Lewis, and its properties are being described elsewhere.

10. Supported in part by grants from NSF and NIH. The original stock of *Drosophila melanogaster* for these experiments derived from a mass culture maintained for many years by Prof. A. G. Steinberg of Western Reserve University. We thank Mrs. Marilyn Ricklin for assistance; Drs. F. Sehnal and P. Lawrence for their helpful comments on the typescript; and Drs. K. Aizawa, J. Briggs, H. M. Mazzone, G. Stairs, and H. Swift for their comments on the electron micrographs.

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Molecular and Thermal Origins of Fast Photoelectric Effects in the Squid Retina

Abstract. *When a short, intense flash of light is absorbed by the outer segments of squid photoreceptors fixed in glutaraldehyde, a voltage appears briefly across the retina. The waveform depends on the relative amounts of rhodopsin and its various stable photoproducts present at the beginning of the flash. Each light-absorbing species present contributes a characteristic voltage component which is summed in the gross waveform. The heating effect of the absorbed light produces a small, long-lasting thermoelectric voltage as well. When this thermal effect is corrected for, interconversion of equal numbers of rhodopsin and acid metarhodopsin molecules by a flash results in a fast voltage waveform whose time integral is zero. Thus the charge flowing in one direction in the retina when rhodopsin is converted to acid metarhodopsin by one photon is apparently exactly reversed when acid metarhodopsin is reconverted to rhodopsin by another.*

Vertebrate retinas (*1*) as well as a variety of other light-absorbing tissues (*2*) show small transient voltages when illuminated with intense short flashes of light. In the vertebrate retina, these fast photoelectric effects depend specifically upon light being absorbed by rhodopsin (*3*), but they are little affected by the physiological state of the retina or by the ionic composition of the solution in which it is bathed (*4*). Moreover, the sizes and shapes of the voltage waveforms are influenced by the kinds and amounts of rhodopsin and its transient photoproducts present at the beginning of a test flash (*5*). Hence it is likely that the origin of fast photovoltages (FPV's) is somehow different from that of the usual electrodiffusive currents and voltages produced by live cells (*6*). Indeed, it has been suggested that FPV's arise from displacement of charges within the photoreceptors' outer segments or even in the rhodopsin molecules themselves (*7*). If so, the structural organization of the parts of the receptors bearing the photopigment and the nature and stability of rhodopsin's photoproducts would be expected to influence the size and shape of the FPV. Thus, it seemed useful to know what fast photoelectric effects would be produced by the squid retina, whose rhodopsin differs photochemically from the vertebrate photopigment and occurs in microvilli within rhabdomeres rather than in the flat lamellae of vertebrate rods (*8*). We find that the squid retina, live or fixed in glutaraldehyde, shows FPV's as large as those of vertebrate retinas and with waveforms which, like those of vertebrates (*5*), bear an apparently simple relation to the states of the photopigment chromophores at the beginning of a test flash. Light also produces an additional thermoelectric voltage which complicates the interpretation of the FPV in the squid retina and perhaps in other tissues as well. We now report a method for distinguishing the photochemical and thermal effects and for studying each separately.

Retinas from live squid adapted to the dark were isolated in darkness as described (*9*, *10*). Disks 4 to 8 mm wide were punched from the retinas and mounted between the two halves of a thin chamber. Cold electrolyte solution was pumped to the upper and lower chamber halves through silver tubes internally coated with AgCl to which electrical connections were made for voltage and current measurements. Electrical and optical shielding was used to reduce flash artifacts to 5 μv or less. A xenon flashtube (Edgerton, Germeshausen, and Grier, type FX33) delivered 100-joule flashes of about 100 μsec duration to the chamber through a set of heat filters, interference filters, and a 25-mm focal length *f*1.4 photographic objective. Only the rhodopsin-bearing inner surface of the retina was illuminated. Flash exposures were measured with a calibrated silicon photovoltaic detector. The rise time of the amplifier system coupled to the direct current was set at 5 μsec.

