

# Heterogenic Components of a Fast Electrical Potential in *Drosophila* Compound Eye and Their Relation to Visual Pigment Photoconversion

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**ABSTRACT** The electroretinogram of the dipteran compound eye in response to an intense flash contains an early, diphasic potential that has been termed the  $M$  potential. Both phases of the  $M$  potential arise from the photostimulation of metarhodopsin. The early, corneal-negative component, the  $M_1$ , can be recorded intracellularly in the photoreceptors and has properties similar to the classical early receptor potential (ERP). The  $M_1$  is resistant to cold, anaesthesia, and anoxia and has no detectable latency. It depends on flash intensity and metarhodopsin fraction in the manner predicted for a closed, two-state pigment system, and its saturation is shown to correspond to the establishment of a photoequilibrium in the visual pigment. On the other hand, the dominant, corneal-positive component, the  $M_2$ , does not behave like an ERP. It arises, not in the photoreceptors, but deeper in the retina at the level of the lamina, and resembles the on-transient of the electroretinogram in its reversal depth and sensitivity to cooling or  $\text{CO}_2$ . The on-transient, which is present over a much wider range of stimulus intensity than the  $M$  potential, has been shown to arise from neurons in the *lamina ganglionaris*. Visual mutants in which the on-transient is absent or late are also defective in the  $M_2$ . It is proposed that the  $M_2$  and the on-transient arise from the same or similar groups of second-order neurons, and that the  $M_2$  is a fast laminar response to the depolarizing  $M_1$  in the photoreceptors, just as the on-transient is a fast laminar response to the depolarizing late receptor potential. Unlike the  $M_1$ , the  $M_2$  is not generally proportional to the amount of metarhodopsin photoconverted, and the  $M_2$  amplitude is influenced by factors, such as a steady depolarization of the photoreceptor, which do not affect the  $M_1$ .

## INTRODUCTION

Fast electrical potentials, arising from photostimulation of rhodopsin of its photoproducts, have been found in the eyes of both vertebrates (Brown and Murakami, 1964; Pak, 1968; Cone and Pak, 1971) and invertebrates (Smith and Brown, 1966; Hagins and McGaughy, 1967; Hillman et al., 1973). When such a potential arises directly from the visual pigment, it is termed an early

receptor potential or ERP (Brown and Murakami, 1964). The ERP has been shown to have the following properties (see review by Cone and Pak, 1971): (a) it is seen intracellularly in the photoreceptors, and there its polarity is opposite to that seen extracellularly; (b) it has no detectable latency; (c) its action spectrum matches that of rhodopsin or (in the case of a photoproduct ERP) one of the photoproducts of rhodopsin; (d) it is very resistant to cold, anoxia, anaesthesia, and a variety of nonphysiological chemical treatments such as high extracellular potassium; (e) its amplitude is proportional to the intensity of the stimulating flash at low and moderate intensities and saturates at very high intensities.

Pak and Lidington (1974) found a fast potential in the electroretinogram (ERG) of *Drosophila* which they termed the metarhodopsin or  $M$  potential. The  $M$  potential is biphasic, and both components have the same action spectrum as the stable metarhodopsin<sub>580</sub> of the peripheral photoreceptors R1-6 (Pak and Lidington, 1974; Grabowski and Pak, 1976). A similar  $M$  potential occurs in the eyes of other dipteran flies (Stark et al., 1977). The corneal-positive component of the  $M$  potential, the  $M_2$ , disappears when the fly is anaesthetized or killed (Pak and Lidington, 1974), suggesting that it may differ in nature from a classical ERP. However, the initial, or  $M_1$ , component appears to be much more resistant to such treatment (Grabowski and Pak, 1976). In this paper we shall present evidence that the  $M_1$  is, in fact, an ERP whereas the  $M_2$  arises from higher order cells.

The primary reason for interest in the  $M$  potential is that, like the ERP, it seems to afford a measure of the state of the visual pigment. Pak and Lidington (1974) had measured the dependence of the  $M_2$  component on flash intensity in the mutant *norpA*<sup>P12</sup>, which has virtually no late receptor potential (LRP), and had found that it was roughly linear for low to moderate stimulus intensity. In view of our conclusion that the  $M_2$  is not an ERP, we reexamined in more detail the suitability of using the  $M_1$  and the  $M_2$  as measures of metarhodopsin level. We will present evidence that the  $M_1$  depends linearly on the amount of metarhodopsin photoconverted by a flash, whereas the  $M_2$  does so only very approximately.

#### METHODS

All flies used in this study were made homozygous for the white-eye mutations *w* or *bw;st* to eliminate the screening pigments which would otherwise have severely attenuated the light stimuli. Thus, "wild type" here refers to the Oregon-R wild-type strain carrying the mutation *w*.

Electrodes for extracellular recording were 5-10 M $\Omega$  in resistance and filled with insect saline (Hoyle, 1955; modified by omission of CaCl<sub>2</sub>) and were driven into the eye to a depth of 40-50  $\mu$ m. The technique for intracellular recording was essentially that of Alawi and Pak (1971). Electrodes for intracellular recording were of 100-300 M $\Omega$  resistance and filled with 2 M potassium chloride or potassium acetate. The cornea was punctured with a gross electrode which was then withdrawn, and a fine electrode was inserted through this hole. A second gross electrode, which also penetrated the cornea, measured the ERG, and a third, inserted in the proboscis, provided the electrical reference. To obtain the depth profile of the ERG the same procedure was followed as for intracellular recording, except that the fine electrode was selected

for lower resistance. The electrode track was radial to the eye, and depth was measured directly from the micromanipulator dial, taking as zero the depth at which the electrode first made contact with the cornea. Recordings were made at 25- $\mu\text{m}$  intervals while advancing and again during withdrawal. ERG depth profiles taken while advancing and receding agreed with each other to within 10  $\mu\text{m}$ , indicating that tissue distortion was very slight.

All electrodes were mounted in plastic holders (W-P Instruments, Inc., New Haven, Conn.) containing a silver-silver chloride bridge which was shielded from light by black plastic. These were then wrapped with black electrical tape. Occasionally, in spite of these precautions, a small, positive photopotential appeared, distinguishable from the  $M$  potential because it was unaffected by prior adaptation and resembled the stimulating flash in time-course. We did not attempt quantitative measurements of the  $M_1$  in experiments where this photopotential was present. The bandwidth of the recording system was normally adjusted for greatest signal-to-noise ratio and extended from 3 to 1,000 Hz. Increasing this bandwidth had little effect on the size or shape of the  $M$  potential. The  $M$  potentials were photographed and their base-line-to-peak amplitudes were measured directly from the photographs.

The stimulus flashes were produced by a 60J photographic strobe lamp (Honeywell Strobonar 65C, Honeywell, Inc., Minneapolis, Minn.) and delivered to the eye by a fiber optics light guide. The strobe unit was enclosed in a copper-lined soft steel box to eliminate artifacts. Light from the strobe lamp passed through two heat filters (KG-1, Klinger Scientific Apparatus Corp., Jamaica, N.Y.), a broad-band color filter, and a variable number of neutral density filters (Balzers Corp., Hudson, N.H.). The color filters were type CS 3-67 or CS 5-56 (Corning Glass Works, Corning, N.Y.), referred to hereafter as "orange" or "blue," respectively. Before each stimulus flash the eye was adapted as necessary by light from a tungsten source. This light was filtered by 460- or 600-nm interference filters (type B-3, Baird-Atomic, Cambridge, Mass.) and delivered to the eye by a branch of the light guide mentioned above.

Inasmuch as *Drosophila* metarhodopsin is thermostable, light of any color will eventually establish a photoequilibrium between rhodopsin and metarhodopsin. It is in this sense that we use the term "adaptation" hereafter. Thus, "460-nm adaptation" means that the pigment is brought to photoequilibrium with 460-nm light, and similarly "orange-adapted" means equilibrated with orange flashes. The amount of adapting light given was always at least  $3/\Lambda$  (the relaxation constant  $\Lambda$  is defined in the section on photoconversion theory) and usually more.

The light intensity at the fly's eye was measured for each flash with a specially designed monitor consisting of fiber optics probe, a silicon photodiode (PIN-3DP, United Detector Technology, Inc., Santa Monica, Calif.), a low noise sample-and-hold amplifier and a readout. The monitor sampled the peak flash intensity at the eye. Its peak sensitivity was adjusted to 480 nm by an internal CS 5-56 filter. The intensity of an unattenuated white (unfiltered) strobe flash was  $\sim 7 \times 10^{16}$  photons/cm<sup>2</sup> at the level of the fly's eye. The intensity of a blue flash was  $\sim 3.4 \times 10^{16}$  photons/cm<sup>2</sup>, and that of an orange flash  $1.5 \times 10^{16}$  photons/cm<sup>2</sup>. These measurements were made with a calibrated photodetector (Lite Mike T.M., Edgerton, Germeshausen & Grier, Inc., Boston, Mass.) and a monochromator (model 33-86-02, Bausch & Lomb, Inc., Rochester, N.Y.). A "flash" hereafter refers to an unattenuated strobe flash unless attenuation is specified.

Experiments were conducted on a stage whose temperature was electronically regulated. The fly was waxed to a glass cover slip which was attached to the temperature stage with thermally conductive paste. Temperatures, where given, are those of the stage. Where no figure is mentioned the temperature was 23°C.

