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.

15. H. J. A. Dartnall, *Vision Res.* **8**, 339 (1968). The mean quantum efficiency of bleaching for rhodopsin in solution in the presence of hydroxylamine for two species of frogs from the genus *Rana* was 0.67.
16. R. D. Penn and W. A. Hagins, *Biophys. J.* **12**, 1073 (1972); J. E. Dowling and H. Ripps, *J. Gen. Physiol.* **60**, 698 (1972); J. Kleinschmidt, thesis, Johns Hopkins University (1974).
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*).
20. F. S. Sjöstrand, *J. Ultrastruct. Res.* **2**, 122 (1958); S. E. G. Nilsson, *ibid.* **11**, 147 (1964); A. I. Cohen, *J. Anat.* **99**, 595 (1965); A. Lasansky, *Philos. Trans. R. Soc. London Ser. B Biol. Sci.* **262**, 365 (1971); E. Raviola and N. B. Gilula, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 1677 (1973); A. Lasansky, *Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci.* **265**, 471 (1973); N. V. Custer, *J. Comp. Neurol.* **151**, 35 (1973); P. Witkovsky, M. Shakib, H. Ripps, *Invest. Ophthalmol.* **13**, 996 (1974).
21. D. A. Baylor, M. G. F. Fuortes, P. M. O'Bryan, *J. Physiol. (Lond.)* **214**, 265 (1971); D. A. Baylor and A. L. Hodgkin, *ibid.* **234**, 163 (1973). Toad rods also show considerable spatial summation [G. L. Fain, *Bull. Mar. Biol. Lab. Woods Hole* **147**, 475 (1974)]. Red rods receive contributions only from other red rods, since their spectral sensitivity for stimuli of diffuse light (even in the presence of bright background illumination) is in close agreement with the absorbance spectrum of red rod photopigment. The waveforms and time courses of red rod responses are invariant with wavelength, as would be expected if they were generated by the bleaching of only one photopigment.
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.
25. G. Gold, G. L. Fain, J. E. Dowling, in preparation.
26. G. D. Pappas and S. G. Waxman, in *Structure and Function of Synapses*, G. D. Pappas and D. P. Purpura, Eds. (Raven, New York, (1972), p. 1; M. V. L. Bennett, *ibid.*, p. 221.
27. G. Falk and P. Fatt, in *Handbook of Sensory Physiology*, H. J. A. Dartnall, Ed. (Springer-Verlag, Berlin, 1972), vol. 7, part 1, p. 200.

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-

Table 1. Effect of 20 mg of *p*-chloroamphetamine (*p*-CA) per kilogram of body weight on whole brain content of serotonin at 3 and 30 days after injection. Each mean is based on an *N* of 3. Whole brain content is for brain minus olfactory bulbs and cerebellum. S.E.M., standard error of the mean.

Group	At 3 days		At 30 days	
	Serotonin (nmole/g ± S.E.M.)	Percent change	Serotonin (nmole/g ± S.E.M.)	Percent change
Control	3.43 ± 0.08		4.02 ± 0.13	
p-CA	1.01 ± 0.11*	−71	1.88 ± 0.14*	−53

* $P < .001$.

age which can be observed in Nissl- and silver-stained sections of the rat midbrain.

Male albino rats (Holtzman, Madison, Wisconsin), 150 to 200 g, were housed two per cage and maintained on Purina Laboratory Chow and tap water. Animals were injected intraperitoneally with 2.5, 5, 10, or 20 mg of DL-*p*-chloroamphetamine per kilogram of body weight, dissolved in sterile physiological saline to give an injection volume of 1 ml/kg. Control animals received an equivalent volume of the saline solution. At 1, 3, 9, 14, and 30 days after injection, rats injected with *p*-CA ($N = 25$) and control rats ($N = 10$) were perfused transcardially under pentobarbital anesthesia by the method of De Olmos and Ingram (*7*), except that formaldehyde was substituted for the paraformaldehyde. Frozen, 30-μm sections were stained by a modification of the cresyl violet stain described by Fernstrom (*8*), or by the silver method (procedure I) of Fink and Heimer (*9*).

