Light-induced Pigment Granule Migration in the Retinular Cells of *Drosophila melanogaster*

Comparison of Wild Type with ERG-defective Mutants

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ABSTRACT The dependence of pigment granule migration (PGM) upon the receptor potential was examined using several strains of electroretinogram (ERG)-defective mutants of *Drosophila melanogaster*. The mutants that have a defective lamina component but a normal receptor component of the ERG (*no on*-transient *A* [*nonA*] and tan) exhibited normal pigment granule migration. The mutants that have very small or no receptor potentials (certain *no* receptor potential *A* [*norpA*] alleles), on the other hand, exhibited no PGM. In the case of the temperature-sensitive *norpA* mutant, $norpA^{H52}$, normal PGM was present at 17° but not at 32°C or above, corresponding to its electrophysiological phenotype. In the *t*ransient *r*eceptor *p*otential (*trp*) mutant, whose receptor potential decays to the baseline within a few seconds during a sustained light stimulus, the pigment granules initially moved close to the rhabdomere when light was turned on but moved away after about 5 s during a sustained light stimulus. All these results lend strong support to the notion that PGM is initiated by a light-evoked depolarization of the receptor membrane, i.e., the receptor potential. However, under certain experimental conditions, the receptor potentials failed to induce PGM in the *trp* mutant. The depolarization of the receptor, thus, appears to be closely associated with PGM but is not a sufficient condition for PGM.

INTRODUCTION

Migration of the pigment granules in the retinular cells of invertebrate compound eyes and the influence of this migration upon light flux transmitted though the rhabdom have been of considerable interest to researchers for more than two decades (for review, see Walcott [1975]). In *Drosophilia*, the existence of fine pigment granules in the retinular cells was first reported by Yasuzumi and Deguchi (1958). Nolte (1961) and Fuge (1967) found evidence that the chromophore of these pigment granules might be ommochrome. Subsequently, it was suggested that these ommochrome granules (0.1–0.2 μm in diameter) migrate radially toward or away from the rhabdomere during light or dark

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adaptation in the apposition eyes of a number of insect species (e.g., housefly: Kirschfeld and Franceschini [1969] and Boschek [1971]; ant: Menzel and Lange [1971]), including *Drosophila* (Franceschini and Kirschfeld, 1971). Kirschfeld and Franceschini (1969) first showed that the transmittance through the rhabdomeres is altered by light or dark adaptation and suggested that the radial migration of ommochrome granules is responsible for regulation of light flux through the rhabdomeres (Kirschfeld and Franceschini, 1969; Snyder and Horridge, 1972; Kirschfeld and Snyder, 1975).

Although a great deal is known about these effects of pigment granule migration upon the light flux through the rhabdomeres, little is known about the mechanisms by which pigment granules move. Kirschfeld and Franceschini (1969) suggested that the depolarization of the photoreceptors triggers the pigment granule migration. One line of evidence these authors presented in support of this hypothesis is that pigment granule migration occurs in the dark in response to an ether or CO_2 treatment, and a CO_2 treatment is known to depolarize the receptor cells in many invertebrates (e.g., Wolbarsht et al. [1966], Baumann and Mauro [1973], and Wong et al. [1976]). A number of other experiments support this interpretation. For example, selective chromatic microstimulation induces pigment granule migration in the excited receptor cells without disturbing the neighboring unexcited cells (fruitfly: Franceschini [1972]; ant: Menzel and Knaut [1972]; butterflies: Stavenga et al. [1977]; bumblebees: Bernard and Stavenga [1978]).

In many dipteran photoreceptors, the receptor cell remains depolarized in the dark after a strong blue stimulus photoconverts a substantial amount of rhodopsin to metarhodopsin but returns to the resting level immediately after a red stimulus reconverts metarhodopsin to rhodopsin Cosens and Wright, 1975; Minke et al., 1975 *a*; Muijser et al., 1975; Razmjoo and Hamdorf, 1976; Tsukahara et al., 1977). Similar prolonged depolarizing afterpotentials (PDAs) have also been observed in other invertebrate photoreceptors (Nolte and Brown, 1972; Hochstein et al., 1973; Brown and Cornwall, 1975). In the case of *Calliphora* photoreceptors, the prolonged depolarization after a strong blue stimulus lasts for >20 s (Muijser et al., 1975). It was found that the pigment granules stay close to the rhabdomere for >20 s after such blue stimuli but move away quickly from the rhabdomere after a red stimulus (Stavenga et al., 1975). This "glueing effect" of pigment granules in *Calliphora* after a strong blue stimulus also supports the hypothesis of Kirschfeld and Franceschini described above. However, the precise mechanism of granule migration remains unclear.

To test the hypothesis that pigment granule migration (PGM) is triggered by the receptor potential, we examined fruitfly mutants with defective photoreceptor potentials (for review, see Pak [1975 and 1979]). Among the mutants examined are: *norpA* (*no* receptor *p*otential *A*), in which the receptor potential is nearly or completely absent, and *trp* (*t*ransient *r*eceptor *p*otential), in which the receptor potential decays toward the baseline during a sustained illumination. Pigment granule migration was also examined in white apricot (w^a), an eye color mutant in which a depolarizing afterpotential persisting for >10 min in the dark can be induced by an intense blue flash (for details see

Materials and Methods). Since this manuscript was submitted Zuidervaart et al. (1979) have also reported on pigment granule migration in the mutants w^a and *trp*.

In *Drosophila*, as in other species of flies, the dynamic properties of pigment granule migration can be studied in vivo by examining the "deep pseudopupil" described by Franceschini and Kirschfeld (1971). The deep pseudopupil is the superposition of the virtual images of corresponding rhabdomere tips of many neighboring ommatidia that can be seen by focusing the microscope near the center of curvature of the compound eye. In muscoid diptera it consists of seven bright spots arranged in a trapezoidal pattern, the six outer ones corresponding to the rhabdomeres of the six peripheral retinular cells (R1-6), and the one in the center corresponding to the rhabdomeres of the two central retinular cells (R7/8). Franceschini (1972) showed that pigment granule migration can be studied quantitatively by measuring the changes in transmitted or reflected light intensities of the deep pseudopupil. This is the technique used in the present study.

MATERIALS AND METHODS

I. *Mutants of Drosophila melanogaster*

In addition to wild-type (Oregon-R) flies, three classes of mutants with defective electroretinogram (ERG) were examined: (*a*) *nonA* (*no on*-transient *A*) and tan, (*b*) *norpA*, and (*c*) *trp*. The ERG phenotypes of wild type and each of these mutants are discussed below.

In the wild type, the ERG (Fig. 1 *a*) consists of three parts: (*a*) the on-transient: a rapid, corneal-positive transient that occurs when the light is turned on, (*b*) the sustained component: a corneal-negative, maintained component that exists while the light is on, and (*c*) the off-transient: a fast corneal-negative transient that occurs when the light is turned off. The sustained corneal-negative component is thought to reflect mainly the ionic activities of the retinular (photoreceptor) cells (Goldsmith, 1965). Thus, this sustained component of the ERG may be used to study many properties of the photoreceptor potential.

