

# The Contribution of a Sensitizing Pigment to the Photosensitivity Spectra of Fly Rhodopsin and Metarhodopsin

BARUCH MINKE and KUNO KIRSCHFELD

From The Hebrew University—Hadassah Medical School, Jerusalem, Israel and the Max-Planck-Institut für biologische Kybernetik, D-7400 Tübingen, West Germany. Dr. Minke's present address is The Hebrew University—Hadassah Medical School, Jerusalem, Israel.

**ABSTRACT** Most of the photoreceptors of the fly compound eye have high sensitivity in the ultraviolet (UV) as well as in the visible spectral range. This UV sensitivity arises from a photostable pigment that acts as a sensitizer for rhodopsin. Because the sensitizing pigment cannot be bleached, the classical determination of the photosensitivity spectrum from measurements of the difference spectrum of the pigment cannot be applied. We therefore used a new method to determine the photosensitivity spectra of rhodopsin and metarhodopsin in the UV spectral range. The method is based on the fact that the invertebrate visual pigment is a bistable one, in which rhodopsin and metarhodopsin are photointerconvertible. The pigment changes were measured by a fast electrical potential, called the *M* potential, which arises from activation of metarhodopsin. We first established the use of the *M* potential as a reliable measure of the visual pigment changes in the fly. We then calculated the photosensitivity spectrum of rhodopsin and metarhodopsin by using two kinds of experimentally measured spectra: the relaxation and the photoequilibrium spectra. The relaxation spectrum represents the wavelength dependence of the rate of approach of the pigment molecules to photoequilibrium. This spectrum is the weighted sum of the photosensitivity spectra of rhodopsin and metarhodopsin. The photoequilibrium spectrum measures the fraction of metarhodopsin (or rhodopsin) in photoequilibrium which is reached in the steady state for application of various wavelengths of light. By using this method we found that, although the photosensitivity spectra of rhodopsin and metarhodopsin are very different in the visible, they show strict coincidence in the UV region. This observation indicates that the photostable pigment acts as a sensitizer for both rhodopsin as well as metarhodopsin.

## INTRODUCTION

### *The Photosensitizing Pigment in Fly Photoreceptors*

Most of the photoreceptors in the compound eyes of flies (the so-called receptors R 1–6) have a spectral sensitivity with a dual peak: one peak is in the green, close to 500 nm, the other in the near ultraviolet at 360 nm. Dual peak sensitivity of this type cannot be explained on the basis of the extinction spectra of known

rhodopsins, which have only a small peak at shorter wavelengths, of <25% of the maximum ( $\beta$ -peak).

Microspectrophotometric results and kinetic measurements of the change in rhodopsin concentration as a function of adapting UV and blue light in normal and vitamin A-deprived flies yielded evidence that the high UV sensitivity is due to a photostable pigment that acts as a sensitizer for rhodopsin (Kirschfeld et al., 1977). According to this model the photostable, UV-absorbing pigment absorbs light quanta and transfers the energy to the blue-absorbing visual pigment.

In contrast to the vertebrate, the invertebrate's metarhodopsin is quasi-thermostable, and does not hydrolyze into opsin and all-*trans*-retinal, but remains in the metarhodopsin state for a long time (Hubbard and St. George, 1958; Hamdorf et al., 1971 *a, b*; 1973; Stavenga et al., 1973; Minke et al., 1973, 1974; Ostroy et al., 1974; Hamdorf and Schwemer, 1975; Lisman and Sheline, 1976; review Goldsmith, 1972). Re-isomerization of rhodopsin is basically due to light absorbed by the metarhodopsin.

An unsolved question is whether the photostable, UV-absorbing pigment transfers energy only to rhodopsin, thus creating its high UV-sensitivity, or whether it also transfers energy to metarhodopsin. In the latter case the photosensitivity spectrum of metarhodopsin must have a second peak in the UV in addition to its peak in the orange range. To answer this question we determined the photosensitivity spectrum of rhodopsin and metarhodopsin in fly photoreceptors R 1-6.

*Methods of Measuring the Photosensitivity Spectra of Rhodopsin and Metarhodopsin in Bistable Pigment Systems In Situ*

Photosensitivity is the product of the absorption coefficient,  $\alpha(\lambda)$ , and the quantum efficiency,  $\gamma(\lambda)$ . There are several approaches available for determining the photosensitivity spectra of the two states of a bistable visual pigment *in situ*. (a) By using spectrophotometrically determined difference spectra, it is possible to derive the rhodopsin and metarhodopsin photosensitivity spectra, if their spectra do not overlap. However, inasmuch as the rhodopsin and metarhodopsin states in most invertebrates do in fact overlap to a high degree, additional information is required in order to derive these spectra (Hamdorf et al., 1973; Stavenga, 1975, 1976; Minke and Kirschfeld, 1978). In general, the use of a difference spectrum is not suitable for deriving the photosensitivity spectrum of a sensitizing pigment which has a high extinction but cannot be changed by illumination. Such a spectrum gives a null difference spectrum. (b) Photosensitivity can be deduced from a criterion action spectrum (CAS). In the CAS of the fly, the receptor potential arises only from activation of rhodopsin (Hamdorf et al., 1971 *a, b*; 1973). For this spectrum, there is evidence that the high UV sensitivity (Burkhardt, 1962; McCann and Arnett, 1972; Horridge and Mimura, 1975) is due to a sensitizing pigment that acts on rhodopsin (Kirschfeld et al., 1977). However, no equivalent data is available for metarhodopsin, because metarhodopsin does not contribute to the receptor potential (Atzmon et al., 1978; Strong and Lisman, 1978). Also, the criterion which is used in measuring the CAS can only be chosen arbitrarily; therefore, the CAS is expressed in

relative units and only the shape of the spectrum is significant. The spectral overlap of rhodopsin and metarhodopsin absorption makes the classical way of deducing photosensitivity spectra from CAS very unreliable in many cases (see Hochstein et al., 1978 for details).

For example Harris et al. (1976), measured spectrophotometrically the efficiency with which lights of different wavelengths create a criterion amount of either metarhodopsin or rhodopsin depending on different preadaptations. From these efficiencies CAS have been calculated in the visible range. The method as applied should yield rhodopsin and metarhodopsin photosensitivity spectra only if the spectra of the two pigments do not overlap (see Analytical Methods). Inasmuch as in fly photoreceptors R 1-6 these spectra in fact do overlap, the "sensitivity" spectra of receptors 1-6 as determined by Harris et al. (1976) do not represent the photosensitivity spectra of rhodopsin and metarhodopsin.

In our approach we used a method similar to the method of "photometric curves" introduced by Dartnall et al. (1936) to analyze vertebrate rhodopsin; that is, we measured the dependence of the rate of approach of the pigment molecules to photoequilibrium on the wavelength of an adapting light. In a second set of experiments we measured in a way similar to that of Hamdorf et al. (1971 *b*, 1973), Stavenga et al. (1973), Stark et al. (1977), Tsukahara and Horridge (1977), and Minke et al. (1978): how the ratio of the concentrations of rhodopsin and metarhodopsin, reached in photoequilibrium, depends on the wavelength of the adapting light. Both sets of data are sufficient to calculate the photosensitivity spectra of rhodopsin as well as of metarhodopsin on an absolute scale, whereby the effect of the sensitizing pigment is directly represented in the photosensitivity. The interpretation of the data has to take into account the bistability of fly visual pigment and the possible existence of several thermolabile states and thermal and photochemical transitions among them. The theory for the behavior of such a system has been worked out by Hochstein et al. (1978), and their results will be used as the tool for the interpretation of our data.

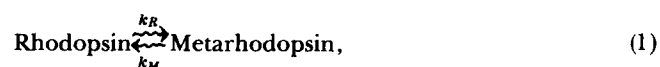
In order to measure the concentration of rhodopsin and metarhodopsin, we applied a signal which allows use of intact flies for the analysis: the so-called *M* potential which arises selectively from activation of metarhodopsin (Pak and Lidington, 1974). In order to be able to do so, we first had to establish that the *M* potential is a linear measure of the visual pigment concentration.

#### EXPERIMENTAL AND ANALYTICAL METHODS

##### *Analytical*

We shall describe briefly the conclusions of the analytical method developed by Hochstein et al. (1978) as applied to our experiments.

**A SYSTEM IN PHOTOEQUILIBRIUM** One of the main conclusions of this analysis is that a complex bistable pigment system can be considered, under certain conditions, as a simple, bistable pigment system with only two photointerconvertible dark stable states, as illustrated in Eq. 1:



where  $k_R$  and  $k_M$  are the phototransition rates from rhodopsin to metarhodopsin and vice versa, respectively.

