Quantum Sensitivity of Rods in the Toad Retina

Abstract. *A dark-adapted toad rod can respond consistently to flashes of light which bleach an average of less than one pigment molecule in its outer segment. These responses are much less variable in amplitude than would be expected if rods were independent quantum detectors. Rods interact with one another by pooling their signals, so that at least 85 to 90 percent of the response recorded from a single rod is generated by pigment molecules bleached in other receptors.*

It has long been known that individual rod photoreceptors in the vertebrate retina can respond to a single quantum of light (*1*). Since neither rods nor second-order (horizontal and bipolar) cells generate action potentials, it is of interest to know how large the single quantum responses are and how they are reliably communicated to more proximal neurons. Although intracellular recordings have been made from rods in several species, few measurements have been made of their responses near threshold. In this report I describe such measurements, made from dark-adapted rods in the retina of the toad, *Bufo marinus*. These measurements indicate that the response produced in a single rod by the bleaching of one rhodopsin molecule is at most only 50 to 100 μv in amplitude. They also show that the bleaching of a pigment molecule in one rod produces responses in many rods. Rods pool their signals, so that at light intensities above threshold most of the response recorded from a single rod is generated by pigment molecules bleached in other receptors.

Eyes from toads dark-adapted overnight were removed in a dim red light, and the cornea and lens were dissected away. The eyecup was placed in a lighttight cage, and the electroretinogram was monitored for 10 to 15 minutes until it reached a stable, dark-adapted threshold. Fine micropipettes, filled with Procion yellow and measuring 800 to 1200 megohms in resistance in the vitreous, were used to penetrate photoreceptors in the central region of the retina. The retina was illuminated with a dual beam photostimulator (*2*), whose absolute intensity was measured by placing a photodiode in the position normally occupied by the eyecup (*3*).

The responses of photoreceptors were identified by their characteristic waveforms to brief flashes of light (*4–6*), by their spectral sensitivities, and by the intracellular injection of Procion yellow. The toad retina contains three spectral classes of receptors —the "red" and "green" rods and a single kind of cone. These can be distinguished both by their different photopigments (*7, 8*) and by their characteristic morphologies (*9*). Receptor responses always showed greatest sensitivity at about 500 nm, indicating that recordings were made exclusively from red rods. The morphology of cells stained with Procion yellow (Fig. 1) confirmed this identification (*10*).

Figure 2A shows the responses of a dark-adapted red rod to 9-msec flashes of diffuse 502-nm light. Rods respond with graded hyperpolarizations, which are quite similar in waveform and time course to those previously recorded from photoreceptors in *B. marinus* (*11*) and from rods in other species (*4–6*). The numbers to the left of each response give the mean number of rhodopsin molecules bleached in the outer segment of the receptor at each intensity, calculated from the absolute calibration of the photostimulator (*12*), the average cross-sectional areas of toad rod outer segments (*13*), the density of red rod photopigment in *B. marinus* and other anurans (*14*), and the quantum efficiency of bleaching in frog rhodopsin (*15*). The transmission of the vitreous and proximal layers of the retina was assumed to be unity.

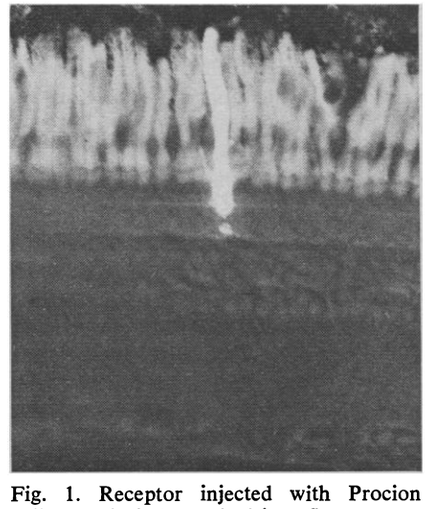

Fig. 1. Receptor injected with Procion yellow and photographed in a fluorescence microscope (*10*). The total length of the cell is about 97 μm. Its long outer segment, together with the spectral sensitivity of its response recorded before the injection, identify it as a red rod (see text). The synaptic terminal (most proximal region of stain) appears detached from the rest of the cell body in this section but can be seen to be connected in an adjacent section. Recordings of the responses of this cell to stimuli near threshold are illustrated in Fig. 2D.

