# Fast Electrical Potential from a Long-Lived, Long-Wavelength Photoproduct of Fly Visual Pigment

WILLIAM L. PAK and KELLIE J. LIDINGTON

From the Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907

ABSTRACT A rapid electrical potential, which we have named the M-potential, can be obtained from the *Drosophila* eye using a high energy flash stimulus. The potential can be elicited from the normal fly, but it is especially prominent in the mutant *norp* $A^{P12}$ (a phototransduction mutant), particularly if the eye color pigments are genetically removed from the eye. Several lines of evidence suggest that the M-potential arises from photoexcitation of long-lived metarhodopsin. Photoexcitation of rhodopsin does not produce a comparable potential. The spectral sensitivity of the M-potential peaks at about 575 nm. The M-potential pigment (metarhodopsin) can be shown to photoconvert back and forth with a "silent pigment(s)" absorbing maximally at about 485 nm. The silent pigment presumably is rhodopsin. These results support the recent spectrophotometric findings that dipteran metarhodopsin absorbs at much longer wavelengths than rhodopsin. The M-potential probably is related to the photoproduct component of the early receptor potential (ERP). Two major differences between the M-potential and the classical ERP are: (*a*) *Drosophila* rhodopsin does not produce a rapid photoresponse, and (*b*) an anesthetized or freshly sacrificed animal does not yield the M-potential. As in the case of the ERP, the M-potential appears to be a response associated with a particular state of the fly visual pigment. Therefore, it should be useful in in vivo investigations of the fly visual pigment, about which little is known.

## INTRODUCTION

For a number of years we have been attempting to dissect genetically the phototransduction process, i.e. the process which links photoexcitation of the visual pigment with ionic events of the photoreceptor membrane (Pak et al., 1969, 1970; Alawi et al., 1972). For this purpose we have been generating *Drosophila* mutants which show defects in the electroretinogram (ERG). In one class of mutants isolated, we found that the ionic events in the photoreceptors are blocked. Hotta and Benzer (1970) and Heisenberg (1971) have

also described a similar class of mutants. We sought to determine whether or not visual pigments exist in the photoreceptors of this class of mutants. It appeared to us that one of the simplest ways to test for the presence of visual pigments is to look for the early receptor potential (ERP). The ERP, in the case of vertebrates and the squid, has been shown to be highly correlated with the photochemistry of the visual pigment (Pak, 1965; Arden et al., 1966; Cone, 1967; Hagins and McGaughy, 1967; Pak and Boes, 1967; Cone, 1969; Gedney et al., 1971). Ercolini et al. (1967) have previously reported that the ERP cannot be obtained from several species of arthropods, including the blowfly, *Calliphora erythrocephala*. There is a possibility, however, that the stimulus energy they used was too low. We, therefore, decided to attempt to elicit the ERP using white-eyed *Drosophila*. Since the eye color pigments are absent in these flies, the amount of light reaching the visual pigments is maximized.

In all animals in which ERP has been detected, it appears as the earliest component of the ERG, providing that a flash stimulus of sufficiently high energy and short duration ($\lesssim$1.0 ms) is used. Although the ERG of higher dipterans has been extensively studied, most investigators utilized rectangular light stimuli of relatively low illuminance and long durations (0.01–10.0 s). Only a few studies have utilized flash stimuli of very short durations (Ercolini et al., 1967; Hotta and Benzer, 1969; Fouchard and Carricaburu, 1970); fewer still have used flash stimuli of high energy (Ercolini et al., 1967; Fouchard and Carricaburu, 1970).

The ERG elicited by a rectangular stimulus consists of three main components: the on-transient, the maintained corneal negative component, and the off-transient. It is now generally agreed that the on- and off-transients arise in the second-order visual cell layer, the lamina ganglionaris, and that most of the maintained component arises as a result of the depolarization of the receptors (see reviews: Goldsmith and Bernard, 1974; Pak, 1974). In the ERG elicited by a flash stimulus ("flash ERG"), the on-transient and the maintained receptor component can be readily recognized, but the off-transient is absent. If the ERP is present in the dipteran flash ERG, it should appear as a new ERG component preceding the on-transient and the maintained component.

Our results showed that dipteran rhodopsin does not produce the classical ERP, confirming the observations of Ercolini et al. (1967). However, we found that a rapid corneal positive potential can be elicited from the *Drosophila* eye by a high energy stimulus under specific experimental conditions. As will be shown, this potential, which we shall call the M-potential, appears to originate from *Drosophila* metarhodopsin. Thus, the purpose of this paper is twofold: (*a*) to describe some of the novel properties of the M-potential and (*b*) to present characteristics of fly metarhodopsin inferred from the

M-potential. The second point is rather important. Langer and Thorell's (1966) microspectrophotometric evidence on the blowfly rhabdomeres suggested that dipteran rhodopsin, upon absorption of light, decays to a blue photoproduct. Recently, however, Hamdorf et al. (1973), Stavenga et al. (1973), and Ostroy and Pak (in preparation) found spectrophotometric evidence that fly rhodopsin decays to a metarhodopsin absorbing in the orange spectral range (560–580 nm). Since the latter results were quite unexpected, an investigation of dipteran metarhodopsin by another technique appeared to be in order. The M-potential permitted such an investigation.