We shall first describe the kinetics of such a system previously analyzed by Hamdorf et al. (1968), Schwemer (1969), Hamdorf et al. (1973), Hamdorf and Schwemer (1975), Stavenga (1975), and Hochstein et al. (1978). We shall indicate the modifications necessary for analyzing a more complex system.

One may define

$$K_M = \frac{k_m}{I}. \quad (2)$$

$K_M$  is actually the photosensitivity, which is the product of the molecular absorbance ( $\alpha_M$ ) and the quantum efficiency ( $\gamma_M$ ) (Dartnall, 1972);  $I$  is the light intensity. The wavelength dependence of  $K_M$  is the *photosensitivity spectrum* of that pigment state. Thus,

$$K_M(\lambda) = \alpha_M(\lambda) \cdot \gamma_M(\lambda). \quad (3)$$

The photoreceptors of the fly are thin long structures; nevertheless, they can be considered to be optically thin, because in our experiments we used diffuse light, and white-eyed animals in which the light is heavily scattered (Razmjoo and Hamdorf, 1976). Therefore, we can use the above formulation for optically thin layers and need not consider "self-screening" effects.

$f_M(It, \lambda)$  and  $f_R(It, \lambda)$  are the fractions of the pigment in the metarhodopsin and rhodopsin states after adaptation with light of wavelength  $\lambda$  and of intensity  $I$  for duration  $t$ . Because the pigment system is a closed one, we have

$$f_M(It, \lambda) + f_R(It, \lambda) \equiv 1. \quad (4)$$

For long stimulus durations (equilibrating stimuli), a photoequilibrium is reached and the fractional concentration of metarhodopsin will be

$$f_M(\infty, \lambda) = \frac{K_R(\lambda)}{K_R(\lambda) + K_M(\lambda)}. \quad (5)$$

The fraction of metarhodopsin  $f_M(\infty, \lambda)$  in photoequilibrium is thus independent of the starting conditions and the light intensity, but depends only on the wavelength of the equilibrating light (Hamdorf et al., 1968). The graph of  $f_M(\infty, \lambda)$  as a function of wavelength is called the *photoequilibrium spectrum* of metarhodopsin (Fig. 6); it corresponds to the "Q function" of Stavenga (1975) and to the "saturation spectrum" of Hochstein et al. (1978). The time-course by which the photoequilibrium of this system (for monochromatic light of constant light intensity) is reached is given by:

$$f_M(It) = f_M(\infty) + [f_M(0) - f_M(\infty)]e^{-(K_M+K_R)It}. \quad (6)$$

If we plot  $f_M$  as a function of  $It$ , we find that the plot has the form of an exponential with an intensity-independent *relaxation constant*,  $\Lambda(\lambda)$ :

$$\Lambda(\lambda) = K_R(\lambda) + K_M(\lambda); \quad (7)$$

that is, for a given  $\lambda$  the sum of the two photosensitivities at that wavelength,  $\Lambda(\lambda)$ , is the reciprocal of the amount of light (number of photons  $\cdot$   $\text{cm}^{-2}$ ) needed to change the pigment concentration by  $(1 - 1/e)$  of the change reached in photoequilibrium.

Eq. 6 can be arranged to the form:

$$\ln \frac{f_M(\infty) - f_M(It)}{f_M(\infty) - f_M(0)} = -\Lambda It. \quad (8)$$

The wavelength dependence of  $\Lambda$  (Fig. 5) is called the *relaxation spectrum* of the pigment. The spectrum in this case is thus the sum of the two photosensitivity spectra. Both states of the pigment approach the photoequilibrium exponentially with one and the same relaxation constant. Thus, the pigment relaxation spectrum characterizes the pigment system and not its separate components.

**DERIVATION OF PHOTOSENSITIVITY SPECTRA FROM RELAXATION AND PHOTOEQUILIBRIUM SPECTRA** There are indications that the visual pigment systems of the invertebrates are more complex than the simple system analyzed above (Fein and Cone, 1973; Hamdorf et al., 1973; Minke et al., 1974; Lisman and Sheline, 1976; Ostroy, 1977). Hochstein et al (1978) have shown that by assuming a closed system with only two dark stable states and using physiological light intensities, the conclusions concerning the photoequilibrium and the relaxation spectra of the simple system are also valid for a more complex pigment system, except that the photosensitivities  $K_R(\lambda)$  and  $K_M(\lambda)$  must be multiplied by wavelength-independent weighting factors  $W_R$  and  $W_M$ , respectively. These weighting factors have a meaning similar to the quantum efficiency ( $\gamma$ ) and they represent the probability that a molecule, once isomerized, will reach the second stable state.

The analysis of Hochstein et al. (1978) has been shown to be valid for the barnacle photoreceptors (Minke et al., 1978). We note that we have recently found that the pigment system of the fly is very different from that of the barnacle (Kirschfeld et al. 1978). In the fly the pigment system can be described by a scheme similar to Eq. 1 without other slow phototransitions. Therefore the weighting factors ( $W_R$ ,  $W_M$ ) in the fly are equal to one and they will be omitted in our equations.

The photosensitivity spectrum of rhodopsin is derived from Eqs. 5 and 7 as the following:

$$K_R(\lambda) = f_M(\infty, \lambda) \cdot \Lambda(\lambda), \quad (9)$$

and that of metarhodopsin as

$$K_M(\lambda) = \Lambda(\lambda)[1 - f_M(\infty, \lambda)]. \quad (10)$$

#### THE GENERAL PARADIGM FOR MEASUREMENT OF THE RELAXATION AND PHOTOEQUILIBRIUM SPECTRA

##### *The Relaxation Spectrum*

Before the relaxation constant of any wavelength was measured, the eye first was preadapted to equilibrium to light of a fixed wavelength, which was usually orange light (590 nm). The orange light brought almost all the pigment molecules to the rhodopsin state (Fig. 6). After a constant dark time of 1 min, the eye received the adapting light which, in general, changed the pigment distribution. This is the stimulation whose effect we determine as a function of its amount and wavelength. Finally, after again resting 1 min in the dark, the eye received a strong constant orange test flash which elicits an  $M$  potential. The dependence of the relative change in amplitude of the  $M$  potential on the amount of adapting light yields the relaxation curve (Fig. 2 c). In Results we shall show that the amplitude of the  $M$  potential ( $M_p$ ) is proportional to the concentration of metarhodopsin:  $M_p(It) = c \cdot M(It)$ , where  $c$  is a constant. Therefore, we can use an equation similar to Eq. 8 to determine the relaxation curve,

$$\ln \frac{M_p(\infty) - M_p(It)}{M_p(\infty) - M_p(o)} = -\Lambda It, \quad (11)$$

where  $M_p$  is the amplitude of the  $M$  potential at a constant time after the onset of the stimulus.

Inasmuch as orange preadaptation brings all the pigment molecules to the rhodopsin state,  $M_p(0)$  is zero.  $M_p(\infty)$  is the amplitude of the  $M$  potential after equilibrating adaptation of a specific wavelength, and  $M_p(t)$  are various amplitudes of  $M$  potentials after various amounts of adapting light at this wavelength. When Eq. 11 is plotted, the negative slope gives  $\Lambda$  in absolute units of  $\text{cm}^2 \cdot \text{photon}^{-1}$ .

#### *The Photoequilibrium Spectrum*

The data for this spectrum was always measured in the same experiments which were used to derive the relaxation spectrum (see Results). Here, in general, the eye was adapted to equilibrating 457-nm blue light, which creates the maximum possible concentration of metarhodopsin. Then, after 1 min in the dark, equilibrating light of a specific wavelength was given. This light brings the pigment system to a photoequilibrium characterized only by the wavelength of the equilibrating light. After 1 min in the dark the fixed orange test flash was given. The amplitude of the  $M$  potential at a constant time as a function of the wavelength of the equilibrating light gives the shape of the equilibrium spectrum. A problem is that we do not know the constant  $c$  that relates the  $M$  potential amplitudes to the metarhodopsin concentration. Therefore, in order to scale the measured photoequilibrium spectrum in terms of fractional metarhodopsin concentration, we used Eq. 5 with the following assumption: the quantum efficiency ( $\gamma_R$ ) of rhodopsin is equal to the quantum efficiency ( $\gamma_M$ ) of metarhodopsin; that is,  $\gamma_R = \gamma_M$ . We assume also that  $\gamma$  is wavelength-independent. Together with Eqs. 3 and 5 we arrive at

$$f_M(\infty, \lambda) = \frac{\alpha_R(\lambda)}{\alpha_R(\lambda) + \alpha_M(\lambda) \cdot \phi} \quad (13)$$

$$\phi = \frac{\gamma_M}{\gamma_R} = 1. \quad (14)$$

We already know from the shape and peak wavelength of the absorption spectra of rhodopsin and metarhodopsin of the fly (Hamdorf et al., 1973; Stavenga et al., 1973) that  $M(t = \infty, \lambda = 600) \approx 0$ . From spectrophotometric measurements we also know that the isobestic point ( $\lambda_{180}$ ) of the fly difference spectrum, at which  $\alpha_R = \alpha_M$ , is close to 510 nm (Hamdorf, Schlecht and Schwemer's most recent and accurate results;<sup>1</sup> see Fig. 7. Therefore  $M(\infty, 510) = 0.5$ , and  $M(\infty, 600) = 0$  give us the scale for the photoequilibrium spectrum with  $\phi = 1$ .