## RESULTS

*Origin of the M Potential*

Previous work in this laboratory<sup>1</sup> has shown that the two components of the *M* potential behave quite differently: only the *M*<sub>1</sub> is present at low temperature, or in certain visual mutants, or intracellularly in photoreceptors. The *M*<sub>2</sub>, moreover, inverts at a greater depth in the retina than does the *M*<sub>1</sub>. We have confirmed these differences and, in addition, have observed that the *M*<sub>2</sub> and the on-transient behave very similarly. In this section we present evidence

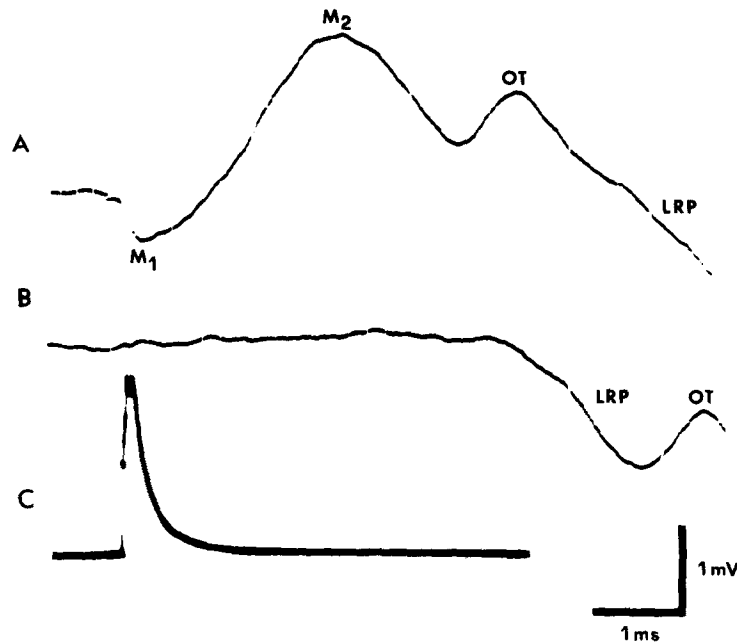


FIGURE 1. ERG of white-eyed *Drosophila* to a broad-band orange strobe flash (see Methods). Corneal positivity is upward. (A) Retina partially blue adapted, showing (in order) *M*<sub>1</sub>, *M*<sub>2</sub>, on-transient (OT), and late receptor component (LRP). LRP onset (which actually precedes OT—see trace B) is obscured by *M*<sub>2</sub>. (B) Retina orange adapted; only LRP component and on-transient are present. Their latency is increased in relation to trace A due to the difference in adaptation. (C) Time-course of stimulus flash.

that with respect to site of origin, resistance to cooling or CO<sub>2</sub> anaesthesia, and effect of certain mutations, the *M*<sub>2</sub> resembles the on-transient much more closely than it does the *M*<sub>1</sub>. On the basis of this evidence, we suggest that the *M*<sub>2</sub> and the on-transient have a common origin. A preliminary account of this has already appeared (Stephenson and Pak, 1978).

Fig. 1 illustrates the components of the *M* potential as they appear in the ERG. Fig. 2 compares the intracellular and extracellular responses to a white

<sup>1</sup> Grabowski, S. R., M-V. C. Lo, M. Wilcox, and W. L. Pak. Manuscript in preparation.

flash. Although previous blue adaptation favors the  $M_1$ , it induces a prolonged depolarizing afterpotential (PDA) in the photoreceptors which interferes with both the late receptor potential (LRP) and the  $M_2$  (discussed further below, see Fig. 12). Thus in Fig. 2 A, as in many of the following experiments, the retina was adapted to white flashes. Fig. 2 A records simultaneously the ERG (lower trace) and the intracellularly recorded response of a photoreceptor (upper trace) to a white flash. The  $M_1$ , though barely visible in the ERG because of the reduced gain (compare with Fig. 1), was relatively large and depolarizing in the photoreceptor. The  $M_2$ , on the other hand, was apparent only in the extracellular record, as was the on-transient. When the retina was orange adapted (visual pigment converted almost entirely to rhodopsin; Fig.

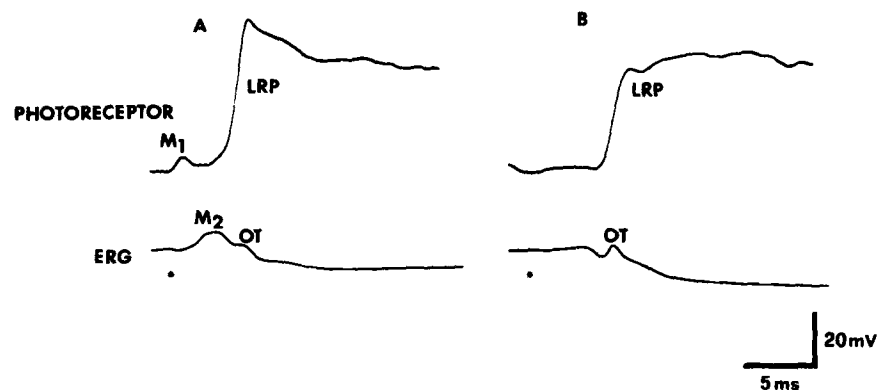


FIGURE 2. ERG (lower) and intracellularly recorded response of photoreceptor (upper) to a white flash, indicated by black dot. (A) Retina adapted to white flashes. Only depolarizing  $M_1$  and LRP are seen in intracellular recording. In the ERG (at same scale) cornea-negative  $M_1$  is hardly visible, but  $M_2$  and on-transient (OT) are prominent, followed by LRP component. Compare with Fig. 1 A, which was recorded under similar conditions. (B) Retina adapted to 600 nm.  $M_1$  absent intracellularly, and no  $M$  potential in ERG. Compare with Fig. 1 B.

2 B), the  $M$  potentials disappeared leaving only the LRP in the photoreceptor and—in the ERG—the on-transient and LRP component.

Fig. 3 shows how the ERG changes with depth below the cornea. In the right-hand column are traces taken at a higher gain and a much higher sweep speed of the ERG to a white strobe flash, showing (in order) the  $M_1$ ,  $M_2$ , on-transient, and LRP component. The traces in the center column, taken at lower gain and on a much slower time scale, are the ERG to a 1-s white light. Since this steady light was much less intense than the strobe flash, it generated only the receptor component (tonic response) and on- and off-transients (phasic responses at on- and offset, respectively, of stimulus). Recordings were made at successive 25- $\mu\text{m}$  intervals on a radial track, beginning  $\sim 10 \mu\text{m}$  below the corneal surface. On the left of Fig. 3, for comparison only, is a camera lucida drawing of a corresponding section through another eye, showing

ommatidia, basement membrane, laminar cartridges (sectioned obliquely), and laminar and medullary rinds (cell body layers).

The traces in Fig. 3 reveal the following important points: (a) the ERG did not change significantly in the first 85  $\mu\text{m}$ ; (b) beyond this depth the  $M_1$  diminished, and reversed sign at a depth of 110–135  $\mu\text{m}$ ; (c) the LRP

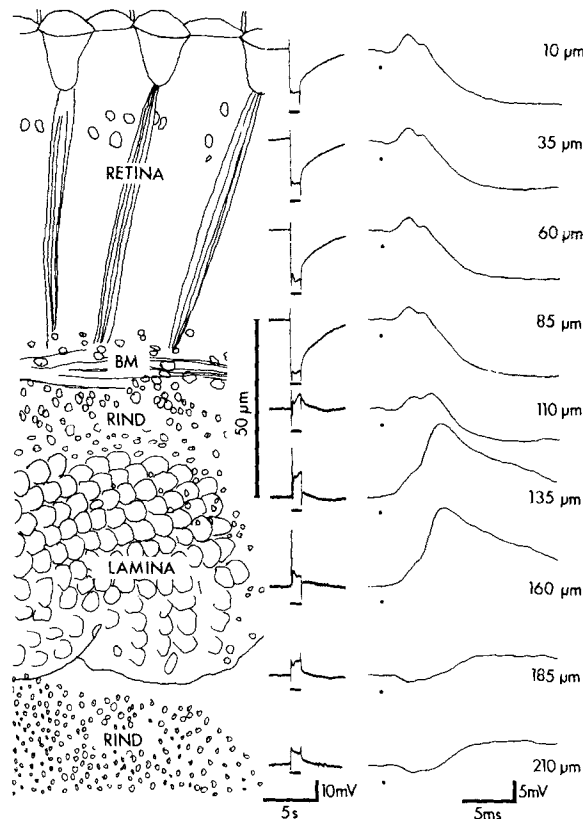


FIGURE 3. Depth profile of ERG of white-eyed fly to a 1-s white stimulus (center column) or a white strobe flash (right-hand column). Stimuli are indicated by bars or dots beneath traces. Traces are arranged vertically in order of depth, with the top trace recorded  $\sim 10 \mu\text{m}$  below cornea, and each subsequent recording 25  $\mu\text{m}$  deeper than the last. At left is a camera lucida drawing of a corresponding section through another eye, indicating retina, basement membrane (BM), laminar rind, laminar cartridges (sectioned obliquely), and medullary rind. See text.

component reversed sign at 85–135  $\mu\text{m}$ ; (d) the  $M_2$  did not reverse sign until a depth of 160–185  $\mu\text{m}$ , (e) the on- and off-transients likewise reversed sign at 160–185  $\mu\text{m}$ . Thus, in relation to the camera lucida drawing, the  $M_1$  and LRP components inverted at a depth that seems to correspond to the basement membrane or the laminar rind, whereas the  $M_2$ , on-, and off-transients inverted near the proximal border of the lamina.

On the fast traces recorded at 135 and 160  $\mu\text{m}$ , the  $M_2$  appeared as a shoulder on the rising edge of a larger peak, composed of the on-transient and LRP component (which at that depth have the same sign). Fig. 4 shows that we are correct in identifying this as the  $M_2$ . Fig. 4 A is identical to the seventh trace in Fig. 3 (160  $\mu\text{m}$ ) and shows the ERG to a white flash. Fig. 4 B shows the response, at the same depth and with the same prior white adaptation, to a blue flash. The blue flash photostimulated rhodopsin (and thus evoked a LRP) almost as efficiently as the white, but was far less effective at stimulating metarhodopsin (and hence at generating an  $M$  potential). Note that the shoulder (attributed to the  $M_2$ ) is negligible in Fig. 4 B.

Thus it appears that the  $M_2$  arises, not in the photoreceptors, but trans-synaptically in the lamina. Since synaptic transmission is severely affected by cold and anaesthesia, we investigated how these affect the  $M_2$  on the on-transient, as shown in Figs. 5 and 6.

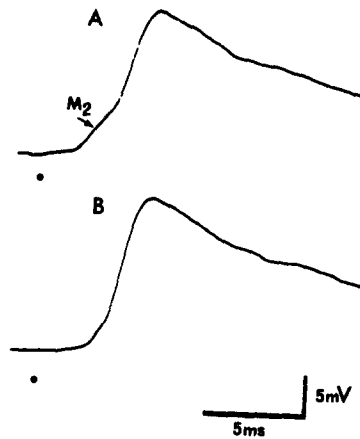


FIGURE 4. ERG at 160  $\mu\text{m}$  in fly of Fig. 3. (A) White flash. (B) Blue flash, which does not generate  $M$  potential. Note that the early component (labeled  $M_2$ ) is present in A but not in B. In both A and B eye was white-adapted.

Fig. 5 shows the effect of temperature on the ERG to successive white flashes. When the fly was cooled to 5°C, only the  $M_1$  and the receptor component were present. As the eye slowly warmed up, the ERG was measured every 30 s. After 90 s the on-transient had returned, although it arose relatively late on the falling slope of the LRP component. A slight  $M_2$  might have been present. After 120 s the on-transient had diminished in latency, and the  $M_2$  was clearly evident as well.