In the *nonA* and tan mutants, the on- and off-transients of ERG are absent or very small in amplitude, but the sustained component is very nearly normal (Pak et al., 1969 and 1970; Hotta and Benzer, 1969 and 1970; Heisenberg, 1971 *a* and 1971 *b*). In intracellular recordings, the receptor potentials obtained from the retinular cells of these mutants are similar to those of wild type (Alawi and Pak, 1971). Thus, the mutations appear to have little or no effect on the receptor potential but affect processes that occur subsequent to the generation of the receptor potential.

In the mutants bearing certain alleles of the *norpA* gene ($norpA^{P12}$, $norpA^{P16}$, $norpA^{P24}$, $norpA^{P39}$, and $norpA^{H44}$), the receptor potential is nearly or completely absent in both the ERG (Fig. 1 *b*) and the intracellular recordings (Alawi et al., 1972). One of the *norpA* alleles, $norpA^{H52}$, is reversibly temperature sensitive. The mutant bearing this allele has a normal ERG at 17°C, but its ERG is abolished after 2 min or more of exposure to temperatures >32°C (Deland and Pak, 1973; Pak et al., 1976; Fig. 4 *a*). The effect of temperature on the ERG is reversible unless the fly has been kept at 32°C or above for longer than 5 min.

The *trp* mutant has a receptor potential that decays to the baseline within a few seconds during a sustained stimulus (Cosens and Manning, 1969; Minke et al., 1975

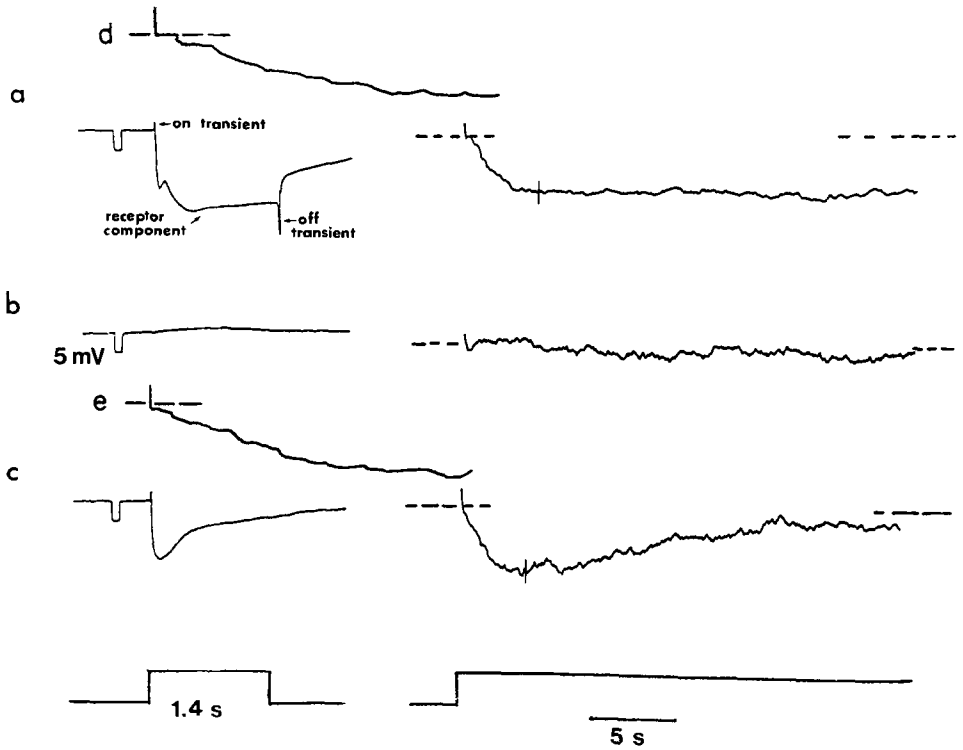

FIGURE 1. Comparison of the PGM and ERG recordings in *Drosophila melanogaster*. On the left are the ERG recordings obtained from (*a*) the wild-type, (*b*) the *norpA*, and (*c*) the *trp* strains of *Drosophila*, and on the right are the PGM recordings obtained from the same strains of flies. The PGM recordings show the light-induced changes in the intensity of light transmitted through the rhabdomeres, measured by antidromic illumination (see text and Fig. 2), as a function of time. The dashed lines indicate the background transmission level in dark-adapted eyes. A downward excursion of the PGM curve indicates a decrease in the transmitted intensity. In all cases, the ERG and PGM responses were obtained from the same fly under nearly identical conditions. The PGM responses are considerably slower than the corresponding ERG responses. In order that full responses may be observed, the two types of responses are presented in different time scales. For comparison purposes, however, the initial portions of the PGM responses of the wild type and *trp* (from the beginning of the responses to the small vertical marks in the right traces of *a* and *c*) have been expanded in the scale to correspond to the ERG time scale and are presented as traces *d* and *e*, respectively, just above the corresponding ERG traces.

b; Fig. 1 *c*). The ultrastructural study of *trp* showed that the pigment granules accumulate about 1 μm away from the rhabdomeres when the eye is exposed to sustained illumination (Cosens and Perry, 1972). Under similar conditions of illumination the granules are found immediately adjacent to the rhabdomere in wild type.

The mutant white apricot (w^a) was used to study the effect of the prolonged depolarizing afterpotential (PDA) on pigment granule migration (PGM). Neither the

wild-type nor the white-eyed (white [*w*]) strain of flies was convenient for this study. The PDA is generated in the R1-6 retinular cells of *Drosophila* by an intense blue stimulus and is terminated by a red stimulus. In wild-type red-eyed flies, the PDA cannot be induced readily because the screening pigments alter the spectral composition of the PDA-inducing stimulus. In the case of the mutant *w*, on the other hand, there are no ommochrome granules to migrate. The mutant w^a was chosen as a compromise because in this mutant the concentration of the screening pigments is not high enough to interfere with the generation of the PDA (see Results), but the concentration of ommochrome granules in the retinular cells is high enough to allow PGM to be observed (Franceschini, 1972).

II. *Maintenance of Flies*

All flies were maintained in 12 h light/12 h dark cycle at 22°C. Experiments were carried out both during the day and the night. Effects of circadian rhythm on rhabdomeric morphology, electrical responses of the photoreceptors, and pigment granule migration have been reported in *Limulus* (Behrens, 1974; Chamberlain and Barlow, 1977; Stavenga, 1979) and *Manduca* (Bennett, 1978). Stavenga (1977) has found, however, that at least in the case of pigment migration, "the day-adapted state can be established [even] at night by either illumination or cooling." Accordingly, in an attempt to minimize any possible circadian effects, we anesthetized all flies by chilling them on ice and prepared them for experiments under white light.

