as measured in digitonin extracts by Larrivee and Goldsmith (1982) to the 530-nm difference spectrum for the total bleach of rhabdoms as measured by Goldsmith and Wehner (1977).

The time-course of change of absorbance near 580 nm, as well as of the fractional concentrations of each metarhodopsin at approach to saturation, $f_M(t)$ and $f_{M'}(t)$, were calculated for various actinic exposures to different wavelengths that drew the hypothetical pigment mixtures to new photosteady states. These calculated time-courses were then compared with measured values of the fluorescence signal and the absorbance change at 580 nm (Fig. 8). Before plotting, the calculated absorbance changes were converted to transmittance changes, since experimental time courses of transmittance and fluorescence are parallel (Fig 7).

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