# KINETICS OF THE PHOTOCURRENT OF RETINAL RODS

R. D. PENN *and* W. A. HAGINS

*From the Laboratory of Physical Biology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014*

ABSTRACT The shapes of the photocurrent responses of rat rods, recorded with microelectrodes from the receptor layer of small pieces of isolated retinas, have been investigated as a function of temperature and of stimulus energy. Between 27 and 37°C the responses to short flashes can be described formally as the output of a chain of at least four linear low-pass filters with time constants in the range 50–100 msec. The output of the filter chain is then distorted by a nonlinear amplitude-limiting process with a hyperbolic saturation characteristic. Flashes producing ~30 photons absorbed per rod yield responses of half-maximal size independently of temperature. The maximum response amplitude is that just sufficient to cancel the dark current. The rate of rise of a response is proportional to flash energy up to the level of $10^5$ photons absorbed per rod, where hyperbolic rate saturation ensues. The responses continue to increase in duration with even more intense flashes until, at the level of $10^7$ photons absorbed per rod, they last longer than 50 min. The time-courses of the photocurrent and of the excitatory disturbance in the rod system are very similar. The stimulus intensity at which amplitude saturation of the photocurrent responses begins is near that where psychophysical "rod saturation" is seen. An analysis of these properties leads to the following conclusions about the mechanism of rod excitation. (*a*) The kinetics of the photocurrent bear no simple relation to the formation or decay of any of the spectroscopic intermediates so far detected during the photolysis of rhodopsin. (*b*) The forms of both the amplitude- and rate-limiting processes are not compatible with organization of rhodopsin into "photoreceptive units" containing more than 300 chromophores. Even at high stimulus intensities most rhodopsin chromophores remain connected to the excitatory apparatus of rods. (*c*) The maximum rate of rise of the photocurrent is too fast to be consistent with the infolded disks of a rod outer segment being attached to the overlying plasma membrane. Most of the disks behave electrically as if isolated within the cell. (*d*) Control of the photocurrent at the outer segment membrane is not achieved by segregation of the charge carriers of the current within the rod disks. Instead, it is likely to depend on control of the plasma membrane permeability by an agent released from the disks.

## INTRODUCTION

In an earlier paper (Hagins et al., 1970) we described a steady electric current (the "dark current") which flows in the interstitial space of the receptor layer of the

retina and enters the rod outer segments in darkness. When the rods are illuminated with a flash of light, the dark current is promptly and transiently reduced. The light-induced change in the dark current (the "photocurrent") and its associated change in interstitial voltage gradient (the "photovoltage") were found to be large enough to account for the visual system's ability to detect single photons absorbed in the rod outer segments. This paper considers the size and shape of the photocurrent responses as functions of intensity and duration of light stimuli. Our aim is to relate the kinetic parameters of the photocurrent to possible cellular mechanisms of rod excitation and to some psychophysical properties of the scotopic visual system.

The findings to be described resemble qualitatively those reported in many previous studies of the a-wave and PIII components of the electroretinogram, responses which have been attributed on indirect grounds to the electrical activity of receptor cells, but accurate kinetic description of the electric currents of rods requires an experimental method which isolates their activity from that of other retinal cells. Therefore, all measurements to be reported here are based upon recordings of voltage differences within the receptor layer of rat retinas by means of Ringer-filled microelectrodes inserted under direct vision by infrared microscopy. This method has previously been shown to isolate the rod responses from those of the retinal neurons (Penn and Hagins, 1969; Hagins et al., 1970).

## METHODS

Dark-adapted rat retinas were isolated and attached by their inner (vitreal) surfaces to Millipore type HA membrane filters (Millipore Corporation, Bedford, Mass.) as previously described (Hagins et al., 1970). A 2 × 4 mm section of retina and filter was placed with the receptor layer uppermost in the shallow chamber shown in Fig. 1. All operations were car-

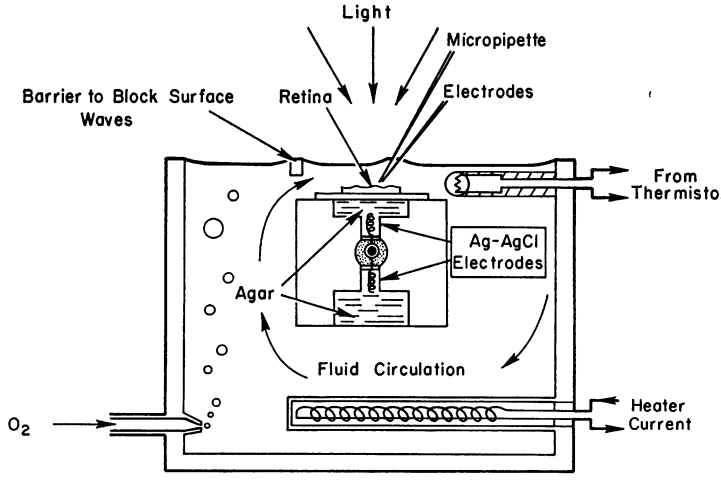


FIGURE 1 Diagram of chamber used to record dark voltage gradients and photovoltage responses of isolated rat retinas.

ried out in deep red or infrared light. The chamber contained a physiological solution (Ringer II, Hagins et al., 1970) maintained at 27–37°C by an electric heater. A rising stream of $O_2$ bubbles at one side of the chamber circulated the fluid past the retina. The fluid velocity was about 100 $\mu$ $sec^{-1}$, 20 $\mu$ above the receptor layer. Two large Ag-AgCl electrodes which were separated from the fluid by agar plugs filled with colloidal carbon grounded the chamber. Radial interstitial voltage gradients in the rod layer were recorded with two glass capillary micropipettes filled with 0.15 M NaCl solution and with tip diameters of 1–2 $\mu$. Each pipette was connected by Ringer-filled fluorocarbon tubing to a gravity-stabilized [saturated KCl-AgCl] | [0.15 M NaCl] junction and then to an Ag-AgCl electrode. The two metal-liquid and liquid-liquid junctions were housed in a heavy aluminum block which stabilized and equalized their temperatures and protected them from light. The DC drift of the voltage difference between the two electrodes was less than 15 $\mu$v $hr^{-1}$. The recording system showed root mean square noise of about 5 $\mu$v over the frequency band 0.1–15 Hz. Its total bandwidth extended from DC to 100 Hz when coupled to the electrodes through capacitance-compensated preamplifiers. For experiments in which fast transient responses of the retina were studied, the microelectrodes were connected to Ag-AgCl electrodes by very short fluid columns which increased the recording system's bandwidth to 0–3000 Hz. The rest of the electronic recording system was as described in Hagins et al., 1970.

An infrared optical system allowed the retina's upper surface to be viewed in profile at $\times$ 100 magnification. The recording electrodes could thus be seen as they entered the receptor layer at an angle of 65° to the long axes of the outer segments. By this means, gross indentation of the tissue could be detected and avoided. Unless otherwise stated, one microelectrode was always placed at the surface of the receptor layer and one at a point radially beneath it at a depth of 50–60 $\mu$. The voltage difference between the two electrodes was therefore a measure of the radial interstitial current in the receptor layer arising from the rods (Hagins et al., 1970) averaged over the interval 0–60 $\mu$.

Stimuli were either steady light from a tungsten lamp or flashes from xenon flashtubes. The light was freed of heat radiation by 1 cm of 6% $CuSO_4$ in $H_2O$, attenuated by neutral density filters, and spectrally shaped by interference filters. In every case, the light was directed downward upon the rod layer as a convergent beam with a half-angle of 20° which uniformly illuminated the entire retinal fragment. Intensities and flash energy densities of the stimuli were measured to an accuracy of $\pm$15% with a calibrated silicon photovoltage detector short-circuited by a wide band operational amplifier. Numbers of photons absorbed by individual rods were calculated from the incident light measurements on the assumptions that (*a*) there are $3 \times 10^7$ rods $cm^{-2}$ of rat retina (Hagins et al., 1970), (*b*) each outer segment has a diameter of 1.7 $\mu$ and a length of 25 $\mu$ (Hagins et al., 1970), (*c*) the absorbance of rhodopsin in the layer of outer segments is 0.01 $\mu^{-1}$ at 500 nm (Hagins, 1957; Liebman and Entine, 1968) and varies with wavelength in accordance with Dartnall's template (Dartnall, 1953) throughout the visible spectrum.

