of the artificial sun rather than the bird's position. The technique will be particularly useful for evaluating the bird's ability to compensate the daily and annual variations of Sun's movement, and in investigation of the accuracy of the bird's internal clock. Similar measurements are possible while the bird is in flight in a wind tunnel.

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References and Notes

1. G. Kramer, *J. Ornithol.* **94**, 201 (1953); G. V. T. Matthews, *J. Exp. Biol.* **30**, 243 (1953); C. J. Pennycuick, *ibid.* **37**, 573 (1960).
2. H. E. Adler, *Ergeb. Biol.* **26**, 235 (1963); M. E. Meyer, *J. Comp. Physiol. Psychol.* **58**, 403 (1964); K. Schmidt-Koenig, in *Advances in the Study of Behavior*, D. S. Lehrman, Ed. (Academic Press, New York, 1964), vol. 1, p. 217.
3. D. S. Blough, *J. Exp. Anal. Behav.* **1**, 31 (1958).
4. Supported by PHS fellowship 1-F1-GM-37,712-01, by NIMH grant MHO4453 to P. H. Klopfer, and by ONR grant NONR 1181 and NASA grant NGR-34-001-019 to P. H. Klopfer and K. Schmidt-Koenig, whom I thank for advice.

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Rhodopsin Photoproducts: Effects on Electroretinogram Sensitivity in Isolated Perfused Rat Retina

Abstract. *Isolated perfused retinas of albino rats were exposed to brief saturating flashes of white light which bleached about 50 percent of the rhodopsin present. Transient photoproducts of the reaction could be detected for about 30 minutes. The b-wave threshold increased by some 3 logarithmic units immediately after the flash and remained stable at this level thereafter. This suggests that the longer-lived intermediate products of rhodopsin photolysis do not influence scotopic visual sensitivity.*

It is now well established from studies on the rat retina, which contains primarily rods (*1*), and cone-deficient retinas of humans (*2*) that the logarithm of the scotopic visual sensitivity is linearly related to the concentration of rhodopsin in the rods. In the isolated perfused retina of the frog—which contains both rods and cones—a similar result has been found under conditions in which electroretinogram b-wave thresholds are determined by the rod system alone (*3*). However, these studies were carried out under steady-state conditions, in which presumably only intact rhodopsin or free retinal (retinaldehyde) and opsin were present in the rods.

After a rhodopsin molecule absorbs a photon, it passes through a series of short-lived intermediate states before the carotenoid chromophore hydrolyzes from the opsin (*4–6*). Whether some or all of these photoproducts affect visual sensitivity is not clear. Recently, Donner and Reuter (*7*) have observed that the rate of thermal decay of one photoproduct, metarhodopsin II, parallels an early increase in the sensitivity of ganglion cells in the frog eye cup during dark-adaptation. These authors have suggested, therefore, that the concentration of metarhodopsin II may determine thresholds in the initial stage of rod adaptation.

To investigate directly such possible relationships, we have examined the effects of brief intense light flashes on the isolated retina of the albino rat. Retinas were mounted in a perfusion chamber inside a recording spectrophotometer, making it possible to measure both electroretinogram (ERG) thresholds and rhodopsin absorption spectra in rapid sequence with the same preparation. A previous report (*8*) describes the preparation in detail and shows that the rapid, "neural" phase of dark-adaptation occurs in the viable, isolated rat retina, but that slow, "photochemical" adaptation does not occur because rhodopsin does not regenerate under such conditions in vitro. Thus, after a partial bleach, the absolute sensitivity of the preparation is permanently decreased in proportion to the amount of rhodopsin bleached. There is a linear relation between rhodopsin concentration and the logarithm of ERG sensitivity in the isolated perfused rat retina, similar to the relation between rhodopsin and ERG sensitivity that exists in the intact rat eye (*1, 8*).

We exposed isolated perfused rat retinas to flashes from a xenon phototube (Ultrablitz Meteor SP-GH). This procedure bleaches about 50 percent of the rhodopsin in the retina (*9–11*), leaving it initially in the form of transient photoproducts whose decay we could measure for comparison with b-wave thresholds determined on the same preparation. Since no rhodopsin regenerates in the isolated rat retina under these conditions (*8*), any changes in sensitivity that occur after the neural component of adaptation is completed may be attributed to the decay of photoproducts.