An intensity-response series similar to the one shown in Fig. 2A was recorded from 40 dark-adapted rods. The rods of *B. marinus*, as well as those of several other species (*5, 16*), give responses to brief flashes of diffuse light whose amplitudes follow the equation

$$V = V_{max} I/(I + \sigma) \qquad (1)$$

where V is the peak amplitude of the response at intensity I, V_{max} is the peak amplitude at the saturation of the response, and σ is the intensity necessary to give a response of $\frac{1}{2} V_{max}$. Equation 1 predicts that, at dim light intensities ($I \ll \sigma$), rod responses will be linearly proportional to I with a proportionality constant equal to V_{max}/σ. This constant is a measure of the sensitivity of a rod since it gives the amplitude of its response at an intensity which bleaches an average of one rhodopsin molecule per rod outer segment. Best values of V_{max}/σ were determined by linear regression from intensity-response data by plotting $1/V$ against $1/I$ (double reciprocal or Lineweaver-Burk plot). The data plotted in this way give a straight line (*17*) whose slope is the inverse of V_{max}/σ. For the ten most sensitive rods V_{max}/σ was 676 ± 129 μv per rhodopsin bleached (Rh*) per receptor [mean ± standard deviation (S.D.)] (*18*). This is somewhat larger than the value of 440 μv/Rh* per receptor previously obtained for the most sensitive mudpuppy rods (*5, 19*).

For the rod of Fig. 2A, V_{max}/σ was about 670 μv/Rh* per receptor. Hence if this rod were a simple quantum detector—that is, if it were responding only to photochemical events occurring in its own outer segment—it would generate a signal of 670 μv for each bleached rhodopsin molecule. It is apparent from the uppermost record in Fig. 2A that this rod was not responding in this fashion, since at an intensity which bleaches less than one rhodopsin molecule per receptor, the rod produced a signal of less than 670 μv. The responses of this rod to dim illumination are examined in more detail in Fig. 2B, which shows three series of consecutive responses at intensities which bleach, on average, 0.65, 0.41, and 0.24 rhodopsin molecule per receptor per flash. It is evident from

these recordings that rods in the toad are able to respond consistently to flashes which bleach less than one rhodopsin molecule per receptor. The mean amplitude of the response decreases approximately linearly with light intensity for these three series of responses, as shown in Fig. 2C.

Figure 2B also shows that the responses of toad rods are much less variable in amplitude than would be expected if rods responded independently of one another. If rods responded independently (and generated signals whose amplitudes near threshold were proportional to the number of molecules bleached), then the peak amplitudes of their responses to dim light intensities should follow a Poisson distribution with variance equal to $V_{max}I/\sigma$. For example, at an intensity of 0.24 Rh* per receptor per flash, the Poisson distribution predicts that only about 20 percent of the flashes should bleach any rhodopsin molecules in the outer segment of the receptor. Yet the receptor of Fig. 2B gave some response to every stimulus at this intensity. The small variability in the amplitudes of rod responses is illustrated for another cell in Fig. 2D, which shows a series of 15 consecutive responses at an intensity of 1.40 Rh* per receptor per flash. At this intensity, about 25 percent of the flashes will bleach no rhodopsin molecules in the outer segment of the receptor, 35 percent will bleach one molecule, 24 percent will bleach two, and the remaining 16 percent will bleach three or more. The amplitudes of the observed responses, however, fall into a very narrow range (460 to 660 μv), as if each flash bleached exactly the same number of pigment molecules. But the chance of this happening in a series of 15 consecutive flashes at this intensity is about 1 in 10^7.