## RESULTS

Short flashes of light cause rod outer segments to produce brief surges of photocurrent whose wave forms vary with stimulus energy density, temperature, and ionic composition of the bathing fluid. The resulting interstitial photovoltage wave forms vary in amplitude but not in shape with electrode position in the rod layer (Hagins et al., 1970). Stimuli of different wavelengths which result in equal numbers of photons being absorbed by rhodopsin produce identical responses; the spectral sensitivity of the responses is that of rhodopsin. The general character of the responses

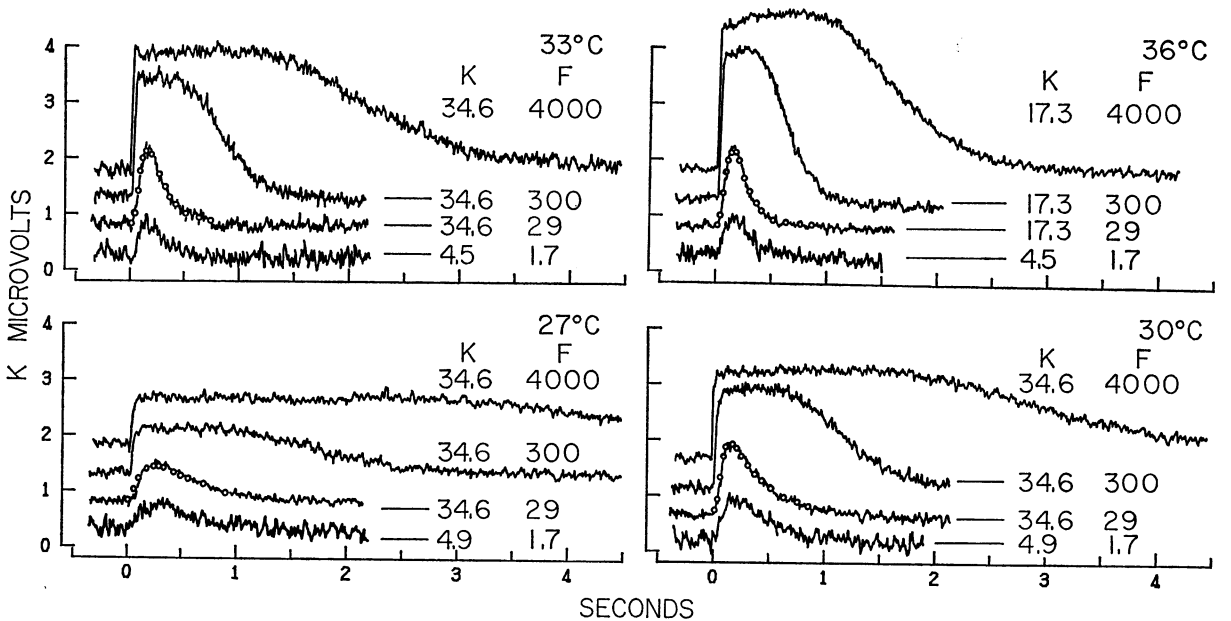


FIGURE 2 Wave forms of photovoltage responses of rat rods to 2-$\mu$sec flashes of green light ($\lambda$ = 560 $\pm$7 mm) at four temperatures and four exposure levels. Electrodes at rod tips (0 $\mu$) and 80 $\mu$ deep in receptor layer. Calibration factors $K$ for response amplitudes to flashes yielding $F$ photons absorbed per rod shown on each curve. Responses to lowest stimulus intensity are averages of four trials summed in a signal averager. Points plotted on responses to flashes of $F$ = 29 photons absorbed per rod are calculated from output of a four-section RC low-pass filter preceding a hyperbolic amplitude limiter (see model of Fig. 5). Filter time constants adjusted in groups of 2 and 2 to give a best fit.

for a range of flash energies and four temperatures are shown in Fig. 2 for a retina in Ringer II. The sign of the membrane photocurrent transient was always outward in the outer segments and inward elsewhere (Hagins et al., 1970). Since the responses were stable and very consistent in shape in this medium, it was used in all experiments to be described in this paper. At each temperature the responses varied in the same general way as the flash exposures were increased from low to high values.

*Kinetics at Low Flash Intensities*

Below 25 photons absorbed per outer segment, each response was a smooth curve which rose to a single rounded maximum and declined asymptotically to zero. The response amplitudes were proportional to flash intensity but their shapes were otherwise unaffected by stimulus energy. Thus the input-output relation at low intensities was linear, and it was of interest to see if the response wave forms could be represented as the output of some linear electric filter network driven by a brief input pulse, since such representations are useful for quantitatively describing the response kinetics and for testing possible models of excitation. It was immediately found that the wave forms, like those of Limulus photoreceptors (Fuortes and Hodgkin, 1964), could be approximated by the output of a chain of low-pass resistance-capacitance (RC) filters. In particular, a wave form consisting of four decaying exponential functions with time constants $\tau_i$ (each exponential representing the impulse response of a single RC filter stage) can be made to resemble a flash response by adjusting properly the $\tau$'s and an amplitude scale factor $L$. It is not necessary for the $\tau$'s to be independently adjustable. Instead, they can be grouped in two pairs, with distinct values

$$\tau_1 = \tau_2 = \tau_A ,$$

and

$$\tau_3 = \tau_4 = \tau_B ,$$

for purposes of curve fitting. Thus only three adjustable parameters, $\tau_A$ , $\tau_B$ , and $L$, are needed to obtain good fits to observed flash wave forms.

In mathematical notation (see also Fig. 5),

$$Y(t) = Lf(t) * E_2(t/\tau_A) * E_2(t/\tau_B), \qquad (1)$$

where $f(t)$ is the instantaneous rate of photon absorption (photons absorbed per rod per second), $E_n(t/\tau_i)$ is the $n$-fold convolution of $[H(t)/\tau_i] \exp(-t/\tau_i)$ with itself, the $\tau_i$'s are arbitrarily adjusted time constants,

$$\begin{aligned} H(t) &= 0, \quad t \leq 0, \\ &= 1, \quad t > 0, \end{aligned} \qquad \text{(Heaviside step function)},$$

and the asterisks denote the normalized convolution operation, i.e.,

$$U(t) * E_1(t) = \frac{1}{\tau_1} \int_0^t U(t - \xi) \exp(\xi/\tau_1)\, d\xi. \quad (2)$$

Now, $L$ is a proportionality constant with the dimensions volt seconds per absorbed photon which represents the total electrical action of an absorbed photon. Thus,

$$L = \int_0^\infty V(t)\, dt \Big/ \int_0^\infty f(t)\, dt = \frac{1}{F} \int_0^\infty V(t)\, dt. \quad (3)$$

For the 1 $\mu$sec flashes used, $f(t)$ can be considered to be an impulse function causing $F$ photons to be absorbed per rod per flash.