#### *Experimental*

**ANIMALS** We used white-eyed *Drosophila*, *Calliphora*, and *Musca* to avoid possible influences of colored screening pigments. Each species was raised on its standard diet medium. For part of the experiments, we used *Drosophila* raised on (vitamin A-free) Sang's synthetic diet medium (Doane, 1967) with 0.8 and 0.4 mg  $\beta$ -carotene per 100 ml medium (The normal  $\beta$ -carotene concentration is about 8 mg/100 ml). In this medium dead adult flies were removed from the bottles.

**ELECTRICAL RECORDINGS** The flies were first anaesthetized slightly with  $\text{CO}_2$  and then fixed with wax on their side to a cooled glass slide. The electrical responses were recorded using glass microelectrodes filled with 2 M  $K^+$  acetate. One electrode was placed on the cornea and the other one on the thorax. Both electrode tips were embedded in a small drop of conducting paste. The voltage signals were simultaneously

<sup>1</sup> Hamdorf, K., P. Schlecht, and J. Schwemer. Personal communication.

displayed on an oscilloscope and recorded in the memory of an averaging computer (NIC-527, Nicolet Instrument Corp., Madison, Wis.) and on a pen recorder. The response was later transferred from the averaging computer to an  $X - Y$  plotter.

**LIGHT STIMULATION** We used a xenon light source (XBO 150 W, Osram, München, West Germany) for the adapting lights in conjunction with monochromatic interference filter (Schott-depal, UV-pil, Mainz, West Germany), quartz neutral density filters (Melles Griot, Arnhem, Netherlands), quartz lenses, and quartz light guide (Schott). The unattenuated intensity of the adapting light at 457 nm at the level of the preparation was  $4.26 \times 10^{15}$  photons  $\cdot$  cm $^{-2}$   $\cdot$  s $^{-1}$ . For the test stimulus we used a xenon photographic flash (Braun, type F 900, Frankfurt, West Germany) in conjunction with a 590 OG edge filter and a KG heat filter (Schott) and neutral density filters (Melles Griot) and a second quartz light guide (Schott). We carefully checked that the whole eye was uniformly illuminated by the adapting light. This was manifested by the exponential function of the relaxation curves. For the test flash, uniform illumination was not essential and we only made sure that the intensity was in the linear range of the  $M$  potential amplitude (see Fig. 2). For criterion  $M$  potential action spectrum measurements (see Results) we used the flash light source with the monochromatic interference filters, and quartz neutral density filters. The duration and amplitude of the flash was recorded by means of a photomultiplier (9558 Q, EMI Electronics, Hays, Middlesex, England) and displayed on a storage oscilloscope. The energy of the light sources was calibrated by means of a photoradiometer (International Light, Inc., Newburyport, Mass., type IL 700).

**MICROSPECTROPHOTOMETRY** The microspectrometric technique has been described elsewhere (Kirschfeld et al., 1977; Minke and Kirschfeld, 1978). The only modification for the present experiments was that the adapting light came from a system very similar to that used for the adapting light in the  $M$  potential measurements. The end of the quartz light guide in the microspectrometer was placed sideways between the objective and the stage of the microscope.

## RESULTS

### *The Use of the M Potential to Measure Pigment Changes*

**THE COMPONENTS OF THE INITIAL PART OF THE ELECTRORETINOGRAM (ERG)** Fig. 1 shows initial parts of electroretinograms on a fast time scale. All the traces were obtained with an orange test flash of maximal intensity after equilibrating 457-nm blue (in trace G: orange) adapting light. The various components of the electrical response are indicated by numbers in trace C: (1) stimulus artifact; (2) the negative phase which is the early receptor potential (ERP) of the fly and arises from activation of metarhodopsin (Grabowski and Pak, 1976); (3) the  $M$  potential; and (4) the on-transient of the ERG arising from activation of the second order neurons in the lamina (Hamdorf and Keller, 1962; review: Goldsmith and Bernard, 1974). The ERP is resistant to extreme media (Brindley and Gardner Medwin, 1966; Hillman et al., 1973), but the  $M$  potential is not (Pak and Lidington, 1974) and can be abolished by hypertonic  $K^+$  as illustrated in trace E. Traces A, B, and C were recorded from *Drosophila*, *Musca*, and *Calliphora*, respectively. The  $M$  potential in *Drosophila* has a time-course somewhat slower than in *Musca* and *Calliphora*. The absence of an on-transient in *Drosophila* (trace A) is due to the fact that the adapting blue light induces a saturated prolonged depolarizing afterpotential (PDA) which saturates

the voltage response of R 1-6 for many minutes and thus abolishes the on-transient (Minke et al., 1975) that normally exists in flies having a short PDA, such as *Calliphora* or *Musca*. The *Musca* response (trace B) seems to lack the ERP component (phase 2) and has a positive phase instead. This positive phase has also been found occasionally in the other species and is illustrated in traces F and G in *Drosophila* after blue (F) and orange adaptation (G). This positive phase, which has no apparent latency, does not change after various adapta-

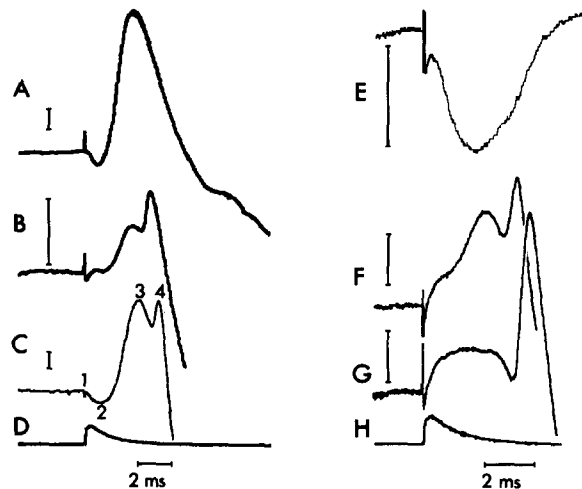


FIGURE 1. The components of the initial part of fly electroretinogram (ERG) which were elicited by a maximum intensity orange (>590 nm) test flash after 457-nm equilibrating blue light. The various components are indicated by numbers in trace C: (1) stimulus artifact; (2) early receptor potential (ERP) which arises from activation of metarhodopsin; (3) *M* potential; (4) on-transient of the ERG. Traces A, B, and C are responses recorded from *Drosophila*, *Musca*, and *Calliphora*, respectively. The ERP phase (2) is cancelled by a fast positive phase in the *Musca* response (see traces F and G). Trace E: ERP response recorded in *Calliphora*. The *M* potential and the on-transient of the ERG were abolished by 2 M hypertonic  $K^+$  acetate. The ERP survived this extreme medium. Traces F and G: A fast positive phase, which appeared in some of the experiments. These recordings are from *Drosophila* raised on vitamin A-reduced medium, after 457-nm equilibrating blue and orange (>590 nm) adaptation (traces F and G, respectively). Traces D and H are photomultiplier responses which indicate the time-course of the test flash. The vertical calibration bars represent 500 and 250  $\mu V$  for the left and right column, respectively. In all the figures only white-eyed flies were used.

tions, and its amplitude depends only on the intensity of the stimulating light. Its photostability differentiates this potential from the *M* potential which is abolished by orange adaptation (trace G). The photostable component may arise from a thermoelectric effect (Hagins and McGaughy, 1967). It probably does not arise from activation of the photostable sensitizing pigment (Kirschfeld et al., 1977), inasmuch as it exists also in totally vitamin A-deprived flies which



show reduced UV spectral sensitivity and no  $M$  potential. One should be careful not to confuse it with the  $M$  potential which has the same polarity but has a certain latency and can be abolished by bleaching with orange light.

