Analysis of the Rhodopsin Cycle in *Limulus* Ventral Photoreceptors Using the Early Receptor Potential

JOHN E. LISMAN and YVETTE SHELINE

From the Harvard Biological Laboratories, Cambridge, Massachusetts 02138, and the Department of Biology, Brandeis University, Waltham, Massachusetts 02154

ABSTRACT The early receptor potential (ERP) was recorded intracellularly from *Limulus* ventral photoreceptors. The ERP in cells dissected under red light was altered by exhaustive illumination. No recovery to the original waveform was observed, even after 1 h in the dark. The ERP waveform could be further altered by chromatic adaptation or by changes in pH. The results indicate that at pH 7.8 there are two interconvertible pigment states with only slightly different λ_{max}, whereas at pH 9.6 there are two interconvertible states with very different λ_{max}. Under all conditions studied the ERPs were almost identical with those previously obtained in squid retinas. This strongly suggests that light converts *Limulus* rhodopsin to a stable photoequilibrium mixture of rhodopsin and metarhodopsin and that, as in squid, the λ_{max} of metarhodopsin depends on pH. This conversion at pH 7.8 is associated with a small (0.7 log unit) decrease in the maximum sensitivity of the late receptor potential. Thus the component of adaptation linked to changes in rhodopsin concentration is unimportant in comparison to the "neural" component.

INTRODUCTION

Limulus photoreceptors have proved to be an excellent preparation for investigating the electrophysiology of visual transduction. Recent studies (Fein and DeVoe, 1973; Fein and Cone, 1973) have attempted to relate electrical activity in the photoreceptor to concentration of visual pigment. However, interpretation of these results was hampered by the fact that the nature of the rhodopsin cycle in *Limulus* was not known.

Hillman et al. (1973) have suggested that *Limulus* rhodopsin rapidly (1 s) regenerates after isomerization, i.e. that there exists only one stable pigment state. If this were so, *Limulus* visual pigment would differ from typical invertebrate pigments which have multiple stable states (Hubbard and St. George, 1958; Brown and White, 1972; Hamdorf et al., 1973; Minke et al., 1973). We present here the results of experiments conducted to determine whether *Limulus* pigment exists in one or more than one stable state.

The existence of multiple pigment states has been most clearly established in the squid. The dark-adapted squid retina contains rhodopsin (λ_{max} 493 nm) with its chromophore in the 11-*cis* configuration (Hubbard and St. George, 1958; Hubbard and Kropf, 1958). Light converts rhodopsin to a stable all-*trans* pig-

ment, metarhodopsin, which can be converted back to rhodopsin by the absorption of a second photon. When the conversion rate of rhodopsin to metarhodopsin is equal to the conversion rate of metarhodopsin to rhodopsin, the population of pigment molecules is at photoequilibrium. The λ_{max} of metarhodopsin (but not of rhodopsin) is sensitive to pH. At low pH, its λ_{max} is 500 nm (acid metarhodopsin), whereas at high pH its λ_{max} is 380 nm (alkaline metarhodopsin). Hagins and McGaughy (1967) showed that the absorption of light by different forms of the squid pigment produced different waveforms of the early receptor potential (ERP). This dependence of the ERP on pigment conformation makes it possible to study visual pigments by electrophysiological techniques.

A short latency response to bright flashes has been previously recorded in *Limulus* photoreceptors and identified as an ERP on the basis of its action spectrum (Brown et al., 1967) and its resistance to fixation (Hillman et al., 1973). We have measured the ERP in *Limulus* in different states of adaptation and at different pHs. Our measurements indicate that *Limulus* visual pigment has more than one stable state and is similar to that of the squid.

We have also measured the change in maximum sensitivity of the late receptor potential associated with the conversion of rhodopsin to a photoequilibrium mixture of rhodopsin and metarhodopsin and found it to be small. Some of our findings have been briefly reported elsewhere (Lisman et al., 1975).

METHODS

Horseshoe crabs obtained from the Marine Biological Laboratories (Woods Hole, Mass.) were kept in artificial seawater for periods of up to 2 mo before use. The lateral olfactory nerve (Clark et al., 1969) was dissected from the animal and desheathed. The nerve was mounted in a transparent chamber and then treated with 2% Pronase (Calbiochem, San Diego, Calif.) for 1 min in order to soften the connective tissue surrounding the photoreceptors. The chamber was placed on the stage of a compound microscope and illuminated through the condenser. Experiments were performed at room temperature (19°–22°C).

Microelectrodes filled with 3 M KCl and having resistances between 4 and 20 MΩ were connected to a unity gain preamplifier with negative capacity compensation. Responses were displayed on a Tektronix, Inc., (Beaverton, Ore.) 565 oscilloscope and photographed. Membrane potential was monitored on a Grass Instrument Co. (Quincy, Mass.) curvilinear chart recorder.

The artificial seawater (ASW) contained 423 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 22 mM $MgCl_2$, and 26 mM $MgSO_4$. At pH 7.8, the pH buffer was 15 mM Tris and 10 mM $NaHCO_3$ (the $NaHCO_3$ was sometimes omitted). At pH 9.6 (or 10) the buffer was 15 mM glycine. The chamber used in experiments that required solution changes had a volume of 0.2 ml. The flow rate of the perfusate was adjusted to be at least five chamber volumes per minute.