The small variability in the amplitudes of rod responses could be explained if the response of a rod were composed of contributions from many receptors. A rod could then respond even though no rhodopsin molecules were bleached in its own outer segment, as long as it received signals from other receptors which were directly stimulated by the light. The spread of signals between receptors has been previously implicated by morphological demonstrations of presumed synaptic contacts between receptors (*20*). In addition, measurements of receptive fields in the turtle retina have shown that both rods and cones can respond to illumination over a larger area than that occupied by their own outer segments (*6*, *21*).

The magnitude of signal pooling in toad roads can be estimated from the variability in the amplitudes of responses recorded from single receptors. To estimate the proportion of the recorded signal coming from other receptors, I use a simple model of receptor interactions based on three assumptions: (i) near threshold the bleaching of rhodopsin molecules in one receptor does not affect the probability of molecules being bleached in other receptors; (ii) the number of rhodopsin molecules bleached in each receptor follows a Poisson distribution; and (iii) in the range from one to four bleached rhodopsin molecules, the receptor responses are linear functions of intensity, and the coupling coefficients between receptors are independent of the amplitudes of receptor responses. It follows from these assumptions that near threshold the

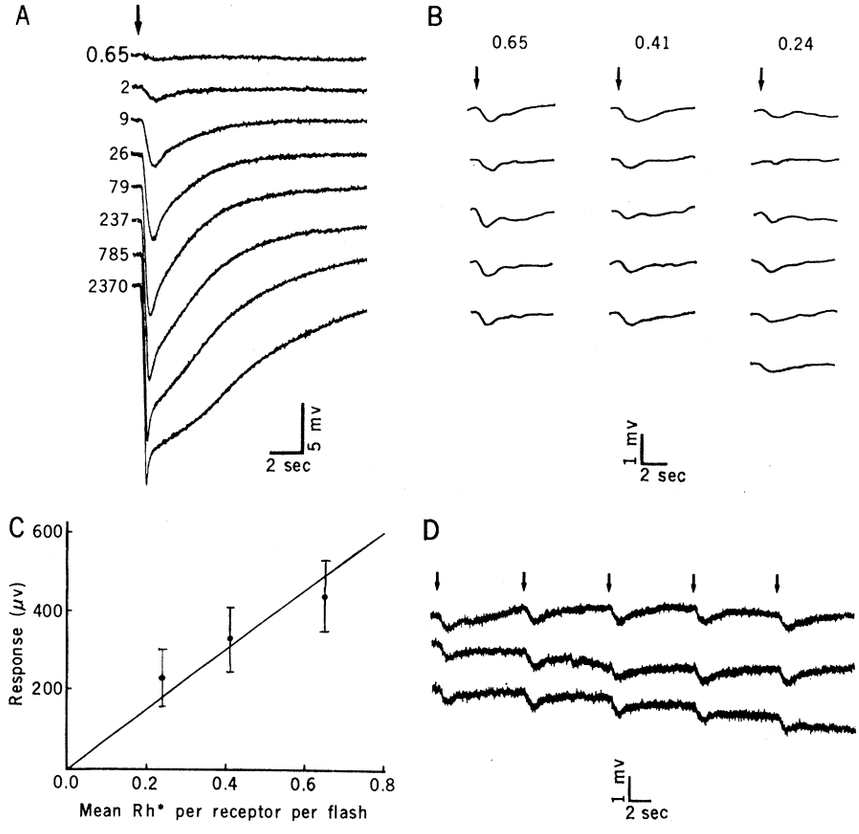

Fig. 2. Responses of dark-adapted toad rods. (A) Intensity-response series for 9-msec flashes of diffuse 502-nm light. Flashes were given at 15-second intervals except for the two at the brightest intensities, which were separated by 30 seconds. Arrow indicates onset of illumination. The number to the left of each response gives the mean number of rhodopsin molecules bleached per receptor per flash (see text). (B) Responses of the rod of Fig. 2A to flashes which bleach fewer than one rhodopsin molecule per receptor. Each column presents a series of consecutive responses to 9-msec flashes of diffuse 502-nm light given at 6-second intervals. Mean intensities of the flashes (in units of Rh* per receptor per flash) are given above the columns. Records have been aligned so that the arrow above each column gives the time of onset of illumination for all the responses in the column. Responses were traced from original records. (C) Mean amplitudes of responses from Fig. 2B plotted as a function of light intensity. Closed circles and error bars give the mean and twice the standard deviation of the peak amplitudes of the responses in each column. The line drawn through the data was placed to intersect the origin, since in the absence of illumination spontaneous fluctuations of membrane potential could not be distinguished from the noise of the recording system. The slope of the line (745 μv per Rh* per receptor) was calculated by the method of least squares from the class of straight lines which pass through the origin. (D) Responses of a rod (see Fig. 1) to 15 consecutive flashes of diffuse 502-nm light at an intensity of 1.40 Rh* per receptor per flash. Flashes were 9 msec long and were given at 8-second intervals. Responses were taken from one continuous record, which was split up into three sections, so that the last response of the first row immediately precedes the first response of the second row, and similarly for the second and third rows. Records were aligned so that the arrows give the onset of illumination for the responses in all three rows.