III. *Analysis of Pigment Granule Migration*

Throughout this work we made use of the deep-pseudopupil technique developed by Franceschini and Kirschfeld (1971). Each fly, immobilized by chilling or anesthetizing with CO_2, was held within an elongated groove in a transparent Plexiglass holder (*F* in Fig. 2) by means of wax with a low melting point (see Fig. 1 of Franceschini, 1972). Care was taken to ensure that the wax did not interfere with the respiratory movement. The head was rotated so that one eye faced the microscope objective (Carl Zeiss, Inc. [New York], Achromat X10, NA = 0.22).[1] If the light beam (from L_1 in Fig. 2) entered the back of the head from below the microscope stage and passed through the eye before reaching the objective lens, the eye was said to be antidromically illuminated (Franceschini, 1972). If the light from a vertical illuminator (Zeiss 11 ST, 6 V, 15 W; L_2 in Fig. 2) passing through the objective lens (Zeiss Epiplan HD X16, NA = 0.35)[1] entered the compound eye through the cornea, then the eye was said to be orthodromically illuminated (Franceschini, 1972). The transmittance of the rhabdomeres was measured with antidromic illumination, while the reflectance was measured with orthodromic illumination. Changes in the transmittance or reflectance were measured with a photomultiplier (RCA Solid State [Sommerville, N. J.] 1P28; *PMT* in Fig. 2). An iris diaphragm was placed in front of the photomultiplier tube to allow only the light coming from the deep pseudopupil image to enter the photomultiplier. The signal from the photomultiplier tube was amplified by a high-input impedance preamplifier (Bioelectric Instruments, Inc. [Hastings-on-Hudson, N.Y.] NF1), displayed on an oscilloscope (Tektronix, Inc. [Beaverton, Oreg.] 502A; *OS* in Fig. 2), and also recorded on a strip-chart recorder (Gould, Inc., Brush Instruments Div. [Cleveland, Ohio] 200; *R* in Fig. 2). We will call the signals observed in these recordings the pigment granule migration (PGM) responses.

[1] Two different objective lenses were used for the two different illumination conditions. The Epiplan objective is designed for use with a vertical illuminator, while the other objective is for use with transmitted light.

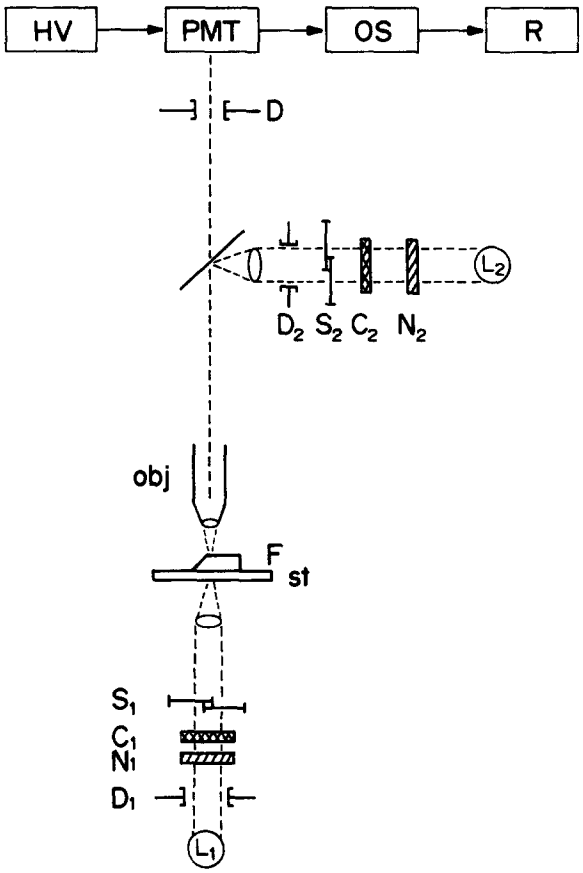

FIGURE 2. Experimental arrangement for PGM measurements. *C*, color filters; *D*, iris diaphragms; *F*, fly holder; *HV*, high-voltage power supply; *L*, light sources; *N*, neutral density filters; *Obj*, microscope objective lens; *OS*, oscilloscope; *PMT*, photomultiplier tube; *R*, strip chart recorder; *S*, shutters; *St*, microscope stage or temperature stage. Subscript 1 refers to those portions of equipment used in antidromic illumination, and subscript 2 to those used in orthodromic illumination. In general, the same light stimulus was used both in inducing pigment granule migration and in measuring the change in transmitted or reflected light intensity due to PGM. Consequently, there is a stepwise change in amount of light reaching the photomultiplier at the onset and offset of stimulus corresponding to the background reflection or transmission (see Figs. 4 *b*, 6 *b*, and 7 *b*). Any change in reflection or transmission due to PGM is superimposed on this rectangular signal (see Figs. 4 *b*, 6 *a* and *c*, and 7 *a* and *d*).

When the eye is light adapted, ommochrome granules move toward the rhabdomeres, and, as a consequence, the transmittance through the rhabdomeres decreases and the reflectance from them increases (Kirschfeld and Franceschini, 1969; Franceschini, 1972). Thus, experimentally, one would observe a decrease in the transmittance of the deep pseudopupil in antidromic illumination and an increase in reflectance in orthodromic illumination. If the eye is dark adapted, on the other hand, the pigment

granules would move away from the rhabdomeres, and, as a consequence, the transmittance would increase and the reflectance would decrease.

The changes in transmittance and reflectance of the deep pseudopupil with light and dark adaptation described above have been interpreted in terms of interactions of ommochrome granules with electromagnetic fields surrounding the rhabdomeres during light propagation through the rhabdomeres (Kirschfeld and Franceschini, 1969; Snyder and Horridge, 1972; Kirschfeld and Snyder, 1975). The interpretation is based on the idea that the rhabdomeres of the fly function as optical waveguides. During light propagation through the waveguide, electromagnetic fields extend beyond the boundary of the waveguide by a small but finite amount. If ommochrome granules are sufficiently removed from the waveguide, as in the dark-adapted eye, the interaction of pigment granules with electromagnetic fields would be negligible. If, on the other hand, pigment granules are located close to the boundary of the waveguide, as in the light adapted eye, they would be within the electromagnetic fields. Consequently, they would interact with the fields, causing light scattering and absorption. Thus, the amount of light transmitted through the rhabdomeres would decrease, because of scattering and absorption, and the amount reflected from them would increase because of scattering.

To study the temperature dependence of PGM in wild type and $norpA^{H52}$, a Peltier-junction temperature stage (*St* in Fig. 2) was placed under the fly holder. The temperature was monitored with a telethermometer (Yellow Springs Instrument Co., Inc. [Yellow Springs, Ohio] 425L) placed on the surface of the fly holder close to the eye. Because the temperature stage is opaque, the fly was illuminated only orthodromically in the temperature-dependence experiments.