A live retina gives a large, slow photocurrent (*9*, *10*) when its inner surface is illuminated, but after 30 minutes at 0°C in darkness without 0_2, this physiological response disappears, leaving only fast photoelectric waveforms of the types shown in Fig. 1. Like the FPV of vertebrate eyes, the responses are not suppressed by substitution of either 1*M* KCl or .01*M* KCl for normal seawater. Fixation for 30 minutes in 5 percent glutaraldehyde in buffered seawater at 0°C (which does not destroy squid rhodopsin) also leaves the waveforms intact, though somewhat more rapid than those of the unfixed retina. Freezing to −80°C and thawing the retina, however, abolishes the FPV without destroying the normal complement of rhodopsin. In all of the experiments to be described, fixed retinas were used since these were available throughout the year. In early work, seawater was used as the bathing solution, while in later experiments a solution with a high electrical resistance containing 1.2 mmole of glycerol, 10 mmole of KCl, and 5 mmole of tris(hydroxymethyl)aminomethane (tris) buffer per liter was used to get the largest possible voltages. Such a solution increased the size of the FPV without appreciably altering its shape.

Illumination of the outer surface of the retina produces neither a slow photocurrent nor an FPV, since the light is totally absorbed by an intraretinal layer of black screening pigment before reaching the outer segments. Light absorbed by the black pigment itself yields no net transretinal voltage. In the dark-adapted retina, a test flash that activates less than 1 percent of the rhodopsin molecules present gives a simple outer-segment-positive wave whose form is independent of wave length (λ) and whose action spectrum is consistent with the absorption spectrum of squid rhodopsin. The voltage rises linearly without detectable latency, reaches a single maximum, and decays exponentially (*11*) to zero. If we allow for the finite duration of the flash, the voltage can be represented mathematically as the content of the second compartment of a three-compartment sequential kinetic system (*12*). The size of the response, called hereafter the "rhodopsin response," or "R wave," is proportional to the number of rhodopsin molecules absorbing one or more quanta from the flash, but the rate constants are independent of light intensity

if only rhodopsin is present at the beginning of the flash. The externally available short-circuit current from the retina during the rhodopsin response, measured with a voltage clamp, is less than 1 electronic charge per 100 chromophores excited. Thus the FPV is nearly 10^9 times weaker than the living retina's receptor current (*10*).

Exhaustive illumination of the retina with 560-nm light changes the FPV waveform to that of Fig. 1b, in which a faster negative phase precedes the persistent positive R wave. After such treatment, the FPV waveform is very stable; its size and shape change little after hundreds of 560-nm flashes. In solutions of squid rhodopsin (*13*, *14*), and in the live retina (*15*), a mixture of rhodopsin and its photoproduct, acid metarhodopsin (M_A), in the molar ratio of about 2 : 3 results from such treatment. Since the two types of chromophore have similar absorption spectra, it would be expected that if the faster negative wave were produced by light absorbed in M_A, all visible light would produce an FPV of about the same shape, with only the relative amplitudes of the negative and positive components varying with wavelength. This was found to be so.

If the retina is titrated to *p*H 9.8, the M_A present should be converted to basic metarhodopsin (M_B) with λ_{max} at 380 nm (*13*, *15*). The absorption due to M_A being removed, the FPV for 560-nm flashes should revert to the R response due to light absorbed by the remaining rhodopsin (Fig. 1c). Now, exhaustive illumination of the retina with green light almost completely (> 95 percent) abolishes the response to 560-nm flashes, since rhodopsin is depleted by this treatment, forming basic metarhodopsin, which has little visible absorption (*13*, *15*). At the same time a new waveform, a large and very fast positive wave, appears when near-ultraviolet flashes are used (Fig. 1d). Since its appearance coincides with the known presence of M_B (*15*), and since it is not found in the dark-adapted retina, it is tentatively identified as the M_B response.

When the *p*H of the seawater is reduced to 7.8 in the dark, the M_B is reconverted to M_A unmixed with rhodopsin. The hypothesis that the negative phase of the waveform in Fig. 1b is due to light absorbed by M_A can then be tested by application of a 560-nm flash. A pure negative wave is seen (Fig. 1e). This reverts to biphasic form when the retina is again exhaustively illuminated with green light and a steady-state mixture of M_A and rhodopsin is reformed (Fig. 1f). When the pure M_A and R responses are added graphically in the proper proportions, a waveform can be calculated which is indistinguishable from the biphasic responses in Fig. 1f.