**THE DEPENDENCY OF THE  $M$  POTENTIAL AMPLITUDE ON THE AMOUNT OF ADAPTING LIGHT** Unlike the ERP, which is a direct linear manifestation of the changes in the visual pigment (Cone, 1967) and therefore can be recorded even in freshly sacrificed animals and under extreme media, the  $M$  potential seems to arise less directly from the pigment changes: it can be abolished by hypertonic  $K^+$  (Fig. 1 E and Grabowski and Pak, 1976), by  $CO_2$ , or by sacrificing the animal.

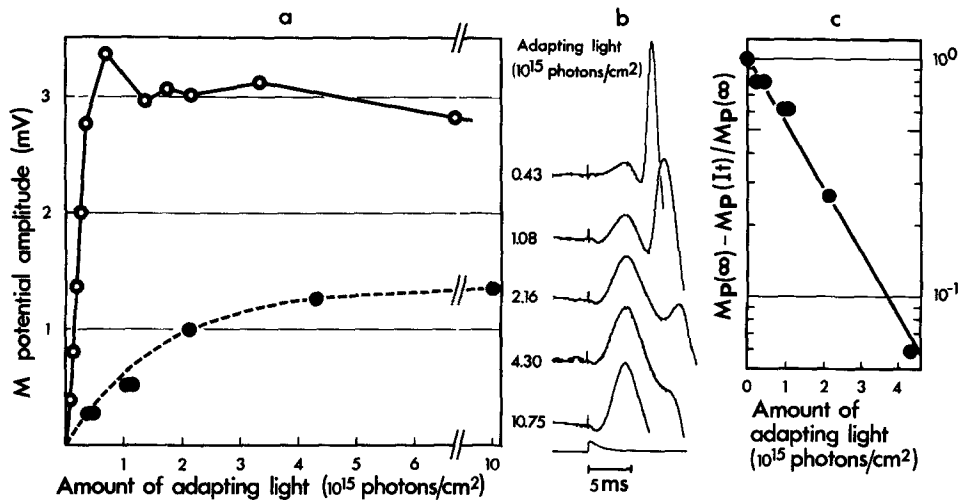


FIGURE 2. The dependence of the  $M$  potential on the amount of adapting light. (a) The amplitude of the  $M$  potential as a function of the amount of adapting blue light (473 and 457 nm in the upper and lower curves, respectively) in two different *Drosophila*. The intensity of the orange test flash with which the  $M$  potential was induced was maximal in the upper curve and one-sixth maximal in the lower one. The broken line is an exponential curve that fits the experimental points. The upper curve clearly is not exponential. (b) The actual  $M$  potentials that were used in a (lower curve); the amount of the adapting blue light is indicated for each trace. (c) A relaxation curve which was derived from the traces in b. The ordinate gives the difference between the peak  $M$  potential amplitude after equilibrating blue light (bottom trace,  $Mp(\infty)$ ) and the  $M$  potential peak amplitudes of the other traces ( $M(I_t)$ ), divided by  $Mp(\infty)$ . The abscissa is the amount of adapting blue (457 nm) light. The straight line is the same exponential that was used in a (lower curve).

Furthermore, in several *Drosophila* mutants which have apparently normal visual pigments and normal receptor potentials, the  $M$  potential is completely missing (Pak and Lidington, 1974). We checked whether the  $M$  potential is nevertheless linearly dependent upon the metarhodopsin concentration in a given animal under constant conditions. Fig. 2 a (lower curve) shows the amplitude of the  $M$  potential in *Drosophila* at a constant time after the onset of the stimulus (which was an orange test flash with one-sixth maximal intensity) as a function of the amount of adapting blue light. Before the adapting blue lights were given, the

eyes had been illuminated with equilibrating orange light. The smooth curve is an exponential which has been drawn for comparison. Fig. 2 *b* shows a sample of the actual *M* potentials used in Fig. 2 *a* (lower curve). The amount of adapting light is indicated at each trace. Fig. 2 *c* is a relaxation curve (Eq. 11) plotted from the same data. The ordinate is the difference between the *M* potential amplitude at a fixed time after equilibrating 457-nm light (bottom trace, Fig. 2 *b*;  $M_p(\infty)$ ) and the *M* potential amplitude [ $M_p(It)$ ] after the various amounts (*It*) of adapting 457-nm light, divided by  $M_p(\infty)$ . The straight line is the same exponential curve as in Fig. 2 *a* on a semi-log plot. It is clear that the change in the *M* potential amplitude as a function of the adapting blue light is exponential. However, this exponential dependence was not always obtained. Many times when we used *maximal* orange test flashes (as in Fig. 2 *a*, upper curve) the above dependence was *not* exponential. As shown in Fig. 2 *a* (upper curve) the *M* potential amplitude function in those cases is initially linear, but suddenly saturates and then may even decay to submaximal amplitudes which, in some animals, reach only half of the maximal amplitude. This dependence seems to arise from the fact that the *M* potential sometimes saturates before the maximal amount of pigment has been shifted to metarhodopsin, a phenomenon which never occurs with the ERP.

We found a systematic way to avoid the above difficulty by reducing either the visual pigment concentration of the fly (with vitamin A-deprived flies—see below, Fig. 8) or by reducing the intensity of the test flash (Fig. 2 *a*). With both methods the saturation of the *M* potential can be avoided. In contrast the ERP can be used to measure pigment concentration at any test light intensity, even in the saturated range of the intensity response curve of the ERP (Hillman et al., 1976; Minke et al., 1973).

**COMPARISON OF THE *M* POTENTIAL WITH THE PDA** In the fly, after orange preadaptation, an intense blue light that shifts rhodopsin to metarhodopsin induces a prolonged depolarizing afterpotential (PDA). The PDA can be very long in *Drosophila* (several hours) and it can be abolished at any time by shifting the pigment back from metarhodopsin to rhodopsin (Minke et al., 1975). Therefore the PDA in *Drosophila* is a good indicator of the fraction of rhodopsin shifted into metarhodopsin.

Fig. 3 compares the dependence of the *M* potential amplitude (●) and the amplitude of the PDA (○) on the amount of adapting blue light in *Drosophila*. The PDA is seen as a prolonged negative phase in the ERG traces, after the cessation of the adapting blue light (Minke et al., 1975). Each *M* potential response was elicited by a constant orange test flash that was given after the blue adapting lights. Some of the responses to the blue adapting lights are the traces illustrated in the inset of Fig. 3. The figure shows that the *M* potential and the PDA both saturate at a similar level of adapting lights. The dotted curve is an exponential curve that fits the *M* potential points. The deviation of the dependence of the PDA amplitude on adapting light from the exponential curve is consistent with a power law dependence of PDA amplitude on pigment shift (Hillman et al., 1976).

**COMPARISON OF THE *M* POTENTIAL AND MICROSPECTROPHOTOMETRIC MEASURES OF PIGMENT SHIFT** To confirm further the linear dependence of the *M*

potential amplitude on pigment activation, we performed microspectrophotometric measurements. These measurements were done on white-eyed *Musca* ommatidia. Since only receptors R 1–6 have visual pigment absorbing in the orange (Harris et al., 1976) we used 590-nm measuring light to avoid the absorption changes in receptors 7/8. Fig. 4 *a* shows two relaxation curves measured microspectrometrically. These curves represent the change in transmission *T* measured at 590 nm after adapting UV (●) and blue (○) lights of various amounts. Fig. 4 *b* presents two relaxation curves which show the relative

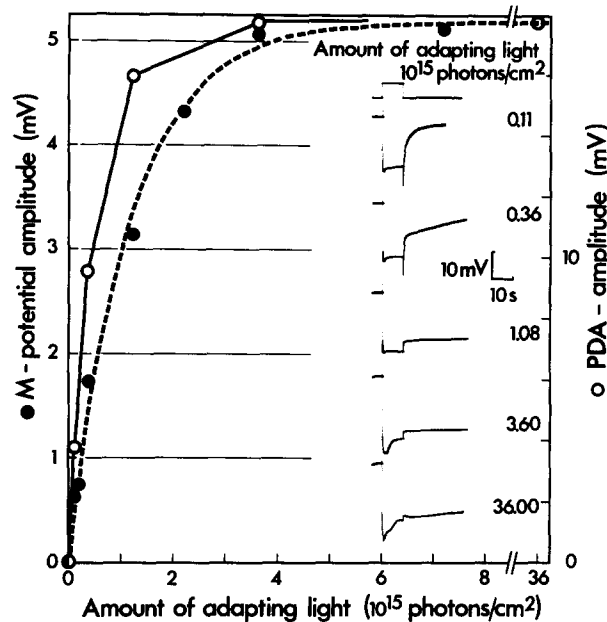


FIGURE 3. Comparison of the *M* potential with the PDA in *Drosophila*. The amplitude of the *M* potential which was induced by a maximal intensity orange test flash (left ordinate ●) and the amplitude of the PDA (right ordinate, ○) as a function of the amount of adapting 473-nm blue light, the PDA-inducing stimulus. The broken curve is an exponential for comparison. *Inset*: A sample of ERG recordings on a slow time scale in which the PDA is manifested as a negative afterpotential. The PDA was measured 10 s after the cessation of the blue light. The amount of PDA-inducing light is indicated for each trace. This light is actually the adapting light for some of the *M* potential points (●). The PDA was recorded in only some of the *M* potential measurements.