The fit of the functions to the wave forms can be seen from the calculated points plotted on the low intensity responses of Fig. 2. Values of $\tau_A$, $\tau_B$, and the first moment $\tau_s(=2\tau_A + 2\tau_B)$ derived from least squares curve fitting by program SAAM 25 (Berman and Weiss, 1967) are given in Table I. Of course, the structure of the filter is not unique. The order of the filter sections can be permuted and $\tau_A$ can be shortened moderately if $\tau_B$ is correspondingly lengthened without a significant decrease in the closeness of the fit. Thus it is not surprising that the individual values of the $\tau$'s do not decrease monotonically as the temperature is increased. Moreover, 2–3 additional filter stages with time constants less than 0.1 $\tau_A$ can be added to the model with little effect (see below). The RC model is thus only a useful interpolation-extrapolation formula for empirically describing the low energy wave forms quantitatively in the time span 0.01–5 sec and for computing the mean delay $\tau_s$ of rod responses. The values of $\tau_s$ actually obtained, however, are almost independent of the physical structure of the model if the fit of curves to observed responses is close.

Estimates of $\tau_s$ are shown in the Arrhenius plot of Fig. 3 for two retinal blocks stimulated with flashes producing 20–30 photons absorbed per rod. $1/\tau_s$ increases

TABLE I

FILTER TIME CONSTANTS FOR LOW INTENSITY FLASH RESPONSES OF RAT RODS

| Temperature | $\tau_A \pm$SE | $\tau_B \pm$SE | $\tau_s = 2(\tau_A + \tau_B)$ |
|---|---|---|---|
| °C | *msec* | *msec* | *msec* |
| 27 | 20.1 ±1.7 | 228.0 ±6.7 | 497 |
| 30 | 18.1 ±2.2 | 157.0 ±5.2 | 350 |
| 33 | 35.2 ±3.7 | 89.3 ±5.4 | 249 |
| 36 | 30.5 ±3.3 | 71.2 ±4.9 | 203 |

* Data were obtained on 6 February 1969 and were derived from least squares curve fitting by program SAAM 25 (Berman and Weiss, 1967).

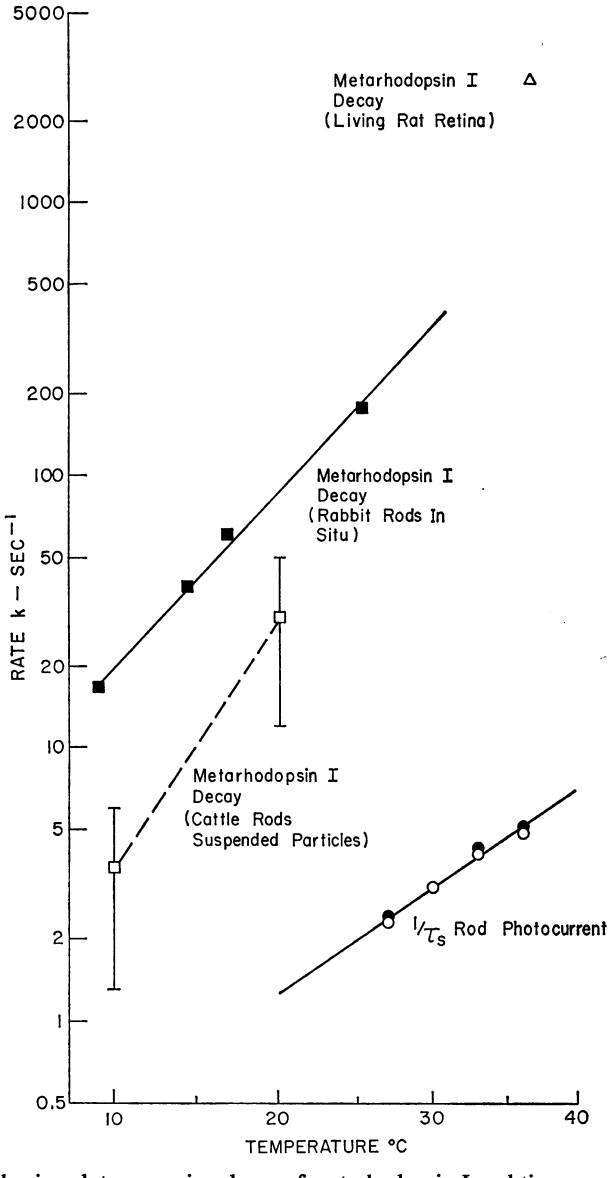


FIGURE 3 Arrhenius plot comparing decay of metarhodopsin I and time-course of photocurrent of rat rods. ○, ●, first moments $\tau_s$ of photocurrent responses for two retinas; ■, rate constants for exponential decay of "intermediate A" (metarhodopsin I) for rabbit rods *in situ* from Hagins (1957); □, decay of metarhodopsin 478 in digitonin solutions of cattle rhodopsin and rod particles in suspension (Abrahamson and Ostroy, 1967). Vertical bars indicate range of exponential time constants into which the observed nonexponential decay curves were resolved. △, observed decay of metarhodopsin I in living rat rods (Hagins and Ruppel, manuscript in preparation).

with an apparent heat of activation of 15.8 ±1.9 (SE) kcal mole$^{-1}$. For comparison with rhodopsin photochemistry, rates of interconversion of mammalian rhodopsin's short-lived intermediates, metarhodopsin 485 and retinene-protein complex 380 ("metarhodopsin 380"), are also shown (Hagins, 1956, 1957; Abrahamson and Ostroy, 1967; see also Cone and Cobbs, 1969). The discrepancies in absolute rate constants and in apparent heats of activation between the photochemical and electrical rate constants are too large to be reconciled by any known change in the parameters of equation 1; the delays $\tau_A$ and particularly $\tau_B$ must be due to processes other than the thermal interconversion of any of rhodopsin's spectrally identified intermediates. Thus the thermal reactions in photolysis of rhodopsin known at present cannot, by themselves, account for the kinetic delays in wave form of the rod photocurrent.

*The Amplitude-Energy Relation*

At flash energies above 30 photons absorbed per rod, the peak amplitudes of the responses become less than that calculated from simple proportionality. Finally the waves become flat topped at about 200 photons/rod. Further increases in stimulus intensity now increase the rate of rise of the photovoltage and prolong its duration, but the amplitude shows little further increase. The limiting amplitude varied from 50 to 300 $\mu$v in a series of 35 experiments, but in each preparation the actual value was closely related to the size of the dark voltage just preceding a test flash. The ratio $\eta$ of the peak amplitude of a response to a flash causing >200 photons to be absorbed per rod to the dark voltage was − 0.93 ±0.04 (SE) with a range of 0.70–1.22. That is, the dark voltage (due to the flow of the dark current) was almost completely abolished at the peak of the photovoltage response. Thus, in the flat retinal blocks used in these experiments, in which uniform axial illumination of the rods was possible, significantly higher values of $\eta$ were found than in the tangentially illuminated slices of Hagins et al. (1970). In slices, $\eta$ usually does not exceed 0.75 with test flashes of 200 photons absorbed per rod. The difference between the two results is not fundamental; it stems from the greater uniformity of illumination which can be realized in flat retinal blocks. The important point is that the limiting response amplitude of the photovoltage is that which cancels the dark voltage almost completely.

Logarithmic plots of the peak amplitudes $A$ of the photocurrent (measured at 0.2 sec after the flash) are plotted vs. flash energy density in Fig. 4 for four preparations at 33 and 37°C. Each curve closely fits a relation of the form

$$A = \frac{F}{F + F_1} \times \text{const}, \qquad (4)$$

where $F_1$ is the absorbed stimulus which produces a response of half-maximal size. Although the saturating amplitudes vary from curve to curve, the half-saturating energies $F_1$ are all about 30–50 photons absorbed per rod.