Both the  $M_2$  and the on- and off-transients disappeared under  $\text{CO}_2$  anaesthesia or  $\text{N}_2$ -induced anoxia. A light dose of anaesthesia increased the latency and duration of both the  $M_2$  and the on-transient until the two overlapped, making it difficult to judge whether they were equally affected or not. To remedy this confusion we made use of a third-chromosome mutant, provisionally designated *t305*, in which the latency of the LRP (and hence of the on-

transient) is much greater than in the wild type. To minimize the LRP component we recorded the ERG at a depth of 150  $\mu\text{m}$  below the cornea, where this component was very small (Fig. 6). The stimulus was a white flash. When the eye was white adapted (Fig. 6 A), both the  $M_2$  and the on-transient can be seen, separated by  $\sim 4$  ms. In Fig. 6 B the eye had been previously orange adapted (photoconverting nearly all metarhodopsin to rhodopsin), and only the on-transient was present. Fig. 6 C shows the ERG of this mutant measured every 30 s before, during, and after  $\text{CO}_2$  anaesthesia. 10 s after  $\text{CO}_2$  was turned on both the  $M_2$  and the on-transient had disappeared. The LRP component disappeared much more slowly. At 180 s, 10 s after  $\text{CO}_2$  was shut off, the LRP component had returned. Both  $M_2$  and on-transient had returned

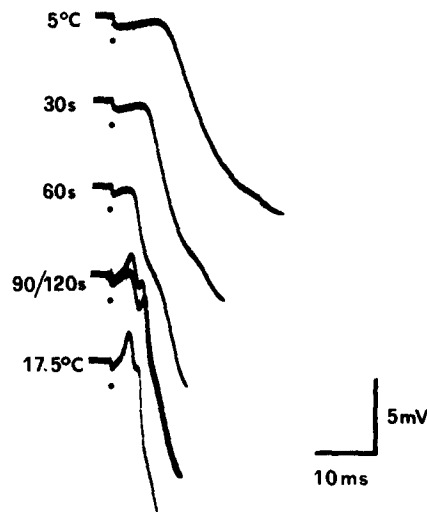


FIGURE 5. Recovery of  $M$  potential during rewarming from  $5^\circ\text{C}$ . At  $5^\circ\text{C}$  only  $M_1$  and receptor component were present. As fly slowly warmed up recordings were made at 30-s intervals. Last trace ( $17.5^\circ\text{C}$ ) was made 2 min after full recovery. Stimuli (dots) were white flashes.

by 240 s, although their height was reduced, especially in the case of the  $M_2$ . In the final trace both had nearly regained their initial height.

The clearest evidence linking the  $M_2$  and the on-transient, however, comes from the study of ERG-defective mutants (Fig. 7). All ERGs in this figure are in response to an orange flash. In each row from left to right are the first and second responses after 460-nm adaptation and the first response after 600-nm adaptation.

In the case of the wild type, the two ERGs following blue adaptation show the  $M_2$  followed by the on-transient while the right hand trace shows only the on-transient.<sup>2</sup> In the mutant *ebony* the on-transient was slower and arose much

<sup>2</sup> Both the on-transient and LRP component are less pronounced in the left-hand traces because prior 460-nm adaption inactivates the photoreceptors. Consequently the first orange flash to a 460-nm-adapted eye produces a much more sluggish response than later flashes.



later (relative to the onset of the LRP) than in the wild type, and the  $M_2$  appeared to be totally lacking. The mutants *tan* and *nonA* seemed to have no  $M_2$  or on-transient at all. In the mutant *nonC* the on-transient had the same latency relative to the LRP as in the wild type, but its amplitude was

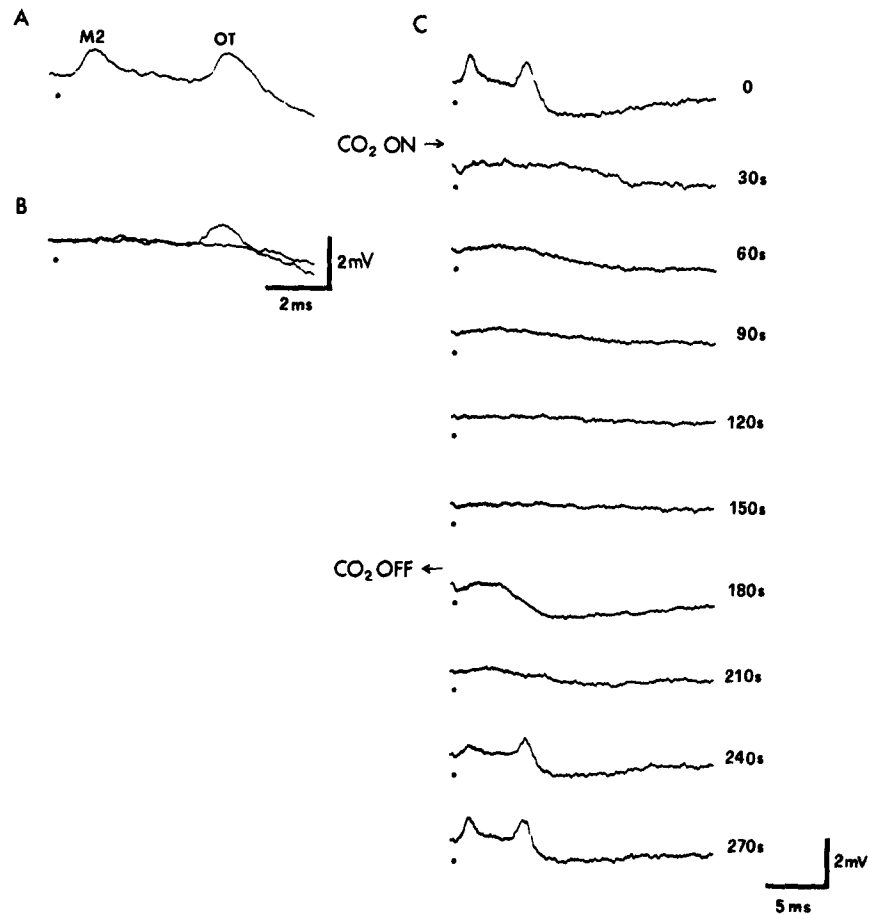


FIGURE 6. Effect of CO<sub>2</sub> anaesthesia on  $M_2$  and on-transient in visual mutant *t.305*, recorded at 150  $\mu\text{m}$  retinal depth. Stimuli (dots) were white flashes. Traces (A) (white-adapted) and (B) (orange-adapted) identify the two peaks as the  $M_2$  and on-transient (OT), respectively. (C) shows effect of CO<sub>2</sub>. Traces were recorded at 30-s intervals. CO<sub>2</sub> was turned on 20 s after first trace, and was turned off at 170 s.

somewhat reduced. The  $M_2$  suffered a corresponding reduction in this mutant. Thus, in mutants where the on-transient was absent or much increased in latency (*ebony*), the  $M_2$  was lacking, and where the on-transient was reduced in amplitude the  $M_2$  was also reduced. Note that the  $M_1$  was unaffected by any of these mutations.

On the basis of the evidence presented above we suggest that the  $M_2$  arises not from the photoreceptors but from higher order cells, possibly those in the lamina that give rise to the on-transient (see Discussion). This casts doubt on the reliability of the  $M_2$  as a measure of metarhodopsin concentration, and we will examine the question presently. But first we must consider whether the  $M_1$  is a linear measure of metarhodopsin level.

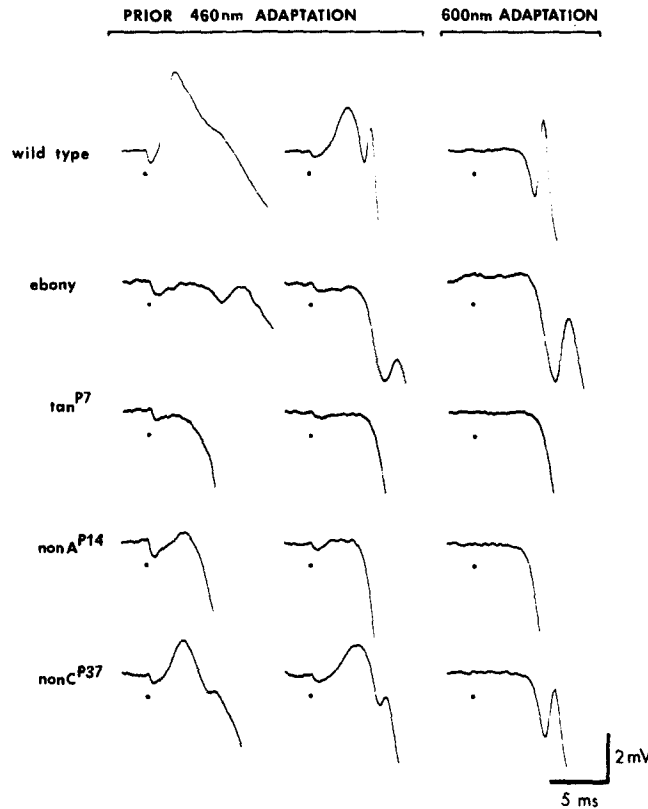


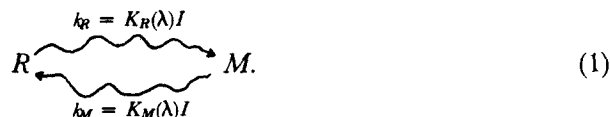
FIGURE 7.  $M$  potentials of wild type and four mutants defective in the on-transient. The traces in each row are the first and second responses after 460-nm adaptation, and the first response after 600-nm adaptation. Stimuli (dots) were orange flashes.

#### *Linearity of the $M$ Potential*

That an ERP response is proportional to the amount of visual pigment photoconverted does not imply that it is proportional to the intensity of the stimulating flash. The reason is that visual pigment conversion saturates at high light levels, and is itself a nonlinear function of intensity. Thus, to determine whether  $M$  potential amplitude is a linear function of the amount of metarhodopsin photostimulated, one needs an independent, *in vivo* measure of the latter. The metarhodopsin level can be measured by microspectropho-

tometry, but for technical reasons it is difficult both to record the  $M_1$  accurately and make accurate microspectrophotometric measurements simultaneously from the same fly. Instead we have measured the incident light flux and assumed that the amount of visual pigment photoconverted from metarhodopsin to rhodopsin, or vice versa, bears a simple relation to the number and wavelength of the photons delivered to the eye. We will spell out this relation and the reasons for assuming it here and in the Discussion, but its ultimate justification comes from the excellent agreement between the  $M_1$  amplitude predicted on this basis and that actually observed.

