# Limulus Rhodopsin: Rapid Return of Transient Intermediates to the Thermally Stable State

Abstract. *Spectral transitions of rhodopsin in single cells of the* Limulus *ventral eye were observed both with flash photometry and by measuring the early receptor potential. Even with repetitive stimulus flashes the rhodopsin did not bleach; after each flash the spectral intermediates decayed rapidly to the initial thermally stable state. The pigment returns to the stable state in a time comparable with the duration of the late receptor potential.*

The ventral photoreceptor of *Limulus*, because of its large size, has proved to be an important preparation for studying the ionic mechanisms in visual excitation. However, little is known of the changes in the visual pigment molecule that may be involved in this excitation process. We have used both flash photometry and measurements of the early receptor potential to observe two transient spectral intermediates in the ventral photoreceptors of *Limulus*, and we compare the lifetime of these intermediates to the time course of excitation and adaptation.

Specimens of *Limulus* (carapace diameter 15 to 20 cm) were obtained from the Marine Biological Laboratory at Woods Hole, Massachusetts. The lateral olfactory nerves containing the ventral photoreceptors were dissected free, desheathed, and placed in a small chamber containing artificial seawater (*1*). Under these conditions the photoreceptor maintains its sensitivity to light, as well as its ability to adapt to light and dark. The absorbance changes in these photoreceptors are very small (less than 0.002) and our observations were limited by the photon noise of the measuring beam. Since photon noise decreases as the square root of the intensity, we operated the measuring light source, a 12-volt quartz-iodide lamp with a tungsten filament, at 14.5 volts off a battery. The measuring beam passed through a broad-band blue filter (Corning 5030; for the wavelength range 360 to 500 nm) and was focused to a spot measuring 40 by 80 $\mu$m by using a 20× microscope objective (numerical aperture 0.4). The spot was centered on cell bodies of individual receptors which had cross sections of about 50 by 100 $\mu$m. After passing through the cell the light was refocused on a photomultiplier. Interference filters peaking at 463 or 427 nm were used to set the measured wavelength and also served to protect the photomultiplier from the intense stimulus flashes (about 0.2 msec) produced by a photographic flash unit (Honeywell Auto/Strobonar 332). A cutoff filter eliminated all short wavelengths (less than 520 nm) from the stimulus flash. With this arrangement continuous recordings of absorbance in the photoreceptor, with negligible flash artifact, could be displayed and recorded on an oscilloscope.

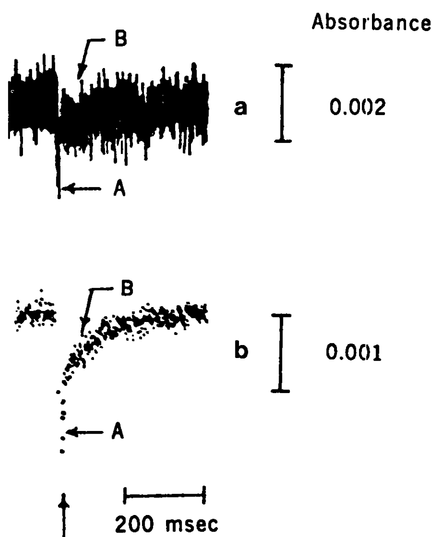


Fig. 1. Decrease in transmitted light observed at 463 nm and 21° to 23°C. (a) Oscilloscope record of photomultiplier output (band pass 0 to 300 hertz). (b) Computer average of photomultiplier output to 40 stimulus flashes (band pass 0 to 1000 hertz). The arrow indicates the occurrence of the stimulus flash (approximately 0.2 msec). The fast and slow components of the absorbance decay are designated *A* and *B*, respectively. The data in (a) and (b) are from different photoreceptors.

At 463 nm each stimulus flash elicited a transient absorbance increase, as shown in Fig. 1a. The increased absorbance decayed to the original baseline with a fast and a slow component, here designated A and B, respectively. At 427 nm a similar transient absorbance increase was observed, but the B component of the decay was much smaller in magnitude than the A component. At both wavelengths the transient absorbance increase always decayed to the original baseline; thus, the visual pigment apparently did not bleach (*2*) but rapidly returned after each flash to the initial thermally stable state (*3*). We attribute the two components of absorbance decay to two photoproducts with different absorption spectra, both of which absorb more strongly in the blue than the initially stable state of the pigment.