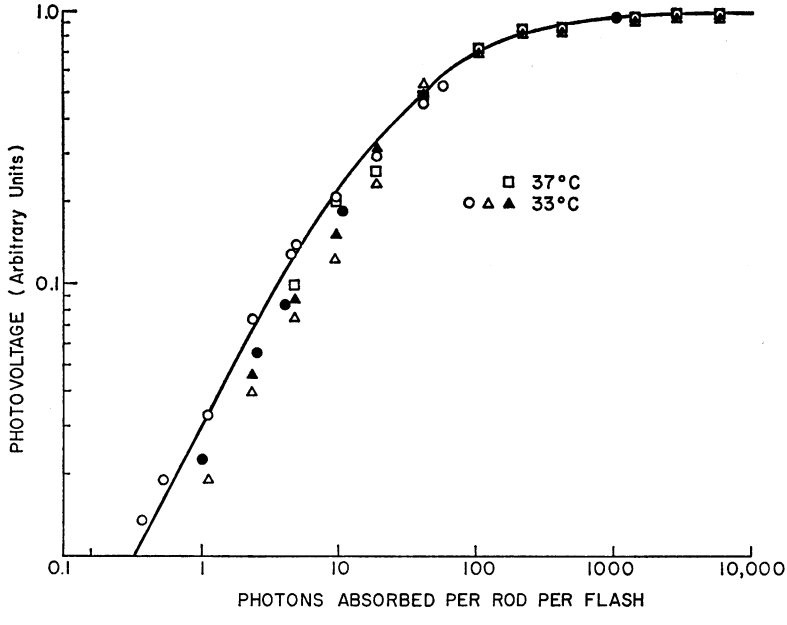


FIGURE 4 Amplitude of photovoltage response vs. photons absorbed from flash. Response amplitudes measured 0.2 sec after flashes of intensities shown. Curve: plot of $A = F/(F+F_1)$, where $F_1$ is the flash exposure which yields a response whose size is half-maximal. $F_1$ is ~35 photons absorbed per rod per flash. Data from three retinas at 33°C and one at 37°C.

Relation 4 suggests that the photocurrent responses be represented at high intensities by the output of a device with a hyperbolic amplitude-limiting characteristic, driven by the four-section low-pass filter previously introduced (Fig. 5). That is, the output response $A(t)$ would be given by

$$A(t) = K_1 \frac{Y(t)}{Y(t) + Y_1}, \qquad (5)$$

where $Y(t)$ is given by equation 1 and $Y_1$ is the value of $Y$ at which $A = K_1/2$.

This simple model fails in several instructive ways which will be considered later, but it is quite sufficient to account for the effect of a steady background $B$ (photons absorbed per rod per second) on the photocurrent produced by a superimposed test flash. If the retina is illuminated by a test flash of energy $F$ photons absorbed per rod and the peak amplitude of the response $v(t)$ is plotted vs. $B$, Fig. 6 is the result. Logarithmic scales are used for both axes. The points are averages of four values of $v$ measured 0.2 sec after a test flash on a steady background turned on 2 sec before.

Curve A is the solution of equations 3 and 5 for steady background with superimposed test flashes. The background intensity $B_1$ which reduces the incremental gain by twofold is about 350 ±80 (SE) photons absorbed per rod per second, but since a single flash of energy $F_1$ yielding 30 photons absorbed per rod yields a half-

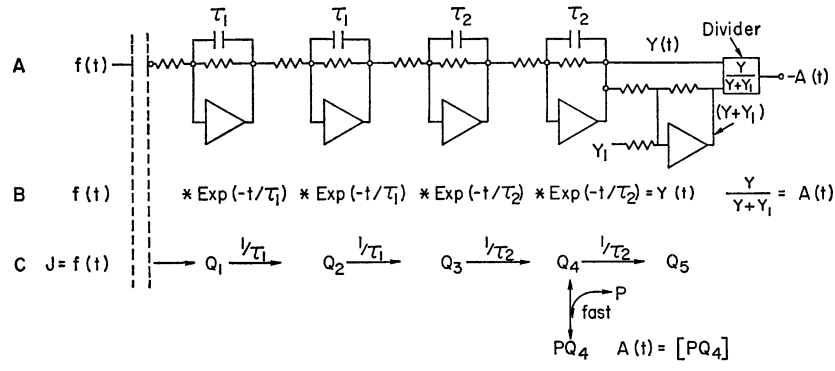


FIGURE 5 Formal model to generate output $A(t)$ which simulates photocurrent responses of retinal rods. (A) Formulation in analogue computer symbolism. (B) Mathematical equivalent (equations 3 and 5). (C) Formulation as a chemical system. $f(t)$, input light intensity; $J$, input chemical flux; $Q_i$, quantity of $i$th chemical intermediate; $P$, a combining site which reacts reversibly with $Q_4$; $\tau_i$, time constant for a first-order decay process.

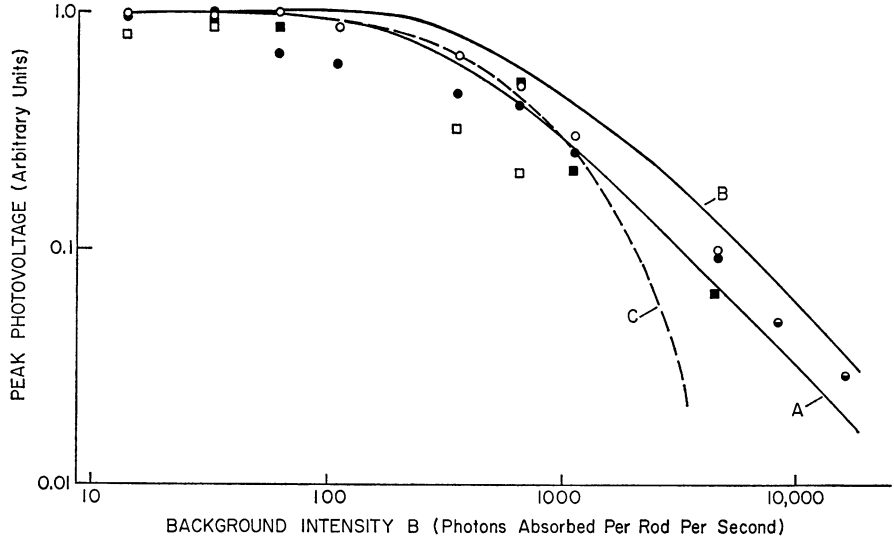


FIGURE 6 Incremental gain of the photocurrent-generating mechanism of rat rods vs. intensity of a steady background light. Gain tested with 1 μsec flashes of energy equivalent to ~20 photons absorbed per rod per flash. Background energy measured in units of photons absorbed per rod per second. Wavelength, 560 ±7 nm for both flashes and background. Data from four retinas at 33°C are shown. Curve A, hyperbolic saturation with half-saturating intensity of ~400 photons absorbed per rod per second. This curve fits the observations best. Curve B, gain vs. background curve derived from observations of sensitivity of human scotopic visual mechanism (Aguilar and Stiles, 1954). Curve C, rod sensitivity vs background predicted for photopigment molecules grouped in photoreceptive units of ~50,000 chromophores each. See Discussion.

maximal response when the background is absent, the ratio $\theta$ ($=F_1/B_1$) is a measure of the duration of the effect of an absorbed photon. For this group of experiments $\theta \cong 0.1$ sec, a value which agrees well with the half width $W$ of a flash response measured directly.

Curve B is the gain of the scotopic visual system of the human eye derived from the measurements of Agüilar and Stiles (1954). Both the psychophysical and the electrical results indicate that rod saturation should lower the incremental gain of the rod system in the presence of background intensities equivalent to a few hundred photons absorbed per rod per second. The significance of curve C will be considered in the Discussion.