IV. *ERG Recordings*

After the PGM was examined, the ERGs were recorded from the same fly under nearly the same conditions of light intensity, temperature, and dark adaptation as were used in pigment migration study. No intracellular recordings were attempted, because it would have been very difficult to obtain recordings from the same fly in which pigment granule migration had been studied. The maximum intensity of the vertical illuminator used in the PGM studies was ~2.6 W/m^2 when measured with a photometer (Lite-Mike, Edgerton, Germeshausen and Grier, Inc. [Boston, Mass.]) lined up 1 mm from the objective lens (Zeiss Epiplan HD 16/0.35). We will designate this intensity $I = 1$, or $\log I = 0$. The light intensity for ERG recordings was matched with the PGM recording conditions by adding neutral density filters (Balzers High Vacuum Corp. [Santa Ana, Calif.]) to the light beam of the ERG setup. Unless specified otherwise, white-light stimuli were used throughout.

RESULTS

I. *Time Constant of PGM in Wild Type*

When the wild-type *Drosophila* eye was illuminated, the transmittance of the deep pseudopupil decreased (Fig. 1 *a*), and its reflectance increased with nearly exponential time-courses, indicating a migration of the ommochrome granules toward the rhabdomeres. Under our experimental conditions (orthodromic illumination; $\log I = 0$), the time constant τ had a value of 2.4 ± 0.3 s at 25°C, which agrees with the value reported by Franceschini and Kirschfeld (1971). We further found that the time constant was significantly temperature dependent. As the temperature was raised from 10° to 35°C, τ

decreased from 7.5 to 1.8 s (Fig. 3), yielding a Q_{10} of nearly 3 in the temperature range between 10° and 20°C. This Q_{10} value is similar to that reported by Srinivasan et al. (1977) for *Eurema* (a butterfly genus of the Pieridae family).

II. *PGM in the Mutants*

The *nonA* and tan (*t*) mutants, in which the on- and off-transients of ERG are

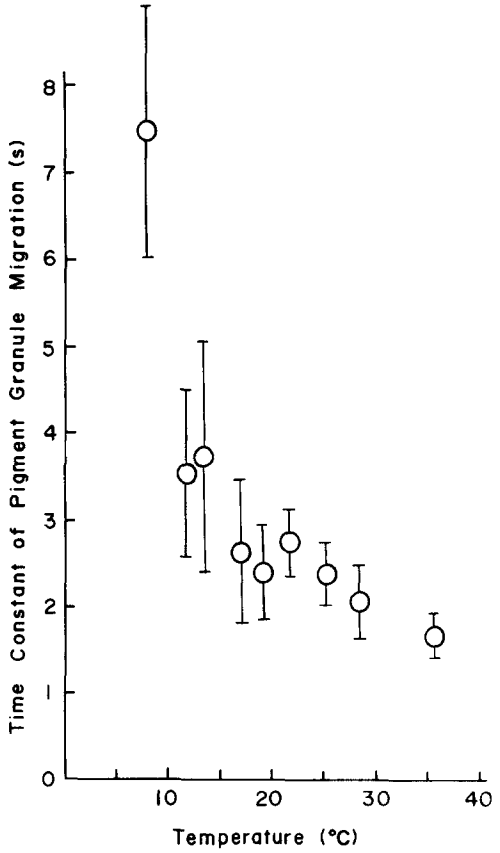

FIGURE 3. Time constant of PGM as a function of temperature. PGM was observed in wild-type flies with the orthodromic illumination arrangement (see text and Fig. 2). The time constant (τ) of change in reflected light intensity, i.e., the time constant of pigment granule migration, was calculated with an exponential regression program, $I = a(1 - e^{-bt})$, where a and b are constants, I is the intensity of the reflected light, t is time, and $\tau = 1/b$. The data were obtained from four flies. The error bars represent standard deviations.

missing but the receptor components are normal, had PGM responses very similar to those of the wild type (Fig. 1 *a*). Qualitative observations of PGM in these mutants have been reported previously by Heisenberg (1971 *a*) and Benzer.[2] Their results also suggested that pigment migration in these mutants is very similar to that of wild type.

[2] Benzer, S. Personal Communication.

Several mutants bearing different alleles of the *norpA* gene have been examined. In $norpA^{P24}$, $norpA^{P39}$, and $norpA^{H44}$, the receptor potential is completely absent. We recorded no PGM responses in these mutants (Fig. 1 *b*). In $norpA^{P16}$, which displays an ERG receptor component of small amplitude ~5 mV under our test conditions) and slow time-course, the PGM was about three times slower than that of wild type. The magnitude of PGM response, on the other hand, was similar to that of wild type.

In $norpA^{H52}$, the PGM responses were reversibly temperature dependent. At 17°C, where the ERG was nearly normal, the PGM was also similar to that of the wild type (Fig. 4). However, at restrictive temperatures (>32°C), which abolish the ERG, PGM was absent (Fig. 4).

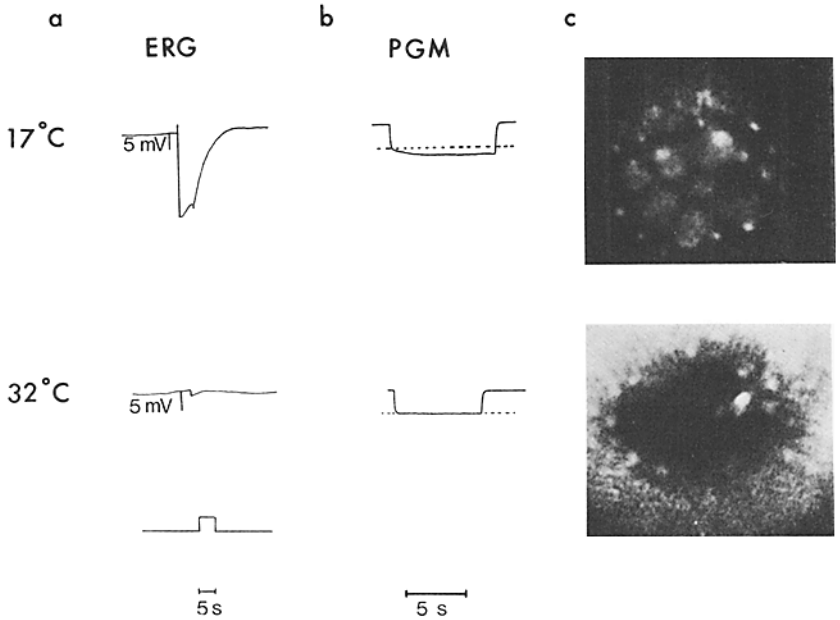

FIGURE 4. Temperature dependence of the ERG and PGM in the temperature-sensitive mutant $norpA^{H52}$. In *a*, *b*, and *c* are shown, respectively, the ERG recordings, the PGM recordings, and photographs of the deep pseudopupil taken at 17° (*top*) and 32°C (*bottom*). The ERG was nearly normal at 17°C but almost abolished at 32°C (*a*). The PGM recordings were taken with an orthodromic illumination arrangement. Unlike the antidromic arrangement used in the recordings illustrated in Fig. 1, this arrangement detects changes in the amount of light reflected from the deep pseudopupil image. The amount of light reaching the photomultiplier increases during migration of pigment granules toward the rhabdomeres (see text). To keep the polarity of the PGM responses the same as those shown in Fig. 1, however, the PGM recordings are inverted so that a downward deflection indicates an increase in the amount of reflected light. At 17°C there was an exponential increase in the intensity of reflected light superimposed on the background reflection. At 32°C, however, only the background reflection was evident. The photographs in *c*, taken with reflected light, show that after the stimulus the amount of reflection from the deep pseudopupil increased at 17° but not as 32°C.