In digitonin solutions, irradiation with near-ultraviolet (UV) light causes M_B to be partly converted to rhodopsin and partly to a fourth chemical species, isorhodopsin (λ_{max} at 480 nm) (*13*). Similarly, UV irradiation of M_B in living retinal slices (1 to 2 photons absorbed per M_B chromophore) produces increased absorbance at 480 to 500 nm, consistent with the formation of isorhodopsin and photoregenerated rhodopsin, though we cannot yet distinguish the two by spectrophotometry in situ. But flashes of 500 nm delivered to an alkaline retina previously illuminated with UV produce an FPV which apparently consists of two superimposed positive waves (Fig. 1g), one of which is identical with the original R wave; the other is smaller and much faster and is tentatively attributed to isorhodopsin. Like the rhodopsin response, it is suppressed by bleaching with blue-green light.

These results suggest that each state of the photopigment chromophores (rhodopsin itself, M_A, M_B, and isorhodopsin) produces its own unique waveform on illumination. But must a chromophore be changed photochemically from one state to another by the light in order for a contribution to the FPV to occur? Or is the FPV produced by some other effect of light? In the latter event, there would not necessarily be any relation between the signs or amplitudes of the various waveforms in Fig. 1, but, on the other hand, if the photochemical changes of the squid's rhodopsin cycle underlie the

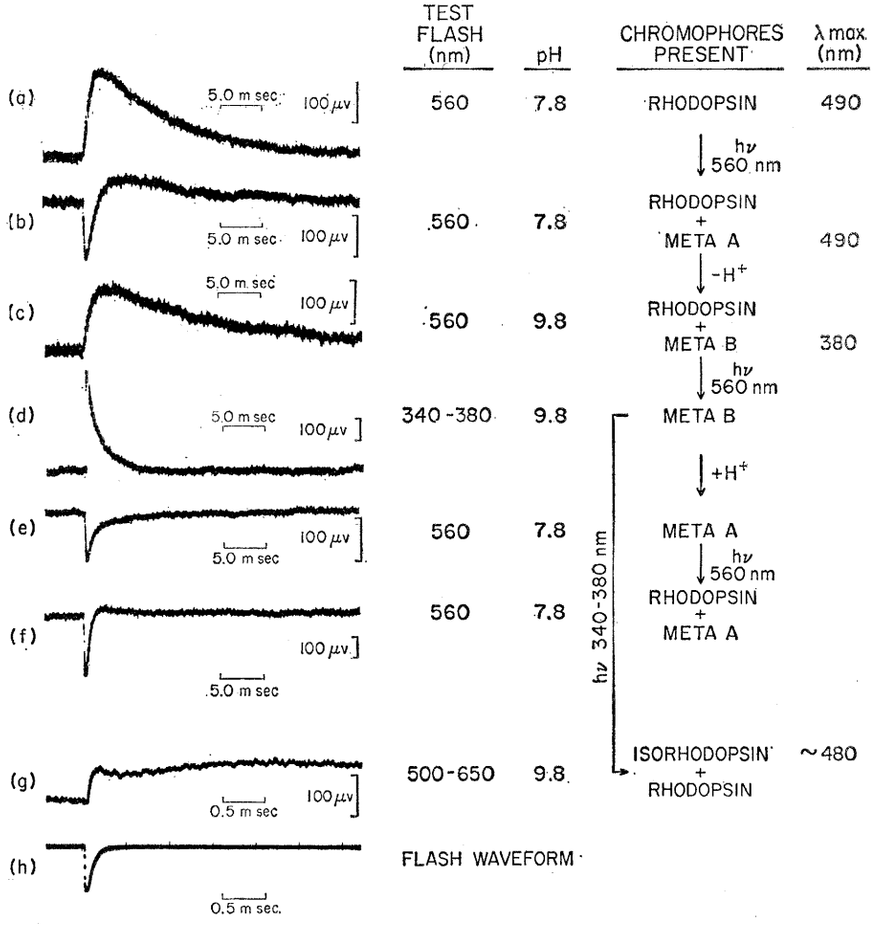

Fig. 1. Waveforms of fast transretinal photovoltages produced by a fixed squid retina with rhodopsin chromophores in states shown at right-hand side. Temperature, 0°C. Solution: 500 m*M* tris(hydroxymethyl)aminomethane-HCl titrated to *p*H shown. Responses are to single test flashes of the following energies (photons per square centimeter): (a) 3×10^{14}, (b and c) 2×10^{15}, (d) 6×10^{14}, (e and f) 8×10^{15}, and (g) 3×10^{16}. Adapting exposures used to convert chromophores in reaction scheme were flashes totaling at least 2 photons absorbed per chromophore in each case.