Light from two sources was combined with a half-silvered mirror before reaching the microscope condenser. The first light source was a tungsten-iodide lamp controlled by an electromechanical shutter and was used to evoke the discrete waves in the experiments cited in Table III. The second light source (xenon flash), used in all other experiments, was a Strobonar 880 (Honeywell Inc., Test Instruments Div., Denver, Colo.). The unattenuated flash from this source was bright enough to saturate the early receptor potential, but if the beam was passed through narrow-band interference filters the ERP was greatly reduced. It was therefore not possible to obtain spectral sensitivity curves. The filters which were used are listed in Table I.

Period of Nonoverlap of ERP and LRP

The question arises of whether measurements of the ERP can be made without contamination by the rising edge of the late receptor potential (LRP). To examine this question we made use of a previous observation that the LRP latency is altered by light adapting the photoreceptor (Brown and Lisman, 1975). The responses to flashes bright enough to evoke saturated ERPs were recorded before and after an adapting stimulus. The initial part of the responses coincided with at most a ±0.25 mV difference. The responses diverged sharply at a later time, which in different cells varied from 5 to 15 ms. This divergence marked the onset of the LRP. We can therefore conclude that the LRP made a negligible contribution to the voltage measured during the first 5 ms of the response.

RESULTS

Effects of Light and pH on the ERP

Animals were dark adapted for 2 or more days. During the dissection and the subsequent insertion of a microelectrode the cells were exposed only to red light

TABLE I

THE CHARACTERISTICS OF FILTERS AND THEIR USES

Make	Model number	Transmission	Experimental use
Wratten	47	400–500 nm	Blue color adaptation
Jena Schott	RG1	λ>610 nm	Orange color adaptation
Wratten	4	λ>460 nm	Stimulation in the visible region of the spectrum
Corning	5970	335–395 nm	Stimulation in the UV and near-UV region of the spectrum
Jena Schott	RG5	λ>665 nm	Dissection
Jena Schott	KG3	λ<800 nm	Heat filter used in all experiments

(RG5 filter). Photoreceptors bathed in artificial seawater (pH 7.8) were stimulated with flashes, each of which was sufficiently bright to saturate the ERP. The ERP evoked by the first flash (Fig. 1*a*) had a small (<1 mV) positive component followed by a larger (4–8 mV) negative component. The amplitudes were measured from the resting potential before the flash to the peak of each component. The ERPs evoked by subsequent flashes had a progressively larger positive component and a progressively small negative component until, after four flashes, further stimulation produced little further change in the ERP (Fig. 1*b*). This stable ERP had a positive component of 1–2 mV and a negative component of 2–4 mV. In five cells, the average increase in the positive component was 0.5 mV; the average decrease in the negative component was 2.8 mV. After 1 h of dark adaptation the ERP showed little if any recovery (Fig. 1*c*). The stable alteration of the ERP suggests that *Limulus* visual pigment can exist in more than one stable state.

Cells which had been previously exposed to bright white light were alternately irradiated with orange (RG1 filter) or blue light (Wratten 47 filter). After each period of adaptation (3 or more min) an ERP was evoked. The peaks of the negative component in successive ERPs were compared: the average differences (ERP_{blue}-ERP_{orange}) for six experiments are listed in Table II. Small but statistically significant differences were obtained in five of the six experiments. In three of these, the amplitude was larger after blue adaptation than after orange

adaptation. In two, the dependence on the color of adaptation was reversed. The factors responsible for this variation are not known, however, the fact that adapting lights do significantly affect the ERP can be taken as a second argument that more than one pigment state contributes to the ERP at pH 7.8. Because the effects were small, the pigments giving rise to the ERP probably have similar absorption spectra, a conclusion consistent with the nearly identical spectral sensitivities of the positive and negative components (Brown et al., 1967).

Squid metarhodopsin is a pH indicator (λ_{max} = 500 nm at low pH; λ_{max} = 380 nm at high pH). Thus, at high pH, squid visual pigment can be converted to a

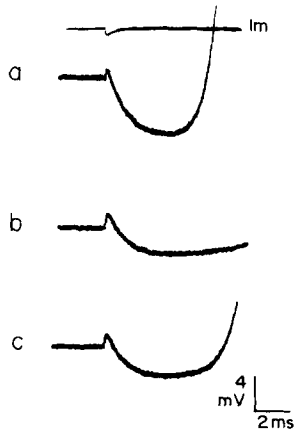

FIGURE 1. The early receptor potential (ERP) before and after exposure to bright illumination. The ERP recorded from a cell dissected under red light (*a*) had a small positive component and a large negative component. After exhaustive illumination (*b*), the ERP had a larger positive and a smaller negative component. The ERP measured after 1 h of dark adaptation (*c*) showed little or no recovery to its original waveform. The upward deflection at the right end of each trace is the onset of the late receptor potential. *lm* = light monitor.

form that has low absorption in the visible region of the spectrum. *Limulus* photoreceptors were perfused with pH 9.6 seawater to determine if their visual pigment is similarly affected by pH. The cells used in these experiments had been previously exposed to white light and gave stable biphasic ERPs in pH 7.8 seawater (Fig. 2*a*). The flashes were filtered to exclude short wavelength light (Wratten 4 filter). After the pH had been raised to 9.6 in the dark, the ERP was nearly monophasic negative (Fig. 2*b*). Subsequent flashes evoked progressively smaller responses, each of which was approximately half the previous response (Fig. 3), until finally the response stabilized at about 0.4 mV (Fig. 2*c*). A large monophasic negative response to visible light could be restored by irradiating the cell with UV light (Corning 5970 filter) (Fig. 2*d*). Further illumination with visible light again reduced the ERP amplitude to approximately 0.4 mV. These results are consistent with the hypothesis that there are two interconvertible pigment states at pH 9.6, one that absorbs visible light and one that absorbs UV light.