**PHOTOCONVERSION OF VISUAL PIGMENT: THEORY** We assume that the photochemical reaction converting rhodopsin to metarhodopsin (or metarhodopsin to rhodopsin) is first-order in both the number of rhodopsin (or metarhodopsin) molecules stimulated and the light flux  $I$  in quanta/cm<sup>2</sup>s incident on the eye, and that the constant of proportionality  $K_R$  (or  $K_M$ ) is a function of wavelength only:



The constant  $K_R(\lambda)$  or  $K_M(\lambda)$  is equal to the photosensitivity—the product of molecular absorptivity and quantum efficiency of rhodopsin (metarhodopsin)—multiplied by a factor that reflects the collecting and filtering properties of the dioptric apparatus and optic media. Let us define  $f_M$  as the fraction of visual pigment in the metarhodopsin state and, similarly,  $f_R$  as the fraction in the rhodopsin state. We assume further that all of the visual pigment is in the form of either rhodopsin or metarhodopsin, that is:

$$f_R + f_M = 1. \quad (2)$$

The first-order rate constants shown in Schema 1 above suppose that the pigment is optically thin; i.e., pigment molecules in the distal part of the rhabdomere do not absorb so much of the incident light that they “shadow” those in the proximal part. This assumption seems to be justified for two reasons. First, the total change in optical density of the rhabdomeres (measured by the deep pseudopupil technique) in *Drosophila* does not exceed 0.16 log unit at any wavelength when the pigment is photoconverted from rhodopsin to metarhodopsin or vice versa.<sup>3</sup> This is in contrast to other dipteran flies such as *Calliphora* where the rhabdomeres are much longer. The second justification for this assumption is that all flies used in this experiment lacked screening pigments, and thus the incident light was not constrained to pass along the rhabdomeres but could also pass in between them and impinge on them from the side. The absence of screening pigments, moreover, means that pigment granule migration was not a factor in these experiments.

<sup>3</sup> Lo, M.-V. C. Personal communication.

Eq. 2 above supposes that the population of any unstable intermediate states between rhodopsin and metarhodopsin is insignificant in relation to the total number of pigment molecules at even the highest intensities used. Since the fraction of visual pigment in intermediate states increases with increasing light intensity, any deviation from this assumption would appear as an intensity-dependent discrepancy between observed and predicted  $M_1$  amplitude. No such intensity-dependent effect was seen; in all cases the size of the  $M_1$  to a test flash appeared to depend only on the total amount and wavelength of the adapting light, and not on its intensity. The spectrophotometric measurements of Kirschfeld et al. (1978), moreover, indicate that the transitions from rhodopsin to metarhodopsin and vice versa are fast (0.125 ms or less in duration) compared to the time necessary for our most intense flashes to photostimulate a major fraction of the visual pigment.

It follows directly from Schema 1 that, when the visual pigment has reached an equilibrium with respect to light of a certain wavelength (i.e., the number of rhodopsin to metarhodopsin conversions per second equals the number in the opposite direction), then

$$f_M^\infty(\lambda)K_M(\lambda) = f_R^\infty(\lambda)K_R(\lambda),$$

where  $f_M^\infty(\lambda)$  indicates the metarhodopsin fraction that is in equilibrium with light of wavelength  $\lambda$ . By making use of Eq. 2, the above equation becomes

$$f_M^\infty K_M = (1 - f_M^\infty)K_R, \text{ or } f_M^\infty(\lambda) = \frac{K_R(\lambda)}{K_R(\lambda) + K_M(\lambda)}. \quad (3)$$

Let us define

$$\Lambda(\lambda) = K_M(\lambda) + K_R(\lambda). \quad (4)$$

A simple kinetic argument (see, for example, Hochstein et al., 1978) shows that when the visual pigment, adapted to one wavelength  $\lambda_i$ , is exposed to light of a new wavelength  $\lambda_f$  and intensity  $I$ , the metarhodopsin fraction  $f_M$  relaxes exponentially from the old equilibrium  $f_M^i = f_M^\infty(\lambda_i)$  to the new one  $f_M^f = f_M^\infty(\lambda_f)$  with a relaxation constant  $\Lambda(\lambda)$ :

$$f_M(It, \lambda_i, \lambda_f) = f_M^f + (f_M^i - f_M^f)e^{-\Lambda It}.$$

In terms of the amount of light  $A = It$  (in photons/cm<sup>2</sup>),

$$f_M(A, \lambda_i, \lambda_f) = f_M^f + (f_M^i - f_M^f)e^{-\Lambda A}, \quad (5)$$

and the metarhodopsin fraction  $f_M$  will have relaxed 63% ( $1 - 1/e$ ) of the way from  $f_M^i$  to  $f_M^f$  when exposed to an amount of light  $1/\Lambda(\lambda_f)$ .

In the case where the stimulating light is not monochromatic, Eqs. 3-5 still hold with the reservation that  $K_R(\lambda)$  and  $K_M(\lambda)$  be replaced by their weighted mean values over the range of incident wavelengths.

AMPLITUDE OF  $M_1$  IF AN ERP: THEORY The intracellularly recorded amplitude of an ERP is presumed to be proportional to the number of

photoconverted visual pigment molecules. More precisely, an ERP presumably arises as a result of integrating a displacement current in the membrane capacitance of the photoreceptor, and this displacement current is proportional to the rate of pigment photoconversion. In our case, an  $M$  potential is generated only on the transition from metarhodopsin to rhodopsin. Thus, if the rate of  $M$  to  $R$  photoconversion is as given in Schema 1, then the amplitude  $P(t)$  of the intracellularly recorded  $M_1$  potential should be given by

$$P(t) = \frac{QK_M(\lambda)}{C} \int_0^t f_M(t') I(t') dt', \quad (6)$$

where  $C$  is the membrane capacitance of the photoreceptor,  $Q$  is a constant relating the amplitude of the photoinduced charge displacement to the amount of photoconversion, and where the effect of the photoreceptor membrane conductance has been neglected (i.e., it is assumed that the input time constant is long compared to the duration of the stimulating flash).

By changing the variable of integration from  $t$  to  $A = It$ , Eq. 6 becomes

$$P(A) = \frac{QK_M(\lambda)}{C} \int_0^A f_M(A') dA'. \quad (7)$$

If the metarhodopsin fraction  $f_M(A)$  is given by Eq. 5 then

$$P(A) = \frac{QK_M(\lambda)}{C} f_M^f A + \frac{QK_M(\lambda)}{C\Lambda} (f_M^i - f_M^f)(1 - e^{-\Lambda A}); \quad (8)$$

or (by Eq. 5)

$$P(A) = \frac{Q(1 - f_M^f)}{C} [f_M^f \Lambda A + f_M^i - f_M(A)], \quad (9)$$

where we have made use of the fact that  $f_R^\infty = 1 - f_M^\infty = K_M/\Lambda$ . If  $A$  is the total amount of light in the flash (proportional, for constant flash time-course and spectral distribution, to flash intensity or energy), then  $P(A)$  is the peak amplitude of the ERP in a photoreceptor. This potential would then decay exponentially at a rate determined by the input time constant (equal to  $C \cdot R_m$ , where  $R_m$  is the cell resistance) of the photoreceptor.

If  $\Lambda \cdot A$  is not large compared to 1 and if  $f_M^f$  (the metarhodopsin fraction in equilibrium with the stimulating flash) is small compared to  $f_M^i$  (the initial metarhodopsin fraction), the first term in Eq. 9 becomes insignificant and the ERP should be proportional to the net shift in metarhodopsin fraction  $f_M^i - f_M(A)$  and even (to the extent that  $f_M^f \ll f_M^i$ ) proportional to  $f_M^i$  itself. In the opposite extreme, where  $\Lambda \cdot A \gg 1$  the ERP should become proportional to the energy of the stimulating flash.

In deriving Eq. 9, we assumed that the pigment transitions occur rapidly with respect to the flash duration, that each  $M$  to  $R$  transition contributes equally to the ERP, and that  $R$  to  $M$  transitions do not contribute. If, on the other hand, one or more of these conditions is not satisfied then it is possible

that a given visual pigment molecule may effectively contribute to the ERP only once during a flash. This leads to a simplification of Eq. 9<sup>4</sup>:

$$P(A) = \frac{Qf_M^i}{C} (1 - e^{-K_M A}) = \frac{Q}{C} [f_M^i - f'_M(A)], \quad (9')$$

where  $f'_M(A)$  is the metarhodopsin fraction remaining after an amount of light  $A$  in the absence of any photoregeneration of metarhodopsin.

Eq. 9' is mathematically equivalent to the equation derived by Williams (1964) for the amount of vertebrate rhodopsin bleached by a flash. Note that for a constant flash energy  $A$ , Eq. 9' predicts that the ERP  $P(A)$  is proportional to  $f_M^i$ , the initial metarhodopsin fraction. For moderate amounts  $A$  of stimulating light and for  $f_M^f$  small compared to  $f_M^i$ , Eqs. 9 and 9' are almost the same. If  $f_M^f = 0$  they are identical. When  $\Lambda \cdot A$  is large compared to 1, however, Eq. 9 continues to grow with  $A$  whereas Eq. 9' saturates at a value

$$P_{\max} = \frac{Q}{C} f_M^i. \quad (10)$$

As we will see below, Eq. 9' is in better agreement with the data.

The preceding discussion concerns the intracellularly recorded amplitude of the ERP. The ERP is most often recorded extracellularly, however, since extracellular recordings are simpler, more stable, and sometimes more accurate. All the quantitative measurements we report here are extracellular, and so we must consider how these are related to the intracellularly recorded amplitude.<sup>5</sup> Murakami and Pak (1970) reported that the time-course of the extracellularly recorded ERP of the vertebrate eye was roughly the first derivative of the intracellularly recorded time-course. This suggests that the extracellularly recorded ERP is proportional to the rate of photoconversion rather than its integral. This is clearly not the case in *Drosophila*, as shown by recordings from the double mutant *tan/norpA* in which both the  $M_2$  and the LRP are very much reduced (Fig. 8). The extracellularly recorded  $M_1$  (Fig. 8 B) peaks later than the light stimulus (Fig. 8 C), nearer to the time of peak of the intracellularly recorded  $M_1$  (Fig. 8 A). But, since the extracellular and intracellular waveforms are not the same shape, the question of their proportionality is not simple and can be resolved only by further experiment.

**AMPLITUDE OF EXTRACELLULARLY RECORDED  $M_1$ : EXPERIMENT** We wish to show whether the extracellularly measured  $M_1$  depends on adaptation and flash intensity in the manner predicted by Eq. 9 or 9' and thus whether, by

<sup>4</sup> To be precise, Eq. 9' assumes that only those metarhodopsin molecules present at the start of the flash may contribute to the  $M_1$  when photoconverted. Alternative assumptions are possible.