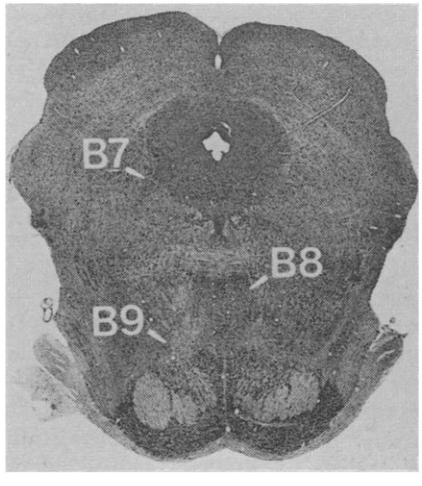

Fig. 1. Coronal section through the midbrain of a rat illustrating the three serotonergic cell groups: B-7, the dorsal raphe nucleus; B-8, the medial raphe nucleus; and B-9, serotonergic cell group within the ventral midbrain tegmentum. Nissl stain, 10 mg/kg *p*-CA, 9 days survival (× 3¼).

Changes in cell bodies, suggestive of degeneration, were consistently observed at 1 and 3 days after injection of 2.5, 5, 10, or 20 mg/kg *p*-CA in either Nissl- or Fink-Heimer–stained sections. The reacting cells were most prominent in an area of the ventral midbrain tegmentum corresponding to the distribution of the B-9 serotonergic cell group (Fig. 1) as described by Dahlström and Fuxe (*10*). The cell reactions to *p*-CA were readily apparent even under low magnification (× 50). In Nissl-stained sections the reacting cells displayed three characteristics: an intense staining of the cytoplasm and nucleus, cellular shrinkage, and a perineuronal space (Fig. 2b). Dosages of 5, 10, or 20 mg/kg *p*-CA produced comparable effects in the B-9 area. However, at the lowest dose of *p*-CA (2.5 mg/kg) there were fewer reacting neurons. Similar results were obtained with the Fink-Heimer method. Cells in the B-9 serotonergic cell group displayed an intense argyrophilia, cellular shrinkage, and a perineuronal space (Fig. 3) at 1 or 3 days after 10 or 20 mg/kg *p*-CA.

A dosage of 5, 10, or 20 mg/kg *p*-CA also produced some abnormally staining cells within the raphe nuclei (B-7 and B-8, see Fig. 1) in Nissl- or silver-stained sections. There were several differences between the effects of *p*-CA on the cells within the B-9 cell group and on cells within the raphe region. In contrast to the intense Nissl staining and argyrophilia described above for the B-9 region, the reacting cells in the B-7 and B-8 regions showed only a mild intensification of Nissl staining and only a mild argyrophilia. While the reacting cells in the B-7 and B-8 areas did show an abnormal shape, there was only an occasional occurrence of perineuronal spaces, and the large number of glial cells observed in the B-9 area (see below) were not present. Although we have not conducted an accurate cell count, it appeared that approximately 80 percent of the cells within the B-9 region demonstrated these neurotoxic changes, while only 10 percent of the cells within the B-7 plus B-8 area were affected. The reacting cells in the B-9 area were typically about 30 μm in diameter, while those in the B-7 and B-8 areas were smaller (about 10 to 15 μm), and were localized to the midline of these cell groups.

Serial sections stained by either the Nissl or silver method failed to reveal any evidence of similar cellular effects of 2.5, 5, or 10 mg/kg *p*-CA in any