response recorded in any one receptor is the linear sum of contributions from all of the receptors in the pool

$$V_j = \alpha_{1j}\mathrm{Rh}_1^* + \alpha_{2j}\mathrm{Rh}_2^* + \ldots + \alpha_{nj}\mathrm{Rh}_n^* = \Sigma\,\alpha_{ij}\mathrm{Rh}_i^* \quad (2)$$

where V_j is the peak amplitude of the response recorded from receptor j, Rh_i^* is the number of rhodopsin molecules bleached in receptor i, and α_{ij} is the potential recorded in receptor j when a rhodopsin molecule is bleached in receptor i. For a diffuse light (one which bleaches, on average, the same number of rhodopsin molecules in each receptor in the pool), the mean and variance of V_j are given by

$$\mathrm{mean}(V_j) = \mathrm{mean}(\mathrm{Rh}^*)\Sigma\alpha_{ij} \quad (3)$$

$$\mathrm{var}(V_j) = \mathrm{mean}(V_j)\Sigma\alpha_{ij}^2/\Sigma\alpha_{ij} \quad (4)$$

where mean(Rh*) has units of rhodopsin molecules bleached per receptor per flash. Equations 3 and 4 can be rearranged to give

$$\Sigma\alpha_{ij}^2 = \mathrm{var}(V_j)/\mathrm{mean}(\mathrm{Rh}^*) \quad (5)$$

Since

$$\Sigma\alpha_{ij}^2 = \alpha_{1j}^2 + \alpha_{2j}^2 + \ldots + \alpha_{jj}^2 + \ldots + \alpha_{nj}^2 \geqslant \alpha_{jj}^2 \quad (6)$$

the square root of $\mathrm{var}(V_j)/\mathrm{mean}(\mathrm{Rh}^*)$ is an upper limit for α_{jj}—that is, for the potential recorded in receptor j when a rhodopsin molecule is bleached in its outer segment. The ratio of the upper limit for α_{jj} to the sum of all the α_{ij} gives an upper limit for the proportion of the signal recorded in receptor j which is generated by the bleaching of its own rhodopsin molecules.

Table 1 summarizes estimates of the extent of receptor pooling for three dark-adapted rods. The second column in Table 1 gives sums of the α_{ij}, which were obtained from intensity-response data for the receptors (since near threshold $\Sigma\alpha_{ij} = V_{max}/\sigma$; compare Eqs. 1 and 3). The third column gives upper limits for α_{jj} calculated from Eqs. 3 to 6 (*22*). The coefficient α_{jj} is at most only 50 to 100 μv, so that the potential generated in a single rod by the bleaching of one rhodopsin molecule is not large enough to have been reliably distinguished from the noise of the high-resistance micropipettes used in these recordings.

The fourth column in Table 1 gives upper limits for the fraction of the response of the three rods which was generated by pigment molecules actually bleached in their own outer segments. These show that if receptors interact according to the model given above (*23*), a single rod in a dark-adapted retina generates at most only about 10 to 15 percent of the signal which can be recorded from it. The remainder of the signal must be generated by photochemical events occurring in other receptors (*24*).