<sup>5</sup> At first glance it might seem obvious that the extracellular  $M_1$  should be proportional to the intracellular  $M_1$ , since the output of any electrical network made up of linear elements (resistors, capacitors, etc.) is necessarily linear. In fact, however, the wave shape as well as the amplitude of the intracellular  $M_1$  changes with intensity, coming to a peak earlier at higher intensities. Thus, if the transfer function between intracellular and extracellular potentials is strongly frequency dependent, this would introduce a departure from linearity. There is also the possibility that the extracellular  $M_1$  peak is contaminated by foreign, nonlinear elements such as the  $M_2$ .

implication, it is proportional to the amount of photoconverted metarhodopsin. The experimental protocol is shown in Fig. 9 A. The retina was 460-nm adapted for 1 min, sufficient to bring metarhodopsin to its equilibrium level,  $f_M^\infty(460)$ . The latter was estimated to be  $\sim 0.80$ . After 1 min of darkness there was an orange flash of variable intensity, and after 1 additional min an orange flash of constant (maximum) intensity. The orange flashes stimulated metarhodopsin much more efficiently than rhodopsin; in fact the metarhodopsin fraction in equilibrium with them was estimated from other  $M$  potential experiments to be  $0.07 f_M^\infty(460)$ , or roughly 0.06. This corresponds (by Eq. 3)

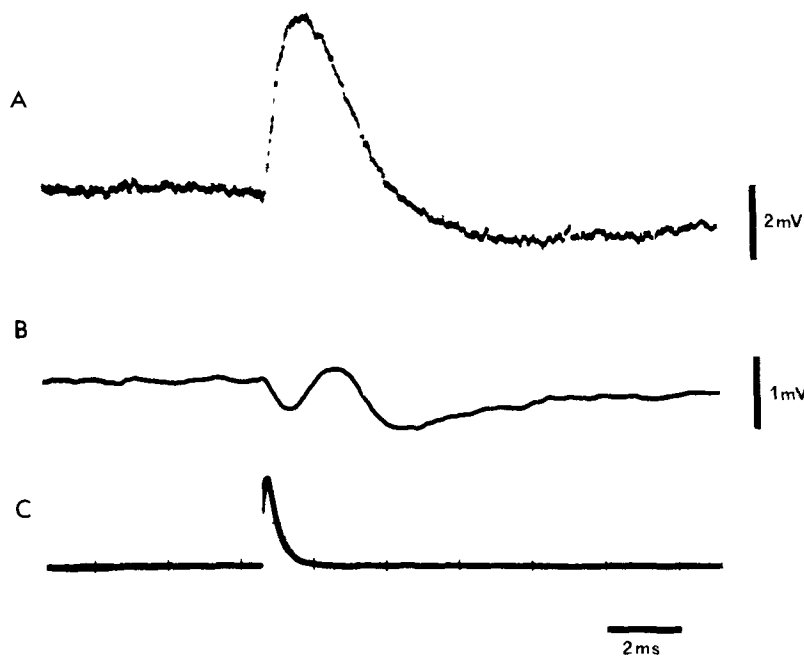


FIGURE 8.  $M_1$  in double mutant  $\tan^{P7}/norpA^{P13}$ . In this double mutant, the  $M_1$  time-course is clear, since the  $M_2$  and on-transient are much reduced by the mutation  $\tan$ , and the LRP by  $norpA$ . (A) Intracellular recording in photoreceptor. (B) ERG. (C) Light monitor.

to a ratio  $K_M/K_R$  for this light of  $\sim 17$ , i.e., the orange flash stimulates metarhodopsin  $\sim 17$  times more efficiently than rhodopsin.

The second, constant flash measured the amount of metarhodopsin remaining after the first, variable flash. We will refer to the  $M_1$  generated by the second flash as  $P_2(A_1)$ , where  $A_1$  is the amount of light in the first flash. Note that, for constant  $A$  and  $f_M^f$ , Eq. 9 is a linear function of  $f_M^i$  and Eq. 9' is proportional to  $f_M^i$ . Thus, if the metarhodopsin fraction remaining after the first flash is given by Eq. 5, then the difference between  $P_2(A_1)$ , the amplitude of the  $M_1$  to the second flash following an adaptation  $A_1$ , and  $P_2(\infty)$ , the corresponding  $M_1$  following complete orange adaptation (several full-intensity

orange fishes), should be an exponential function of  $A_1$ :

$$P_2(A_1) - P_2(\infty) = pe^{-\Lambda A_1}, \quad (11)$$

where  $\Lambda$  is the relaxation constant for the orange flash and  $p$  is a factor

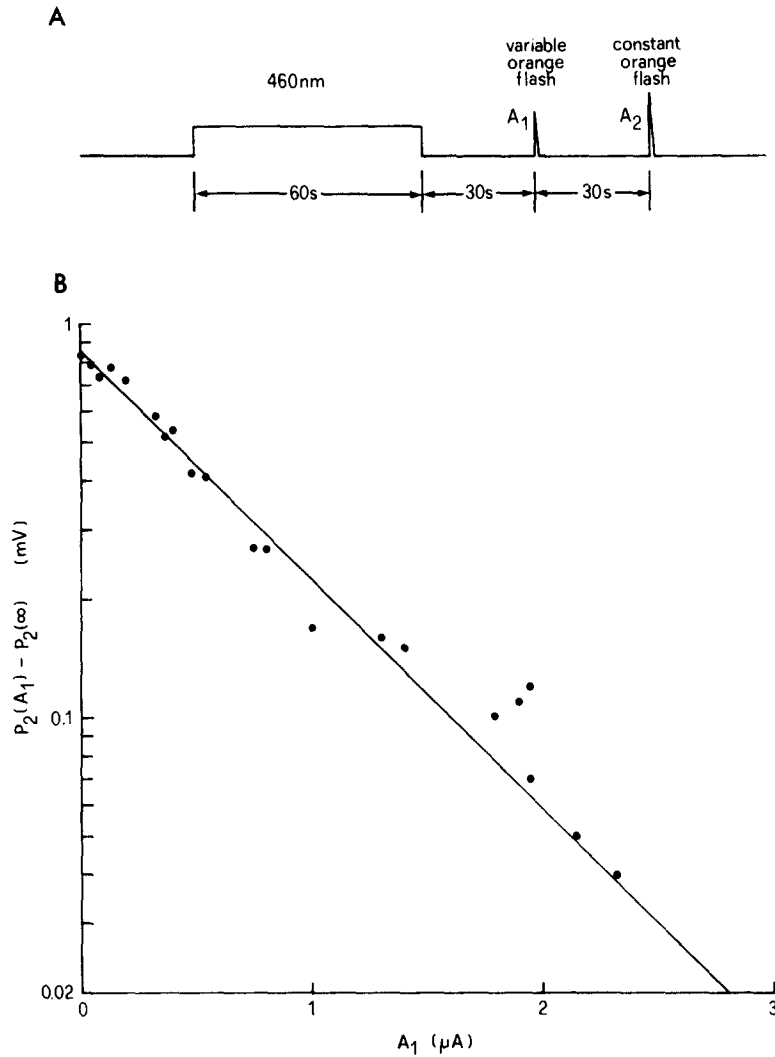


FIGURE 9. (A) Protocol for two-flash experiment. (B) Dependence of  $M_1$  on state of adaptation of visual pigment for a constant stimulus intensity. Data are measurements from a single wild-type fly. Ordinate is  $P_2(A_1) - P_2(\infty)$  (see text), where  $P_2(A_1)$  is the amplitude of  $M_1$  to the second, constant flash. Abscissa is intensity  $A_1$  of first flash, measured as microamperes of photometer output. The straight line, fitted by eye to the data, corresponds to an exponential decay with relaxation constant  $\Lambda = 1.33 \mu A^{-1}$ .



independent of  $A_1$ . This is shown in Fig. 9 B, which plots  $P_2(A_1) - P_2(\infty)$  vs.  $A$  on a semilog scale.

The abscissa of each point is actually the output (in microamperes) of the light monitor during flash no. 1 and is thus a relative measure of the amount

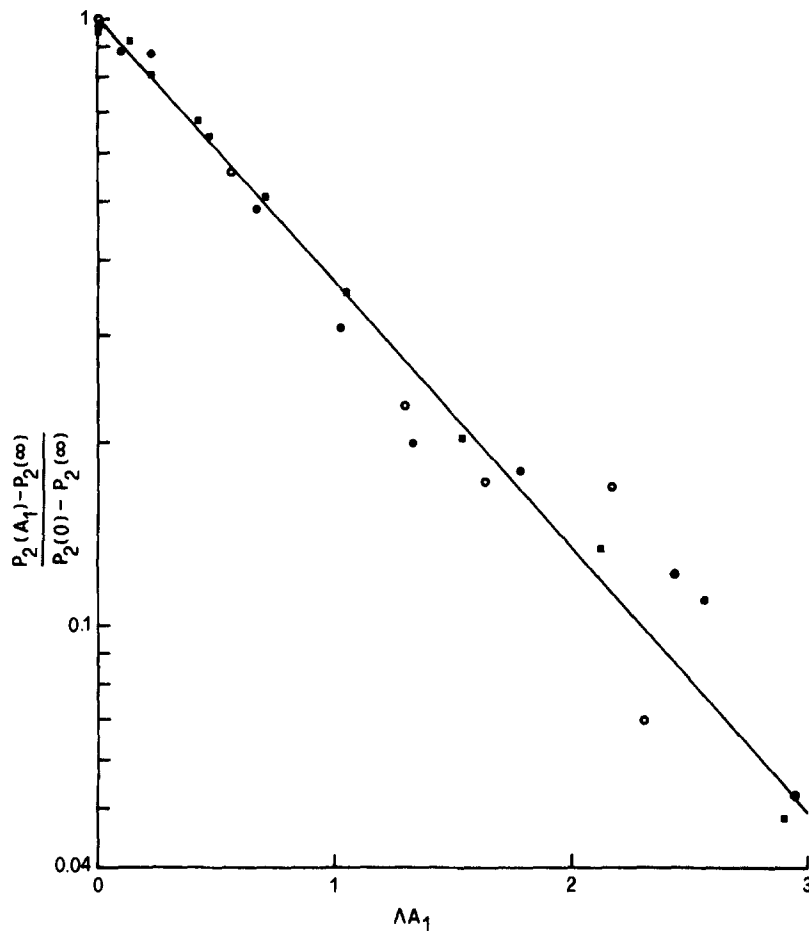


FIGURE 10. Dependence of  $M_1$  on state of adaptation of visual pigment. Data from three flies. (●, ○) Wild-type flies. (■) *tan/norpA* double mutant. Ordinate is  $P_2(A_1) - P_2(\infty)$  as in Fig. 9, but normalized for each fly so that maximum is 1. Abscissas for each fly are normalized by relaxation constant  $\Lambda$ . Most points are means of two to three  $M_1$  measurements at that abscissa. Straight line is locus of equation  $y = \exp(-x)$ .

of light  $A_1$ . The data in Fig. 9 B come from a single wild-type fly and lie roughly along a straight line as predicted by Eq. 11. The slope of this line is proportional to  $-\Lambda$ . Fig. 10 is a similar plot pooling the data from three flies (including the one above), where for each fly the abscissae were normalized by  $\Lambda$  and the ordinates by  $P_2(0) - P_2(\infty)$ .