Since photoproducts A and B both decayed rapidly, and since the visual pigment did not bleach, we were able to use a computer of average transients to sum the absorbance changes produced by many stimulus flashes (Fig. 1b). Absorbance changes were observed in more than 25 cells. In some cells absorbance changes were too small to be seen clearly in a single trace, but computer averaging showed that both components, A and B, were present. At room temperature (21° to 23°C) the decay half-times of A and B were 4 ± 1 and 70 ± 20 msec, respectively. The measured half-times were not altered by variations in the intensity, wavelength, and duration of exposure of the measuring beam. We have looked for and not found any other faster or slower components of

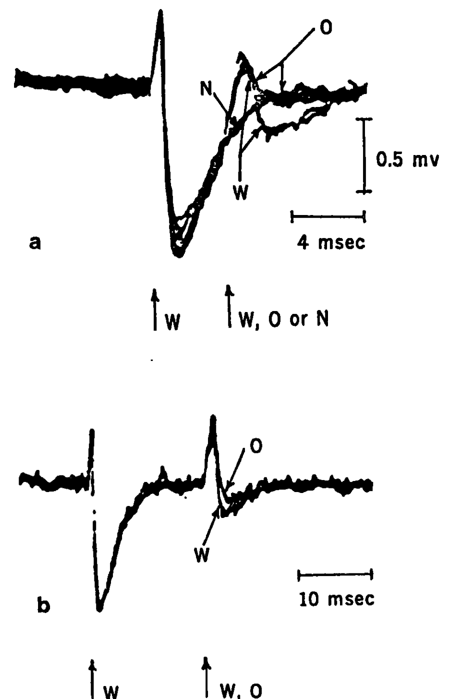


Fig. 2. Intracellular ERP elicited by two flashes (approximately 0.2 msec). (a) The downward deflection indicates increasing intracellular negativity. In each case the first flash was white. The second flash was either white (*W*; no filter), orange (*O*; Wratten 16 filter; above 520 nm), or absent (*N*). The arrows indicate the occurrence of the stimulus flashes.

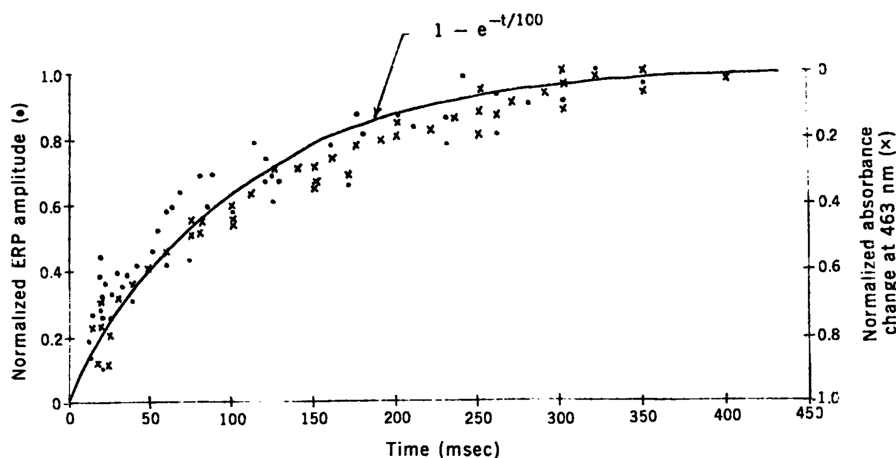


Fig. 3. Comparison of recovery of ERP amplitude and decay of absorbance at 463 nm. The normalized ERP amplitude (dots) is the ratio of the negative component of the ERP elicited by the second flash to that elicited by the first flash (*5*). Amplitudes were measured from the base line. The absorbance decay at 463 nm (crosses) was normalized because different cells gave a different initial absorbance change. The points represent composite data of six cells for ERP measurements and six cells for absorbance measurements. A 100-msec exponential (that is, a 70-msec half-time) is included for comparison: $t$ = time.

absorbance change in the time interval between 100 μsec and 1 second.