In a typical experiment, threshold b-wave responses (20 μv criterion) and absorption spectra were first recorded from the dark-adapted preparation. After these base-line measurements were made, the retina was exposed to a single white flash from the xenon flash gun. Immediately thereafter, absorption spectra and b-wave thresholds were recorded in alternate succession until no further changes were observed. The retina was then exposed for 10 minutes to a bright white light from a tungsten source, which bleached nearly all the remaining rhodopsin. After this bleach, measurements of threshold and absorption spectra were continued for about 20 minutes.

Figure 1 shows the absorption curves for one experiment. Beginning 45 seconds after the flash, there is a sharp decrease in absorption above 430 nm and an increase in absorption at lower wavelengths. Five minutes after the flash, absorption decreases in the near-ultraviolet, maximally at about 380 nm, and there is a significant increase at longer wavelengths, maximal in the vicinity of 465 to 475 nm. Later there are progressive decreases in absorption at all wavelengths below about 550 nm. This fading continues for about 30 to 50 minutes, after which the tracings stabilize. After the final prolonged bleach, the spectra remain stable for at least 20 minutes, from about 420 to 650 nm. Below 420 nm, sequential spectra often reveal a slight decrease in absorption. This probably represents free retinal being reduced to retinol (vitamin A_1).

The difference spectra of the transient dark processes occurring after a flash (Fig. 1, inset) were obtained by subtracting the stabilized tracings recorded 40 to 60 minutes after the flash (curves 9 to 11) from the tracings recorded 45 seconds after the flash (curve 3), when the near-ultraviolet absorption was maximum, and the curve beginning 5 minutes after the flash (curve 4), when the longer wavelength absorption reached its peak. These spectra reveal the existence of two photoproducts, one absorbing maximally at about 380 nm and the other at about 470 nm. These probably correspond to metarhodopsin II and a later intermediate with maximum absorption, as determined in digi-

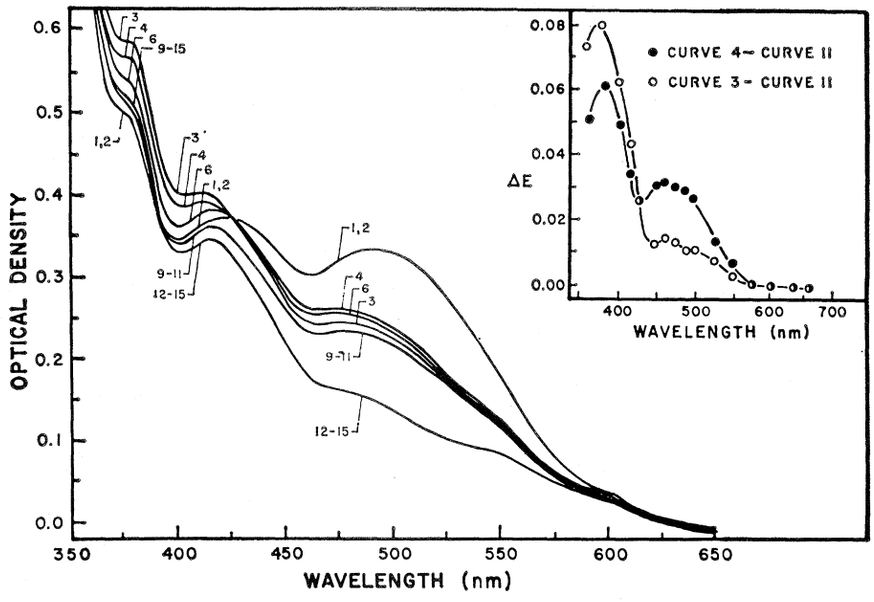

Fig. 1. Sequential absorption spectra from an isolated and perfused albino rat retina after exposure to a single white flash. Curves 1 and 2, dark-adapted controls; curve 3 begins 45 seconds after the flash: curve 4, 5 minutes; curve 6, 15 minutes; curve 9, 40 minutes; curve 11, 60 minutes. The retina was then exposed for 12 minutes to bright tungsten light. Spectra 12 through 15 were recorded at intervals during the 45 minutes after cessation of the bleaching light. For clarity, some spectra were omited when this figure was drawn from the original spectrophotometer tracing. Hence, numbering is not sequential. Spectra were recorder at 2.5 nm/sec. The inset shows the difference spectra for the principal intermediate photoproducts in this experiment.

tonin extracts of cattle rods, at 467 nm (*5, 6, 11, 12*).