*The Duration of Responses to Bright Flashes*

Although the photovoltage responses to bright flashes are limited in amplitude, they last longer and longer as the flash energy is increased. The model of Fig. 5 predicts such an effect, but the quantitative agreement with observation is poor. Equations 3 and 5 yield voltage transients which all die away ultimately with exponential time-courses whose time constants are given by the larger of $\tau_A$ or $\tau_B$ ; but the flash responses of Fig. 2 show progressively slower and slower decays as the flash energies increase from 1000 to 5000 $h\nu$/rod. A concise way to show this is to compare the areas under the responses with flashes of various light intensities. This is done in Fig. 7. The points are obtained from two retinas at 33°C while the curve represents the model of Fig. 5. The straight line indicates the charge flow to be expected if each absorbed photon caused a transient photocurrent whose total charge flow was $\sim 9 \times 10^5$ electronic charges. The model of Fig. 5 predicts responses whose areas ultimately increase logarithmically with flash exposure $F$, while the observations increase in area somewhat more rapidly. Thus, in the formal sense, the rods behave as if the decay process is slower after flash exposures exceeding 1000 $h\nu$/rod than with weaker stimuli. The integrated electrical effect of an absorbed photon at high flash energies, however, is not conserved. If the photons absorbed from bright flashes were as effective electrically as those from dim flashes, the response areas would follow the straight line in Fig. 7. Clearly the total charge flow in the photocurrent response to a bright flash is less per absorbed photon than it is for dimmer flashes.

The recovery process after a bright flash contains still a further complication not shown in Figs. 2 and 6. If a retina is stimulated by a regular sequence of test flashes each yielding 20 photons absorbed per rod, a regular sequence of responses of uniform size and shape results (Fig. 8 A). If a flash yielding 30,000 photons absorbed per rod is now delivered in the midst of the test flashes, a large flat-topped response of saturating amplitude occurs. During this response, the test flashes produce deflection too small to see. As the large response declines, however, the test responses reappear and grow to their former height, but the large response declines more rapidly than the test responses grow. The delay in recovery of responsiveness is

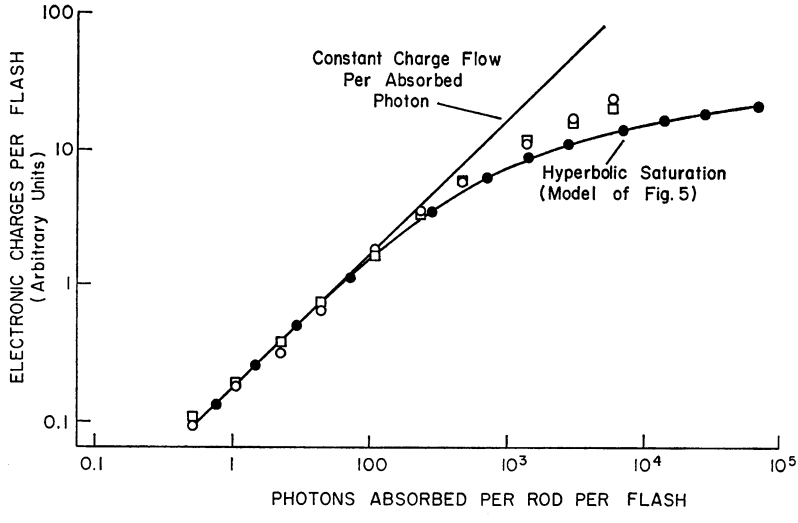


FIGURE 7 Total charge flow in photocurrent responses to flashes of various intensities. Ordinate scale in arbitrary units. For the two retinas shown the time integrals of the responses were $\sim 9 \times 10^5$ (circles) and $1.1 \times 10^6$ (squares) electric charges per absorbed photon. For comparison of the two preparations, the data for the preparation with the greater current gain (squares) were shifted downward to coincide with the circles. Curve, charge flow predicted for the amplitude-limited model of Fig. 5 with $F_1 = 30$ photons absorbed per rod per flash. Total charge flows in the responses were calculated from the areas under the flash responses in volt seconds, corrected for the average transfer resistance of 30 MΩ for rat rods with electrodes across receptor layer (Hagins et al., 1970).

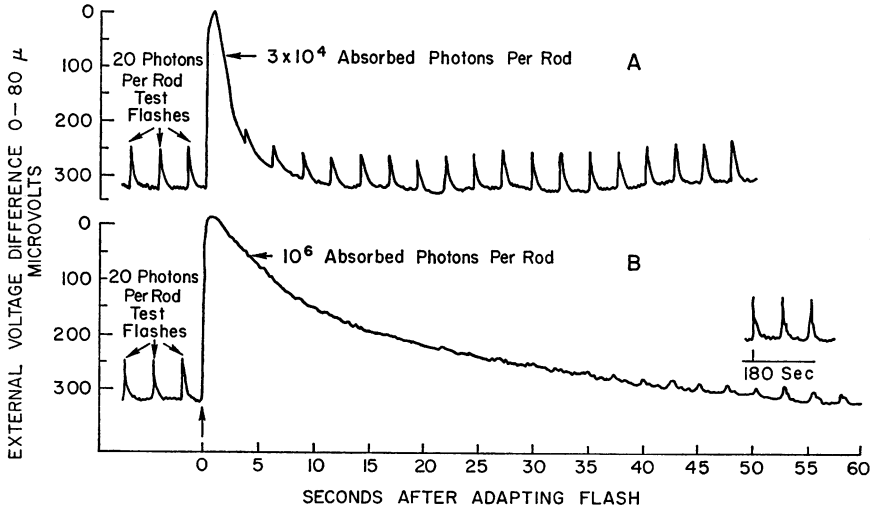


FIGURE 8 Effects of adapting flashes on rod responses to test flashes. Both test and adapting flashes of wavelength 560 ±7 nm. Test flash duration, 1 μsec; adapting flash, 80 μsec. Dark voltage gradient reduced to ~0 at peak of responses to adapting flashes. Recovery of responses to test flashes occurs more slowly than return of dark voltage gradient to its normal value.

even more obvious after a flash from which each rod absorbs about a million photons (Fig. 8 B). This behavior was observed consistently in retinas in Ringer II at 33°C. Clearly the amplitude of a response to a test flash cannot be predicted simply from the size of any preexisting photovoltage due to an earlier adapting flash. This result conflicts with the predictions of equations 3 and 5, in which the output of the sequence of low-pass filters is the linear superposition of its inputs. In the model this output is then flattened by the hyperbolic saturation process (equation 5) without distinction between prolonged effects of a bright flash absorbed in the past on one hand, and the immediate effect of a weak test flash on the other. For such a simple model, the gain at any time depends only upon the output of the nonlinear element at that instant. Fig. 8 shows that this rule is not followed by rods.

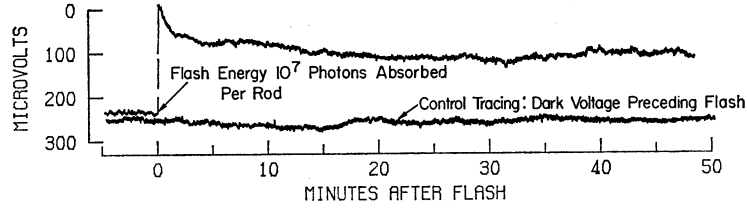


FIGURE 9 Effect of intense flash on voltage gradient in rod layer of rat retina. Electrodes at 0 and 80 $\mu$. Flash produced $\sim 10^7$ photons absorbed per rod (560 $\pm$7 nm). Flash duration, 80 $\mu$sec.