## MATERIALS AND METHODS

In these experiments we used a control strain derived from the Oregon-R wild strain and a class of ERG defective mutants, *norp* $A^{P12}$ (formerly *x*-12) (see review: Pak, 1974). *Norp* A is a recessive mutation on the X chromosome. The alleles of the *norp* A locus are characterized by either a complete lack of the receptor potential or by receptor potentials of unusually small amplitude and slow time-course (Pak et al., 1970; Hotta and Benzer, 1970; Heisenberg, 1971). Our previous studies suggested that a phototransduction event is impaired in this class of mutants (Pak et al., 1970; Alawi et al., 1972; Deland and Pak, 1973).

Almost all of the experiments were performed on flies having white eyes, i.e. on flies from which the eye color or "screening" pigments have been genetically removed. This was accomplished by placing the mutant or its wild type allele on a genetic background consisting of a combination of the mutations, *brown* (on the second chromosome) and *scarlet* (on the third chromosome) (Lindsley and Grell, 1968). We found no evidence that the genes used to provide the white eye background have any effect on the ERG except to increase its sensitivity to light. Elimination of the eye color pigments served two purposes: (*a*) It maximized the amount of stimulus light reaching the visual pigment, and (*b*) it eliminated the eye color pigments as a possible source of the M-potential.

The stimulus flashes were produced by a 60-J photographic strobe lamp (Honeywell Strobonar 65C, Honeywell, Inc., Minneapolis, Minn.). They were delivered to the eye through a pair of lenses and a foot long, flexible lightpipe of ¼-inch diameter. The strobe lamp was housed in a copper box of ¼-inch wall thickness, and the copper box in turn was enclosed in a soft steel box. With these precautions and proper grounding of the instruments, the stimulus artifact was negligible.

The energy of the flash stimulus was controlled by using a set of Kodak wratten neutral density filters (Eastman Kodak Co., Rochester, N. Y.). Monochromatic flashes were produced by using a set of Baird-Atomic B-3 interference filters (Baird Atomic, Inc., Bedford, Mass.) having half peak bandwidths of 18–25 nm. In order to eliminate infrared components in the stimulus flash, two heat-absorbing filters (KG-1, Klinger Optical, Klinger Scientific Apparatus Corp., Jamaica, N. Y.) were placed in the light path. All measurements, including spectral sensitivity and flash energy measurements, were made with these filters in place. The flash duration at half peak energy was 0.25 ms. The flash energy densities at the plane of the preparation when 460-, 500-, 540-, 600-, and 650-nm filters were in place were 6.8, 6.7, 5.9, 6.2,

and $4.9 \times 10^{-4}$ J/cm$^2$, respectively, corresponding to 1.6, 1.7, 1.6, 1.9, and $1.6 \times 10^{15}$ photons/cm$^2$.

The background or adapting light originated from a 150-W Xenon arc lamp (GmbH, Osram, Berlin, Germany) and reached the preparation through a Bausch and Lomb High Intensity Monochromator, (Bausch & Lomb Inc., Scientific Instrument Div., Rochester, N. Y.) a system of lenses, a shutter, and a diaphragm. The background luminance was varied using a pair of neutral density wedge filters.

The fly was prepared for recording by lightly anesthetizing with $CO_2$ and then fixing the intact living animal on a glass coverslip with low melting point carbowax. The coverslip was then mounted on the stage so that the eye from which recordings were to be made was positioned about 1 cm from the tip of the lightpipe.

All recordings were made extracellularly using glass capillary electrodes of large tip diameters ($\sim$1.0 $\mu$m). The electrodes were pulled from Pyrex capillaries of 1.0-mm OD and 0.7-mm ID and filled with a saturated solution of Niagara Sky Blue. The dye solution was used to avoid exposing the silver leads in the electrodes to scattered light from the stimulus flash. In the absence of the dye, the scattered light often caused a photoartifact. The dye filled electrodes had resistances of approximately 20–30 M$\Omega$. In some cases, electrodes having resistance of about 5 M$\Omega$ were used.

The recording electrode was inserted into the cornea and the reference electrode into the proboscis. The electrodes were connected to a DC preamplifier which had its high cut-off frequency set at 30 kHz. The preamplifier was usually DC coupled to the oscilloscope, but sometimes the AC mode (time constant = 47 ms) was also used.

For some experiments the animals were thoroughly dark adapted. This involved dark adapting the fly for 15 h or longer, preparing for recording in dim red light, and then further dark adapting on the recording stage for 20–30 min. These flies will be referred to as "completely dark adapted" preparations. For most experiments, however, the M-potential was elicited with an orange flash (600 nm) from an eye that had been pretreated either with several blue flashes (400 or 460 nm) or a few minutes of illumination with a steady blue light. These procedures were found to be optimal in evoking the M-potential.