In addition to these photometric observations, we have investigated transient photoproducts in the ventral photoreceptors by observing the early receptor potential (ERP) (*4*). The ERP was measured intracellularly in photoreceptors which were bathed in isotonic KCl to eliminate the late receptor potential (*5*). A white flash (approximately 0.2 msec) always elicited a biphasic, predominantly negative ERP. Changes in the color of the flash changed only the amplitude and not the time course of the ERP. Observations by Brown *et al.* (*6*) have shown that the ERP of the ventral photoreceptor is produced by the visual pigment, which in this receptor absorbs maximally at about 530 nm. When an ERP was elicited by a second flash presented to the preparation shortly after the first, the results shown in Fig. 2, a and b, were obtained. As shown in Fig. 2a, the wave form of the ERP elicited by the second flash depended strongly on whether this flash was white or had been made orange with a cutoff filter. Evidently the blue light (less than 520 nm) contained in the white flash elicited a monophasic negative response. This blue-sensitive response diminished when the time interval between the two flashes was increased (Fig. 2b), and was not observed when the interval exceeded about 20 msec. Since this response is blue sensitive and can be elicited only during the first few milliseconds after a flash, it may possibly arise when photoproduct A absorbs light from the second flash. With a further increase of the time interval between the flashes, the predominantly negative component of the ERP grew until the second white flash elicited an ERP identical to that elicited by the first flash (*5, 7*). In Fig. 3 we compare this recovery of ERP amplitude to the decay of photoproduct B (*8*). Within experimental error both processes have the same time course.

After the first 20 msec and throughout the decay of photoproduct B shown in Fig. 3, the ERP had the same wave form for both white and orange flashes. If photoproduct B contributes to the ERP we would expect to observe a component of the ERP which decays with the same time course as photoproduct B. However we have not observed such a component. Instead, the ERP grew in amplitude as photoproduct B decayed (see Fig. 3). Thus it appears that photoproduct B contributes little if at all to the ERP. In this case, Fig. 3 shows the correlation between the decay of a precursor (photoproduct B) and the formation of its ERP-producing product.

We have found that the late receptor potential (LRP) observed in receptors bathed in artificial seawater had a latency of 9 to 12 msec following a saturating stimulus flash and about 100 msec following a dim flash. The duration of the LRP was about 200 to 500 msec with these flashes. Thus, most of photoproduct A decays before the LRP arises, whereas photoproduct B persists throughout most of the interval during which the pigment molecule may actively excite the receptor. During this time the molecule does not appear to produce an ERP response, a situation which may also obtain in vertebrates (*9*).

In vertebrate (*10*) and molluscan (*11*) photoreceptors a direct correspondence has been observed between the ERP response and the state of the pigment molecule. Our results extend this correspondence to an arthropod photoreceptor.

In vertebrate photoreceptors, long after the excitatory event represented by the LRP, rhodopsin continues to undergo a series of spectral transitions (*12*). In contrast, in *Limulus* both the photometric and the ERP observations indicate that the pigment attains a thermally stable state (at least in the chromophore region of the molecule) within a time comparable to the duration of the late receptor potential. Since the visual pigment of the ventral photoreceptor attains a thermally stable state within this short interval, the adaptation effects which far outlast the LRP (*5*) probably arise from other sites in the receptor.