In the mutant *trp*, both the PGM and the ERG responses first increased in amplitude and then returned to baseline during sustained illumination. When light was turned on, the transmittance of the deep pseudopupil decreased, indicating that ommochrome granules migrated toward the rhabdomere (Fig. 1 *c*). After about 5 s, the transmittance gradually recovered toward the dark level, indicating that the granules were moving away from the rhabdomere, even though the light was still on. (Zuidervaart et al. [1979] have also reported that PGM is transient in *trp*.) The time-course of pigment migration response, however, was considerably slower than that of the ERG (Fig. 1 *c* and *e*).

ERG recordings (Fig. 5 *A*) showed that in the eye color mutant w^a, a bright blue stimulus induced a PDA that was maintained many minutes after the cessation of stimulus. A red stimulus, however, terminated the PDA. Correspondingly, PGM recordings (Fig. 5 *B*) showed that the increase in reflectance of rhabdomeres induced by the blue stimulus was maintained for >10 min after the termination of the stimulus. After a red stimulus, however, the

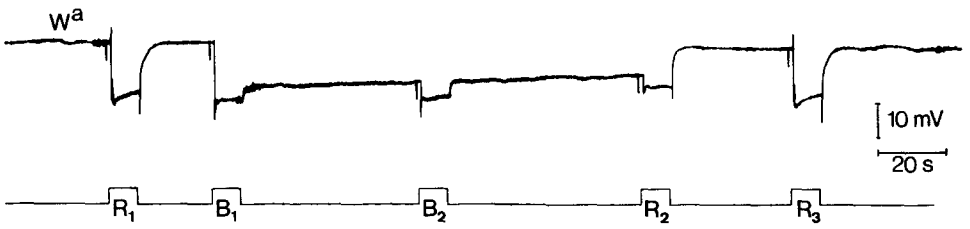

FIGURE 5 A. Prolonged depolarizing afterpotential (PDA) recorded in the eye color mutant w^a. The first red stimulus R_1 (Corning Glass Works [Corning, N. Y.] filter 2-73) evoked a normal ERG response (cf Fig. 1 *a*), which decayed to the baseline within 10 s after the stimulus. The first blue stimulus B_1 (Corning filter 5-60), on the other hand, induced a PDA. The second blue stimulus B_2, delivered during the PDA, had little effect on the PDA time-course. The second red stimulus R_2, which followed B_2 by about 1 min, terminated the PDA. The third red stimulus R_3, delivered after the termination of the PDA, evoked once again an ERG response very similar to that to the first red stimulus R_1.

reflectance returned to the dark level with a time constant of ~19 s at 25°C. Similar results have also been reported by Zuidervaart et al. (1979). The results are consistent with the interpretation that during the PDA the pigment granules remain close to the rhabdomeres and that a red stimulus, which abolished the PDA, causes the pigment granules to move away from the rhabdomeres upon its cessation.

III. *Cases in Which Depolarization of Receptor Does Not Trigger PGM*

All of the results described above are consistent with the hypothesis that the depolarization of photoreceptor triggers the PGM. However, the results described below suggest that the depolarization of the photoreceptor cell alone is not sufficient to guarantee pigment granule migration. Under certain conditions, the light stimulus evoked a receptor potential, but the receptor

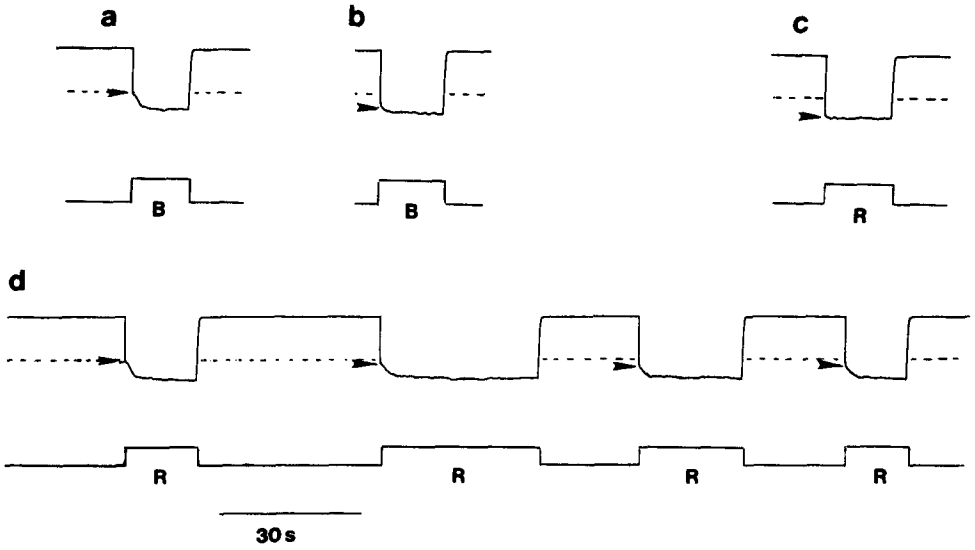

FIGURE 5 B. PGM recordings in w^a. As in the recordings of Fig. 4, the recordings were obtained with an orthodromic illumination arrangement and are presented in such a way that a downward deflection indicates an increase in the amount of reflection. The stimulus protocol was as follows: a fly that had been dark adapted for 10 min was first exposed to a blue stimulus (*a*) and then to another blue stimulus (*b*) 5 min later. The fly was then dark adapted for 10 min, presented with a red stimulus *c*, and then 10 min later, with the series of red stimuli shown in *d*. The blue stimuli were obtained with Corning filter 5-60, and the red stimuli with Corning filter 2-73. The dashed lines indicate the level of background reflectance in the dark-adapted state, and the arrowheads indicate the level of reflectance at the onset of the stimulus. It can be seen that during the first blue stimulus the reflectance increased from the "dark-adapted level" to the "light-adapted level" (*a*), indicating migration of pigment granules toward the rhabdomeres. During the second blue stimulus (*b*) and the first red stimulus (*c*), however, the reflectance of the rhabdomeres changed very little, because it was already at the light-adapted level at the onset of stimuli (see arrowheads in *b* and *c*), indicating that the reflectance did not return to the dark-adapted level 5 and 10 min after the blue stimuli *a* and *b*, respectively. The results suggest that after a bright blue stimulus the pigment granules that have migrated toward the rhabdomeres remain in that position for many minutes. The arrowhead in the first PGM response in *d* shows that the previous red stimulus (*c*) has caused the reflectance to return to the dark-adapted state during the dark interval after the red stimulus. The series of four red stimuli shown in *d* was intended to determine the time required for the reflectance to return to the dark-adapted state after a red stimulus. The dark intervals between the red stimuli in *d* are 38, 20, and 21 s, in that order. It can be seen that the reflectance very nearly returned to the dark-adapted state in 38 s (arrowhead in the second response of *d*) but not in 20 or 21 s (arrowheads in the last two responses in *d*) after a red stimulus. All recordings were taken from the same fly.