## RESULTS

Typical electroretinograms obtained with high energy flashes from normally pigmented eyes of the control strain are shown in Fig. 1 A, B, and C. Fig. 1 A was obtained by presenting a completely dark-adapted eye with a maximum energy white stimulus. The eye was then treated with a series of three blue flashes (460 nm) followed by 5 min of dark adaptation. Then three, maximum energy orange flashes (600 nm) were administered to the eye 1 min apart. Fig. 1 B was obtained with the first orange flash, and Fig. 1 C with the third orange flash. The on-transient and the sustained receptor component can be readily recognized. After treatment with blue flashes, a small, corneal positive potential appears in addition to the above two components (Fig. 1 B). As seen in Fig. 1 B and C, a succession of orange flashes

greatly reduces the amplitude of the potential. This is the M-potential to be discussed in this paper.

One can immediately eliminate the possibility that the M-potential may originate from the eye color or screening pigments in the eye, since it can be

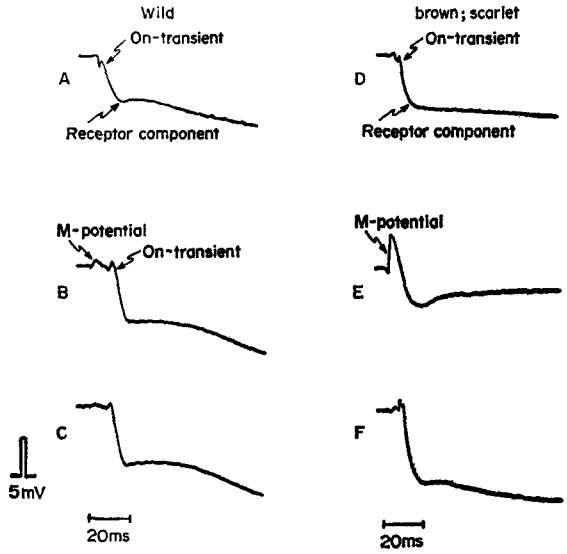



FIGURE 1

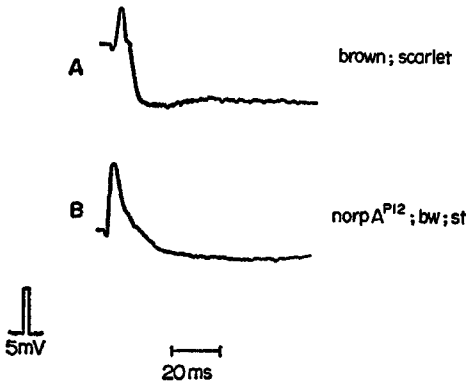



FIGURE 2

FIGURE 1. Electroretinograms obtained with high energy flashes from normally pigmented eyes and white eyes of *Drosophila*. The oscilloscope traces A, B, and C were obtained from a normally pigmented (red) eye of wild type *Drosophila* and traces D, E, and F from a white-eyed, but neurologically normal, fly carrying the homozygous recessive mutations *brown* and *scarlet*. The top traces A and D were obtained from completely dark adapted flies (see text). The flies were then subjected to three maximum energy blue flashes (460 nm) followed by 5 min of dark adaptation. Then three, maximum energy red flashes (600 nm) were delivered to the eye 1 min apart. Traces B and E were obtained with the first red flash, and traces C and F with the third. Note the M-potential obtained with the first red flash in each fly (B and E).

FIGURE 2. Comparison of flash ERG obtained from the mutant *norp* $A^{P12}$, with the wild type ERG. In each case, the ERG response was elicited with a maximum energy 600-nm flash after pretreatment with three blue flashes (460 nm) and 5 min of dark adaptation. Trace A was obtained from a fly carrying no neurological mutations (wild type). Trace B was obtained from the mutant *norp* $A^{P12}$. In both cases the homozygous recessive mutations, *brown* and *scarlet*, were used to remove the eye color pigments.

obtained from the mutant strains in which the eye color pigments are missing. This is shown in Fig. 1 E. The ERGs in Fig. 1 D, E, and F were obtained from an electrophysiologically normal fly, but with the homozygous recessive mutations *brown* and *scarlet*. Experimental conditions were identical to those used to obtain Fig. 1 A, B, and C. It may be seen by comparing Fig. 1 B with 1 E that the amplitude of the M-potential is much larger in the white-

eyed fly than in the red-eyed wild type. Similar results can also be obtained from another strain of white-eyed mutant, *white*. Thus the eye color pigments cannot be responsible for the generation of the M-potential. One reason for the larger amplitude of the M-potential in the white-eyed strains presumably is that in the absence of the screening pigments more light can reach the visual pigment.

In both the white-eyed strains and the red-eyed control strain, the M-potential was absent or very small if the eye was completely dark adapted (Fig. 1 A and D). The amplitude of the M-potential was greatly enhanced if the eye was pretreated with either a few minutes of steady blue illumination (~460 nm) or several blue flashes (460 nm) (Fig. 1 B and E). Moreover, an orange stimulus (600 nm) appeared to be optimal in eliciting the M-potential.

In all experiments to be described below, we used a *brown-scarlet* background in both the mutant and control strains to eliminate the eye color pigments. In order to abbreviate the nomenclature, the mutant will be referred to simply as *norp* $A^{P12}$ and the control strain as "wild type," with the understanding that both strains carry the homozygous recessive mutations *brown* and *scarlet*.