Fig. 11 plots  $P_1(A_1)$ , the amplitude of the  $M_1$  to the first flash, as a function of  $\Lambda A_1$ , for the same three flies as in Fig. 10. Data for each fly were adjusted vertically to give the best agreement with the predicted curves within the range where the latter overlap. The dotted curve is a plot of Eq. 9, with the values  $f_M^i = 0.80$ ,  $f_M^f = 0.06$  mentioned earlier. Note that the shape of this curve depends only on the ratio  $f_M^f/f_M^i$ , which was experimentally determined from the  $M_1$  data. The solid curve plots Eq. 9', where the parameter  $K_M$  has the value  $0.94 \Lambda$ , based on  $K_M/K_R = 17$  for the orange flashes (see above)

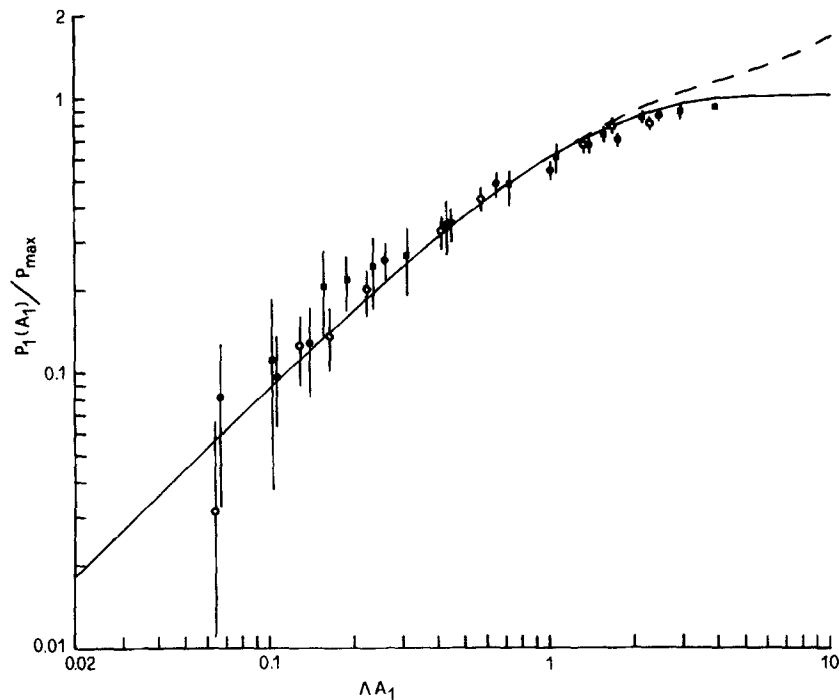


FIGURE 11. Dependence of  $M_1$  on intensity of stimulating flash. Same three flies as in Fig. 10. Ordinate is normalized  $M_1$  amplitude. Abscissa as in Fig. 10. Each point is mean of two to six  $M_1$  measurements at same intensity. Vertical bars represent standard error of this mean. Dashed and solid lines plot Eqs. 9 and 9', respectively.

and on Eq. 6. The ordinates of the two curves were arbitrarily adjusted so that  $P_{\max} = 1$  for the solid curve and that they approach the same low intensity asymptote.

Each point in Fig. 11 is the mean of two to six measurements, and an error bar indicates the standard error of this mean. The data agree remarkably well with Eq. 9', but seem to differ from the curve generated by Eq. 9 at high intensities. The value of the apparent saturation level  $P_{\max}$  in absolute terms ranged from 0.7 to 1.2 mV in the different experiments. It should be stressed

that only one parameter was adjusted to fit the data from each fly to the theoretical curves, namely the vertical scaling factor. As in Fig. 10, the agreement between the data and Eq. 9' or, to a lesser extent, Eq. 9 is excellent and we take this as evidence that the  $M_1$  as measured extracellularly (and presumably intracellularly as well) is proportional to the amount of metarhodopsin converted by a flash or, for a constant stimulus intensity, to the amount of metarhodopsin present at the start of the flash. Moreover, we take this proportionality in conjunction with the arguments cited above to confirm that the  $M_1$  arises directly from the visual pigment and is indeed an ERP.

**LINEARITY OF THE  $M_2$  COMPONENT** The corneal-positive  $M_2$  component is approximately 10 times larger in amplitude than the  $M_1$ . Because of this it is tempting to use it as a metarhodopsin assay, and it has been so used in the past, but its origin in higher order cells makes such an application suspect. Having determined that the  $M_1$  is a linear measure of metarhodopsin photo-conversion we have used this to show that the  $M_2$  is not.

The most obvious shortcoming of the  $M_2$  as a metarhodopsin assay is that it also depends on receptor potential. This is most easily seen during a prolonged depolarizing afterpotential (PDA). In *Drosophila* as in many other invertebrates, a stimulus that converts a substantial amount of the visual pigment to the metarhodopsin state also causes a depolarization in the photoreceptors which long outlasts the stimulus (Cosens and Briscoe, 1972; Minke et al., 1975). Fig. 12 A is a record of the ERG during such a PDA. The stimulus in this case was less than that needed to induce a full-size PDA and the afterpotential decayed gradually over the following several minutes. An orange flash, if given a few seconds after the 480-nm PDA-inducing stimulus, would catch the photoreceptors when they were still depolarized. If the same orange flash were given minutes later, the PDA would have decayed and the photoreceptors would no longer be depolarized, but in the two cases the fraction of the visual pigment in the metarhodopsin state would be the same. By varying the interval in this paradigm we can investigate the effect on the  $M$  potential of a PDA in the photoreceptors, under conditions where the metarhodopsin fraction does not change.

Fig. 12 B shows the result of such an experiment. The abscissa plots the amount of afterpotential remaining in the ERG at the time of the flash. This is an accurate indication of the amount of depolarization in the photoreceptors. When the photoreceptors were depolarized, the  $M_2$  component was typically reduced by more than 40%. Notice that the  $M_1$  amplitude was not affected by the depolarization. The fact that the same conditions of blue adaptation that give a large  $M$  potential in *Drosophila* also cause a PDA is enough to compromise seriously the value of the  $M_2$  as a metarhodopsin assay in many cases.

Even in cases where no PDA is present, however, the relation between  $M_1$  and  $M_2$  components is not linear. In the mutant *norpA*<sup>P12</sup> (formerly called x-12; see Alawi et al., 1972), the receptor potential (and hence the PDA) is almost totally absent. Fig. 13 plots the amplitude of the  $M_2$  in such a fly against the  $M_1$  amplitude under conditions of varying flash intensity and constant adaptation or vice versa. A line of regression on the  $M_1$  amplitude

has a slope of 1.51 for these data. Although system noise caused considerable scatter in the  $M_1$  measurements at the lower end of this double-log plot, it cannot explain the deviation from linearity at the upper end. The degree of nonlinearity in the  $M_2$  in this experiment was typical, but the slope and even

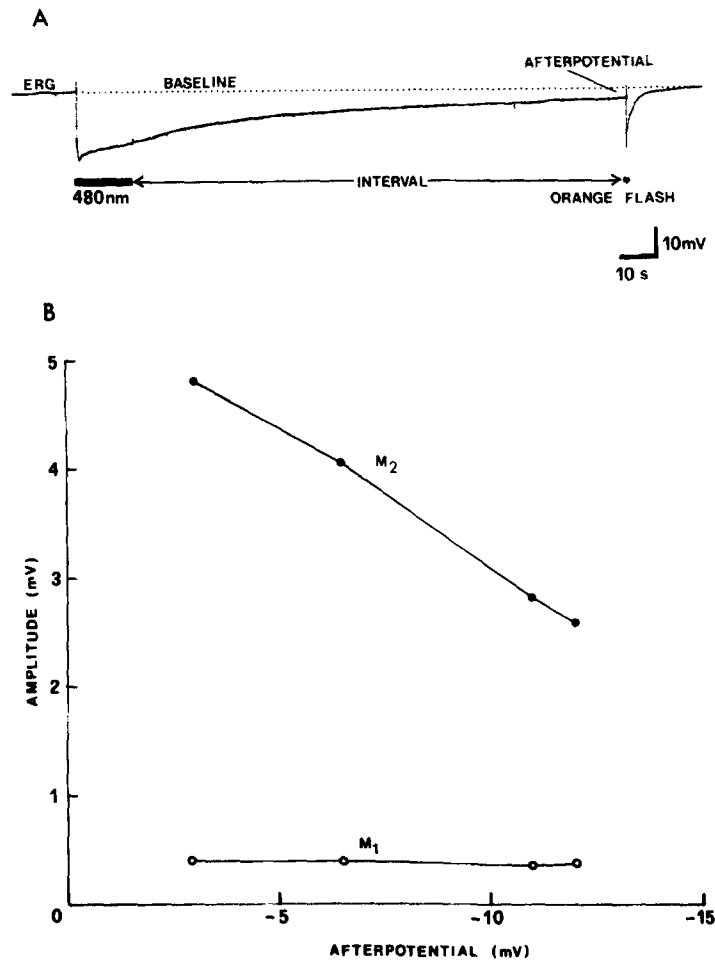


FIGURE 12. Effect of prolonged depolarizing afterpotential (PDA) on  $M_2$  and  $M_1$ . (A) ERG evoked by a 15-s 480-nm light. This light induced a PDA which decayed over several minutes. After varying intervals, corresponding to varying amounts of PDA remaining, an orange flash evoked an  $M$  potential. (B) Amplitude of  $M_2$  and  $M_1$  components. The abscissa is amount of afterpotential in ERG at time of flash.

shape of the relation varied considerably from fly to fly. Sometimes the curve flattened at the top, suggesting a saturation in the  $M_2$ .

Most of the scatter at the upper end of the curve in Fig. 13 is due to variation in the  $M_2$ . Although the signal-to-noise ratio is much worse for the

$M_1$  than for the  $M_2$ , systematic variations are much larger in the latter in both absolute and relative terms. In the course of an experiment during which the  $M$  potential was evoked half a hundred times, the mean  $M_1$  to a given stimulus usually diminished slowly by 10% or less, whereas the  $M_2$  often changed by 20% and occasionally as much as 30%. Thus, the  $M_2$  is, in general, not a linear measure of metarhodopsin photoconversion and is subject to considerable variability due to PDA and other factors.