observed charge displacements, interconversion of equal numbers of chromophores between two states by a flash might be expected to yield an FPV whose positive and negative waves would be equal in area and would therefore sum to zero (*16*).

The waveforms attributable to rhodopsin's various derivatives do not all fit this zero-integral prediction. Basic metarhodopsin, which is thought to arise from M_A with loss of one or more protons (*13*), produces outer-segment-positive waves when illuminated with UV flashes that partly convert it to rhodopsin and isorhodopsin. Yet illumination of these latter products with a green flash, which should reconvert them ultimately to M_B, also produces an apparently unidirectional positive wave. Thus a full photochemical cycle which apparently restores M_B to its original form seems to yield a net one-way flow of charges across the fixed retina. The FPV of the squid retina in basic solution thus cannot comprise a series of photoreversible charge displacements unless additional slow after-voltages, which we have not yet found, are present (*17*).

The rhodopsin and M_A responses at *p*H 7.5, being of opposite polarities, are more promising as candidates for the two parts of a photoreversible charge displacement. When a retina is adapted to blue flashes each containing about 10^{17} photons cm^{-2} of wavelengths in the range 420-500 nm, the FPV's produced by test flashes of the same spectral composition are extremely stable from flash to flash, as was noted above. In the mixtures of rhodopsin and M_A thus established, a flash interconverts equal numbers of chromophores from one form to the other (*13*). Yet the time integral of the FPV is not zero but rather shows a large net positive area (Fig. 2a and b).

Could the failure of the voltage integral to vanish in this case be due to a component in the FPV with a net positive area which does not arise from photochemical changes in the photopigment chromophores? Apparently so. Three kinds of evidence suggest that such a component of thermoelectric origin (*18*) exists. First, if a thin disk of black membrane filter (Millipore type HA) is placed against the outer segments of a retinal disk mounted in the experimental chamber and a flash containing the equivalent of 10^{17} 500-nm photons is absorbed by the filter, the temperatures of filter and retinal surface jump 2 to 4°C, and an outer-segment-positive voltage transient appears across the retina (Fig. 2c). The shape of the transient is roughly that