As described above, at pH 9.6 the response to visible light was 0.4 mV after

TABLE II

EFFECT OF CHROMATIC ADAPTATION* ON THE ERP AT pH 7.8

Date of experiment(s)‡	No. of trials	Average difference in amplitude of negative component ($ERP_{blue}-ERP_{orange}$)±SD	*t* value	Statistically significant (level)
		mV		
October 1973§	23	−0.19±0.09	−10.1	yes (0.001)
10 June 1976‖	11	−0.079±0.079	−3.3	yes (0.01)
17 June 1976‖	6	0.10±0.12	2.0	no (0.05)
20 July 1976‖	4	0.135±0.07	3.9	yes (0.05)
21 July 1976‖	14	0.11±0.09	4.6	yes (0.001)
2 August 1976‖	27	0.19±0.085	11.6	yes (0.001)

* 3 or more min adaptation (equivalent [at 530 nm] intensity at nerve surface was approximately 10^{-3} W/cm^2).

‡ These experiments were done during a period when the article was being revised; therefore, they bear a date later than that of the original submission of the article.

§ Pooled data from five cells with comparable ERP amplitudes. ERP evoked by full spectrum flash.

‖ ERP evoked by light restricted to visible region of spectrum (Wratten 4 filter).

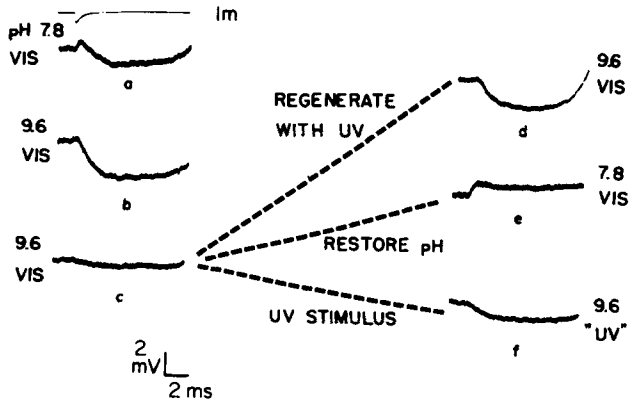

FIGURE 2. Effects of high pH on the ERP. A stable biphasic response (*a*) to visible light was recorded at pH 7.8. The first ERP (*b*) 60 s after the pH had been raised to 9.6 was primarily negative. The responses to subsequent flashes were progressively smaller until only a small response (*c*) remained. Cells in this condition could then be treated in three different ways: (i) if the cell was irradiated with UV light, a large negative response (*d*) to visible light was again recorded; (ii) if the pH was lowered to 7.8 in the dark, a visible flash evoked a positive ERP (*e*); (iii) if the cell was stimulated with a UV flash (visible light was also present; see text), the response (*f*) was negative. *lm* = light monitor. All the recordings shown are from the same cell.

exhaustive illumination with visible light. If the cell was then returned to pH 7.8 seawater in the dark, the ERP evoked by the first flash was monophasic positive (Fig. 2*e*). Subsequent flashes evoked ERPs which were progressively more biphasic. After four flashes, the waveform became stable and was identical to that recorded before exposure to pH 9.6 seawater.

We then sought to evoke an ERP by stimulating the presumed UV-absorbing pigment. The preparation was perfused with pH 9.6 seawater and irradiated

with visible light until the ERP evoked by visible light was ≤0.4 mV. Cells were then stimulated with flashes filtered to transmit primarily UV light (Corning 5970). There appeared to be a small negative ERP but the response could not be reliably distinguished from the noise. Since the UV filter attenuated the UV component of the flashes, the filter was removed and, under these conditions, monophasic negative responses approximately 1 mV in amplitude were recorded (Fig. 2*f*). We believe that this response resulted from the absorption of UV radiation, since flashes filtered to transmit only visible light saturated the negative component of the ERP at a response amplitude of less than 0.4 mV (Fig.

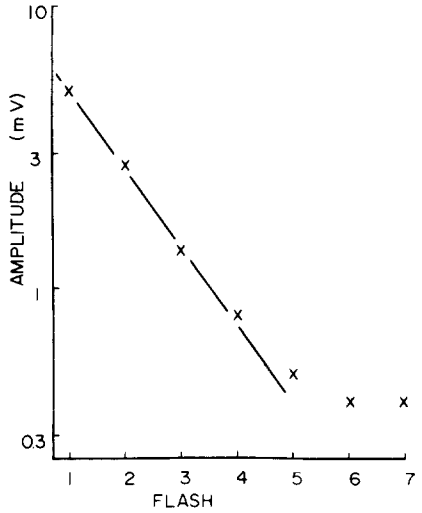

FIGURE 3. The peak amplitude of the negative ERP evoked by ther first seven flashes (visible light; W4 filter) after the pH had been raised from 7.8 to 10. Each flash was bright enough to saturate the ERP but reduced the ERP by only 54%. The line, fit (by eye) to the first four points, has a slope of 0.54. Similar limitations on the flash bleaching of vertebrate rhodopsin were first reported by Hagins (1955). The data are not from the same cell as Fig. 2.

2*c*). Since the response to the combination of short and long wavelength light was larger than 0.4 mV we tentatively conclude that the additional negative component was generated by a short wavelength-absorbing pigment.