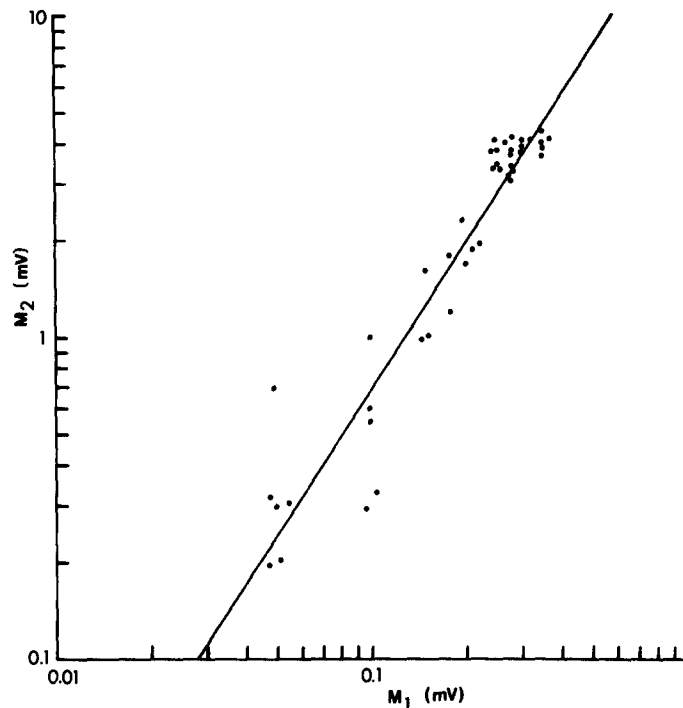


FIGURE 13. Dependence of  $M_2$  on  $M_1$  for mutant  $norpA^{P12}$ . Stimuli were orange flashes of variable intensity after constant adaptation, or orange flashes of constant intensity after varying adaptation. Line of regression on  $M_1$ , plotted, has equation  $(M_2) = 22.7 (M_1)^{1.51}$ .

#### DISCUSSION

##### $M_1$ Component

Since the  $M_1$  potential is present in the photoreceptors and is resistant to cold, anaesthesia, and anoxia, and the  $M_1$  amplitude is proportional to the amount of visual pigment photoconverted by the stimulus, there seems little doubt that it is an ERP.<sup>6</sup> As shown in Fig. 11, the  $M_1$  amplitude depends on flash

<sup>6</sup> This is not meant to imply that all ERPs are necessarily generated by the same mechanism.

intensity in a way consistent with Eq. 9'. This agreement seems to validate the assumptions underlying this equation that

- (a) the visual pigment in white-eyed *Drosophila* compound eye is optically thin;
- (b) the amplitude of the  $M_1$  is proportional to the amount of metarhodopsin photoconverted to rhodopsin; and
- (c) extracellularly recorded  $M_1$  amplitude is proportional to intracellularly recorded  $M_1$  amplitude.

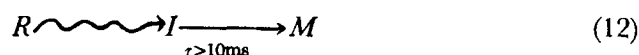
Lo and Pak (1978) have reported that the brightness of the rhabdomeric images in the deep pseudopupil (DPP) of white-eyed *Drosophila* decreases when the eye is blue-adapted. This optical effect occurs at wavelengths where the absorbance of the visual pigment is either negligible (656 nm) or constant (isosbestic) when the pigment is photoconverted from rhodopsin to metarhodopsin. They interpreted this DPP darkening effect as a change in transmission of the rhabdomeres that is dependent on the visual pigment photoconversion but not entirely due to absorption by the pigment. If this interpretation is correct, the effect should have produced in Fig. 10 a vertical displacement—presumably a lowering—of the points with abscissae  $\Lambda A_1$  less than about 0.2 relative to the rest of the curve. For these points the retina was still almost totally blue-adapted when the  $M$  potential was evoked, and the stimulating flash should have been attenuated by any DPP darkening effect present. Since such a displacement is not evident in Fig. 10, we may conclude that the DPP darkening effect does not alter the intensity of light reaching the rhabdomeres by more than 0.05 log units (corresponding to the scatter in Fig. 10). This is consistent with direct measurements of the magnitude of the DPP darkening effect which indicate an average value of 0.034 density units.<sup>3</sup>

The  $M_1$  amplitude in Fig. 11 appears to saturate at high intensity and, thus, to be in better agreement with Eq. 9' than with Eq. 9. Assuming that the apparent saturation in Fig. 11 is significant, what does it imply? We should note that the ERP of other animals has been found to saturate whenever its intensity dependence has been examined, and in fact saturation has been used as a criterion to distinguish the ERP from other photopigment responses such as those from the pigment epithelium (Cone and Pak, 1971). Thus, the ERP has been shown to satisfy Eq. 9' in rat rods ( $R_2$  component: Cone, 1964), turtle cones (Hodgkin and O'Bryan, 1977), and *Limulus* ventral photoreceptors (Lisman and Bering, 1977). The reason Eq. 9 continues to grow is that it assumes that a given visual pigment molecule may cycle from  $M$  to  $R$  and back several times during a single flash, if the latter is sufficiently intense, and contribute to the ERP once each cycle. As mentioned above, Eq. 9 assumes that (a)  $R$  to  $M$  transitions are silent, (b) all pigment transitions are rapid compared to the flash duration, and (c) each  $M$  to  $R$  transition contributes equally. The fact that the  $M_1$  satisfies Eq. 9' better than Eq. 9 suggests that one or more of these conditions may be violated.

In many cases where an ERP-generating transition or transitions induced by a first flash can be reversed by a second, the second flash produces a charge displacement opposite (rat: Cone, 1967; Pak and Boes, 1967; *Limulus*: Lisman

and Sheline, 1976) and equal (squid rhodospin-acid metarhodopsin: Hagins and McGaughy, 1967) to the first. If the inverse transitions in rat and *Limulus* produce charge displacements that are not only opposite but equal to the forward transitions, this is sufficient to explain why the ERP saturates in these animals. Any visual pigment molecule that completes a full photochemical cycle in such a system makes no net contribution to the ERP. Such is not always the case, however; transitions between squid rhodopsin and isorhodopsin on the one hand and basic metarhodopsin on the other generate outer segment-positive ERPs regardless of the direction in which they proceed (Hagins and McGaughy, 1967). In *Drosophila* we have seen no evidence of any "rhodopsin potential" whatsoever accompanying the inverse, *R* to *M* transition, although we cannot exclude a potential so small and so slow as to be undetectable.

The saturation seen in Fig. 11 could also be explained by the accumulation of a transient intermediate. In fact, if there were an unstable intermediate state *I* between *R* and *M*



with a lifetime greater than, say, 10 ms, this would explain not only why a given pigment molecule contributes to the  $M_1$  only once each flash (Eq. 9') but also why no *R* potential is detectable; the latter would be generated too slowly to be above the noise level assuming that the transition from *R* to *I* is silent. There are several arguments that rule out a scheme such as Schema 12, however. First, from spectrophotometry Kirschfeld et al. (1978) estimated the time constant of the *R* to *M* transition as only 0.125 ms in *Drosophila*. Second, Eq. 11, which assumes that transient intermediates are insignificant, fits the data very well (Fig. 10). Third, an intense white flash delivered to a 600-nm-adapted [ $f_M^\infty(600) \cong 0$ ] eye does give an  $M_1$ , indicating that a substantial number of visual pigment molecules can complete the transition from *R* to *M* in less than the duration of the flash, or less than 0.7 ms.<sup>7</sup>

Since the assumptions of silent *R* to *M* photoconversion and rapid pigment transitions appear to be valid for *Drosophila*, in order to explain the  $M_1$  saturation we need to consider the possibility that each *M* to *R* transition may not contribute equally to the ERP. If the  $M_1$  showed a refractoriness in the sense that a visual pigment molecule contributes to the ERP only once during a flash, although it may cycle from *M* to *R* and back several times, then saturation would be assured. There is some evidence that this may be the case.<sup>7</sup>

Although the nature of the charge displacement underlying the  $M_1$  may be uncertain, its magnitude is not. The amplitude of the intracellularly recorded  $M_1$  is  $\sim 5$  mV (see Fig. 8 A) under conditions where  $\sim 50\%$  of the pigment is photoconverted from *M* to *R*. The charge displacement is a function of photoreceptor membrane capacitance, which may be calculated in either of two ways. We may assume, as mentioned above, that the intracellularly

<sup>7</sup> Stephenson, R. S., and W. L. Pak. Manuscript in preparation.

recorded  $M_1$  decays with the membrane time constant of the photoreceptor. Since the time constant of  $M_1$  decay is  $\sim 3$  ms (see Fig. 8 A) and the cell input resistance is  $\sim 30$   $M\Omega$ , we obtain a capacitance value of 100 pF. A calculation of photoreceptor membrane area, based on measurements of the plasma membrane and rhabdomere in electron micrographs and assuming close packing of microvilli in the rhabdomere, yields a total membrane area (including the rhabdomeric membrane) of  $1 \times 10^4$   $\mu\text{m}^2$ . If we assume a specific capacitance of 1  $\mu\text{F}/\text{cm}^2$ , we arrive at the same value, 100 pF, for the membrane capacitance.

The total charge displacement across the membrane due to the above stimulus is then  $5 \times 10^{-13}$  C. The particle density in the membrane is 3,000/ $\mu\text{m}^2$  as determined by freeze fracture.<sup>8,9</sup> If we assume this to be the density of rhodopsin itself, we obtain  $3 \times 10^7$  rhodopsin molecules per photoreceptor. Therefore, the net charge displacement across the photoreceptor membrane when a single metarhodopsin molecule is photoconverted is 0.2 electronic charge, and the direction of the displacement current is inward. This charge displacement may result from a change in dipole moment or total charge of a membrane protein and needs not imply that charge is transferred across the entire membrane. In fact, it does not even necessitate a charge movement in the membrane; a transfer of charge across the Helmholtz double layer on either face of the membrane would be sufficient. The value 0.2e per pigment molecule converted is consistent with recent ERP measurements in other species: about  $-0.4e$  for the  $R_2$  component of the ERP in rat rods (Ruppel and Hagsins, 1973),  $0.07e$  and  $-0.12e$  for the  $R_1$  and  $R_2$  components of the ERP in red sensitive turtle cones (Hodgkin and O'Bryan, 1977),  $0.03e$  and  $-0.14e$  for the two components of the ERP in *Limulus* ventral eye (Lisman and Bering, 1977).

### *M<sub>2</sub> Component*

The  $M_2$  component does not arise in the photoreceptors and is not an ERP. We have suggested that the  $M_2$  and the on-transient may arise from the same or similar groups of cells on the basis that they invert at the same depth in the eye and are similarly affected by cooling or anaesthesia, and that mutations affecting one affect the other. According to this hypothesis, a flash generates a depolarizing  $M_1$  in the photoreceptors which, in turn, triggers the  $M_2$  response in second-order neurons, in the same manner that the onset of a depolarizing LRP triggers the on-transient. A similar phenomenon presumably occurs in the vertebrate retina where an ERP-like potential has been observed in second-order horizontal cells (Hodgkin and O'Bryan, 1977).