In Fig. 2 a response obtained from the ERG defective mutant *norp* $A^{P12}$ is compared with that from wild type. In both cases the responses were elicited with 600-nm flashes of maximum available energy following pretreatment with three blue flashes (460 nm) and 5 min of dark adaptation. The M-potential can be readily recognized in the ERGs of both wild type and *norp* $A^{P12}$ (Fig. 2 A and B). Indeed, the amplitude of the M-potential in *norp* $A^{P12}$ is often larger than in wild type (Fig. 2 B). The reason for this is not entirely clear. Part of the explanation appears to be that in this mutant the M-potential can be observed in essential isolation, because the on-transient and the receptor component of the *norp* $A^{P12}$ ERG are grossly underdeveloped (Pak et al., 1970; Hotta and Benzer, 1970; Heisenberg, 1971). In wild type, on the other hand, the large negative going receptor component of the ERG would tend to subtract from the amplitude of the M-potential. In any event, the results displayed in Fig. 2 make it clear that *norp* $A^{P12}$ is well suited for the study of the M-potential. Thus, most of our studies were carried out using *norp* $A^{P12}$.

The dominant component of the M-potential is corneal positive. Typically however, the M-potential is preceded by a small corneal negative wave (Figs. 1 E, 2 A and B). If a maximum energy orange flash (600 nm) is used, the corneal positive M-potential emerges from the small corneal negative wave in ~1.1 ms. It reaches its maximum amplitude of ~7 mV in ~2.4 ms and then decays with a time constant of ~3.5 ms. The small corneal negative wave preceding the dominant, positive component of the M-potential appears to be an integral part of the M-potential. However, no systematic investigations of this component have yet been made.

The M-potential and the on-transient have the same polarity. If the amplitude of the M-potential is large, its temporal overlap with the on-transient makes it difficult to recognize the latter as a separate entity (Figs. 1 E and 2 A). One can show, however, that aside from the polarity, the two potentials have entirely different properties.

We present in Figs. 3 and 4, respectively, the amplitudes and peak latencies of the M-potential and the on-transient plotted against the stimulus energy. The on-transient was obtained from the wild type fly using stimulus flashes

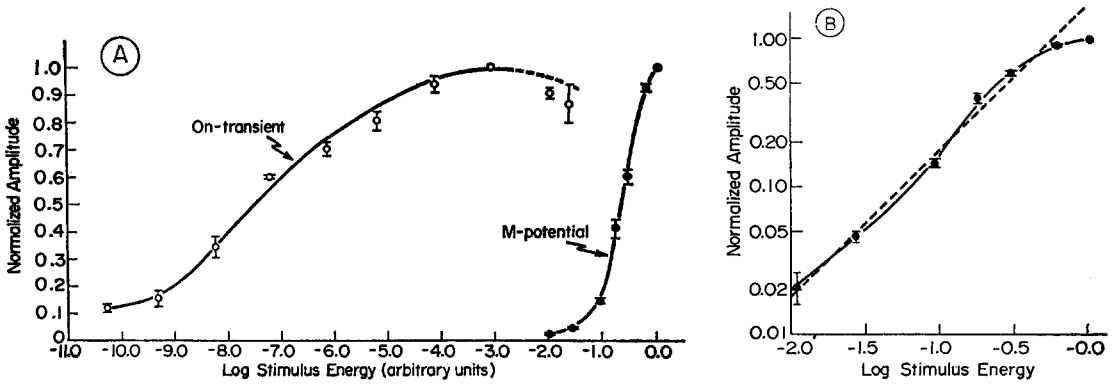



FIGURE 3. (A) Dependence of the on-transient and M-potential amplitudes on the stimulus energy. The on-transient was obtained from the wild type fly (+; *brown; scarlet)* using 500-nm flashes, and the M-potential was obtained from *norp* $A^{P12}$ (*norp* $A^{P12}$; *brown; scarlet*) using 600-nm flashes. The stimulus wavelengths were chosen to optimally stimulate the respective responses. The unattenuated flash (0.0 on the stimulus energy scale) cast $1.7 \times 10^{15}$ photons/cm$^2$ on the specimen at 500 nm and $1.9 \times 10^{15}$ photons/cm$^2$ at 600 nm. The on-transient data (open circles) were obtained from two flies, and each data point is based on four measurements. Each data point on the M-potential (filled circles) is based on 12–31 measurements obtained from 6–16 different flies. The error flags represent the standard errors of the mean. (B) Dependence of the M-potential amplitude on the stimulus energy. The M-potential data displayed in Fig. 3 A are replotted in a double log plot. The dashed line is a line of unit slope.

of 500 nm, while the M-potential was obtained from *norp* $A^{P12}$ using orange stimulus flashes (600 nm). The stimulus wavelengths were chosen to optimally stimulate the respective responses. With no attenuation of stimulus, the numbers of quanta impinging on the specimen at the two wavelengths were comparable ($1.7 \times 10^{15}$ photons/cm$^2$ at 500 nm and $1.9 \times 10^{15}$ photons/cm$^2$ at 600 nm).