Effect of Pigment States on the Late Receptor Potential

As demonstrated in Fig. 1, the ERP recorded from a cell dissected under red light is altered by exposure to white light. This stable change probably reflects the conversion of rhodopsin to a photoequilibrium mixture of rhodopsin and its photoproduct, metarhodopsin (see Discussion). Since all previous studies of the late receptor potential in *Limulus* ventral photoreceptors were performed on cells dissected under bright white light (personal communications: A. Mauro, J. E. Brown, A. Fein, S. Yeandle, and R. Srebro), it seemed important to determine whether and to what degree such changes in pigment states affected the sensitivity to light.

The dissection and the insertion of a microelectrode were done in red light

(RG5 filter). After 15 min of dark adaptation, the average amplitude and number of discrete waves evoked by dim stimuli (2-s duration) were measured. Discrete waves (quantum bumps) are the elementary units of the receptor potential and are thought to arise from the absorption of single photons; their frequency varies linearly with intensity (Fuortes and Yeandle, 1964). The average number of light-evoked discrete waves was defined as the difference between the average number of discrete waves occurring during a stimulus minus the average number occurring spontaneously (Adolph, 1964) during an equal period in the dark. The cells were then stimulated by four or more flashes, each of which was sufficiently bright to saturate the ERP. As described previously, the first of these flashes evoked a nearly monophasic negative ERP; subsequent responses revealed a stable reduction in the amplitude of the negative component. The cell was then dark adapted (ventral photoreceptors have a slow phase of sensitivity recovery with a time constant of 4.5 min; a stable value of sensitivity is reached within 10–15 min. [Fein and DeVoe, 1973]). After 15–30 min in the dark, the averge number and amplitude of light-evoked waves was again measured. Table III summarizes the data from three cells. The change in pigment states had little or no effect on the average discrete wave amplitude. However, the average number of light-evoked discrete waves, normalized to equal intensities, was reduced (by factors of 4.3, 4.6, and 5.0, respectively). If, after alteration of the pigment states, the intensity had been raised by factors of 4.3, 4.6, and 5.0, respectively, the frequency and amplitude of discrete waves would have been the same as before alteration of the pigment. This means that the alteration of pigment states is associated with a fall in the maximum sensitivity of about 0.7 log unit.

The 0.7 log unit loss of sensitivity is associated with a reduction of about 50% in the amplitude of the negative (rhodopsin) component of the ERP (Fig. 1; Table III). The amplitude of the ERP is commonly thought to be directly proportional to the number of pigment molecules in the plasma membrane. Therefore, our data might indicate a nonlinear relationship between rhodopsin concentration and sensitivity; however, we believe this conclusion is premature. The amplitude of the ERP is also dependent on membrane capacitance and thus on membrane area. Anatomical studies on other photoreceptors show that there are light-induced changes in membrane area (White and Lord, 1975). If this were so in *Limulus*, the reduction in rhodopsin content of the plasma membrane might be more than 50%. Furthermore, since we have not shown that the full sensitivity is recovered if the original rhodopsin concentration is restored, we cannot exclude the possibility that part or all of the loss in maximum sensitivity is related to factors other than the loss of rhodopsin. For these reasons, the 0.7 log unit value is an upper limit for the pigment-related reduction of sensitivity, and 50% is a lower limit for the reduction in rhodopsin concentration.

Stimuli bright enough to alter the pigment states also affected the duration of the depolarizing after-potential evoked by a saturating flash. In cells (which presumably contained primarily rhodopsin) not previously exposed to bright white light, a saturating flash evoked after-potentials lasting as long as 100 s (Fig. 4). Subsequent flashes evoked much shorter after-potentials (Fig. 4). Flashes given after 1 h of dark adaptation also evoked shorter after-potentials. In the

latter two states, the cell presumably contained a photoequilibrium mixture of rhodopsin and metarhodopsin.

The above relationship between pigment states and the duration of afterpotentials can be plausibly explained by the theory of Hochstein et al. (1973). According to their theory, stimulation of rhodopsin produces a long afterpotential which can be antagonized or inhibited by stimulation of metarhodopsin. Thus the antagonistic processes observed in *Limulus* median eye (Nolte and Brown, 1972), in barnacle (Hochstein et al., 1973), and, more recently, in insects (Minke et al., 1975; Muijser et al., 1975), may also occur in *Limulus* ventral photoreceptors. The failure of colored stimuli to evoke depolarizing after-

TABLE III

EFFECT OF PIGMENT STATES ON DISCRETE WAVE FREQUENCY AND AMPLITUDE

	Cell no.	Before photoequilibrium	After photoequilibrium		
Intensity	1	I_0	5.2 I_0		
	2	I_0	1.6 I_0		
	3	I_0	2.3 I_0		
Discrete waves (per second) (mean±SD)	1	1.9±0.2	3.8±0.25		
	2	0.95±0.09	0.70±0.08		
	3	1.3±0.1	0.46±0.06		
Spontaneous discrete waves (per second) (mean±SD)	1	0.41±0.10	2.0*±0.2		
	2	0.30±0.05	0.48±0.07		
	3	0.65±0.10	0.16±0.04		
Light-induced discrete waves (per second) (mean±SD)	1	1.5±0.3	1.8±0.5		
	2	0.65±0.14	0.22±0.15		
	3	0.65±0.20	0.30±0.10		
Light-induced discrete waves normalized for equal intensity (per second) (mean±SD)	1	1.5±0.3	0.35±0.1	4.3	Proportional reduction in light-induced discrete waves
	2	0.65±0.14	0.14±0.09	4.6	
	3	0.65±0.20	0.13±0.04	5.0	
Average amplitude of discrete waves (mV) (mean±SEM)	1	2.6±0.2	3.2±0.3		
	2	2.3±0.15	2.4±0.25		
	3	6.3±0.34	5.6±0.48		
Amplitude of negative component of ERP (mV)	1	8.0	3.8	2.1	Proportional reduction in amplitude of negative component
	2	3.6	2.0	1.8	
	3	3.2	2.0	1.6	

* In some cells a large increase in the rate of spontaneous discrete waves occurred after stimulation with bright brief flashes. This also occurred in cells that had been dissected in white light.

potentials in ventral photoreceptors dissected under white light (Hochstein et al., 1973) may be due to the fact that rhodopsin cannot be stimulated without stimulating acid metarhodopsin because the two have such similar λ_{max}.