Table 1. Estimates of the size of single quantum events and the extent of signal pooling in toad rods. Data in each row were calculated from measurements made on a different rod. Results for the rods in the first two rows are based on measurements of 5 responses at the intensity given in the first column, and those in the last row on 15 responses.

Intensity (mean Rh* per receptor)	$\Sigma\alpha_{ij}$ (μv per Rh* per receptor)	α_{jj} (μv per Rh*)	$\frac{\alpha_{jj}}{\Sigma\alpha_{ij}}$
0.41	714	52	0.07
0.65	668	109	0.16
1.40	544	44	0.08

It does not appear that contributions from neighboring receptors provide a mechanism for increasing the sensitivity of single rods, since rods in *B. marinus* (*25*) and in other species (*20*) appear to be connected through gap junctions, which provide only for the passive spread of current between neurons (*26*). It is possible, however, that the pooling of receptor signals may improve the sensitivity of the visual system as a whole, perhaps by increasing the effectiveness of transmission between receptors and second-order neurons (*27*). An understanding of the function of receptor pooling must await a more quantitative description of its morphological and physiological properties.

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3. The output of the diode (United Detector Technology, Pin-5) was calibrated in the unbiased mode against a Kipp and Zonen compensated thermopile, which had been calibrated by the manufacturer. The calibration of the thermopile was verified to within 20 percent of its nominal value by comparing its output to that of a calibrated Eppley thermopile. The diode sensitivity in the region for which its output was proportional to light intensity was 1.76 ± 0.13 watt/amp (mean $\pm$ S.D., $N = 15$). The absolute quantum flux of the stimulator at the position of the eyecup was set to the same value (1.2×10^{13} quanta per square centimeter per second in the absence of neutral density filters) before the beginning of each experiment by adjusting the voltage across the source for the appropriate diode current. The source was driven by a regulated power supply, and drift in its intensity during the experiment was less than ± 5 percent. Since the diode was quite sensitive to infrared (IR) illumination, IR blocking filters (KG-1 and KG-3) were placed in the beam during all measurements. The contribution of IR under these conditions was assessed by inserting a second set of IR filters into the beam. The ratio of the diode currents with and without this second set in the stimulator was within ± 0.01 log unit of the transmittance of these filters at 502 nm, so that the contribution of IR must have been less than 3 percent of the measured intensity. Variations of intensity across the field of stimulation were less than ± 5 percent of the mean, and their effects were minimized by always placing the eyecup in the center of the field and making penetrations only in the central region of the retina. The final lens in the photostimulator had a focal length of 65 mm and was about 150 mm from the focal point. Hence small errors (1 to 2 mm) in focusing the light onto either the eyecup or the photodiode would have produced negligible error in the absolute calibration. Since most of the vitreous was drained away, it is doubtful that the eyecup itself could have focused or defocused the light enough to produce a measurable change in the absolute intensity illuminating the photoreceptors. Taking all of these factors into consideration, I estimate the absolute calibration to be within 35 percent (or about 0.15 log unit) of the value given above.

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10. Rods were stained, fixed, and embedded as previously described for retinal cells in the carp retina [A. Kaneko, *J. Physiol. (Lond.)* **207**, 623 (1970)]. Of six Procion yellow marks recovered from 11 injections, four were located in single red rods stained in isolation, one was found in three contiguous red rods, and the last filled two red rods and an adjacent cone. In these latter two cases, the spectral sensitivity of the response was that of a red rod receptor, and the fluorescence was brighter in one of the red rods than in the other cells. It is not known why more than one cell was stained, but the poor fixation of these two retinas may well have been a contributing factor. Dye was never found in cells proximal to the photoreceptors.

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12. The neutral density filters which were used to attenuate the light in the photostimulator were calibrated in situ by using a photomultiplier tube placed in the position normally occupied by the eyecup and a lock-in voltmeter (Brower Laboratories, model 131). They were also calibrated with a recording spectrophotometer, and for all of the filters these two calibrations agreed within 0.05 log unit (or about 12 percent). The length of the flashes was measured by displaying on an oscilloscope the voltage of a photocell placed after the shutter. The waveform of this voltage was very nearly square and was 9.0 msec long, with very little variation in time course (less than ± 2 percent) from flash to flash.