As may be seen in Fig. 3, the M-potential can be obtained only at the highest two log unit ranges of stimulus energy. The on-transient, on the other hand, can be obtained over a stimulus energy range of over 10 log units. The amplitude of the on-transient attains saturation some 3 log units below the maximum available stimulus energy (Fig. 3). At higher energies it becomes

increasingly difficult to measure the amplitude of the on-transient accurately because of its overlap with the M-potential (see Figs. 1 E and 2 A). For this reason the amplitude of the on-transient in the highest 1.5-log unit range of stimulus is not given in Fig. 3. We have replotted in Fig. 3 B the dependence of M-potential amplitude on stimulus energy in a double log plot. The experimental points may be fitted with a straight line of unit slope (dashed line), perhaps over a range of about 1.5 log units of stimulus energy. Above this energy, the response begins to saturate.

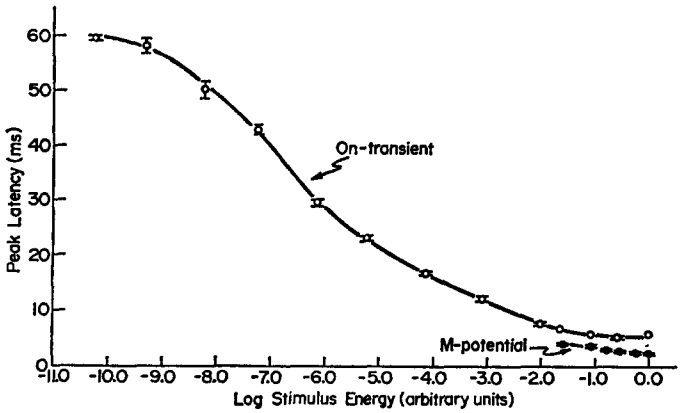



FIGURE 4. Dependence of the on-transient and M-potential peak latencies on the stimulus energy. All data plotted here were taken from the same oscilloscope traces from which the amplitude data (Fig. 3) were taken. However, some of the traces used for amplitude measurements of the M-potential were not used for peak latency measurements. Each of the on-transient data points (open circles) is based on four measurements from two flies. Each M-potential data point (filled circles) is a mean of 9–19 measurements obtained from 5 to 10 different flies. The error flags show the standard errors of the mean.

The peak latencies of both responses vary with stimulus energy (Fig. 4). By "peak latency" we mean the time interval between the onset of the stimulus and the peak of the response. At the highest 1.5-log unit range of stimulus energy, the peak latency of the M-potential is roughly half that of the on-transient. With further attenuation of stimulus, the M-potential can no longer be observed. The on-transient, on the other hand, occurs later and later until its peak latency can be as large as 60 ms.

The spectral sensitivity of the M-potential peaks at a wavelength of about 575 nm (Fig. 5, circles, right curve). On the other hand, the spectral sensitivity of the fly receptor potential has two peaks, at around 350 nm and at, or slightly below, 500 nm. This holds true whether the receptor potential is recorded extracellularly as a component of the ERG (Goldsmith, 1965; Goldsmith and Fernandez, 1968; Pak et al., 1970; Stark and Wasserman, 1972) or recorded intracellularly (Burkhardt, 1962; McCann and Arnett,

1972; Alawi and Pak, unpublished observations). Spectral sensitivity studies based on the optomotor response also gave similar results (Eckert, 1972). These results suggest that different pigments or pigment states are responsible for the receptor potential, and hence the on- and off-transients, on the one hand and the M-potential on the other.

The pigment responsible for the M-potential can be shown to be photointerconvertible with a pigment(s) having its absorption maximum at around 480–490 nm. The M-potential is thermally stable in the dark. Once formed, its amplitude changes little when left in the dark for over an hour. The M-potential pigment is, however, readily bleached away by a succession of orange

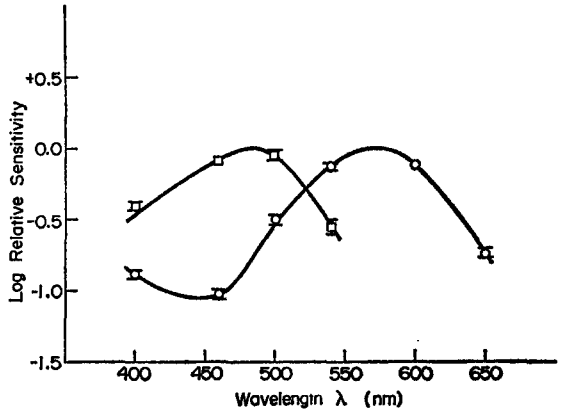



FIGURE 5. Spectral sensitivity of the M-potential and spectral sensitivity of photoconversion. The spectral sensitivity of the M-potential is shown by the open circles (curve on the right). Each data point is a mean of 8 (at 400 nm) to 19 (at 600 nm) measurements taken from a total of 10 different flies. The open squares represent the data points for the spectral sensitivity of photoconversion (see text). Each point is a mean of 11 measurements from 6 different flies. The error flags represent the standard errors of the mean.