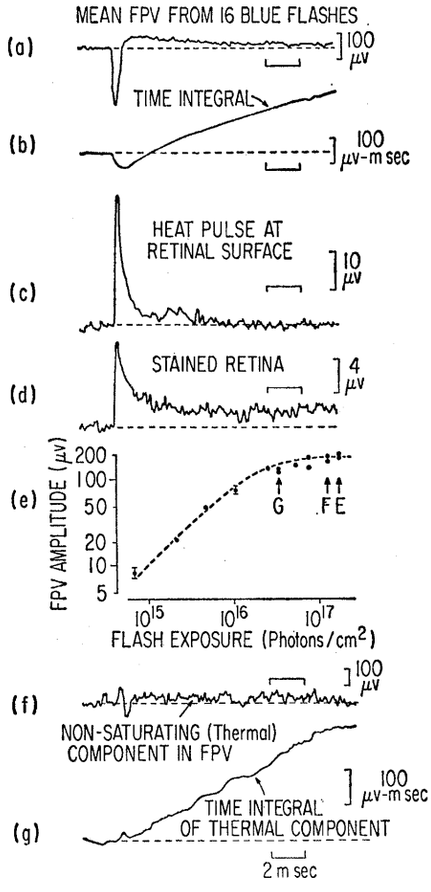

Fig. 2. Thermoelectric contributions to the FPV. All waveforms are summed responses to 10 to 20 flashes from the output of a computer of average transients. Voltage scales normalized to single flashes. Temperature, 0°C; *p*H, 7.40. Solution: 1.2*M* glycerol, 10 m*M* KCl, 5 m*M* tris(hydroxymethane)aminomethane buffer. (a) FPV from a blue flash of exposure about 10^{17} photons cm^{-2}. (b) Time integral of waveform in (a). (c) Transretinal voltage due to flash containing 10^{17} blue photons cm^{-2} absorbed by a black Millipore filter disk in contact with the outer segments of the retina. (d) Voltage transient due to about 10^{17} photons cm^{-2} 550 to 650 nm absorbed by methylene blue dye with which outer segments of a retina were stained. Absorbance of dye was greater than 2 per 100 μm thickness of outer segments at 650 nm. (e) Points: Peak to peak amplitude of biphasic FPV as a function of flash energy. Blue flashes (420 to 500 nm). For points without vertical bars the standard error is too small to be shown. Line: Saturation curve calculated for absorption of one or more photons by photopigment with a mean extinction coefficient of 20,000 cm^2 $mmole^{-1}$. (f) FPV waveform from flash of energy *E* minus waveform from energy *F*. (g) Time integral of (f).

to be expected for the output of a thermocouple consisting of a plane layer 400 μm thick of a substance with the thermometric conductivity of water in contact at its lower and upper surfaces with a dissimilar substance of similar thermal conductance, the heat being applied at the junction nearest the light. If the retina is not present, the filter alone yields no voltage transient. The retina thus has a thermoelectric coefficient of appreciable size relative to the electrolyte solution.

Second, if a retinal disk is heavily stained with methylene blue or crystal violet so that orange light (600 to 650 nm) is totally absorbed within 50 μm of the surface, an orange flash produces an outer-segment-positive voltage transient (Fig. 2d) that also conforms to the predicted behavior of a differentially heated thermocouple junction pair. Blue flashes that are still absorbed by the photopigment give the normal FPV response. Light absorbed by either dye thus produces a photovoltage different from that due to illumination of the photopigment. But since the two dyes are chemically quite different, their common action is probably to convert light to heat.

Third, while the amplitude of the FPV apparently reaches a limiting size at high flash energies (Fig. 2e), a small positive component is present which does not saturate. This is shown in Fig. 2f, in which 20 FPV's produced by flashes of energies *F* (Fig. 2e) are subtracted from 20 produced by flashes of higher intensity *E*. The ordinary biphasic components, which are of maximum amplitude at both flash energies, are canceled by this procedure, leaving the small, long-lasting component whose integral (Fig. 2g) is large enough to account for the deviation from zero seen in Fig. 2b. There is as yet no direct proof that this nonsaturating component is a thermoelectric one, but since rhodopsin converts at least 90 percent of absorbed light energy into heat (*19*), an appreciable thermoelectric voltage (TEV) should be expected from the flashes used in Fig. 2a. Moreover, the laws of heat flow predict that an initial spatial distribution of heat like that of the rhodopsin in the retina will yield a TEV without the pronounced initial peak seen in Fig. 2, c and d. Instead, the transient should decay more slowly from its initial value, in agreement with the result in Fig. 2f (*20*).