13. The cross-sectional area of red rod outer segments varies from 52 μm^2 near the bases to 42.5 μm^2 near the tips. The average cross-sectional area is about 47 μm^2.

14. P. K. Brown, personal communication. The density of red rod photopigment obtained from macrospectrophotometry on *B. marinus* retinas in the presence of hydroxylamine was 0.64 ± 0.07 (mean $\pm$ S.D., $N = 8$), giving a mean absorption of 77 percent. Since this density is not corrected for light passing between receptors or through green rods and

cones, it is a lower limit for the density of pigment in the photoreceptors themselves. The value of the pigment density actually used to estimate the proportion of quanta absorbed by the red rods is that calculated from the specific density given by Liebman and Entine (*8*) for red rods in *Rana pipiens*. This gives a total pigment density in the receptors of 0.96 (corresponding to an absorption of 89 percent), which is larger than the value given above and hence more conservative for the calculations of Table 1. The specific density of frog rods (0.014 μm^{-1}) was multiplied by the lengths of red rod outer segments in *B. marinus*, which were measured in fixed material to be 68.3 ± 2.1 μm (mean $\pm$ S.D., $N = 93$). Shrinkage of the outer segments during fixation, dehydration, and embedding was estimated to be less than 10 percent by comparing the lengths of the longest rod outer segments in fixed material to the lengths of the longest unfixed rod outer segments isolated from the retina of the other eye in the same animal.

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17. Coefficients of correlation (r^2) for the fit of intensity-response data to a straight line on a double reciprocal plot were between .981 and .999 for the 40 rods, with a mean value of .997. This implies that the peak amplitudes of toad rod responses can be closely fitted with Eq. 1 at dim light intensities and hence that peak amplitude is directly proportional to intensity near threshold. However, this does not necessarily show that the intensity-response data can be satisfactorily fitted with Eq. 1 at all levels of illumination, since the double reciprocal plot places disproportionate emphasis on the data at dim intensities.
18. The values of σ for the ten most sensitive toad rods, calculated from the best values of V_{max}/σ and the largest amplitude responses actually recorded from the photoreceptors, were 34 ± 11 quanta absorbed per receptor per flash, or 23 ± 7 Rh* per receptor per flash (mean $\pm$ S.D.). These are in close agreement with values previously reported for rods in other species (*5*). If it can be assumed that rods in all vertebrates have about the same sensitivity, this agreement provides an independent confirmation of the absolute calibration of the photostimulator.
19. The quantum efficiency for mudpuppy porphyropsin was assumed to be the same (0.64) as that for porphyropsins in other species (*15*).