change in *M* potential amplitude as a function of adapting light for UV (●) and blue (○) adapting lights in white-eyed *Musca*. The adapting light source and setting were very similar in the two experiments. It is clear that the slopes of the relaxation curves in Fig. 4 *a* are similar to the corresponding slopes of Fig. 4 *b*. This agreement between the relaxation curves measured photometrically and by the *M* potential indicates that the *M* potential (when sufficiently low light intensities were used) is a reliable measure of the metarhodopsin concentration.

*The Photoequilibrium and the Relaxation Spectra*

THE RELAXATION SPECTRUM Fig. 5 illustrates the relaxation spectrum,  $\Lambda(\lambda)$  measured in *Calliphora*. The main curve shows the dependence of the rate of approach of the pigment to the photoequilibrium on the wavelength of the adapting light. As has been shown in Methods (Eqs. 6 and 8), the result of

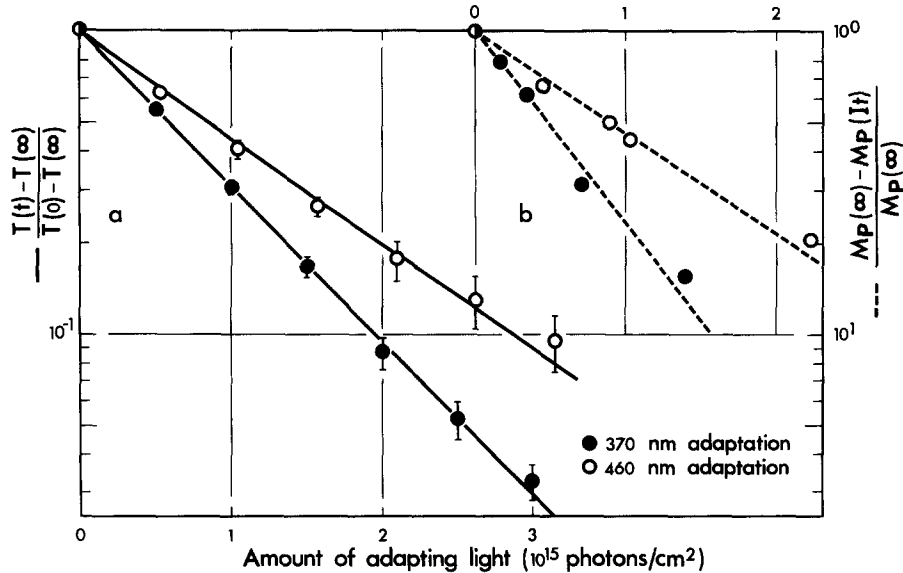


FIGURE 4. Comparison of the  $M$  potential and microspectrophotometric measurements of pigment shift. (a) Two microspectrophotometrically derived relaxation curves from eyecup preparations (*Musca*, white) (Kirschfeld et al., 1977). Transmission at  $\lambda = 590$  nm has been measured of five ommatidia at one and the same time in each preparation. Since only rhabdomeres 1-6 have a visual pigment absorbing at 590 nm, only their contribution is recorded. Each preparation has been preadapted first to equilibrium with light of wavelength  $\lambda = 590$  nm. Then the transmission  $T$  was measured after different amounts ( $It$ ) of 460-nm blue ( $\circ$ ) or 370-nm UV ( $\bullet$ ) light have been given. As ordinate the normalized difference is plotted between the transmission measured after an amount ( $It$ ) of adapting light was given and the transmission measured after equilibrating adaptation ( $It = \infty$ ). The points are means with standard error of the mean of two measurements from three eyes (three different flies). Experiments were performed at 18°C. (b) Relaxation curves obtained using the  $M$  potential (see Fig. 2 b) to 460-nm blue ( $\circ$ ) and 370-nm UV ( $\bullet$ ) adapting lights, both derived from one and the same *Musca* fly. The points are averages of two relaxation curves measured in two different flies.

measuring a relaxation constant  $\Lambda(\lambda)$  (Eq. 7) is independent of the starting conditions. This fact is illustrated in the inset of Fig. 5, which plots two sets of relaxation curves against the same adapting green light (545 nm), after two different preadaptations which give two different pigment distributions (starting conditions) between rhodopsin and metarhodopsin. These measurements were

carried out in the same fly. After orange preadaptation (●), most of the pigment population is in the rhodopsin state (see Fig. 6). The change in  $M$  potential amplitude was from zero to the maximal amplitude that can be obtained with 545-nm adapting light. After the blue preadaptation (○), the

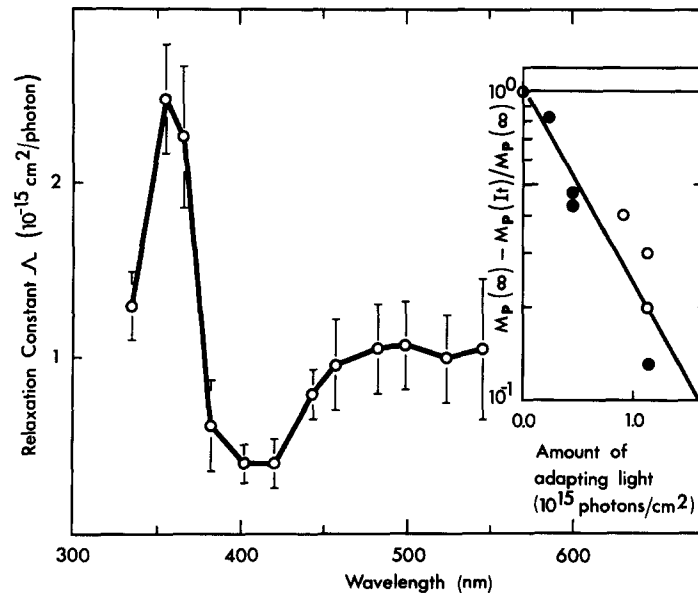


FIGURE 5. The relaxation spectrum  $\Lambda(\lambda)$ . The figure shows the dependence of the rate of approach of the pigment populations to photoequilibrium on the wavelength of the adapting light. Each point represents the (negative) slope, of a relaxation curve (Fig. 2 c) determined at different wavelengths. The ordinate (in absolute units) indicates relaxation constants (in  $\text{cm}^2/\text{photon}$ ); the abscissa indicates the wavelength of the adapting light. The vertical bars are standard errors of the mean. Each bar was calculated from four different experiments. Inasmuch as each relaxation curve is determined by many measurements, we could not hold the fly in constant conditions long enough to complete the measurements over the whole spectrum. Therefore we used each fly only for "half" of the spectrum, that is, from 335.5 to 420 nm or from 420 to 545 nm. The spectrum of the figure thus represents measurements from eight flies. In each half spectrum we measured one point of the other half again to get the ratio of the UV-visible relaxation slope in every experiment. *Inset*: Example of a relaxation curve, determined with 545-nm green adapting light, which was measured after two different preadaptations: one in the orange ( $\lambda > 590$  nm, ●) which shifts all the pigment to the rhodopsin state, and the other in the blue ( $\lambda = 457$  nm, ○) which shifts the maximal percentage of pigment to the metarhodopsin state. The two sets of points fit one relaxation curve. This indicates that the starting conditions do not affect the slope of the relaxation curve.

change in  $M$  potential amplitude was from the maximum possible  $M$  potential amplitude to about one-fifth of it, which is the maximal amplitude that can be obtained with 545-nm adapting light (Fig. 6). The fit of the two sets of points to the same relaxation curve shows that the theory presented in Methods is

applicable. Inasmuch as the relaxation spectrum has an absolute scale, we did not normalize the curves obtained from different animals (presented in the figure) for the best fit, but we only averaged the absolute values. However, in one experiment where the distance between the edge of the light guide and the eye was larger than usual so that the light intensity at the level of the preparation was weaker than usual, we multiplied all the points by a factor of 1.7. The vertical bars, which are the standard error of the mean, probably reflect primarily the variability in the effective absolute light intensities in the various experiments and not interindividual differences, except in the green region where the  $M$  potential amplitude was relatively small.