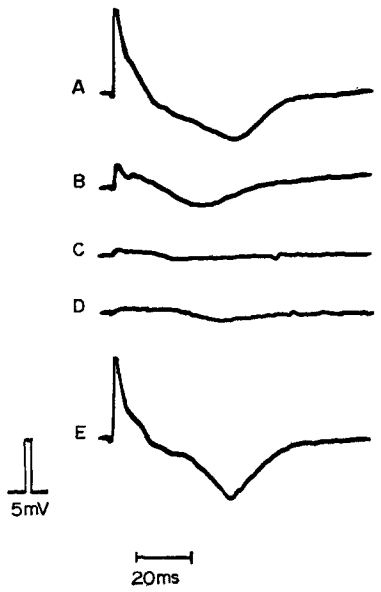
flashes. The response displayed in Fig. 6 A was obtained from an eye that had been pretreated with a series of three blue flashes (460 nm). In the next two orange flashes (bleaching flashes), delivered 1 min apart, the response was almost completely eliminated (Fig. 6 B and C). If the preparation was left in the dark, little, if any, recovery of the M-potential could be obtained. The oscilloscope trace shown in Fig. 6 D was obtained 30 min after the last orange flash had been delivered to the eye. If now a set of three blue flashes was delivered to the bleached eye, an orange test flash after these blue flashes elicited a fully recovered M-potential (Fig. 6 E). Indeed, the above sequence of experiments could be repeated many times on a given eye. The time interval between the bleaching orange flashes and the test orange flash did not seem to affect the results very much. The only important experimental

parameter appeared to be the presence or absence of the blue flashes between the bleaching and test flashes. These results showed that the pigment responsible for the M-potential absorbs maximally at about 575 nm and that this pigment can be photoconverted back and forth with another pigment or pigments absorbing maximally in the greenish blue spectral range.

What is the absorption spectrum of the greenish blue pigment(s)? If the greenish blue pigment is the native fly visual pigment, rhodopsin, its absorption spectrum can be inferred from the spectral sensitivity of the mutant ERG. Unfortunately, one does not know a priori that rhodopsin is the only pigment with which the M-potential pigment (575 pigment) photointerconverts. In order to be certain that we were measuring the absorption spectrum of the pigment(s) which exists in a relationship of mutual photoconversion with the M-potential pigment, we resorted to the following strategy. We measured the spectral sensitivity of photoconversion of the unknown blue pigment to the M-potential pigment. At the start of each experiment the eye was treated with a steady 600-nm background light for 10 min to be certain that no 575 pigment (M-potential pigment) remained. We then determined the effectiveness of photoconversion of the unknown pigment to the M-potential pigment at each of the following four wavelengths, 400, 460, 500, and 540 nm. This was done by presenting the eye with several flashes of different energies at each of these wavelengths and following each flash with a maximal energy 600-nm flash. From the amplitude of the M-potential produced by the 600-nm flash, we could infer the amount of the 575 pigment photoconverted from the unknown pigment. The idea was to determine, at each of the four wavelengths, the flash energy needed to photoconvert just enough blue pigment to the M-potential pigment so that an M-potential of criterion amplitude (0.5 mV) is obtained by the succeeding full energy 600-nm flash. The "spectral sensitivity of photoconversion" was obtained by plotting the reciprocal of the above determined energies on a log scale (Fig. 5, left curve). The sensitivity peak occurs at a wavelength of approximately 480–490 nm. This presumably corresponds to absorption maximum of the pigment(s) with which the M-potential pigment enters into mutal photoconversion.

We next sought to determine the degree of stability of the 575 pigment at room temperature. The following results showed that the M-potential does decay in the dark, albeit very slowly. In the first place, if the eye was completely dark adapted, the M-potential could not be elicited even with 600-nm flashes of maximum available energy ($6.2 \times 10^{-4}$ J/cm$^2$). This result thus suggested that, given enough time, the M-potential pigment decays away in the dark. The decay of the M-potential could be observed more directly using the following protocol. The eye was first treated with a series of blue flashes to leave the pigment in the 575 pigment state initially. Next, a 600-nm flash was used to monitor the amplitude of M-potential. Immediately after-

wards a series of blue flashes was used to restore the pigment to the 575 state. The preparation was then left in the dark for varying lengths of time to allow the 575 pigment to decay, after which a 600-nm flash was used to monitor the amplitude of the M-potential. Once again, a series of blue flashes



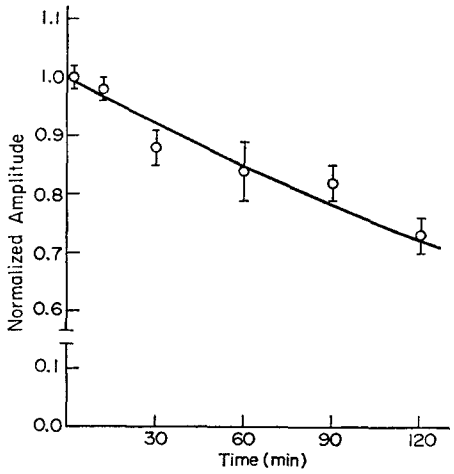




FIGURE 6

FIGURE 7

FIGURE 6. Photoconversion between the M-potential pigment and a pigment(s) absorbing at shorter wavelengths. This series of experiments was performed on eyes that had been pretreated with three 460-nm flashes. All responses were elicited with maximum energy 600-nm flashes from the white-eyed *norp* $A^{P12}$ mutant (*norp* $A^{P12}$; *bw; st*). Responses A, B, and C were produced, respectively, by the first, second, and third 600-nm flash delivered to the pretreated eye, 1 min apart. The preparation was then left in the dark for 30 min, and response D obtained. The preparation was then subjected to three 460-nm flashes. Response E shows the full recovery of the M-potential 1 min after treatment with the blue flashes.