potential failed to induce a PGM. This was the case when the *trp* mutant was exposed to a conditioning or adapting stimulus before the test stimulus.

In one series of experiments, white stimuli of maximum intensity (log I = 0) and 20–30-s duration were delivered to the *trp* mutant with various intervals between the stimuli. The test stimulus delivered to a dark-adapted animal evoked a characteristic *trp*-like PGM response (Fig. 6 *a*) and an ERG response of maximum peak amplitude for the stimulus intensity used (Fig. 6 *d*). A second stimulus, following the first by <50 s, invariably failed to induce a PGM, even though it evoked a receptor component of up to 8 mV peak

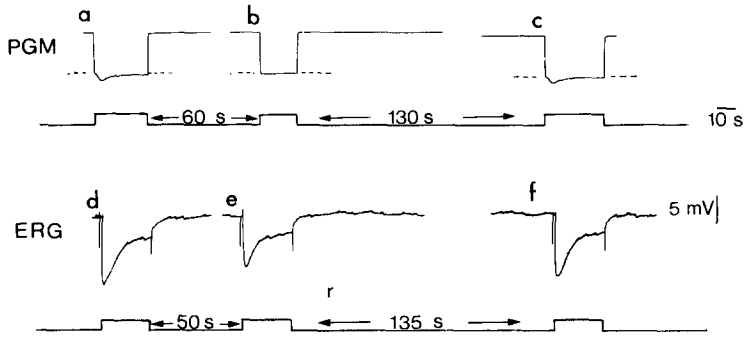

FIGURE 6. Dependence of the PGM and ERG responses of the *trp* mutant on stimulus intervals. The PGM recordings are shown in the top row (*a–c*) and the ERG recordings in the bottom row (*d–f*). As in the recordings of Fig. 4, the PGM recordings were taken with orthodromic illumination arrangement and are presented in such a way that a downward deflection indicates an increase in the amount of reflection. The dashed lines indicate the level of background reflection in the dark-adapted state. Any change in the amount of reflection due to PGM shows up as deflections of the signal trace below the dashed lines. The PGM recording (*a*) and ERG recording (*d*) were taken after the eye was dark adapted for 4 min. There was a clear *trp*-type PGM response consisting of initial increase in reflection and then gradual relaxation of the signal to the dark level (*a*). The peak amplitude of the ERG was about 10 mV (*d*). The PGM recording (*b*) and ERG recording (*e*) were taken, respectively, 60 and 50 s after those in *a* and *d* were taken. The PGM recordings showed no sign of ommochrome granule movement (*b*). The peak amplitude of ERG was ~8 mV (*e*). The PGM recording (*c*), taken 130 s after *b*, again showed a clear *trp*-type PGM response. The peak amplitude of ERG (*f*), taken 135 s after *e*, was ~10 mV. All measurements were made on the same fly. The test intensity was ~2.6 W/m^2.

amplitude in the ERG (Fig. 6 *e*). In some flies, even a 60-s interval was not long enough to restore a PGM (Fig. 6 *b*). A PGM response typical of *trp* could be observed reliably only if the dark interval between two stimuli was 2 min or longer (Fig. 6 *c*). Under these conditions, the receptor component of the ERG evoked by the test stimulus again had the maximum peak amplitude for the stimulus intensity used (Fig. 6 *f*).

One possible interpretation of these results is that the ERG and PGM responses are always generated together in any given photoreceptor, but, because different populations of photoreceptors are sampled in ERG and

PGM recordings, the two responses—the ERG and PGM—may not always appear together when mass recordings are compared. In other words, the population of photoreceptors giving rise to the ERG in Fig. 6 *e* is not the same as the one with no PGM in Fig. 6 *b*. In the deep pseudopupil technique, PGM is observed in a relatively small population of 20–30 ommatidia "looking directly into" the microscope objective. The ERG, on the other hand, is composed of contributions from essentially all photoreceptors in the eye because the long wavelength component of the stimulus is capable of reaching even those receptors oriented sideways to the stimulus. Thus, one might argue that if the second stimulus follows the first by 50 s or less, the 20–30 ommatidia that are looking directly into the objective respond with neither the ERG nor PGM because of the adapting effect of the first stimulus, but the photoreceptors in the remaining ommatidia respond with both and give rise to the ERG seen in Fig. 6 *e*. This possibility was tested by examining PGM responses in several areas of the eye around the area originally examined. A total of ~180 ommatidia (one-fourth of the eye) that were not looking directly into the objective during the preceding stimulus were examined. No PGM was observed in any of the areas examined. If the above explanation for the disparity in ERG and PGM recordings were correct, then none of the areas examined would contribute to the ERG. Moreover, the remaining untested regions of the eye should make smaller than normal contributions to the ERG (and PGM), because any stimulus that abolishes PGM and ERG responses in one-fourth of the eye is expected to have a major effect on the remaining part of the eye as well. The amplitude of the ERG recorded 50 s after the first stimulus, however, was ~80% of that of the first response (Fig. 6 *e*), suggesting that many photoreceptors that do not show PGM to the second stimulus, nevertheless, make substantial contributions to the ERG.

In another series of experiments (Fig. 7), the *trp* mutant was pre-illuminated with light of moderate or weak intensity ($\log I = -2$ in Fig. 7 *b* and *f* and $\log I = -3$ in Fig. 7 *c* and *d*) for various durations (4.5 s, 2 s, and 1 s in Fig. 7 *b*, *c*, and *d*, respectively) before it was subjected to a test stimulus of maximum intensity ($\log I = 0$). The transition from pre-illumination to test illumination was accomplished by simply removing the neutral density filters used in pre-illumination. Thus, in this protocol, the test stimulus of maximum intensity immediately followed a weaker, pre-illuminating (or conditioning) stimulus, without being interrupted by a dark interval. Under these conditions, the test stimulus generally failed to induce PGM (Fig 7 *b* and *c*), even when it was capable of evoking an ERG of up to 8 mV amplitude from the same fly under very similar stimulus conditions (Fig. 7 *f*). A PGM response could be reliably observed only when the pre-illuminating light intensity was sufficiently weak ($\log I \leq -4$) and/or if the duration of illumination was sufficiently short (Fig. 7 *d*).