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**References and Notes**


1. The ventral photoreceptors have been described in A. W. Clark, R. Millechia, A. Mauro, *J. Gen. Physiol.* **54**, 289 (1969). The artificial seawater consisted of 435 m*M* NaCl, 10 m*M* KCl, 10 m*M* $CaCl_2$, 20 m*M* $MgCl_2$, and 25 m*M* $MgSO_4$.
2. R. Hubbard and G. Wald [*Nature* **186**, 212 (1960)] found that digitonin solutions of rhodopsin from the *Limulus* lateral eye could be bleached, and G. C. Murray [*Science* **154**, 1182 (1966)] was able to bleach rhodopsin in situ in the ventral photoreceptors of *Limulus*. However, under our experimental conditions *Limulus* rhodopsin in the ventral photoreceptors did not appear to bleach.
3. Many invertebrate rhodopsins have a thermally stable photoproduct (metarhodopsin), and in some cases the pigment can be photically driven back and forth between rhodopsin and the stable metarhodopsin. For a discussion see T. H. Goldsmith, in *Handbook of Sensory Physiology, Photochemistry of Vision*, H. J. A. Dartnall, Ed. (Springer-Verlag, Berlin, 1972), vol. VII/1, pp. 685–719. If a stable metarhodopsin occurs in ventral photoreceptors, the thermally stable state we observe should consist of a mixture of rhodopsin and stable metarhodopsin. However, as yet there have been no observations which clearly indicate the presence of a stable metarhodopsin in these receptors.
4. R. A. Cone and W. L. Pak, in *Handbook of Sensory Physiology, Principles of Receptor Physiology*, W. R. Lowenstein, Ed. (Springer-Verlag, Berlin, 1972), vol. 1, pp. 345–365.
5. A. Fein and R. D. DeVoe, *J. Gen. Physiol.* **61**, 273 (1973).

6. J. E. Brown, J. R. Murray, T. G. Smith, *Science* **158**, 665 (1967).
7. P. Hillman, F. A. Dodge, S. Hochstein, B. W. Knight, B. Minke, *J. Gen. Physiol.* **62**, 87 (1973).
8. In Fig. 3 the recovery of the ERP was measured in isotonic KCl and the photometric measurements were made in artificial seawater. The time course of the absorbance changes remains the same when the cells are in isotonic KCl.
9. The "free retinal" state of rat rhodopsin appears not to produce an ERP response (*10*). This state has yet to be distinguished photometrically from metarhodopsin II (which does produce an ERP response). Spectrophotometric observations do not yet exclude the possibility that both the free retinal state and metarhodopsin II arise from the decay of metarhodopsin I. Hence the free retinal state might form before the development of the LRP.
10. R. A. Cone and W. H. Cobbs, *Nature* **221**, 820 (1969).
11. W. A. Hagins and R. E. McGaughy, *Science* **157**, 813 (1967).
12. See discussion in W. A. Hagins, *Annu. Rev. Biophys. Bioeng.* **1**, 131 (1972).
13. We thank J. Lisman, P. Brown, R. DeVoe, T. H. Goldsmith, and J. Brown for useful criticisms and suggestions on preparing the manuscript. Supported in part by grants from the National Institutes of Health to R.A.C. and by NIH special fellowship 5FO3 GM 42438 to A.F. Part of this work was done at the Marine Biological Laboratory, Woods Hole, while A.F. was a Grass Foundation fellow.
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## Fatty Acyl–Coenzyme A Elongation in Brain of Normal and Quaking Mice

Abstract. *Microsomal enzyme systems from mouse brain that catalyze, respectively, the elongation of palmitoyl-coenzyme A (palmitoyl-CoA), stearoyl-CoA, or arachidyl-CoA appear and reach maximal activity at different times after birth of the animal. A specific $C_{20}$-CoA elongating system exists in mouse brain in addition to the previously recognized $C_{16}$-CoA and $C_{18}$-CoA elongating enzymes. The $C_{20}$-CoA elongation system is severely reduced in the mutant quaking mouse.*

The quaking mouse is a recessive autosomal mutant characterized by defective myelination of the central nervous system (*1*). Baumann and collaborators (*2–4*) have noted that the cerebroside (galactolipid) and sphingomyelin content in the myelin lipids of quaking mice is much less than normal and that the nonhydroxylated long-chain fatty acids typical of these myelin lipids are also drastically reduced in the mutant. Brain lipids of adult quaking mice were found to contain at most one-tenth as much nonhydroxylated $C_{24}$ acids as did normal controls (*3*). In efforts to localize the block in myelin galactolipid and sphingomyelin biosynthesis in the quaking mouse, Baumann and colleagues made two important observations: (i) the activities of the cytoplasmic de novo fatty acid synthetase [which produces palmitate ($C_{16}$)] and of the microsomal enzyme system that catalyzes elongation of palmitoyl-coenzyme A (palmitoyl-CoA) to stearoyl-CoA were only slightly below normal in quaking mice (*5–7*) and (ii) the rate of stearoyl-CoA elongation in mutant brain extracts was also near normal (*6*), but the products of this enzymatic process were not (*6, 7*). Brain microsomes from normal mice incubated with stearoyl-CoA afforded 78.5 percent arachidate ($C_{20}$), 10.5 percent behenate ($C_{22}$), and 11 percent lignocerate ($C_{24}$), while the corresponding enzyme preparation from the quaking mutant produced 99 percent $C_{20}$, 0.8 percent $C_{22}$, and 0.2 percent $C_{24}$ (*6, 7*). Baumann and collaborators have also furnished evidence for the existence of two separate microsomal elongating systems in normal mouse brain, one converting palmitoyl-CoA to stearate ($C_{18}$) and the other converting stearoyl-CoA to longer ($C_{20}$ to $C_{24}$) acids (*8*).