A variation of the above procedure for demonstrating the TEV can be used

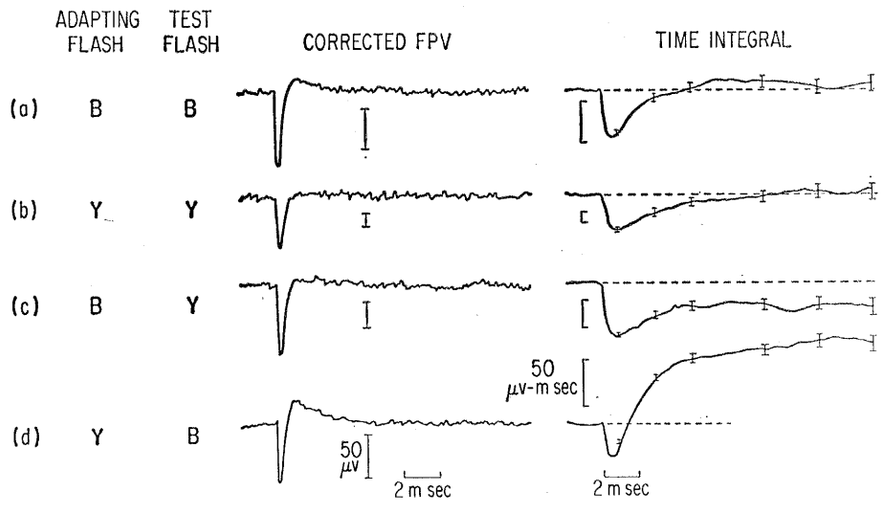

Fig. 3. Voltage waveforms and their time integrals for the saturating (photochemical) component of the FPV. Traces obtained by the subtraction of a response to one bright flash (exposure E) from n responses to flash exposures E/n. For blue flashes, $E = 1.7 \times 10^{17}$ photons cm^{-2}, and for yellow, $E = 8.6 \times 10^{16}$ photons cm^{-2}. Voltage scales normalized to single flashes. Vertical bars on time integral traces represent standard error due to amplifier noise. (a) $n = 5$, (b) $n = 9$, (c) $n = 9$. Each yellow flash preceded by a bright blue flash, (d) $n = 5$. Each blue flash preceded by a bright yellow flash. Experimental conditions as in Fig. 2.

to obtain the shape the FPV would have if the thermoelectric effect were absent. If the FPV's produced by N flashes of energy G (Fig. 2e) are summed and the response to one flash of energy $G \cdot N$ is then subtracted from the sum, the resulting curve is corrected for voltage components, such as the TEV, which increase linearly between the two flash energies, since the total light energy in the intense flash is equal to that in the weaker ones. This method has been used to obtain the results of Fig. 3 in which the photoreversibility of the charge displacements in interconverting rhodopsin and M_A is shown.

If the retina is adapted to flashes containing 10^{17} photon cm^{-2} of blue light (420 to 500 nm), the corrected FPV shows zero net area with an uncertainty of ± 20 percent of the area in the fast negative wave (Fig. 3a). If the retina is then adapted to yellow light (500 to 600 nm), similar yellow flashes also yield a corrected FPV with zero net area (Fig. 3b). But if the retina is adapted to saturating blue flashes and the FPV is elicited with yellow flashes, the area in the fast negative wave is greater than that in the slow positive one (Fig. 3c). Conversely, blue test flashes produce an FPV with a net positive area if the retina has been previously adapted with yellow flashes. These results further support the idea that the rhodopsin and M_A components of FPV arise from a photoreversible charge shift, since the proportions of M_A and rhodopsin in a mixture will depend in general on the spectral composition of the adapting flashes. This rule applies to vertebrate rhodopsin and its derivatives (*13*), as well as to the squid photopigment system (*14*, *15*). From the curves of Fig. 3, c and d, it appears that short flashes of blue light produce mixtures which are relatively rich in M_A, while yellow flashes favor the formation of rhodopsin. There is not yet enough known about the stability and spectra of squid lumirhodopsin and other metastable intermediates in the retina to permit the FPV's of Fig. 3 to be quantitatively predicted from the photochemical effects of the adapting flashes, however.

From these results we conclude that the rhodopsin response in the FPV probably arises from a change in molecular charge distribution in those excited rhodopsin molecules that are subsequently converted to acid metarhodopsin by thermal reactions, while the M_A response is due to a restoration of rhodopsin's original charge distribution. Furthermore, the externally detectable photovoltage is not likely to result simply from asymmetrical orientation of the rhodopsin molecules relative to the surfaces of the retina, as originally suggested by Brindley and Gardner-Medwin (*4*) and by Lettvin (*7*). Indeed, both the transition moment of the rhodopsin chromophores (*15*) and the long axes of the radially symmetrical microvilli of squid photoreceptors (*8*) lie parallel with the retinal surfaces. However, it is possible that the important orientation is that of the photopigment relative to the photoreceptor cell membranes, as suggested by the results of Smith and Brown (*21*).

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17. Nor is the observed unidirectional charge flow likely to be due to rectification of an internally symmetrical electrical process by nonlinearities in the retina's interstitial impedances. Direct alternating-current bridge measurements at 20 hz to 40 khz on fixed squid retinas yield rectification ratios that differ from unity by less than ± .00004.
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22. We thank the staff of the Marine Biological Laboratory, Woods Hole, Massachusetts, for their hospitality during much of this work.

4 May 1967