The results described above, and illustrated in Figs. 6 and 7, suggest that PGM does not occur in the *trp* mutant if a conditioning stimulus of sufficient intensity precedes the test stimulus, even though the test stimulus evokes a receptor potential of sizable amplitude. Under similar stimulus conditions, wild-type flies did not show a similar absence of PGM. With the stimulus

protocol displayed in Fig. 6, wild-type flies showed typical wild-type PGM responses when the dark interval between test stimuli was 40 s or longer. With a shorter dark interval, a smaller PGM response was induced by the second stimulus, presumably because the pigment granules had not yet completely returned to the dark-adapted state from the previous illumination. With the stimulus protocol displayed in Fig. 7 *b* or *c*, test stimuli generally induced PGM responses typical of wild type but of smaller amplitude than those

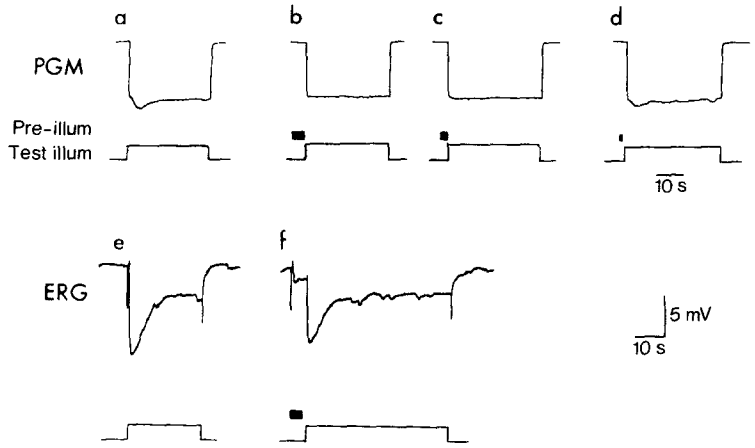

FIGURE 7. The effects of pre-illumination on the PGM and ERG recordings of *trp* mutant. Shown in the top row are the PGM recordings (*a–d*), and in the bottom row the ERG recordings (*e* and *f*). The stimulus protocol is shown just below each recording. In the protocols, the test stimuli (maximum intensity: log $I = 0$) are indicated by the thin lines. The dark bars preceding the test stimuli in *b–d* and *f* are the pre-illuminating stimuli of moderate or weak intensity. Every recording was taken after the fly was dark adapted for 150–180 s. In *a* and *e*, the test stimulus was not preceded by adapting light. Under this condition, a typical *trp*-type PGM response (Fig. 6) was induced by the test stimulus (*a*), and peak amplitude of ERG was ~10 mV (*e*). As shown in *b*, when the pre-illuminating stimulus ($I = -2$ log unit for 4.5 s) preceded the test light, the test stimulus failed to induce granule migration. In the ERG recording obtained under the same condition, the test stimulus evoked a 7.6-mV receptor response superimposed on the 1.2 mV caused by pre-illumination (*f*). In *c*, the pre-illuminating stimulus was reduced both in intensity (log $I = -3$) and duration (2s). However, the test light still failed to induce an observable migration of granules. When the pre-illumination of the same intensity as in *c* was further reduced in duration to 1 s, the test light finally succeeded in inducing a PGM (*d*). All measurements were made on the same fly.

induced without pre-illumination, apparently because the conditioning stimulus had already triggered a small PGM.

IV. *PGM Induced by a Small ERG*

One might infer from the results in section III that an ERG of sufficiently small amplitude (peak amplitude ≤8 mV) is incapable of inducing a PGM.

In other words, there might be a threshold amplitude of the receptor potential for triggering the migration of granules. The following results argue against such a hypothesis.

In the *trp* mutant, a PGM response could be induced even when the stimulus intensity was varied to produce an ERG of 5 mV or less, if the fly had been dark adapted for a few minutes. Fig. 8 *a* shows a PGM response obtained

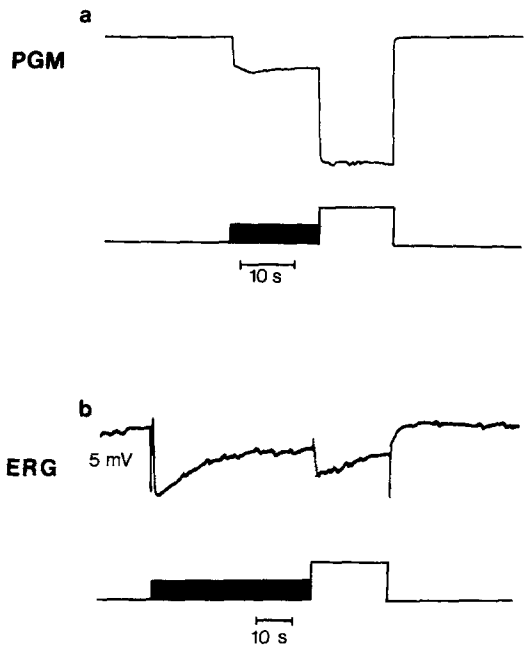

FIGURE 8. PGM and ERG responses obtained from the *trp* mutant using a stimulus of moderate intensity. (*a*) PGM responses to a stimulus of moderate intensity (dark bar; log $I = -0.64$) immediately followed by a full-intensity stimulus (log $I = 0$) recorded from a fly that had been dark adapted for 3 min. [Because the light intensity for ERG recording was reduced to match that for PGM recording (see Materials and Methods, section IV ERG Recordings), the full intensity stimulus used (log $I = 0$) was over a log unit below the intensity needed to elicit an ERG of saturated amplitude from a red-eyed fly.] A PGM response was induced by the moderate-intensity stimulus but not by the full-intensity stimulus. (*b*) ERG responses to a stimulus sequence similar to that in *a* obtained from the same fly after it had been dark adapted for 2 min. The ERG amplitude elicited by the moderate-intensity stimulus was ~5 mV. The above results were obtained from the same fly used in the conditioning stimulus experiment illustrated in Fig. 6. Calibration pulse, 5 mV.

from a *trp* mutant (the same fly used in the conditioning stimulus experiment illustrated in Fig 6) with a stimulus of moderate intensity (log $I = -0.64$) after the fly had been dark adapted for 3 min. Because of the relatively low stimulus intensity, the absolute magnitude of the response is smaller than those induced by bright stimuli (Figs. 6 *a* and *c* and 7 *a* and *d*). (Its amplitude relative to the

background reflectance is comparable to the latter, however). Nevertheless, it is clear that a PGM has been induced by the stimulus. The ERG obtained from the same fly under similar experimental conditions had an amplitude of only ~5 mV (Fig. 8 *b*). It may be recalled that the same fly, if exposed to a conditioning stimulus, failed to evoke a PGM in response to a stimulus that evoked an 8-mV ERG (Fig. 6 *b* and *e*).