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22. The variability in the response amplitudes is the result of both fluctuations in the numbers of rhodopsin molecules bleached in the various receptors in the pool and low-frequency noise in the recording system. No attempt was made to measure low-frequency noise for the recordings from the three receptors of Table 1. Since the noise of the recording system should be subtracted from the total variance before calculating the upper limit for α_{jj}, the values for the upper limits should be smaller than those given in Table 1. This may explain why the upper limits for the cell in the second row of Table 1 are more than twice as large as those for the other two cells, since the responses of this cell (which are shown in Fig. 2B) showed more low-frequency noise than those of the other two (see, for example, the responses in Fig. 2D, which were used for the data in the last row of Table 1).
23. The third assumption of the model is not needed for the calculation of the upper limit for α_{jj} for the receptor in the first row of Table 1, since at an intensity of 0.41 Rh* per receptor per flash, on the average only about 7 percent of the receptors will have more than one rhodopsin molecule bleached in their outer segments.
24. It might be supposed that the results in the fourth column of Table 1 are critically dependent on the absolute calibration of the photostimulator and the estimate of the number of rhodopsin molecules bleached by the flashes. Equations 3 to 6 show, however, that the error in the upper limit for $\alpha_{jj}/\Sigma\alpha_{ij}$ goes only as the square root of the error in the estimate of mean(Rh*). This means that even if the quantum flux illuminating the retina were as much as 0.3 log unit (or 100 percent) greater than that indicated by the absolute calibration—and this is probably the largest plausible error considering the care with which this calibration was made (*3*, *12*)—the mean value of the upper limit of $\alpha_{jj}/\Sigma\alpha_{ij}$ for the three receptors would increase from 0.10 only to 0.15. If in addition it were supposed that every one of the incident quanta in this illumination bleached a rhodopsin molecule in some red rod [so that mean(Rh*) was given only by the ratio of the number of incident quanta to the density of red rod receptors—about 17,000 mm^{-2} in the toad], the mean value of $\alpha_{jj}/\Sigma\alpha_{ij}$ would still increase only 0.21. The number of rhodopsin molecules bleached per receptor would have to be 25 times greater than those given in Table 1 before rods could be thought to be generating even half of their own response, and 100 times greater before they could be assumed to be producing all of it.
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28. Preliminary results of these experiments were reported at the meeting of the Association for Research in Vision and Ophthalmology, Sarasota, Florida, 25 to 29 April 1974. I thank Paul K. Brown, Joel E. Cohen, and Mark Lurie for helpful advice and assistance, John E. Dowling and John E. Lisman for critical readings of the manuscript, and Patricia A. Sheppard for technical assistance. Supported in part by NIH research fellowship award EY-54840 and by NIH grant EY-00824 to J. E. Dowling.

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p-Chloroamphetamine: Selective Neurotoxic Action in Brain

Abstract. *Injection of 2.5, 5, 10, or 20 milligrams of* p-*chloroamphetamine per kilogram of body weight into rats produced evidence of cytopathological changes in sections of brain stained by a Nissl or silver method. As early as 1 day after drug injection cells demonstrated an intense Nissl staining, intense argyrophilia, cellular shrinkage, and perineuronal spaces. At 30 days after injection both stains revealed cellular debris and glial reactions characteristic of cellular dissolution. The neurotoxic effects of 2.5, 5, or 10 milligrams of* p-*chloroamphetamine per kilogram were primarily restricted to an area of the ventral midbrain tegmentum corresponding to the distribution of the B-9 serotonergic cell group. After 20 milligrams of* p-*chloroamphetamine per kilogram there was also evidence of neurotoxic effects on cells within the substantia nigra. These results confirm previous suggestions that the long-term reduction in serotonin content of brain, tryptophan-5-hydroxylase activity, and uptake of serotonin after injection of* p-*chloroamphetamine is due to a neurotoxic effect of the drug or some metabolite on serotonergic cell bodies.*

A number of halogenated arylalkylamines have been reported to produce a decrease in brain content of serotonin accompanied by a decrease in activity of tryptophan-5-hydroxylase (*1*). Some of these compounds, such as *p*-chlorophenylalanine (*p*-CP), have been extensively used to examine the role of serotonin in brain, while others such as *p*-chloroamphetamine (*p*-CA) and fenfluramine have been used clinically as an antidepressant and an anorexogenic agent, respectively. Some of these halogenated derivatives, including *p*-CA, have been shown to have long-term and apparently irreversible effects on serotonergic systems of brain. Serotonin content of brain remains significantly reduced up to 4 months after a single dose of *p*-CA in the rat (*2–4*). There is also a long-term reduction in serotonin uptake by brain slices or synaptosomal fractions isolated from rat brain (*4*, *5*) and a reduction in tryptophan-5-hydroxylase activity (*2–4*). These findings have led to the speculation that *p*-CA may have some neurotoxic action on serotonergic neurons in brain, either directly, or indirectly through the formation of a toxic metabolite (*3*, *4*). Such a toxic action might be similar to that observed on serotonergic and catecholaminergic neurons of brain after the intraventricular administration of 5,6-dihydroxytryptamine or 6-hydroxydopamine, respectively (*6*). In the present study, we have found that *p*-CA produces cellular dam-