The on-transient in *Drosophila* and other muscoid diptera is known to arise from the lamina (Alawi and Pak, 1971; Heisenberg, 1971, Goldsmith and Bernard, 1974). Histological studies of the lamina in diptera (Boschek, 1971; Trujillo-Cenoz, 1972; Strausfeld, 1976) have shown that the peripheral pho-

<sup>8</sup> Harris et al. (1977) obtained a slightly higher figure, but their value, unlike that cited above, was not corrected for curvature of the fracture face.

<sup>9</sup> Schinz, R., and W. L. Pak. Manuscript in preparation.



toreceptors R1-6 establish synapses on the large monopolar neurons L1 and L2 as well as on the smaller L3 in the lamina. In view of their primary visual input as well as their radial orientation, anatomically these laminal neurons appear likely candidates for generating the rapid  $M_2$  and on-transient components of the ERG.

In the lamina of *Drosophila* Alawi and Pak (1971) obtained depolarizing intracellular responses that resembled the on-transient in time-course and latency. They were unable, however, to exclude the possibility that these might have been due to glial cells. In *Calliphora* the monopolar cells L1 and L2 generate hyperpolarizing responses to light with sharp on- and off-responses (Autrum et al., 1970, Järvilehto and Zettler, 1971). These cells are highly sensitive to small increments in stimulus intensity (Järvilehto and Zettler, 1971), a distinguishing feature of the ERG on-transient (Heisenberg, 1971). It seems likely that these cells are at least partly responsible for the on-transient, and it is possible that they are also responsible for the  $M_2$ .

We have attempted to identify the cells generating the  $M_2$  in the lamina of *Drosophila*, but this proved to be very difficult due to the small size of these cells. Since the experiments reported here on the  $M_2$  were completed, however, Minke and Kirschfeld (1980) have recorded intracellularly an  $M_2$ -like potential in the lamina of the fly *Calliphora*.

Since the  $M_2$  is not an ERP, one should be extremely cautious about using it as a quantitative measure of visual pigment. In cases where a PDA is present or where the photoreceptor membrane potential may vary (due, for example, to background illumination), such a use would seem to be ruled out entirely. In other dipteran flies such as *Calliphora* and *Musca* PDAs are not as prolonged as in *Drosophila* and this problem is correspondingly less severe. Indeed, Minke and Kirschfeld (1979) have been able to use the  $M_2$  in these flies with consistent results. In *Drosophila*, however, we have found that, even in the absence of a PDA,  $M_2$  measurements may be subject to an error as large as 30–40% due to nonlinearity (see Fig. 13), and may also change by as much as 30% with time in an unpredictable manner. The  $M_1$  is free of these drawbacks and appears to be better suited for use as a metarhodopsin assay, in spite of its lower signal-to-noise ratio.

We thank Doctors Mei-Ven Lo, Rudolf Schinz, and Sandra Grabowski and Mr. Mike Wilcox for permission to quote their results before publication. We are grateful to Jessica Miller for taking care of the flies and to Lucy Winchester for typing the manuscript. Supported by National Institutes of Health grants EY-00033 and EY-07008.

*Received for publication 4 June 1979.*

#### REFERENCES

- ALAWI, A., V. JENNINGS, J. GROSSFIELD, and W. L. PAK. 1972. Phototransduction mutants of *Drosophila melanogaster*. *Adv. Exp. Biol. Med.* **24**:1–21.
- ALAWI, A., and W. L. PAK. 1971. On-transient of insect electroretinogram: its cellular origin. *Science (Wash. D.C.)*. **172**:1055–1057.
- AUTRUM, H., ZETTLER, F., and M. JÄRVILEHTO. 1970. Postsynaptic potentials from a single

- monopolar neuron of the Ganglion Opticum I of the blowfly *Calliphora*. *Z. Vgl. Physiol.* **70**: 414-424.
- BOSCHEK, C. B. 1971. On the fine structure of the peripheral retina and lamina ganglionaris of the fly *Musca domestica*. *Z. Zellforsch. Mikrosk. Anat.* **118**:369-409.
- BROWN, K. T., and M. MURAKAMI. 1964. A new receptor potential of the monkey retina with no detectable latency. *Nature (Lond.)*. **201**:626-628.
- CONE, R. A. 1964. Early receptor potential of the vertebrate retina. *Nature (Lond.)*. **204**:736-739.
- CONE, R. A. 1967. Early receptor potential: photoreversible charge displacement in rhodopsin. *Science (Wash. D.C.)*. **155**:1128-1131.
- CONE, R. A., and W. L. PAK. 1971. The early receptor potential. In *Handbook of Sensory Physiology*. Vol. I. Principles of Receptor Physiology. W. R. Lowenstein, editor. Springer-Verlag, Berlin. 345-365.
- COSENS, D., and D. BRISCOE. 1972. A switch phenomenon in the compound eye of the white-eyed mutant of *Drosophila melanogaster*. *J. Insect Physiol.* **18**:627-632.
- GOLDSMITH, T. H., and G. D. BERNARD. 1974. The visual system of insects. In *Physiology of Insecta*, Vol. II. M. Rockstein, editor. Academic Press, Inc., New York. Second edition. 165-272.
- GRABOWSKI, S. R., and W. L. PAK. 1976. Fast electrical responses from *Drosophila* metarhodopsin. The Association for Research in Vision and Ophthalmology Abstracts. **16**: no. 3. (Abstr.)
- HAGINS, W. A., and R. E. MCGAUGHY. 1967. Molecular and thermal origins of fast photoelectric effects in the squid retina. *Science (Wash. D.C.)*. **157**:813-816.
- HARRIS, W. A., D. F. READY, E. D. LIPSON, A. J. HUDSPETH, and W. S. STARK. 1977. Vitamin A deprivation and *Drosophila* photopigments. *Nature (Lond.)*. **266**:648-650.
- HEISENBERG, M. 1971. Separation of receptor and lamina potentials in the electroretinogram of normal and mutant *Drosophila*. *J. Exp. Biol.* **55**:85-100.
- HILLMAN, P., F. A. DODGE, S. HOCHSTEIN, B. W. KNIGHT, and B. MINKE. 1973. Rapid dark recovery of the invertebrate early receptor potential. *J. Gen. Physiol.* **62**:77-86.
- HOCHSTEIN, S., B. MINKE, P. HILLMAN, and B. W. KNIGHT. 1978. The kinetics of visual pigment systems I. mathematical analysis. *Biol. Cybern.* **30**:23-32.
- HODGKIN, A. L., and P. M. O'BRYAN. 1977. Internal recording of the early receptor potential in turtle cones. *J. Physiol. (Lond.)*. **267**:737-766.
- HOYLE, G. 1955. The effects of some common cations on neuromuscular transmission in insects. *J. Physiol. (Lond.)*. **127**:90-103.
- JÄRVILEHTO, M., and F. ZETTLER. 1971. Localized intracellular potentials from pre- and postsynaptic components in the external plexiform layer of an insect retina. *Z. Vgl. Physiol.* **75**:422-440.
- KIRSCHFELD, K., R. FEILER, and B. MINKE. 1978. The kinetics of formation of metarhodopsin in intact photoreceptors of the fly. *Z. Naturforsch. Teil C. Biochem. Biophys. Biol. Virol.* **33C**: 1009-1010.
- LISMAN, J. E., and H. BERING. 1977. Electrophysiological measurement of the number of rhodopsin molecules in single *Limulus* photoreceptors. *J. Gen. Physiol.* **70**:621-633.
- LISMAN, J. E., and Y. SHELINE. 1976. Analysis of the rhodopsin cycle in *Limulus* ventral photoreceptors using the early receptor potential. *J. Gen. Physiol.* **68**:487-501.
- LO, M.-V., C., and W. L. PAK. 1978. Desensitization of peripheral photoreceptors shown by blue-induced decrease in transmittance of *Drosophila* rhabdomeres. *Nature (Lond.)*. **273**:772-774.

- MINKE, B., and K. KIRSCHFELD. 1979. The contribution of a sensitizing pigment to the photosensitivity spectra of fly rhodopsin and metarhodopsin. *J. Gen. Physiol.* **73**:517-540.
- MINKE, B., and K. KIRSCHFELD. 1980. Fast electrical potentials arising from activation of metarhodopsin in the fly. *J. Gen. Physiol.* **75**:381-402.
- MINKE, B., C-F. WU, and W. L. PAK. 1975. Isolation of light-induced response of the central retinula cells from the electroretinogram of *Drosophila*. *J. Comp. Physiol.* **98**:345-355.
- MURAKAMI, M., and W. L. PAK. 1970. Intracellularly recorded early receptor potential of the vertebrate photoreceptors. *Vision Res.* **10**:965-975.
- PAK, W. L. 1969. Rapid photoresponses in the retina and their relevance to vision research. *Photochem. Photobiol.* **8**:495-503.
- PAK, W. L., and R. J. BOES. 1967. Rhodopsin: responses from transient intermediates formed during its bleaching. *Science (Wash. D.C.)*. **155**:1131-1133.
- PAK, W. L., and K. J. LIDINGTON. 1974. Fast electrical potential from a long-lived, long-wavelength photoproduct of fly visual pigment. *J. Gen. Physiol.* **63**:740-756.
- RÜPPEL, H., and W. A. HAGINS. 1973. Spatial origin of the fast photovoltage in retinal rods. In *Biochemistry and Physiology of Visual Pigments*. H. Langer, editor. Springer-Verlag, New York. 257-261.
- SMITH, T. G., and J. E. BROWN. 1966. A photoelectric potential in invertebrate cells. *Nature (Lond.)*. **212**:1217-1219.
- STARK, W. S., A. M. IVANYSHYN, and R. M. GREENBERG. 1977. Sensitivity and photopigments of R1-6, a two-peaked photoreceptor, in *Drosophila*, *Calliphora*, and *Musca*. *J. Comp. Physiol.* **121**:289-305.
- STEPHENSON, R. S., and W. L. PAK. 1978. Metarhodopsin potential of *Drosophila*: corneal-positive component due to second-order neurons. *Biophys. J.* **21**:172a. (Abstr.)
- STRAUSFELD, N. J. 1976. Atlas of an insect brain. Springer-Verlag, Berlin. 214 pp.
- TRUJILLO-CENOZ, O. 1972. The structural organization of the compound eye in insects. In *Handbook of Sensory Physiology*, Vol. VII/2. Physiology of Photoreceptor Organs. M. G. F. Fuortes, editor. Springer-Verlag, Berlin. 5-62.
- WILLIAMS, T. P. 1964. Photoreversal of rhodopsin bleaching. *J. Gen. Physiol.* **47**:679-689.