The conclusions drawn in the paragraphs above must be considered preliminary. Given the possibility of radically altering the rhodopsin and metarhodopsin concentrations (as in Fig. 2), it should be possible in future studies to examine more critically the relationship between pigment states and properties of the late receptor potential.

DISCUSSION

The dependence of the ERP waveform on the wavelength of adaptation indicates that *Limulus* ventral photoreceptors contain a visual pigment with more

than one stable state. This is most clearly demonstrated at pH 9.6. Illumination with visible light reduces the amplitude of subsequent ERPs evoked by visible light (Fig. 2*c*). The ERP amplitude can then be restored by illumination with UV light (Fig. 2*d*). These results indicate that at pH 9.6 there are two interconvertible pigment states which absorb in different regions of the spectrum. At pH 7.8 the color of preadaptation also affected the ERP (Table II) but the effects were small, suggesting two pigment states with nearly identical absorption spectra. Because effects of adaptations were not previously observed (Minke et al., 1973), it was suggested (Hillman et al., 1973) that ventral photoreceptors contained a single thermally stable pigment which is regenerated rapidly in the dark. This model is no longer applicable in view of the data presented here.

The ERP waveforms shown in Figs. 1 and 2 can be readily interpreted in terms of a squid-like rhodopsin cycle (Hubbard and St. George, 1958). Squid rhodopsin (λ_{max} 493 nm) is converted by light to a stable photoproduct, metarhodopsin, from which rhodopsin can be photoregenerated. Metarhodopsin is a pH indica-

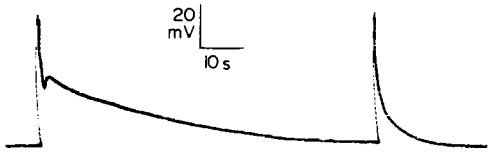

FIGURE 4. Long depolarizing after-potentials in cells not previously exposed to bright light (first response). The second and all subsequent responses to the same intensity flashes had much shorter depolarizing after-potentials. Each flash was bright enough to saturate the ERP.

tor; acid metarhodopsin has a λ_{max} at 500 nm; alkaline metarhodopsin has a λ_{max} at 380 nm. We assume here that, as in the squid, the pigment in the dark-adapted *Limulus* photoreceptor is mainly rhodopsin (Hubbard and St. George, 1958) and that stimulation of single pigment states gives rise to monophasic ERPs (Hagins and McGaughy, 1967). On the basis of these assumptions we infer that the large negative component of the ERP recorded from the dark-adapted cell (Fig. 1*a*) is due to stimulation of rhodopsin. The smaller positive component must be due to another pigment, possibly to acid metarhodopsin. After exhaustive illumination, the negative (rhodopsin) component is smaller and the positive component is larger, suggesting that the positive component is produced by stimulation of a photoproduct of rhodopsin, presumably acid metarhodopsin. Under these conditions, the ERP is not altered by further illumination and does not recover to its original waveform even after 1 h in the dark (Fig. 1*c*). Presumably light has produced a stable photoequilibrium mixture of rhodopsin and acid metarhodopsin. As in other invertebrate photoreceptors (Brown and White, 1972; Minke et al., 1973), regeneration of rhodopsin in vitro is either very slow or absent. After the pH is raised to 9.6, the ERP evoked by visible light is nearly monophasic negative (Fig. 2*b*). The positive component generated by acid metarhodopsin has almost wholly disappeared because acid metarhodopsin has been converted to alkaline metarhodopsin which does not absorb long wavelength light. With further illumination the negative component is reduced in amplitude (Figs. 2*c* and 3) as more and more rhodopsin is converted to

alkaline metarhodopsin. A small residual ERP probably occurs because the pH is not high enough to convert all metarhodopsin to the alkaline form. (Meaningful experiments at pHs higher than 10 were not possible due to deterioration of the cells.) Exhaustive irradiation with UV light converts alkaline metarhodopsin back to rhodopsin and a large negative response to visible light can then be evoked (Fig. 2*d*). If, on the other hand, the rhodopsin is converted to alkaline metarhodopsin, as described above, and the pH is then lowered to 7.8 in the dark, the cell contains primarily acid metarhodopsin and the ERP is thus monophasic positive (Fig. 2*e*). After further illumination the ERP becomes biphasic, as acid metarhodopsin is converted to a photoequilibrium mixture of acid metarhodopsin and rhodopsin.