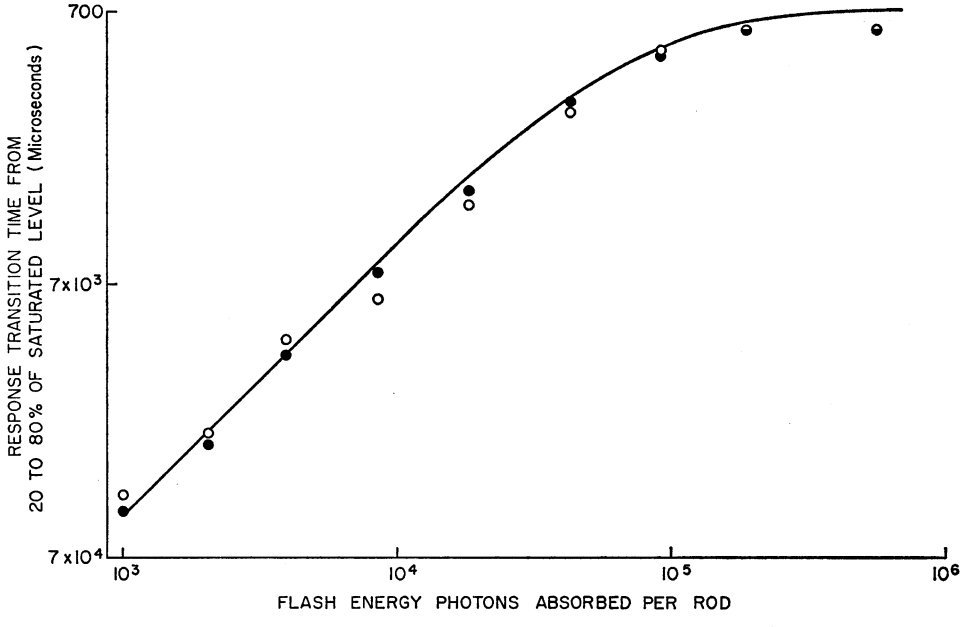


FIGURE 10 Time required for flash response to rise from 20 to 80% of its final (saturated) level vs. flash intensity. All flashes of duration 80 $\mu$sec and wavelength 560 $\pm$7 nm. Points, measurements from two retinas at 33°C. Curve, hyperbolic saturation relation of the form of equation 4. Electrodes at rod tips and 80 $\mu$ depth in rod layer.

Since bright flashes yield long responses, is there an upper limit to the length? Fig. 9 shows that if there is such a limit, it exceeds 50 min in Ringer II. The response shown was produced by a flash causing about $2 \times 10^7$ $h\nu$ to be absorbed per rod, thus activating ~70% of its rhodopsin chromophores and bleaching about 35% (Hagins, 1957; Williams, 1964). Many such long responses were obtained, all of which consisted of immediate total suppression of the dark current followed by return of about 10–20% during the first 5 min after the flash. During the entire 50

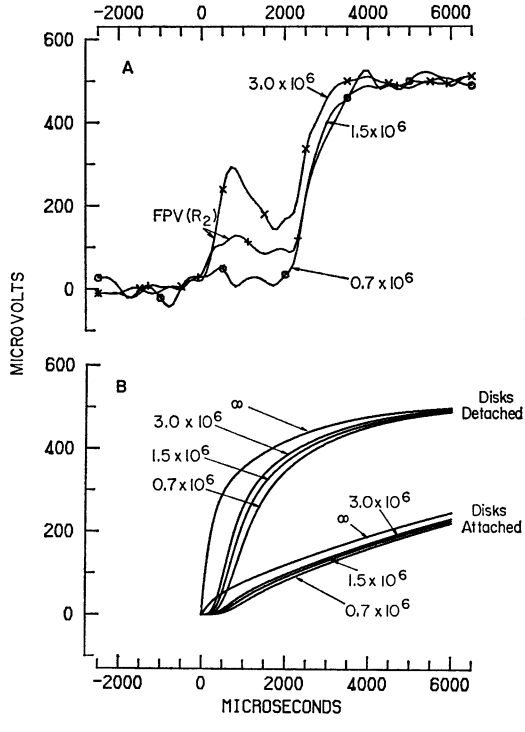


FIGURE 11 Rate saturation. (A) Responses of three retinas to intense 80 $\mu$sec flashes of wavelengths 560 ±7 nm. Each curve was normalized to a standard height of 500 $\mu$v. The actual amplitudes varied from 300 to 600 $\mu$v from retina to retina, without relation to flash intensity. Each response rose asymptotically to a level at which the dark voltage gradient vanished. Flash energies marked on each curve. Although the three flash intensities were in the ratio 1:2:4, the 20–80% rise times are virtually identical. The $R_2$ waves of the fast photovoltage can be seen at the higher flash energies. Electrodes at 0 and 60 $\mu$ depths in rod layer. (B) Computed external voltage transients for rods with cable properties shown in Table I. Resistance of outer segment envelope for charge carriers of dark current assumed to increase in proportion to $V(t)$ of equation 1. Curves computed for flash energies shown on each curve. Two sets of solutions are plotted. The upper group is for the case of rod disks isolated from the plasma membrane. The lower group is for disks attached to the envelope and contributing to its capacitance. Both groups ultimately rise to a final asymptote of 500 $\mu$v. Only the upper curves match those of A. The initial delays in the curves for flashes of finite intensity are due to the four-stage filter proposed to explain the response shapes of Fig. 2. Clearly the observed curves have an initial delay which is threefold larger.

min, no additional responses could be produced by additional flashes, however bright. Thus the prolonged photocurrent was accompanied by prolonged reduction of the rod sensitivity to zero. The two phenomena, however, are experimentally distinct. Both could be important in the phenomenon of dark and light adaptation.

### *Saturation of the Rate of Rise*

As the flash exposures increase, the flash responses of Fig. 2 show steadily increasing rates of rise at short times, but there is an exposure value beyond which they all rise to the saturating level at the same rate. The transition from rate proportionality to rate saturation occurs at about $10^5$ photons absorbed per rod per flash (Fig. 10). The ordinate shows the time required for the photocurrent to rise from 20 to 80% of its final saturated level. Each curve was obtained from a different retinal fragment. The responses, which were all in the range 300–600 $\mu$v in size, were reduced to the same nominal amplitude of 500 $\mu$v for plotting. Actual wave forms of the responses are shown in Fig. 11 A for exposures of 0.69, 1.48, and $3.0 \times 10^6$ $h\nu$ absorbed per rod. At the higher of the two exposures an early peak produced by a different kind of electrical phenomenon, the $R_2$ component of the fast photovoltage (FPV) or "early receptor potential" can be seen. This fast early response, whose properties are considered elsewhere (Cone and Cobbs, 1969; Hagins and Rüppel, 1971; Hagins, Rüppel, and Yoshikami, manuscript in preparation), serves to show that the limiting rate of rise is not due to limitations in the amplifying system. Rate saturation was observed in all preparations in which it was looked for, and the limiting rate was always such that the transition from 20 to 80% of final saturating level required about 0.7 msec at 33°C whatever the original value of the dark voltage preceding the flash. Fig. 11 A also shows that the rise of the response is preceded by a delay of about 2 msec. Its origin is considered in the Discussion together with the theoretical curves of Fig. 11 B.

## DISCUSSION

### *Mechanisms of Amplitude Saturation*

Several properties of the photocurrent allow useful deductions about the action of light in rod outer segments. The results shown in Fig. 2, in particular, suggest that the processes which shape the time-course of responses to dim test flashes occur earlier in the excitatory sequence than the process or processes responsible for amplitude saturation. If the nonlinear process were placed earlier than the low-pass filters of Fig. 5, amplitude saturation would still occur, but the responses to both bright and dim flashes would be of about the same shape and would differ only in amplitude. This simple fact is important because it forms a powerful argument against the concept of photoreceptive units as the cause of saturation in the rod system.

Photoreceptive units can be defined as groups of rhodopsin chromophores within which a single absorbed photon produces no larger output than multiple hits. Such units, which would be the analogue of photosynthetic units responsible for saturation effects in chloroplasts, were originally proposed by Wald (1954) to account for the great light-adapting effect of flashes which bleach a relatively small proportion of the rhodopsin in a region of the human retina. Rushton (1963) has pointed out that photoreceptive units provide an unsatisfactory explanation of the threshold-elevating effect of bleached rhodopsin in rods. For if the number of chromophores $N$ in a unit is adjusted so as to predict correctly the adapting effect of dim flashes, the effect of bright flashes is greatly overestimated.