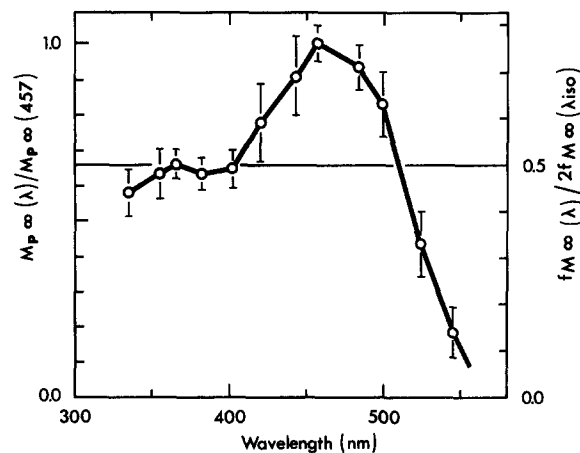


FIGURE 6. The photoequilibrium spectrum which was measured in *Calliphora*. The left ordinate is the normalized amplitude of the  $M$  potential  $M_p(\infty, \lambda)$  measured at a fixed time. The stimulus was a constant, one-sixth of maximum intensity, orange test flash after equilibrating lights of different wavelengths  $\lambda$ . The normalization was to the  $M_p(\infty, 457)$ . The right ordinate gives the fraction of metarhodopsin. This fraction was derived from the left ordinate by assuming that  $\gamma_R = \gamma_M$  (see Eqs. 13 and 14) and that the isosbestic point ( $\lambda_{iso}$ ) is at 510 nm. Thus,  $M(\infty, 510)$  is defined as 0.5. The vertical bars are standard error of the mean. Each bar was calculated from four different experiments.

**THE PHOTOEQUILIBRIUM SPECTRUM** Fig. 6 shows the photoequilibrium spectrum  $f_M(\infty, \lambda)$  measured in *Calliphora* (Eq. 5). The ordinate (left) is the normalized  $M$  potential amplitude induced by a constant (one-sixth maximum intensity) orange test flash plotted as a function of the wavelength of equilibrating adapting lights. The data for Fig. 6 was in fact derived from the same experiments which were used in Fig. 5. Fig. 2 *a* (bottom curve) is indicative of the experimental procedure: for each adapting wavelength the amplitude of the  $M$  potential was plotted as a function of the amount of adapting light ( $It$ ). As a rule we measured two additional points at the saturated level (not illustrated in Fig. 2 *a*) to be sure that photoequilibrium was obtained. The  $M$  potential amplitude at a fixed time from the onset of the red flash after saturated

adaptation was plotted in Fig. 6 as a function of the adapting wavelength. For several adapting wavelengths we used orange as well as blue preadaptations (as in the inset of Fig. 5) and found that the same amplitude of  $M$  potential was obtained at photoequilibrium, which indicates that the initial distribution of the pigment does not affect the photoequilibrium spectrum. The right ordinate of Fig. 6 gives the fraction of metarhodopsin reached in equilibrium. It is derived by assuming: (a) the quantum efficiency of rhodopsin ( $\gamma_R$ ) is equal to the quantum efficiency of metarhodopsin ( $\gamma_M$ ) and (b) that the isosbestic point is at 510 nm. As can be seen, in the red region the metarhodopsin concentration is zero, and in the blue region (at 460 nm) the metarhodopsin concentration is maximal. This is in general agreement with the electrophysiological results of Hamdorf et al. (1973), Hamdorf and Rosner (1973), Rosner (1975), and with the spectrophotometric results of Hamdorf et al. (1973) and Stavenga et al. (1973). It is interesting to note that in the UV region the curve is rather flat and has a value similar to that of the isosbestic point. These facts indicate that the photosensitivity spectra of rhodopsin and metarhodopsin should have similar shapes and peak wavelengths in the UV.

#### *The Derived Photosensitivity Spectra of Rhodopsin and Metarhodopsin*

The product of the photoequilibrium spectrum and the relaxation spectrum gives us the photosensitivity spectrum of rhodopsin according to Eq. 9 (with the assumption that the  $\gamma_R = \gamma_M$ ); i.e.,

$$K_R(\lambda) = f_M(\infty, \lambda)\Lambda(\lambda).$$

This spectrum as a function of wavelength ( $\lambda$ ) is illustrated by circles in Fig. 7. The photosensitivity spectrum of metarhodopsin, was calculated by eq. 10 (see Methods):

$$K_R(\lambda) = \Lambda(\lambda)[1 - f_M(\infty, \lambda)].$$

This spectrum is illustrated as  $\times$  in Fig. 7. The smooth curves (Fig. 7) are photometrically derived photosensitivity spectra of rhodopsin (left) and metarhodopsin (right) in *Calliphora* as measured by Hamdorf, Schlecht, and Schwemer.<sup>1</sup> The shape of our calculated rhodopsin spectrum fits a Dartnall nomogram peaking at 485 nm well. The peak of our rhodopsin spectrum is slightly different from the spectrum obtained by Hamdorf et al.<sup>1</sup> which also has Dartnall nomogram shape.

The photosensitivity spectrum of metarhodopsin in the orange was previously derived by in vivo measurements of Pak and Lidington (1974) by using the criterion action spectrum (CAS) of the  $M$  potential in *Drosophila*. The absorption spectrum of metarhodopsin in the orange was also derived from photometric measurements in *Calliphora* (Hamdorf et al., 1973; Stavenga, 1976). We repeated the measurements of Pak and Lidington (1974) by measuring the CAS of the  $M$  potential in *Calliphora*, but we extended the measurement to the UV region. The purpose of these measurements was to determine directly the ratio of the photosensitivities of metarhodopsin in the UV and at the longer wavelengths (see Discussion).

The reciprocal of the light intensity needed to produce a criterion 0.1 mV *M* potential as a function of the wavelength of the flash light is plotted as + in Fig. 7. All the flash test lights were given after a constant equilibrating 457-nm blue light. Inasmuch as the intensities of the monochromatic flashes were relatively

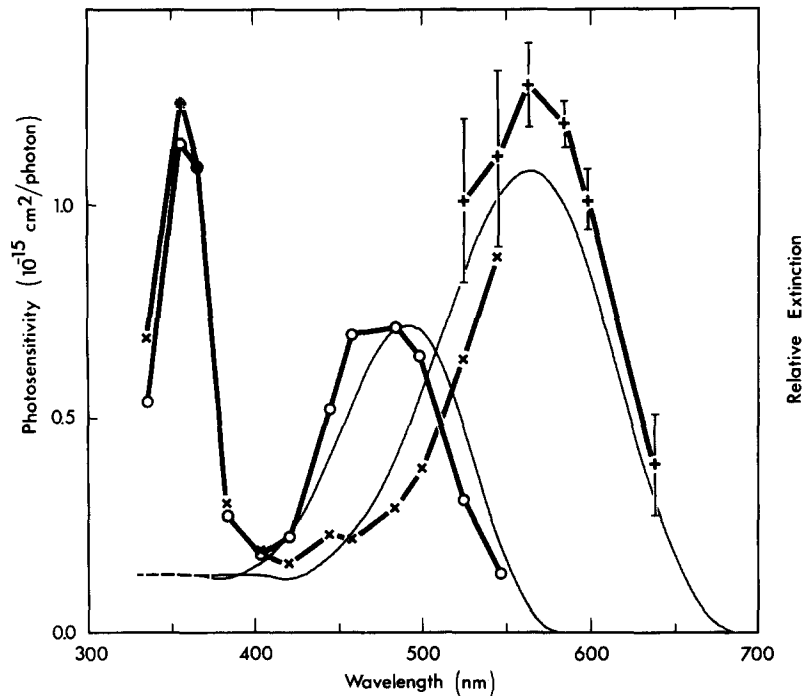


FIGURE 7. The derived photosensitivity spectra of rhodopsin and metarhodopsin. The figure shows the photosensitivities (in  $\text{cm}^2/\text{photon}$ ) of rhodopsin (O) and metarhodopsin (X) as a function of wavelength  $\lambda$  as derived from eqs. 9 and 10, respectively, and by using the data of Figs. 5 and 6 (see Methods). The crosses (+) are the reciprocal of the flash light intensity of different wavelength that was needed to produce a criterion 0.1 mV *M* potential. These flashes were given after equilibrating 457-nm adapting blue light. Thus, the crosses represent the criterion action spectrum (CAS) of the *M* potential which was measured in the orange range with monochromatic filters and in the UV range with broad band UV filter (UG-11, see CALIBRATION in Results). The *M* potential CAS was normalized at the UV point to the peak photosensitivity spectrum of metarhodopsin (+ at 355 nm). The vertical bars are standard errors of the mean calculated from three different experiments. The smooth curves are recent extinction spectra of rhodopsin (left) and metarhodopsin (right) which were derived by Hamdorf, Schlecht, and Schwemer<sup>1</sup> from the photometrically measured difference spectrum of *Calliphora*. These spectra were normalized to the peak photosensitivity of rhodopsin (O).