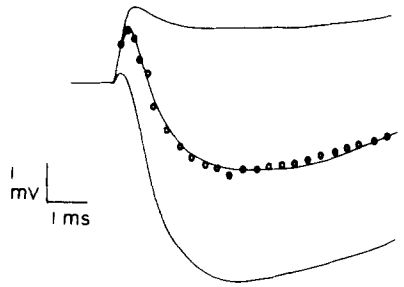

FIGURE 5. Reconstruction (circles) of a biphasic ERP by addition of the scaled negative and positive responses generated by pure or nearly pure rhodopsin and acid metarhodopsin, respectively. The solid lines are tracings of the ERPs recorded under conditions similar to those in Fig. 2*a*, *b*, and *e*. The scaling factor was 0.8 for the positive ERP and 0.66 for the negative ERP. In three other cells scaling factors for the positive and negative components were: 0.7, 0.64; 0.62, 0.59; 0.66, 0.57. The number of rhodopsin molecules stimulated during the biphasic response recorded before raising pH and the number stimulated during the first response at pH 9.6 should be the same since the rhodopsin concentration should not change in the absence of illumination. Since the amplitude of each ERP component is the product of the number of molecules stimulated and the light-induced change in dipole moment per molecule, the scaling factor for the negative (rhodopsin) component ought therefore to be 1.0 provided that the change in dipole moment was independent of pH. The fact that the scaling factor was not 1.0 suggests that the change in dipole moment of *Limulus* rhodopsin is dependent on pH. Light-induced dipole changes in solubilized vertebrate rhodopsin are pH dependent (Petersen and Cone, 1975).

According to this explanation, the stable biphasic ERP at pH 7.8 is the summation of negative and positive components due to rhodopsin and acid metarhodopsin, respectively. To examine further this model, reconstructions of the biphasic ERP were made by linear summation of a predominantly negative and a monophasic positive response (such as in Fig. 2*b* and *e*). These responses are thought to be generated by pure or nearly pure rhodopsin and acid metarhodopsin, respectively. Scaling factors were chosen to give a best fit. This procedure yielded a good approximation of the biphasic ERP (Fig. 5).

The above interpretation of *Limulus* ERPs in terms of the squid pigment cycle does not depend on any a priori assumptions about the polarity of the ERPs

caused by stimulation of particular pigment states. The polarities deduced from this analysis are compared to those in the squid in Table IV. The complete correspondence of polarities lends further support to the hypothesis that *Limulus* and squid visual pigment are similar. With the exception of the small amount of a second pigment (probably acid metarhodopsin) present in the dark-adapted *Limulus* photoreceptor, we have found no qualitative differences between *Limulus* and squid visual pigment.

The data presented in recent studies of pigment kinetics in *Limulus* (Fein and Cone, 1973; Fein and DeVoe, 1973; Hillman et al., 1973) can be reinterpreted in terms of the squid model. In these studies dissections were done under bright white light (personal communication, A. Fein). Therefore, rhodopsin and metarhodopsin were in photoequilibrium at the start of the experiments. Fein and

TABLE IV

COMPARISON OF *LIMULUS* AND SQUID VISUAL PIGMENT

	Limulus	Squid (*Loligo*)
Polarity of rhodopsin ERP	negative	negative*
Polarity of acid metarhodopsin ERP	positive	positive*
Polarity of alkaline metarhodopsin ERP	negative (?)	negative*
Polarity of first component of ERP	positive	positive*
λ_{max} of rhodopsin	520–530‡	493§
λ_{max} of acid metarhodopsin	515–535‖	500§
λ_{max} of alkaline metarhodopsin	330–400¶	380§

* The ERP recorded by an intracellular electrode has a polarity opposite the ERP recorded by an extracellular electrode (Smith and Brown, 1966). The polarities of the squid ERP listed here are the expected intracellular responses based on the extracellular measurements of Hagins and McGaughy (1967).

‡ Murray, 1966; Nolte and Brown, 1970.

§ Hubbard and St. George, 1958.

‖ The relatively small effect of chromatic adaptation on the ERP (Table II) suggests that the λ_{max} of metarhodopsin must be very close to that of rhodopsin.

¶ The absorption of alkaline metarhodopsin must substantially overlap the transmission of the Corning 5970 filter used to photoregenerate rhodopsin.

DeVoe (1973) used an initial flash to isomerize the pigment. A second flash was used to measure the extent to which the ERP, and thus presumably the pigment, had returned to its original state. For short interflash intervals (10 ms), the negative component of the ERP evoked by the second flash was reduced. Responses to flashes at longer interflash intervals showed that the negative component recovered to its initial state with a time-constant of 100 ms. Fein and Cone (1973) made photometric measurements during this recovery period and found an absorption increase at 463 nm due to a thermally unstable intermediate. The intermediate decayed with a time course identical to that for the recovery of the negative component of the ERP. Since the nature of the rhodopsin cycle in *Limulus* was not understood, it was unclear whether the return to the initial state was due to the rapid regeneration of rhodopsin or to the completion of the transitions between two thermally stable states. It now seems clear that Fein and Cone (1973) measured an unstable intermediate(s) involved in the transition between two thermally stable pigment states. There-

fore, the return of the pigment to its initial state was actually the return of the populations of pigment to photoequilibrium, rather than the return of individual molecules to their starting condition.

We think that the substance which increased in absorbance at 463 nm in Fein and Cone's experiments is an intermediate in the light-induced transition from acid metarhodopsin to rhodopsin rather than in the reverse transition. We base this on the following argument: since the negative component of the biphasic ERP is generated by rhodopsin, and since the time constant for recovery of the negative component after an initial flash closely matches the disappearance of the pigment absorbing at 463 nm, the unstable pigment is probably an intermediate in the light-induced transition from metarhodopsin to rhodopsin. Evidence for transient intermediates in the metarhodopsin to rhodopsin transition has been inferred from measurements of the ERP in barnacle (Minke et al., 1974).