Moreover, the amplitude of the receptor component of the ERG is temperature dependent. At 10° and 35°C, the amplitude of the ERG in wild-type flies was only ~30% (≤5 mV) of that obtained at 19°C. The amplitude of PGM obtained at 10° and 35°C, however, was comparable to that obtained at 19°C. Thus, the small receptor potentials observed at 10° and 35°C (5 mV or less) appear to be entirely capable of inducing PGM.

DISCUSSION

The main objective of the experiments described in this paper was to reexamine the hypothesis that the depolarization of the receptor cell triggers the migration of pigment granules toward the rhabdomere (Kirschfeld and Franceschini, 1969). The experiments involved the use of a number of *Drosophila* mutants that are defective in the photoreceptor potential. The results summarized below lend strong support to the hypothesis that the depolarization of the photoreceptor is intimately involved in pigment migration. (This conclusion is not intended to exclude the possibility that a precursor step(s) of depolarization, rather than depolarization itself, triggers the migration process.)

(1) There is no PGM in those *norpA* mutants that entirely lack the receptor potential (Fig. 1 *b*).

(2) In the case of the temperature-sensitive mutant, $norpA^{H52}$, the PGM is normal when the ERG is normal, but is absent when the ERG is absent (Fig. 4).

(3) In the case of the *trp* mutant, pigment granules first move toward and then away from the rhabdomere during a sustained stimulus (Fig. 1 *c*), which is consistent with the electrophysiological observation that during such stimuli the receptor first depolarizes and then repolarizes (Fig. 1 *c*; Cosens and Manning, 1969; Minke et al., 1975 *b*).

(4) If the PDA is induced by an intense blue stimulus, the pigment granules remain close to the rhabdomeres after the termination of the stimulus (observed in the eye color mutant w^a; Fig. 5 *A* and *B*).

If the receptor potential triggers pigment granule migration, one possible mechanism of migration is that the electric field created by the receptor potential itself directly acts as the electromotive force for movement of pigment granules (cf. Kinosita [1963] for movement of chromatophores; Stavenga [1971]). The observations discussed below suggest that this model of migration is not a likely possibility.

Both the PGM and ERG response first increase in amplitude and then return to the dark level during a sustained illumination in the *trp* mutant (Figs. 1 *c*, 6, and 7). The time-courses of the two responses, however, are very

different. For the preparation illustrated in Fig. 1 *c* and *e*, it takes ~3 s for the PGM response to reach its peak at room temperature, whereas under nearly identical conditions in the same fly, the receptor component of the ERG peaks within 0.2 s and returns to baseline within ~2 s. The peak amplitude of the *trp* PGM response, on the other hand, does not differ significantly from that of the wild-type PGM response (Fig. 1 *a* and *c*). The implication of the results is that in these flies pigment granules continued to move toward the rhabdomere after the receptor depolarization had already peaked and returned to its baseline. The granules began dispersing only after they have migrated as much as in wild type under similar stimulus conditions (Fig. 1 *a* and *c*). If the difference in time-course between the PGM and ERG responses were due to a diffusion lag, one would not expect a continued migration of pigment granules after the electromotive force had already disappeared. Thus, a simple electrophoretic model that requires the receptor potential to directly provide the electromotive force for pigment granule movement is inconsistent with the results of the present series of experiments. An explanation of PGM more consistent with the observed results is that the receptor potential triggers an intermediate step(s) having its own time-course, and the intermediate step(s) in turn acts on the granules.

A possible weakness of the above argument is that it is based on the ERG time-courses and that these may not reflect the time-course of the transmembrane potential changes accurately enough. In many parallel intracellular and extracellular measurements performed on the same *Drosophila* eyes in this laboratory, however, the time-to-peak of the intracellularly recorded receptor potential and that of the extracellularly measured ERG receptor component were found not to differ by more than a few milliseconds. Still another point to consider in comparing the time-courses of ERG and PGM is that PGM is observed in a relatively small number of the photoreceptors that are directly illuminated, whereas the ERG is seen as a mass response contributed by essentially all photoreceptors in the eye (cf. Results, section III). Thus, one might attempt to ascribe the difference in the time-courses of PGM and ERG to the difference in the population of cells in which the two processes were observed. As may be seen in Figs. 1, 6, 7, and 8, however, the ERG time-course is always much faster than that of PGM. Because the ERG time-course becomes faster with increasing stimulus intensity, the time-course of an ERG recorded from only the directly illuminated cells—the population of cells from which the PGM is observed—would be, if anything, faster than that recorded from the entire eye. Thus, the observed difference in the time-courses of ERG and PGM cannot arise from the fact the ERG and PGM are recorded from different populations of photoreceptors.

The above interpretation of pigment granule migration is further supported by the results displayed in Figs. 6 and 7. These results obtained from the *trp* mutant show that whenever the *trp* eye is sufficiently light adapted, no PGM occurs even though an ERG of sufficiently large amplitude can be elicited from the eye. The evidence again suggests that the generation of the receptor potential alone is not sufficient to guarantee an induction of PGM. In addition

to the generation of the receptor potential, some other conditions need be met (or some other step(s) generated) before the pigment granules are triggered to migrate.

Both microtubule-microfilament systems and intracellular calcium concentration have been suggested as important controlling factors for the pigment migration in photoreceptors of many arthropods (e.g., Miller and Cawthon [1974], Frixione et al. [1977], and Olivo and Larsen [1978]). Olivo and Larsen (1978) have suggested that a light-induced increase in intracellular calcium concentration of retinular cells either activates a transport mechanism or disassembles microtubules that are holding granules away from rhabdomeres. A similar hypothesis for the pigment aggregation and dispersion in chromatophores of the squirrel fish was suggested by Byers and Porter (1977). The results of our experiments and the discussion above do not exclude the possibility that microtubules or microfilaments actively transport ommochrome granules in the retinular cells of flies. However, to our knowledge, there exists no evidence that such a structure exists in the retinular cells of *Drosophila*. On the other hand, the granule migration induced by light, ether, or CO_2 could possibly be linked to an increase in intracellular calcium concentration, since exposure to light (Brown and Blinks, 1974; Brown et al., 1977) or to a metabolic inhibitor (Lo et al., 1980) has been shown to raise the intracellular calcium concentration in *Limulus* photoreceptors. If this were true, one might speculate that the regulation of intracellular calcium concentration may be defective in *trp* mutants. The dependence of pigment migration on ATP and Mg^{++} has also been reported in fish melanophores (Miyashita, 1975). The possible involvement of intracellular concentrations of Ca^{++}, Mg^{++}, and/or ATP in pigment granule migration remains to be investigated.

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