FIGURE 7. Decay of the M-potential. The plot shows the decrease in the amplitude of the M-potential when left in the dark for varying lengths of time (see text for details). The line is an exponential curve having a time constant of 367 min. Each data point is a mean of five to eight independent measurements. A total of 16 flies were used. The error flags represent the standard errors of the mean.

was used to restore the pigment to the 575 state, and then a 600-nm flash was used to determine the amplitude of the M-potential. The average of the first and last measurements of the M-potential amplitude was taken as the amplitude when no decay of the 575 pigment had yet taken place. This was then compared to the amplitude after the decay in the dark. The results obtained in this manner are plotted in Fig. 7. An exponential decay curve

having a decay constant of 367 min has been drawn through the points. The experimental errors are large. Nonetheless, it is clear that a reduction in the amplitude of the M-potential does occur in the dark.

## DISCUSSION

Any suggestion that the M-potential is identical with, or is a component of the on-transient can be disposed of very quickly. In the first place, the M-potential is very prominent in the flash ERG of the mutant *norp* $A^{P12}$. From previous studies, *norp* $A^{P12}$ is known to lack the on-transient in its ERG (Pak et al., 1970; Hotta and Benzer, 1970; Heisenberg, 1971). Moreover, as was shown in the previous section, the properties of the two potentials are very different. Their spectral sensitivities are entirely different (Fig. 5), and the dependence of their amplitudes and peak latencies on stimulus energy is not the same (Figs. 3 and 4).

If the M-potential is not the on-transient, could it possibly be the early receptor potential (ERP) of the *Drosophila* retina? Some of the properties displayed by the M-potential are reminiscent of those of the photoproduct components of the ERP. These components arise as result of the photoreversal from transient intermediates of rhodopsin back to rhodopsin, isorhodopsin, or other intermediates (Arden et al., 1966; Cone, 1967; Pak and Boes, 1967; Hagins and McGaughy, 1967; Minke et al., 1973). Similarly, the M-potential is apparently generated from a slowly decaying intermediate photoproduct of a fly visual pigment (see Results). Moreover, like the ERP, the M-potential requires high energy flash stimuli for its generation, and it has a relatively rapid time-course.

One of the main characteristics of the ERP is the linearity of its amplitude with respect to the amount of visual pigment bleached by a flash (Cone, 1964). Although the amplitude data on the M-potential are not as clean as one would like, they do suggest that the M-potential is linear in the lower 1.5 log unit range of stimulus energy (Fig. 3 B). The response then begins to saturate, probably because at high enough stimulus energies, the receptors begin to run out of visual pigment molecules available for photoexcitation. Moreover, the amplitude of the M-potential in successive flashes decreases (Fig. 6), because the previous flashes have decreased the number of available pigment molecules. These results support the interpretation that the amplitude of the M-potential, like the ERP amplitude, is proportional to the amount of pigment excited by the stimulus flash.[1]

[1] One consequence of this observation is that each pigment molecule need absorb only one photon to contribute to the generation of the M-potential, i.e. multiple absorptions of photons by a given molecule are not required for M-potential generation. If multiple absorptions were needed for M-potential generation, one would expect the slope of the curve in Fig. 3 B in the low energy range to exceed unity (e.g. slope of two for double absorptions).

In contrast to the classical ERP, however, the M-potential cannot be obtained from an animal that has been anesthetized with either ether or $CO_2$, nor can it be obtained from a freshly sacrificed fly. Moreover, the M-potential apparently does not have a counterpart corresponding to the forward reaction from the unbleached pigment state (rhodopsin). Thus, no rapid potential originating from fly rhodopsin has yet been identified. In the case of the classical ERPs of both vertebrates and invertebrates, the most conspicuous component is the forward reaction component originating from rhodopsin (Brown and Murakami, 1964; Cone, 1964; Pak, 1965; Smith and Brown, 1966; Hagins and McGaughy, 1967; Hillman et al., 1973). On the other hand, a transient intermediate pigment state which does not produce an ERP component has been reported for the *Limulus* ventral photoreceptors (Fein and Cone, 1973).

These results suggest that the M-potential may be generated by a mechanism different from that responsible for the classical ERP. Nevertheless, the M-potential appears to be closely related to the ERP. Quite possibly, it is a new form of the ERP. Like the ERP, it is a response associated with a particular state of *Drosophila* visual pigment. Therefore, the M-potential lends itself for use in exploration of the sequence of bleaching and regeneration of the *Drosophila* visual pigment in vivo, just as the ERP has been used for the vertebrate pigment in situ (Ebrey, 1968; Cone and Cobbs, 1969; Goldstein, 1970; Goldstein and Wolf, 1973). In particular, the M-potential may be used to study dipteran metarhodopsin.