From the analytical results just mentioned and the fact that the fatty acids having long chains ($C_{22}$ and $C_{24}$) are greatly diminished in the brain lipids of quaking mice, these investigators concluded that the stearoyl-CoA elongating system is defective in the mutant (*6*). We offer an alternative interpretation of their data. Since stearoyl-CoA elongation, as judged by incorporation of [$^{14}$C]malonyl-CoA, is not diminished in the mutant (*6*) but stops at the $C_{20}$ stage (*6, 7*), it seemed to us improbable that, in normal mouse brain, one and the same enzyme system elongates $C_{18}$-CoA not only to but also beyond $C_{20}$. If it did, the mutation would have resulted in an altered chain-length specificity. It was more likely, in our view, that the conversion of $C_{18}$ to $C_{20}$ is the function of one enzyme system and the extension of $C_{20}$ to $C_{22}$ and $C_{24}$ is the function of another, separate from either of the two elongating activities described by Baumann and co-workers. Diminished levels of the postulated third acyl-CoA elongating system rather than impaired stearoyl-CoA elongation would satisfactorily explain the reduced content of $C_{24}$ acids in mutant mouse brain. The results reported here support our proposal.

Brains from normal mice (Charles River Breeding Laboratory, Wilmington, Massachusetts) and from the quaking mutant (strain C57BL/6J-*qk*, Jackson Laboratory, Bar Harbor, Maine) were washed in cold 0.1*M* potassium phosphate buffer, *p*H 7.0, containing 1 m*M* dithiothreitol, 0.3*M* sucrose, and 0.9 percent NaCl. Three pooled brains were homogenized for 2 minutes with a Potter-Elvehjem homogenizer fitted with a Teflon pestle. Microsomes prepared as described (*8*) were washed, suspended in buffer, and stored at − 70°C. The conditions for enzyme assays are described in the figure legends.

In Fig. 1A are plotted activities for de novo fatty acid synthesis and for elongation of $C_{16}$-CoA, $C_{18}$-CoA, $C_{20}$-CoA, and $C_{22}$-CoA in extracts of brains taken from normal mice at various ages. The soluble fatty acid synthetase (*9*) fluctuates somewhat, rising slightly between 3 and 9 days after birth and declining slowly thereafter. Palmitoyl-CoA elongation activity is first detectable at 3 days and reaches a maximum 12 days after birth. The stearoyl-CoA elongating system appears later; it is not measurable until 5 days after birth and its activity rises to a maximum at 17 days. These differences in time course are additional and confirmatory evidence for the nonidentity of the elongating systems for palmitoyl-CoA and stearoyl-CoA (*8*). Elongation activity for $C_{20}$-CoA and $C_{22}$-CoA as a function of age is detectable 3 days after birth and rises steadily but relatively slowly thereafter. This pattern, which is distinct from that for either $C_{16}$-CoA or $C_{18}$-CoA elongation, strongly indicates the existence of a separate elongation system for the final steps in the synthesis of $C_{24}$ acids. As shown in Fig. 1B [data taken from Baumann *et al.* (*2*)], $C_{24}$ acids begin to appear in normal mouse brain only after all three elongating activities have become measurable by enzymatic assay.

The steep rise in the content of $C_{24}$ fatty acids in normal mouse brain 15 to 20 days after birth, which coincides with the active phase of myelination (*2*), fails to occur in the quaking mouse, as shown by analysis of mutant total brain lipids (*2*) (Fig. 1B). Proceeding from the assumption that this