An argument similar to Rushton's can be applied to the present experiments. To explain amplitude saturation in rat rods with a half-saturating stimulus level of 30 photons absorbed per rod would require that each outer segment contain only about 60 photoreceptive units, each with about $5 \times 10^5$ chromophores in it. This is unreasonable on structural grounds: a rod disk in rats contains no more than about $4 \times 10^4$ chromophores (unpublished measurements). Moreover, if photoreceptive units were as large as $5 \times 10^5$ chromophores, the form of the gain vs. background curve (Fig. 6, curve C) would be quite incompatible with observation. If an ensemble of units absorbs an average of $M$ photons each during a summation time of 0.1 sec, the fraction $P$ of units which remain unhit is

$$P = \exp(-M).$$

Thus if 500 photons were absorbed per summation time in a rod whose rhodopsin was organized into only 60 units, its threshold would be elevated by $\exp(500/60) \cong 4000$. In fact, Fig. 6 shows only a $\sim$15-fold reduction in gain with such background exposures.

An industrious model builder can modify the hypothesis of photoreceptive units to bring it into closer harmony with the [flash energy]-[peak photovoltage] relation by introducing distributions of unit sizes and special relations for the kinetics of regeneration of bleached chromophores, but the central feature of the unit hypothesis is that a unit, once hit, has all of its remaining chromophores disconnected from the excitatory apparatus until complete resynthesis has occurred. Clearly this is not so for rat rods. Flashes yielding 1000–10,000 photons absorbed per rod produce photocurrents whose amplitudes are maximal but whose rise times to the plateau continue to decrease as the stimulus flashes are made more energetic. The additional absorbed photons are as effective in controlling the photocurrent as are those of dimmer flashes.

Then what physical process is responsible for the hyperbolic input-output relation for rods? There are many formal mechanisms, such as saturation of binding sites by photochemically produced substances, which can be made to produce a suitable nonlinearity (see Fig. 5), but an attractive alternative is a mechanism involv-

ing ionic permeability changes. Suppose that the dark current can be represented as the discharge of some electrochemical electromotive force (E.M.F.) through some series and parallel combination of membrane conductances and electrolytic resistances of cytoplasm and extracellular fluid. Now, if the value of any one light-sensitive conductance $g$ through which the dark current flows is proportional to the output $Y$ of the low-pass filters of Fig. 5, a hyperbolic input-output relation will result for the output $A(t)$. A hyperbolic relation also is found if $r(= 1/g)$ varies linearly with $Y$! If one now adds the condition that $r$ (or $g$) must vary in such a way that the dark current decreases as $Y$ increases, the desired amplitude-intensity relation falls out.

Obviously the hyperbolic forms of equations 4 and 5, in themselves, tell little about which conductance is changed by light. But microelectrodes located internally in cones show small increases in resistance during the light response (Bortoff and Norton, 1967; Toyoda et al., 1969; Baylor and Fuortes, 1970). From this Tomita (1970) argues that the membrane conductance of the outer segment decreases in light, and since the $Na^+$ conductance would control an inwardly flowing dark current, a simple explanation for the photocurrent as a reduction in the $Na^+$ influx results. The present work supports the idea since the maximal photocurrent just cancels the dark current, and it is a useful working hypothesis which is supported by ionic substitution experiments (Sillman et al., 1969; Yoshikami and Hagins, 1970, and manuscript in preparation). More conclusive tests of it will be reported elsewhere.

*Rate Saturation*

The onset of rate saturation at stimulus levels of $>10^5$ photons/rod per flash suggests that a second form of nonlinearity in the rod response exists. At any time $t$ after flash, the $\dot{v}$ $(= \partial v/\partial t)$ vs. $F$ curve is hyperbolic like that of $v$ vs. $F$, so that, by a repetition of the previous argument, saturation due to any simple organization of the chromophores into units of, say, 300 chromophores each, is an unsatisfactory explanation for rate saturation, but if one accepts the hypothesis that the photocurrent is a light-induced suppression of the dark current resulting from a decrease in the $Na^+$ conductance of the outer segment's plasma membrane (Yoshikami and Hagins, 1970; Tomita, 1970), a simple explanation is possible.

Consider the electrical analogue of a retinal rod shown in Fig. 12. Let the dark current be represented as a differential flow of membrane current between inner and outer segments (Hagins et al., 1970). Despite its probable origin in ionic batteries and conductances, linear network theory allows $I_D$ to be represented as originating from current generators in parallel with the membrane of inner or outer segment or both. Now if a flash of light were to set in motion a process which suppressed $g_{Na}$ in the outer segment, even instantaneously, the external voltage gradient produced by $I_D$ would fall from its initial value to zero, not instantaneously, but with a time-course

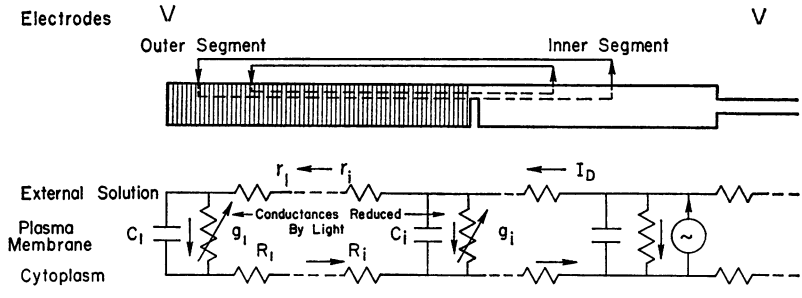


FIGURE 12 Electrical model of a retinal rod used to explain rate saturation in the photocurrent response.

which depends upon the speed with which the charge in the membrane capacitance along the cable adjusts from a distribution characteristic of a low membrane potential in the outer segment (with $I_D$ on) to a new distribution appropriate to a higher membrane potential (when $I_D$ has been turned off). For a rod of given length with electrodes at given positions, the time-course of the redistribution depends upon the sum $R$ of longitudinal cytoplasmic and external fluid resistances (ohms per centimeter) and upon the membrane capacitance (farads per centimeter), but the rise time of the transient is independent of the size of the initial $I_D$ and is only weakly influenced by the initial membrane conductance as long as the membrane time constant $v$ is larger than 500 $\mu$sec. Values of external voltage transients derived from solutions of the cable equation for the model of Fig. 12 (Hagins, Rüppel, and Yoshikami, manuscript in preparation) are plotted in Fig. 11 B for the membrane parameters of retinal rods given in Hagins and Rüppel (1971). All membranes are treated as having a specific capacitance of 1 $\mu$F cm$^{-2}$ (Cole, 1968) and time constants of 1 msec. The computed external voltage differences between an electrode at the rod tips and one 60 $\mu$ deep in the receptor layer are plotted for two variants of the model. In the lower group of curves, the rod disks are assumed to be attached to the envelope membrane and to contribute to its capacitance in proportion to their area. In the upper group, the envelope is assumed to be smooth and free of infolded disks. In both groups, the distorting effects of the low-pass filters of Fig. 5 are included to show how little they affect the 20–80% rise times. The filters produce initial delay, however, which is seen also in the observed curves. The observed delays are greater than the computed ones because the model of Fig. 5 has too few RC sections. At least eight are required for a good fit, but a detailed treatment of this point is outside the scope of this work.

The results in Fig. 11 B establish an approximate relation between the 20–80% rise time $T_r$ (microseconds) of the flash response of a retina with electrodes at 0 and 60 $\mu$ depths in the receptor layer on one hand, and the membrane capacitance $C_{os}$ (microfarads per square centimeter) of the outer segment envelope and cytoplasmic resistivity $\rho$ (ohm centimeters) on the other. Measurements on computed

solutions of the cable model of Fig. 12 yield the equation

$$T_r \approx 3.3\, \rho C_{os}\ \mu\text{sec}, \tag{6}$$

for a rod with outer and inner segment diameters of 1.7 $\mu$ and the outer segment 90% obstructed by the disks (Hagins et al., 1970). If $\rho$ is assumed to be 210 $\Omega$ cm (i.e., 3 times that of Ringer II), the match between the curves of Figs. 11 A and 11 B (disks attached) indicate that $C_{os}$ is about 1 $\mu$F cm$^{-2}$. Thus the envelope apparently has few infolded disks, for these would increase the apparent membrane area (and $C_{os}$) by 20 to 30-fold. If $C_{os}$ were actually 20 $\mu$F cm$^{-2}$, rise times as short as those of Fig. 11 A would require that $\rho$ be less than 20 $\Omega$ cm, a value comparable with seawater and much too small for mammalian cells.