Recently Hamdorf et al. (1973) and Stavenga et al. (1973) have obtained spectrophotometric evidence that most of the *Calliphora* visual pigment absorbs maximally at about 480–490 nm and that it is converted by light to a stable photoproduct (metarhodopsin) of $\lambda_{max} \approx 560$–580 nm (560 nm: Hamdorf et al., 1973; 570–580 nm: Stavenga et al., 1973, and private communication). Moreover, the pigment can be photoconverted back and forth between the two states. Our recent spectrophotometric studies of the whole eye of *Drosophila* showed that the *Drosophila* photopigment behaves similarly (Ostroy and Pak, in preparation).

In view of these results, it appears almost certain that the M-potential is produced by *Drosophila* metarhodopsin. The $\lambda_{max}$ of the fly metarhodopsin agrees reasonably well with the wavelength at which the spectral sensitivity of the M-potential peaks (Fig. 5). The photointerconvertability between rhodopsin and metarhodopsin is consistent with the properties of the M-potential (Fig. 6). Moreover, the M-potential is stable, and so is fly metarhodopsin (Hamdorf et al., 1973; Stavenga et al., 1973; Ostroy and Pak, in preparation). The M-potential, however, does decay very slowly. At room temperature (25°C) it decays with a time constant of approximately 367 min (Fig. 7). The decay is probably due to a chemical process converting metarhodopsin to

rhodopsin. The process, however, is exceedingly slow and does not appear to be the main mechanism of photopigment regeneration in vivo. In the case of *Calliphora*, Stavenga et al. (1973) have found spectral evidence for much faster conversion of metarhodopsin to rhodopsin in the dark, the time constant being approximately 25 min.

To many workers, the existence of thermostable metarhodopsin absorbing in the orange came as a complete surprise. This was particularly true since Langer and Thorell (1966; Langer, 1972), in their pioneering work on microspectrophotometry of single blowfly (*Calliphora*) rhabdomeres, had reported (*a*) that the rhabdomeres of the six peripheral retinula cells ($R_{1-6}$) contain visual pigment with a primary absorption peak between 510 to 530 nm and a secondary peak at around 380 nm, and (*b*) that light converts *Calliphora* rhodopsin to a thermostable photoproduct having absorption maximum between 460 and 500 nm. However, our M-potential results strongly support the recent spectral data on metarhodopsin. The problem with Langer's preparations might have been that they contained some metarhodopsin in addition to rhodopsin, thus shifting the apparent rhodopsin peak toward the red. (See Langer's comment in Stavenga et al., 1973.) Moreover, the spectral composition of the bleaching light might not have been optimal for photoconversion of rhodopsin to metarhodopsin.

Still another line of evidence suggests that the fly visual pigment has a thermostable metarhodopsin absorbing in the orange. In several different species of arthropods, including the dipterans, the presence of a thermostable, photoreversible metarhodopsin has been found to affect the physiology of the photoreceptor membrane (Nolte et al., 1968; Hillman et al., 1972; Nolte and Brown, 1972; Cosens and Briscoe, 1972; Hamdorf et al., 1973; Hochstein et al., 1973). Briefly, a saturating stimulus light with a spectral distribution favoring the stimulation of rhodopsin produces a receptor potential which does not turn off even after the stimulus light is extinguished. The sensitivity of the receptor also remains reduced long after the stimulus has been turned off. These effects are either reversed or prevented from appearing by preferential photoexcitation of metarhodopsin, but not of rhodopsin. In the case of both *Drosophila* (Cosens and Briscoe, 1972) and *Calliphora* (Hamdorf et al., 1973), these effects are induced by blue stimuli and are reversed by yellow, orange, or red stimuli. Thus, these results also suggest that fly rhodopsin absorbs in the greenish blue and exists in photoreversible equilibrium with metarhodopsin absorbing in the orange.

The pigment with which the M-potential pigment (575 pigment) photointerconverts is probably rhodopsin. The spectral sensitivity of photoconversion shown in Fig. 5 peaks at about 485 nm. This value is consistent with the absorption peak of the rhodopsins of *Calliphora* (Hamdorf et al., 1973; Stavenga et al., 1973) and *Drosophila* (Ostroy and Pak, in preparation). It is also con-

sistent with the spectral sensitivity peak of the fly receptor potential (e.g. Burkhardt, 1962; Goldsmith and Fernandez, 1968; McCann and Arnett, 1972). On the other hand, one cannot rule out the possibility that there is another stable photoproduct peaking at about 485 nm and that the M-potential pigment photointerconverts with this 485 photoproduct as well as rhodopsin. It is our hope that further work along the present line will shed light on this point.

The properties of fly metarhodopsin inferred from the study of the M-potential generally agree with those obtained from the spectrophotometry of the whole eye. The study of the M-potential has the advantage that is performed on preparations in vivo. Thus, for example, the study of the M-potential allows the lifetime of the photoproduct to be measured in vivo by relatively simple means. Moreover, the spectral sensitivity of the M-potential should yield the "true" absorption peak of the pigment in question, while the spectrophotometry requires substraction procedures resulting in a difference spectrum. Thus, there are reasons to feel that, like the vertebrate ERP, the M-potential and other similar potentials are useful alternative means of studying the nature of dipteran pigments and their photoproducts, about which little information exists. This information is needed to better understand visual pigments in general, and to proceed with genetic dissection of the phototransduction process.

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