weak and the photosensitivity of metarhodopsin was relatively low in the blue region, we could not measure this spectrum for wavelengths shorter than 520 nm. It was essential, however, to measure at least one point of the *M* potential CAS also in the UV region. Therefore, we used a broad band UV filter (Schott



UG 11, Mainz, West Germany) together with a heat protecting filter KG 1, 1 mm). With these filters we could get a measureable  $M$  potential. The procedure of calibrating the amount of effective UV photons of this broad band UV flash was the following.

**CALIBRATION** First the emitted quantum intensity distribution  $dQ/d\lambda$  ( $\lambda$ ) of the UV flashes was measured by means of a monochromator of known relative efficiency (GM 100, Schoeffel Instruments Div., Kratos Corp., Westwood, N. J., half-width of 4.25 nm) combined with a radiometer (11 700 Research Radiometer, International Light, Inc.). The same measurement was performed for the 580-nm interference filter which has a half-width of 15 nm. Because the photosensitivity spectrum of the metarhodopsin is approximately constant in the spectral range covered by the 580-nm interference filter, the relative effective quantum intensity  $Q$  (580) of the orange test flash can be determined by integrating over  $dQ/d\lambda$ . In the UV stimulus the half-width of the emitted quantum-intensity distribution  $dQ/d\lambda$  of 50 nm is wide compared with the photosensitivity spectrum in the UV (Fig. 5). Therefore, to calculate the efficient quantum content of the UV stimulus, the integral

$$\int_{-\infty}^{+\infty} \frac{dQ}{d\lambda}(\lambda)\Lambda(\lambda) d\lambda = Q(\text{UV})$$

has to be calculated. The result was that at maximal flash intensities the number of usable quanta  $Q$  (UV) was 0.72 of the number of usable quanta  $Q$  (580). The ratio of the amplitudes of the  $M$  potentials induced with these stimuli was  $M(\text{UV})/M(580) = 0.56$  (average of three determinations).

Insofar as these stimuli are rather weak, we are still in the linear range of the exponential function which describes the increase of  $M$  potential amplitude with intensity. Therefore we calculate tentatively the relative height of the UV to 580 sensitivities as  $0.56/0.72 = 0.78$ . This is a preliminary estimate, however, because we have to consider that the photostable UV pigment does activate not only the transition of  $M \rightarrow R$  but also that of  $R \rightarrow M$ . Before the stimuli were given, the eyes had been preadapted to 457-nm blue light, which produced a mixture of approximately 25% rhodopsin and 75% metarhodopsin present at the onset of the stimulus (see Fig. 6). Therefore not all the light quanta in the UV flash can be used for the  $M \rightarrow R$  transition. If we make the plausible assumption that these UV quanta will be used proportionally to the percentage of  $R$  and  $M$  present, respectively, only 75% will be available for the transition  $M \rightarrow R$ . The relative heights of the UV to 580 photosensitivity hence becomes  $0.78/0.75 = 1.04$ . To this ratio the data of the CAS had been normalized in Fig. 7.

Since the CAS has a relative ordinate only, we normalized the UV point (+) to the UV peak of the derived photosensitivity spectrum of metarhodopsin ( $\times$ ).

#### *The Use of Vitamin A-Deprived Drosophila*

A close similarity between the absorption spectra of rhodopsin ( $\alpha_R$ ) and metarhodopsin ( $\alpha_M$ ) in the UV can explain the null difference spectrum of the fly in the UV (Kirschfeld et al., 1977). However, the double peak photosensitivity of both rhodopsin and metarhodopsin, presented in Fig. 7 is very unusual for visual pigments. Because it is known that vitamin A deprivation affects selectively the UV peak in the spectral sensitivity of the fly (Goldsmith et al., 1964; Stark et al., 1977) we used vitamin A-deprived flies to examine the effect of vitamin A deprivation. Fig. 8 *a* shows two relaxation curves measured for 365 nm UV and 457 nm blue adapting light in *Drosophila* raised on a vitamin A-deficient medium (5 and 10% of normal vitamin A concentration). The figure

shows that in vitamin A-deprived flies we need more UV light than blue light to cause the same fractional shift of pigment. In terms of the relaxation spectrum this means that in contrast to the normal fly where the UV region is the highest in this curve (Fig. 5), in the vitamin A-deprived flies the UV peak is lower than the blue region of the spectrum. This fact corresponds to a selective reduction of the UV peak relative to the visible peak in the photosensitivity spectra. Fig. 8 *a* shows another phenomenon which has been observed in some of the "deprived" flies. In these flies the relaxation curve for UV adapting light was not exponential, whereas that for blue light remained exponential. The significance of these results for determining the origin of the UV peak will be dealt with in the Discussion.

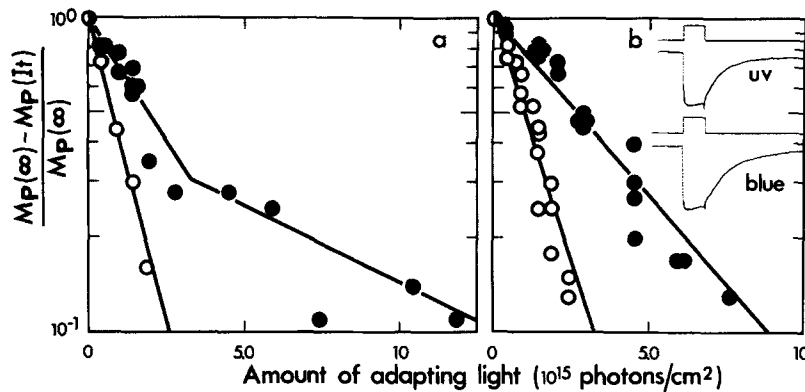


FIGURE 8. The effect of vitamin A deprivation on the relaxation curve in the visible and the UV spectral ranges. Sections *a* and *b* represent relaxation curves which were measured in *Drosophila* that were raised on vitamin A deficient media with 10 and 5% of normal vitamin A content for *a* and *b*, respectively (see Methods). The adapting lights of the relaxation curves are 365-nm UV (●) and 457-nm blue (○) lights. The vitamin A deprivation decreases the slope of the relaxation curves to UV adapting light so that it is no longer steeper than the slope of the blue adapting light as seen in normal flies (Fig. 4). (*a*) In some flies the relaxation curve of UV adapting light is no longer exponential in contrast to the relaxation curve to blue light. (*b*) *Inset*: ERG recordings to strong 365-nm UV and 457-nm blue light which in normal *Drosophila* induce a very long PDA (of several hours), but in the vitamin A-deprived fly no obvious PDA is induced by these wavelengths.

We found a considerable variability in the effect of the same vitamin A-deficient medium on the relaxation curves measured in different deprived flies. Often the deprived flies were indistinguishable from normal. This variability seems to arise from the inhomogeneous consumption of vitamin A by the individual flies raised in the same bottle. We found a systematic way to examine quickly whether the fly has the characteristics illustrated in Fig. 8. This examination is indicated in Fig. 8 (*inset*) which shows the ERG response to strong equilibrating UV and blue light after orange preadaptation. These lights in normal or weakly deprived flies induce a very long PDA (expressed as a prolonged negative phase at the cessation of the light, Fig. 3). In totally vitamin

A-deprived flies, we found no PDA and no *M* potential (see also Razmjoo and Hamdorf, 1976; Stark et al., 1977).

The phenomena illustrated in Fig. 8 can be observed only in deprived flies that show no (or a short) PDA to either blue or UV lights (inset) but still give an *M* potential to an orange test flash.