In this paper we have given an interpretation of the pigment cycle based on recordings of the ERP. Both the ERP and flash photometry (Fein and Cone, 1973) indicate that *Limulus* rhodopsin does not bleach at physiological pH. This conclusion is difficult to reconcile with two reports indicating that it does. The pigment extracted from *Limulus* lateral eye bleaches to a 370 nm photoproduct (Hubbard and Wald, 1960). Given the similarity of ERPs recorded from the lateral and ventral eyes (Smith and Brown, 1966; Brown et al., 1967), there is no reason to suspect that the two eyes have fundamentally different pigments. It might be argued that *Limulus* rhodopsin bleaches in extracts but not in the membrane, as has been found in *Deilephila* (Schwemer and Paulson, 1973), but this seems unlikely in view of the microspectrophotometric data on intact ventral photoreceptors which also indicate that bleaching occurs (Murray, 1966). Very recently, results similar to Murray's have been obtained with the additional observations that the stable photoproduct is a UV-absorbing pigment from which rhodopsin can be photoregenerated, and that bleaching is not increased at high pH.[1] Possible resolutions of the conflicts in these data will be discussed in the manuscript cited in footnote 1.

Adaptation Linked to Rhodopsin Concentration

The results in Fig. 1 indicate that irradiating a dark-adapted photoreceptor that has been dissected under red light causes a decrease in the rhodopsin concentration and an increase in the metarhodopsin concentration. During such stimulation the sensitivity of the late receptor potential is greatly reduced. When the photoreceptor is returned to darkness, sensitivity gradually increases (3 or more log units) until it reaches a stable level (see Fein and DeVoe, 1973, for recovery kinetics). We have found that this stable value is approximately 0.7 log unit less sensitive than before illumination (Table III). The ERP indicates that rhodopsin had not been regenerated during this recovery period (Fig. 1); (see also Fein and DeVoe, 1973). Therefore this recovery of sensitivity must be related to "neural" mechanisms, possibly removal of calcium from intracellular sites that regulate sensitivity (Lisman and Brown, 1972*a*, *b*; Brown and Blinks, 1974; Lisman and Brown, 1975). The existence of the residual loss of sensitivity suggests that there

[1] J. E. Lisman, P. K. Brown, and M. G. Dubin. Manuscript in preparation.

is also a relatively small component of adaptation linked to changes in rhodopsin concentration as has been found in other invertebrates (Hamdorf et al., 1973; Barnes and Goldsmith, 1973). Thus, in vertebrates (Wald, 1958; Dowling, 1963) and in invertebrates there appears to be both "neural" and "photochemical" adaptation. What we interpret to be the photochemical component of adaptation in *Limulus* affects only the probability that an incident photon will evoke a discrete wave without affecting the amplitude of the wave (Table III). In contrast, neural adaptation in *Limulus* affects primarily the amplitude of the discrete waves (Dodge et al., 1968).

The relative unimportance of photochemical adaptation in invertebrates vs. vertebrates can be illustrated by comparing our results to adaptation studies of the rat retina in which the regeneration of rhodopsin was blocked by removal of the pigment epithelium (Weinstein et al., 1967). In the rat, bleaching half the rhodopsin lowered the maximum sensitivity by 3 log units. In *Limulus* reducing the rhodopsin concentration by at least 50% lowered the maximum sensitivity by no more than 0.7 log units. Thus, any photochemical component of adaptation is relatively unimportant in *Limulus*, not because changes in rhodopsin concentration do not occur, but because the highly nonlinear relation between sensitivity and concentration found in vertebrates does not hold in *Limulus*.

The authors wish to thank P. K. Brown, G. Fain, A. Fein, R. Hubbard, and G. Wald for their comments on the manuscript and for helpful discussions.

This work was supported by National Institutes of Health Research Grants EY 00508 to G. Wald and EY 01496 to J. Lisman.

Received for publication 22 September 1975.

REFERENCES

ADOLPH, A. 1964. Spontaneous slow potential fluctuations in the *Limulus* photoreceptor. *J. Gen. Physiol.* **48**:297–322.

BARNES, S. N., and T. H. GOLDSMITH. 1973. Dark adaptation of lobster. *Biol. Bull. (Woods Hole).* **145**:423.

BROWN, J. E., and J. R. BLINKS. 1974. Changes in intracellular free calcium during illumination of invertebrate photoreceptors: detection with aequorin. *J. Gen. Physiol.* **64**:643–665.

BROWN, J. E., and J. E. LISMAN. 1975. Intracellular Ca modulates sensitivity and time scale in *Limulus* ventral photoreceptors. *Nature (Lond.).* **258**:252–253.

BROWN, J. E., J. R. MURRAY, and T. T. SMITH. 1967. Photoelectric potential from photoreceptor cells in ventral eye of *Limulus*. *Science (Wash. D. C.).* **158**:665–666.

BROWN, P. K., and R. H. WHITE. 1972. Rhodopsin of the larval mosquito. *J. Gen. Physiol.* **59**:401–414.

CLARK, A. W., R. MILLECCHIA, and A. MAURO. 1969. The ventral photoreceptor cells of *Limulus*. I. The microanatomy. *J. Gen. Physiol.* **54**:289–309.

DODGE, F., B. W. KNIGHT, and J. TOYODA. 1968. Voltage noise in *Limulus* visual cells. *Science (Wash. D. C.).* **160**:88–90.

DOWLING, J. E. 1963. Neural and photochemical mechanisms of visual adaptation in the rat. *J. Gen. Physiol.* **46**:1287–1301.

FEIN, A., and R. CONE. 1973. *Limulus* rhodopsin: rapid return of transient intermediates to the thermally stable state. *Science (Wash. D. C.).* **182**:495–497.