Can it be that the rod disks are attached to the envelope membrane through a high series access resistance much like that of the sarcoplasmic reticulum of vertebrate striated muscle? If so, the access resistance must produce a time constant for charge equalization between disk and envelope which is much longer than 1 msec. Given that the capacitance of a single infolded half-disk (at 1 $\mu$F cm$^{-2}$) is $\sim$0.002 pF, the access resistance would have to exceed $\sim 2 \times 10^{11}\ \Omega$. By comparison, the resistance of a single Na channel in a squid axon membrane is less than $10^{11}\ \Omega$ (Hille, 1970). Thus it is not likely that the interiors of the disks are attached by conducting channels to the external medium over an appreciable length of rat rods outer segments. This conclusion supports the anatomical observation of Cohen (1965, 1968, 1971) that most of the disks of mammalian rods are separated from plasma membranes. The significance of this observation in assessing possible excitatory mechanisms for the photocurrent is obvious: an additional transmission mechanism is needed to explain the spread of sensory signals from the internal disks to the plasma membrane. A possible agent for transmission is suggested by Yoshikami and Hagins (1971).

### *Duration of the Photocurrent and the Excitatory Mechanism*

In the preceding discussion the photocurrent was considered to arise from a light-induced change in conductance of the outer segment's envelope to ions capable of acting as major carriers of charge through it. Several workers have proposed a fundamentally different scheme for current control based on segregation of charge carriers within the rod disks. Bonting (1969), among others, suggested that the primary action of light is to change the permeability of the disk membranes so that gross exchange between their contents ($Na^+$ and $K^+$, for example) and the cytoplasm takes place. Since the disks occupy a large fraction of the space in the outer segments, a large change in composition of the fluid in contact with the inside of the rod envelope would result and the E.M.F.'s of one or more of the batteries driving the dark current would change. Thus the current through the envelope membrane would change even without any alteration in its specific ionic permeabilities.

The results of this paper conflict with the predictions of such an idea at two points. First, responses of almost saturating amplitude are observed when no more than 100 photons are absorbed in a rod. Since a rat rod contains about 1000 disks (Dowling and Gibbons, 1961; Penn and Hagins, unpublished observations), less than 10% would absorb photons from such stimuli and thus the predicted change in ionic composition of the rod cytoplasm would be less than 10% of the maximum possible change when all disks had been hit. Some other explanation of the amplitude–flash energy relation would thus be needed. Second, the "carrier segregation" hypothesis limits the total current flow in response to a stimulus flash to something less than the total ionic contents of the rod disks, but flash responses like that shown in Fig. 9 produce total charge flow through the rod envelope of more than $3 \times 10^{-12}$ moles/rod. If a rod outer segment were solid crystalline NaCl, it could contain only about $2.2 \times 10^{-12}$ moles of $Na^+$, which is too little to sustain such a current. These observations make carrier segregation an unlikely control mechanism, but they do not conflict with the idea that the disks release an agent which controls the permeability of the envelope membrane (Yoshikami and Hagins, 1971).

*Psychophysics and the Rod Photocurrent*

The photocurrent has been previously shown to be large enough and to have the right spatial distribution to be the agent for transmitting information about absorbed photons from outer segments to synapses in rat rods (Hagins et al., 1970). The results of this paper add three further arguments favoring the excitatory role of the photocurrent: (*a*) the time-course of the current agrees roughly with the temporal distribution of excitatory disturbances in the human scotopic visual system studied by psychophysical methods (Hallett, 1969 *b*); (*b*) the photocurrent saturates in amplitude within the same range of light levels (~500–2000 *hν* absorbed per rod per second) at which psychophysical rod saturation occurs (Aguilar and Stiles, 1954; Hallett, 1969 *a*), (*c*) exposures which bleach an appreciable part of the rhodopsin drastically reduce rod sensitivity for many minutes just as they do that of the scotopic system. At the same time, the long-lasting reduction in dark current can be taken to be the electrophysiological manifestation of the dark light (Rushton, 1965). Thus several important properties of the "rod system" of psychophysics can be directly linked to the behavior of the receptor cells.

Yet the correspondences between rods and the rod system of psychophysics are imperfect. The measured electrical responses of rods in isolated rat retinas are somewhat slower than those inferred by Hallett for the living human eye (Fig. 2). The discrepancy may be partly due to slowing of the responses in the isolated retina. During the first 2–3 min after the retina is removed, the rod layer shows flash responses whose peaks are 20–30% earlier than those seen when the preparation has stabilized in Ringer II. Then, after 2–3 hr, further slowing usually takes place. Thus physiological and psychophysical responses cannot be compared precisely

until conditions have been found for maintaining isolated retinas nearer to their state in the living eye. Similarly, the stimulus level at which psychophysical rod saturation sets in human eyes in increment threshold studies is about twice as high as that necessary to reduce the gain of the photocurrent of rat rods by twofold. In fact, the rod saturation described by Alpern et al. (1969) is found to require flashes nearly 30 times as bright as those which half-saturate the rat rod photocurrent. This discrepancy is apparently much too large to be due to error in estimation of the absolute light energies used in the two kinds of experiments or to species differences. Instead, it is possible that the retinal mechanism which assesses the size of rod responses to flashes in human eyes contains an integrator or a smoothing operator whose output is proportional to the area under the photocurrent response to a flash and not its peak amplitude. If this were so, it can be seen by reference to Fig. 7 that the charge flow in the photocurrent per photon absorbed would be reduced twofold from its value at low light levels by stimuli of about $10^3$ photons absorbed per rod. This figure is close to that of Alpern et al. and yet it is also consistent with Aguilar and Stiles's (1954) increment threshold measurements on steady backgrounds. Moreover, it is shown elsewhere (Yoshikami and Hagins, manuscript in preparation) that conditions can be found in which the saturating level for rod photocurrent responses can be raised by several factors of 10 in an appropriate ionic environment.

Finally, the long-lasting photocurrent (i.e., suppression of the dark current) produced by a flash which bleaches appreciable amounts of rhodopsin is not yet known to occur in the living eye. Since mammalian retinas deprived of circulation do not regenerate appreciable amounts of rhodopsin (Hagins, 1957) except under special conditions not applicable in the present experiments (Cone and Brown, 1969), we cannot yet show that the decay of the photocurrent in an isolated bleached rat retina tracks the resynthesis of its photopigment with the required logarithmic relationship. Nevertheless, the close similarity of the photocurrent to the known properties of the scotopic visual system make it likely that it is the primary sensory output of rods. If so, its characteristics must set bounds on the performance of the eye in dim light.

It remains to consider the possible role of conelike photoreceptors to the electrical responses described in this work. There are now several reports in the literature suggesting that at least some strains of rats may have receptors with (*a*) greater red sensitivity than rhodopsin-bearing rods, (*b*) faster responses to light flashes, and (*c*) ability to discriminate light stimuli on a background more intense than that at which the scotopic visual system saturates (Dowling, 1967; Muntz et al., 1969; Green, 1971). The receptor currents of such cells will undoubtedly contribute to voltage gradients in the receptor layer, but only in proportion to their relative numbers in the entire population of sensory cells. Thus one cone in, say, 100 rods could achieve appreciable control of the electroretinographic b-wave and of the

neural layers of the retina while producing photocurrent which would be undetectable by the technique used in this work.

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