#### DISCUSSION

##### *The Use of the M Potential to Measure Metarhodopsin Concentration*

It is widely accepted that the ERP arises directly from pigment conformational changes and thus reflects the changes in visual pigment after illumination (Cone, 1967). The *M* potential, on the other hand, seems to arise from the second order neurons (the lamina). It seems to be initiated by the positive (intracellular) ERP of the receptors R 1-6 (Stephenson and Pak, 1978; Minke and Kirschfeld<sup>2</sup>). We have shown that the saturation of the *M* potential amplitude at high light intensities does not necessarily arise from a saturation of shifting metarhodopsin to rhodopsin. However, if we compare microspectrophotometric measurements with *M* potential amplitudes which are sufficiently weak, it becomes obvious that the *M* potential amplitude in this case linearly reflects the concentration of metarhodopsin. We know already that there is no *M* potential arising from activation of rhodopsin (Pak and Lidington, 1974) and that the *M* potential arises only from activation of receptors R 1-6 (Grabowski and Pak, 1976). Thus, the *M* potential is an ideal tool for investigating in vivo changes in metarhodopsin concentration in receptors R 1-6 of the fly.

##### *Photosensitivity Spectra of Rhodopsin and Metarhodopsin Derived from Photoequilibrium and Relaxation Spectra*

If one tries to derive the photosensitivity spectrum of rhodopsin and metarhodopsin in fly photoreceptors 1-6 from the difference spectrum in the UV, one is faced with the difficulty that there is a null difference spectrum in this spectral range (Kirschfeld et al., 1977). However, inasmuch as it is possible to shift visual pigment by UV adaptation, relaxation and photoequilibrium spectra can be measured and photosensitivity spectra can be derived. There are three facts illustrated in Results that support the validity of using the relaxation and photoequilibrium spectra in order to derive the photosensitivity spectra in our experiments: (a) there is an excellent fit of the derived rhodopsin spectrum in the visible range to a Dartnall nomogram peaking at 485 nm which fits very well the CAS of the receptor potential of *Calliphora* (see e.g. McCann and Arnett, 1972; Dörrscheidt-Käfer, 1972; Horridge and Mimura, 1975), and there is also a satisfactory coincidence with the derived rhodopsin extinction spectrum of Hamdorf et al. as calculated from the difference spectrum (Fig. 7). (b) There is a good fit of two sets of relaxation measurements to one relaxation curve for an adapting light of one and the same wavelength after two different preadaptations, one where all the pigment is shifted to the rhodopsin state and the other where a maximal amount of pigment is shifted into the metarhodopsin state

<sup>2</sup> Minke, B., and K. Kirschfeld. Manuscript in preparation.

(Fig. 5, *inset*). This fit supports the theory behind the relaxation measurements which predicts no dependence of the relaxation curve on the initial pigment distribution. (c) The relaxation spectrum in the UV (Fig. 5) and the derived photosensitivity of rhodopsin in the UV (Fig. 7) have a very similar shape (half-width and peak wavelength) to the action spectrum of the receptor potential of *Calliphora* in the UV (McCann and Arnett, 1972; Horridge and Mimura, 1975) which reflects the photosensitivity spectrum of rhodopsin.

It is clear from Eq. 9, that the shape of the photosensitivity spectrum of rhodopsin can be derived without knowing the quantum efficiencies ( $\gamma_M$ ) and ( $\gamma_R$ ) or their ratio ( $\phi = \gamma_M/\gamma_R$ ). This is due to the fact that we can use the shape of the photoequilibrium spectrum (in relative units) without any scaling of pigment concentration for the derivation of this function. However, for the derivation of metarhodopsin photosensitivity, even for the shape, we have to scale the photoequilibrium spectrum in terms of pigment concentration due to the  $[1 - f_M(\infty)]$  term in Eq. 10. In order to get this case we assumed that  $\phi = 1$ , as other investigators usually do (Hamdorf et al., 1973; Stavenga, 1976).

By using the  $M$  potential CAS we could check the validity of our statement in Methods that  $W_R = W_M = 1$ , namely that we did not find evidence for a thermal return of isomerized molecules to the original stable state, unlike the situation in the barnacle (Hochstein et al., 1978). In Fig. 7 we normalized the  $M$  potential CAS (+) in the one point at the UV region to the derived metarhodopsin spectrum (×) in the UV peak. By this normalization we get a rhodopsin (○) to metarhodopsin (+) peak ratio in the visible similar to the ratio of data in Hamdorf et al. (Fig. 7). This similarity suggests that  $W_R$  and  $W_M$  in the fly are in fact close to unity.

Stark and Zitzmann (1976) and Stark et al. (1977) derived the photosensitivity spectrum of the fly metarhodopsin by using the photoequilibrium spectrum together with the CAS of the ERG as a measure of rhodopsin photosensitivity (see Introduction). Their derived metarhodopsin spectrum in the visible range does not have the shape of metarhodopsin absorption and is very different in the peak absorption from the metarhodopsin peak absorption obtained by Pak and Lidington (1974) and by us. It has also a metarhodopsin-to-rhodopsin peak ratio of less than one which is also very unusual. However, they also found that the derived metarhodopsin spectrum has a pronounced peak in the UV.

#### *The Photostable UV Pigment as a Sensitizer for Rhodopsin and Metarhodopsin*

Kirschfeld et al. (1977) presented evidence for the hypothesis that the high UV peak in R 1-6 spectral sensitivity arises from a photostable sensitizing pigment that absorbs the light quanta and transfers the energy to the blue rhodopsin. The results presented in this paper further support this hypothesis for the following reason. According to our knowledge of energy transfer on the basis of the concept of Förster (1951), we expect that if there were energy transfer from a photostable pigment to rhodopsin, there would also be an energy transfer to metarhodopsin, since both extinction spectra are sufficiently close on the wavelength scale. Hence, metarhodopsin as well as rhodopsin should show a high photosensitivity in the UV, whereby the UV spectra should coincide. And

this is exactly what we find (Fig. 7). If there were some other reason for the high UV sensitivity, we should rather expect a different location of the rhodopsin and metarhodopsin UV photosensitivity maxima, if we realize that their maxima in the visible are separated by some 90 nm.

The *M* potential CAS which reflects the shape of the photosensitivity spectrum of metarhodopsin indicates directly that metarhodopsin has a pronounced peak in the UV which is as high as the orange peak. This result supports the validity of the derived photosensitivity spectrum of metarhodopsin (see Fig. 7).

The shape of the derived photosensitivity spectrum of rhodopsin in the UV is much narrower than the photosensitivity spectrum of the UV rhodopsin found in the *Ascalaphus* (Hamdorf et al., 1971 *b*). However, this derived UV spectrum fits the absolute extinction spectrum of the photostable pigment measured by Kirschfeld et al. (1977), and fits the CAS of the fly receptor potential that was measured by several investigators (Burkhardt, 1967; Dörrscheidt-Käfer, 1972; McCann and Arnett, 1972; Horridge and Mimura, 1975). Therefore, the UV sensitivity of the fly R 1-6 does not seem to arise from the existence of a UV rhodopsin.

From the results presented so far, one might suggest that the fly has an unusual rhodopsin and metarhodopsin with double peak absorption spectra. The experiments with vitamin A-deprived flies are against this possibility (Kirschfeld et al., 1977, and Fig. 8). From a pigment with double peak absorption spectrum we expect that reduction in visual pigment concentration will reduce the visible and the UV peaks to the same degree. The selective reduction in UV photosensitivity with vitamin A deprivation and the deviation from an exponential dependence of the relaxation curve of UV adaptation in some flies support the sensitizing pigment hypothesis for the following reason: the energy transfer from the excited photostable pigment to the visual pigment is a bimolecular reaction. It is first order, provided that the energy transfer is independent of concentration. This is the case in normal rhabdomeres. However, at low pigment concentration, the energy transfer might become concentration dependent with different quantum efficiencies for *R* and *M* and therefore the reaction is no longer necessarily first order. This fits the result in Fig. 8 *a*.

The deviation of the relaxation curve from exponential, however, can also be explained in a different way. The high UV sensitivity could be due to a second chromophore of the visual pigment. Low vitamin A concentration might result in two populations of rhodopsin: one with a high UV absorption due to a second chromophore and another one having low UV absorption lacking the second chromophore. This second chromophore might also be part of the opsin molecule itself. Energy from UV quanta, absorbed e.g., by tryptophane in the bovine opsin, is known to be transferable to the retinylidene chromophore, which then is isomerized in the usual way (Rosenfeld and Ottolenghi, 1977; see also Ashmore, 1977).

Any model for UV sensitization predicts selective reduction in the UV sensitivity when the concentration of the visual pigment and (or) the sensitizing pigment are reduced. The detailed mechanism of the sensitization, however, still has to be worked out.

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