Figure 2 shows typical time courses of tne formation and decay of these photoproducts. Absorption at 380 nm is maximum by the time the first spectrum is recorded, and then it decays over the next 30 to 40 minutes. The 465- to 475-nm peak behaves somewhat differently. Absorption at these wavelengths rises rapidly over the first 5 to 10 minutes, and then falls slowly over the next 30 to 50 minutes. It is our impression that the substance which absorbs maximally at 465 to 475 nm decays somewhat more slowly than the substance absorbing maximally at 380 nm, although variations of the time course of fading of these peaks in different experiments make a definite conclusion difficult.

Also shown in Fig. 2 are b-wave thresholds, recorded from the preparation at various times after the flash, for comparison with the photoproduct decay curves. After the flash, the threshold rises by an amount which corresponds to the bleaching of about 50 percent of the rhodopsin present (*1, 8*). In our experiments, the first threshold is recorded 4 minutes and 30 seconds after the flash, by which time the neural phase of adaptation is completed (*1, 8*). From this time until the retina is subjected to a final bleach, the b-wave threshold remains stable despite continued photoproduct decay. When virtually all of the rhodopsin has been bleached, the threshold stabilizes some 6 log units above its initial value in the dark. This total range of sensitivity, and the approximately 3-log-unit rise in threshold seen in most experiments after a 50 percent bleach, are consistent with previous results in the eye of the intact rat and in the isolated rat retina (*1, 8*).

Further experiments confirmed that the slow spectral changes seen after a single white flash are due to the fading