FEIN, A., and R. P. DEVOE. 1973. Adaptation in the ventral eye of *Limulus* is functionally independent of the photochemical cycle, membrane potential, and membrane resistance. *J. Gen. Physiol.* **61**:273–289.

FUORTES, M. G. F., and S. YEANDLE. 1964. Probability of occurrence of discrete potential waves in the eye of *Limulus*. *J. Gen. Physiol.* **47**:443–463.

HAGINS, W. A. 1955. The quantum efficiency of bleaching of rhodopsin. *J. Physiol. (Lond.).* **139**:22P–23P.

HAGINS, W. A., and R. E. MCGAUGHY. 1967. Molecular and thermal origins of fast photoelectric effects in the squid retina. *Science (Wash. D. C.).* **157**:813–816.

HAMDORF, K., R. PAULSEN, and J. SCHWEMER. 1973. Photoregeneration and sensitivity control of photoreceptors of invertebrates. *In* Biochemistry and Physiology of Visual Pigments. H. Langer, editor. Springer-Verlag, Berlin. 155–165.

HILLMAN, P., F. A. DODGE, S. HOCHSTEIN, B. W. KNIGHT, and B. MINKE. 1973. Rapid recovery of invertebrate early receptor potential. *J. Gen. Physiol.* **62**:77–86.

HOCHSTEIN, S., B. MINKE, and P. HILLMAN. 1973. Antagonistic components of the late receptor potential in the barnacle photoreceptor arising from different stages of the pigment process. *J. Gen. Physiol.* **62**:105–128.

HUBBARD, R., and A. KROPF. 1958. The action of light on rhodopsin. *Proc. Natl. Acad. Sci. U. S. A.* **44**:130–139.

HUBBARD, R., and R. C. C. ST. GEORGE. 1958. The rhodopsin system of the squid. *J. Gen. Physiol.* **41**:501–528.

HUBBARD, R., and G. WALD. 1960. Visual pigment of the horseshoe crab, *Limulus polyphemus*. *Nature (Lond.).* **186**:212–215.

LISMAN, J. E., and J. E. BROWN. 1972*a*. The effects of intracellular Ca^{++} on the light response and on light adaptation in *Limulus* ventral photoreceptors. *In* The Visual System: Neurophysiology, Biophysics and their Clinical Applications. G. B. Arden, editor. Plenum Publishing Co., New York. 23–33.

LISMAN, J. E., and J. E. BROWN. 1972*b*. The effects of intracellular iontophoretic injection of calcium and sodium ions on the light response of *Limulus* ventral photoreceptors. *J. Gen. Physiol.* **59**:701–719.

LISMAN, J. E., and J. E. BROWN. 1975. The effects of intracellular injection of calcium buffers on light-adaptation in *Limulus* ventral photoreceptors. *J. Gen. Physiol.* **66**:489–506.

LISMAN, J., Y. SHELINE, and P. K. BROWN. 1975. Localization of a pH-sensitive site of metarhodopsin. *Biophys. J.* **15**(2, Pt. 2):172 a. (Abstr.).

MINKE, B., S. HOCHSTEIN, and P. HILLMAN. 1973. Early receptor potential evidence for the existence of two thermally stable states in the barnacle visual pigment. *J. Gen. Physiol.* **62**:87–104.

MINKE, B., S. HOCHSTEIN, and P. HILLMAN. 1974. Derivation of a quantitative kinetic model for a visual pigment from observations of early receptor potential. *Biophys. J.* **14**:490–512.

MINKE, B., C. WU, and W. L. PAK. 1975. Isolation of light-induced response of the central retinula cells from the electroretinogram of *Drosophila*. *J. Comp. Physiol.* **98**:345–355.

MUIJSER, H., J. T. LEUTSCHER-HAZELHOFF, D. G. STAVENGA, and J. W. KUIPER. 1975. Photopigment conversions expressed in receptor potential and membrane resistance of blowfly visual sense cells. *Nature (Lond.).* **254**:520–522.

MURRAY, G. 1966. Intracellular absorption difference spectrum of *Limulus* extra-ocular photolabile pigment. *Science (Wash. D. C.).* **154:**1182–1183.

NOLTE, J., and J. E. BROWN. 1970. The spectral sensitivities of single photoreceptor cells in the lateral, ventral and median eyes of normal and white-eyed *Limulus*. *J. Gen. Physiol.* **55:**787–801.

NOLTE, J., and J. E. BROWN. 1972. Ultraviolet induced sensitivity to visible light in ultraviolet receptors of *Limulus*. *J. Gen. Physiol.* **59:**186–200.

PETERSEN, D., and R. A. CONE. 1975. The electric dipole moment of rhodopsin solubilized in Triton X-100. *Biophys. J.* **15:**1181–1200.

SCHWEMER, J., and R. PAULSEN. 1973. Three visual pigments in *Delephila elpenor* (Lepidoptera, Sphingidae). *J. Comp. Physiol.* **86:**215–229.

SMITH, T. G., and J. E. BROWN. 1966. A photoelectric potential in invertebrate cells. *Nature. (Lond.).* **212:**1217–1219.

WALD, G. 1958. Retinal chemistry and the physiology of vision. *In* Visual Problems of Colour. National Physical Laboratory symposium no. 8. Her Majesty's Stationery Office, London.

WEINSTEIN, G., R. HOBSON, and J. E. DOWLING. 1967. Light and dark adaptation in the isolated rat retina. *Nature (Lond.).* **215:**134–138.

WHITE, R. H., and E. LORD. 1975. Diminution and enlargement of the mosquito rhabdom in light and darkness. *J. Gen. Physiol.* **65:**583–598.