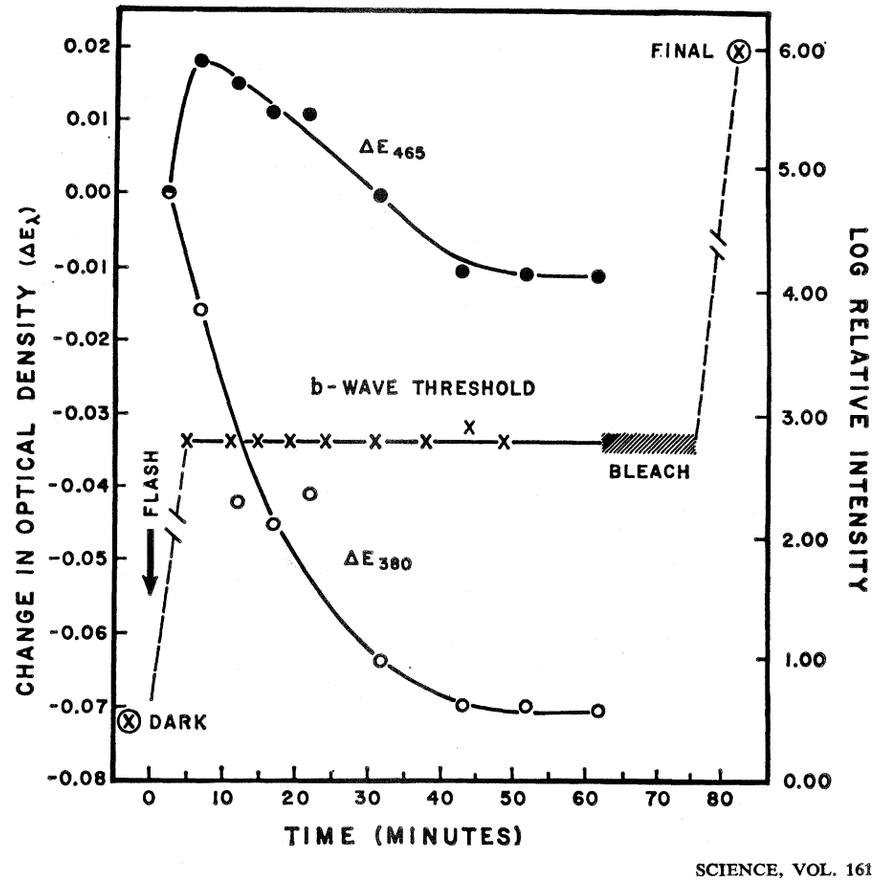

Fig. 2. Time course of photoproduct decay in the dark at 30°C for the experiment shown in Fig. 1. The ordinate on the left indicates changes in optical density relative to the first absorption spectrum recorded after the flash (Fig. 1, curve 3). The line connecting the symbols (X) shows the time course of changes in the b-wave threshold following a flash. Relative light intensity values are shown on a logarithmic scale on the right-hand ordinate. The circled X's at the lower left and upper right indicate light intensities required to produce a criterion b-wave response in the totally dark-adapted state before the bright flash (dark control) and after a 12-minute exposure to a bright tungsten light which bleached nearly all of the rhodopsin present.

of photoproducts. Retinas were exposed first to a saturating white flash and then, at 10-second intervals, to two near-ultraviolet flashes produced by interposing a Corning CS 7-51 filter between the xenon flash gun and the preparation. The photoproduct difference spectra in such experiments showed significantly less fading of the 380-nm photoproduct, whereas the fading of the 465- to 475-nm peak was greater than that in those experiments in which retinas were exposed to a single white flash.

These observations agree with those of Matthews *et al.* (*5*), who showed that a flash of intense near-ultraviolet light rapidly converts metarhodopsin II to a mixture of rhodopsin, isorhodopsin, and the 467-nm intermediate. This procedure differentiates metarhodopsin II from a mixture of free retinal and opsin, which has a similar absorption spectrum but does not undergo rapid spectral shifts immediately after near-ultraviolet irradiation.

Our results are also consistent with recent studies of the early receptor potential (ERP) in the rat eye (*12*). These studies correlated components of the ERP with the presence of metarhodopsins I and II and the 467-nm intermediate, and determined the time courses of formation and decay of these photoproducts. In the rat, metarhodopsin I is formed almost immediately after a saturating flash and decays in a few milliseconds at physiological temperatures. This explains why we did not observe this intermediate in our experiments. On the other hand, at room temperature in the rat or rabbit eye, metarhodopsin II forms with a half-time of 1 msec and decays over several minutes, whereas the 467-nm intermediate forms and decays even more slowly (*11, 12*).

Our results demonstrate that the presence of metarhodopsin II and the 467-nm intermediate after a bleaching exposure does not affect scotopic visual sensitivity in the rat retina. This conclusion differs from that of Donner and Reuter (*7*), who have proposed that metarhodopsin II affects scotopic sensitivity in the frog. Our results suggest that, after the completion of neural adaptation, the rod threshold is simply determined by the fraction of intact rhodopsin in the retina.

It may be argued that neural adaptation is somehow related to photoproduct decay [see, for example, (*7*)], but our results make this suggestion unlikely. Although most of the neural recovery takes place almost immediately upon cessation of the adapting light, it usually continues for some 4 to 5 minutes (*1, 8*). The photoproducts seen in our experiments are still present and decaying long after neural adaptation has ended. On the other hand, earlier photoproducts—prelumirhodopsin, lumirhodopsin, and metarhodopsin I—disappear in the rat retina within milliseconds after the flash at physiological temperatures (*4, 11, 12*), long before neural recovery is complete. In addition, neural adaptation may change electroretinographic thresholds by several logarithmic units even though negligible rhodopsin has been bleached (*1, 8*). In such a situation, the concentration of intermediates must be extremely small, yet neural adaptation can be as extensive as when considerable bleaching has occurred.

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References and Notes

1. J. E. Dowling and G. Wald, *Proc. Nat. Acad. Sci. U.S.* **44**, 648 (1958); J. E. Dowling, *Amer. J. Ophthalmol.* **50**, 875 (1960); *J. Gen. Physiol.* **46**, 1287 (1963).
2. W. A. H. Rushton, *J. Physiol.* **156**, 193 (1961).
3. Ch. Baumann, *Pflügers Arch. Ges. Physiol.* **298**, 70 (1967).
4. G. Wald, *Annu. Rev. Biochem.* **22**, 497 (1953); in *Handbook of Physiology*, H. W. Magoun, Ed. (American Physiological Society, Washington, D.C., 1959), vol. 1, pp. 671–692; R. A. Morton and G. A. J. Pitt, *Fortsch. Chem. Org. Naturst.* **14**, 244 (1957); R. Hubbard, D. Bownds, T. Yoshizawa, *Cold Spring Harbor Symp. Quant. Biol.* **30**, 301 (1965).
5. R. G. Matthews, R. Hubbard, P. K. Brown, G. Wald, *J. Gen. Physiol.* **47**, 215 (1963).
6. S. E. Ostroy, F. Erhardt, E. W. Abrahamson, *Biochim. Biophys. Acta* **112**, 265 (1966).
7. K. O. Donner and T. Reuter, *Vision Res.* **7**, 17 (1967).
8. G. W. Weinstein, R. R. Hobson, J. E. Dowling, *Nature* **215**, 134 (1967).
9. By "bleaching" we mean here that the visual pigment chromophore has been photoisomerized to the all-*trans* form, so that the molecule is rendered unstable and, after passing through a series of intermediate thermal reactions, will hydrolyze to free retinal and opsin. Since most of these intermediates are highly colored, this is not bleaching in the more traditional sense, which implies loss of color. In the sequence of intermediate photoproducts following absorption of a photon, true bleaching does not occur until the stage of metarhodopsin II.
10. J. E. Dowling and R. Hubbard, *Nature* **199**, 972 (1963).
11. W. A. Hagins, *J. Physiol.* **129**, 22P (1955); thesis, University of Cambridge, Cambridge, England (1957); T. P. Williams, *J. Gen. Physiol.* **47**, 679 (1964).
12. R. A. Cone, *Science* **155**, 1128 (1967); W. L. Pak and R. J. Boes, *ibid.* p. 1131.
13. Supported in part by PHS grant NB-05336 to J.E.D., by PHS postdoctoral fellowship 1 F2 NB-36070-01 to R.N.F., and by Research to Prevent Blindness, Inc. Contribution No. 18 from the Augustus C. Long Laboratories of the Alan C. Woods Building, Wilmer Institute.

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Enzymatic Synthesis of Melatonin in Avian Pineal Body: Extraretinal Response to Light

Abstract. *In the chick pineal body, activity of the melatonin-forming enzyme hydroxyindole-O-methyl transferase is greater in the light than in darkness. Neither bilateral enucleation of the eyes nor sympathetic denervation prevented this light-induced elevation of enzyme activity. This fact indicates that in the bird, in contrast to mammals, neither the retinas nor sympathetic innervation of the pineal body are essential for environmental control of melatonin formation.*

Enzymatic synthesis of melatonin by hydroxyindole-*O*-methyl transferase (HIOMT) in the pineal organs of birds is enhanced in the light and suppressed by darkness (*1*). In the rat, on the other hand, pineal activity is greater in the dark than in the light (*2*). Information about lighting appears to reach the pineal body via retinal receptors and sympathetic nerves in the rat (*3*). We here report experiments with birds indicating that this light-influenced pineal HIOMT activity is independent of the eyes and of sympathetic innervation.

Male White Rock chicks were reared from the day of hatching under a 14-hour photoperiod (LD 14:10), given free access to food (chick starter) and water, and provided with heat without light during the brooding period, according to standard rearing practice. Pineal bodies were denervated by removal of both superior cervical sympathetic ganglia within the first 4 days after hatching (*4*). Birds were anesthetized with Combuthal (*5*). Removal of the ganglion could be readily confirmed by the development of permanent ipsilateral ptosis; transitory erection of the head feathers sometimes occurred during the first hours after ganglionectomy (*6*). In some controls both ganglia were exposed but not removed. Both eyes were removed from other chicks (1 to 3 days old). In some cases, the second eye was removed 1 day after the first; in others, both eyes were removed during the same operation. After enucleation, the orbits were packed with gelatin foam, and the eyelids were sutured shut. Survival was good after both operations.

When 4 weeks old, all chicks were transferred to either continuous light (LD 24:0) or constant darkness (LD 0:24) in environment chambers so designed that they could be completely