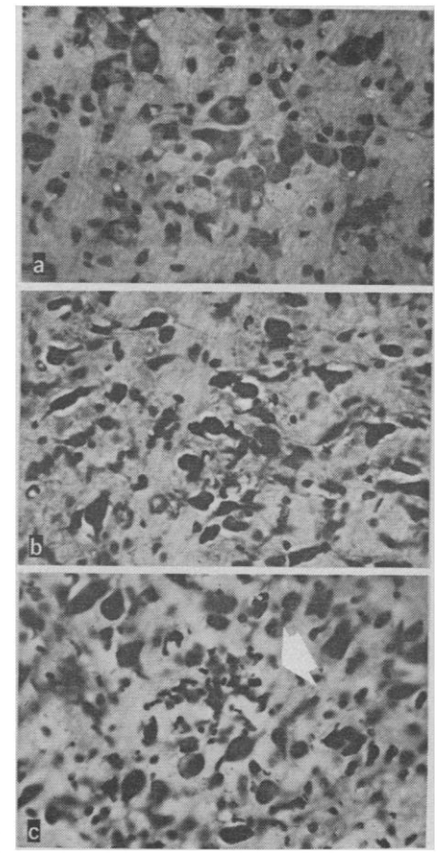

Fig. 2. Appearance of cells in the B-9 cell group in Nissl-stained sections (× 108). (a) Control animal. (b) Three days after 20 mg/kg *p*-CA. Note the shrunken appearance and dark staining of the cells. A perineuronal space is clearly visible around each reacting neuron. The smaller dark-staining bodies appear to be glia. (c) Thirty days after 20 mg/kg *p*-CA. Note the continuing increased staining of some cells, the occurrence of glial reactions (arrow), the relative absence of perineuronal spaces, and the more normal appearance of some cells.

other area of the brain. These dosages of *p*-CA appear, therefore, to have a highly selective action on cells within the B-9 serotonergic cell group in the ventral midbrain tegmentum.

The intense reactions of the cells in the B-9 region as revealed by both the Nissl and silver methods strongly suggests an irreversible degenerative change. Moreover, the abnormal staining exhibited by these cells after *p*-CA was identical to that described by Hedreen and Chalmers (*11*) for cells in the substantia nigra following the intraventricular administration of 6-hydroxydopamine. In both cases there is a clumping of argyrophilic deposits in the cytoplasm and a dense argyrophilia of the nucleus in silver-stained sections, that is observable by 1 day after drug administration. In addition we found a clumping of Nissl substance in cytoplasm and nucleus. Also, both drugs appear to produce cellular shrinkage and a perineuronal space (*11*, and Figs. 2b and 3).

Raising the dosage of *p*-CA to 20 mg/kg produced the same changes in the B-9 cell group as that seen after 5 or 10 mg/kg, and there did not appear to be an increase in the number of cells affected. However, 20 mg/kg *p*-CA also produced an appearance of neurotoxicity in cells of the substantia nigra by 1 day after injection. The reacting cells in substantia nigra presented the same appearance as that described for the reacting cells in the B-9 area.

The silver stain of Fink and Heimer (*9*) is capable of impregnating degenerating axons and their synaptic endings. However, we found no evidence of either degenerating axons (see Fig. 3) or of degenerating terminals within the brains of animals given 10 or 20 mg/kg *p*-CA. These results are in agreement with other studies that have failed to detect terminal degeneration even after lesions destroying the serotonergic cell bodies or their ascending axons in the medial forebrain bundle (*12*). It is commonly agreed that these negative results are due to the small size of the unmyelinated serotonergic fibers, since degenerative changes in axons and their terminals have been seen by histochemical fluorescence or electron microscopy (*13*). Hedreen and Chalmers (*11*) also failed to observe a 6-hydroxydopamine–induced degeneration of noradrenergic axons and terminals in brain sections stained by the Fink-Heimer method.

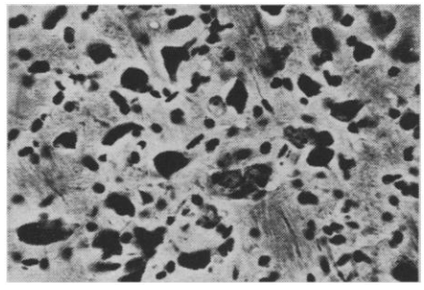

Fig. 3. Appearance of cells in the B-9 cell group at 3 days after the injection of 20 mg/kg of *p*-CA in a Fink-Heimer–stained section (× 108). Note the intense argyrophilia of cytoplasm and nucleus, shrunken appearance of the cells, and perineuronal spaces. Normal-appearing fibers can be seen coursing through this region.

The cytopathological reaction of B-9 cells to 10 or 20 mg/kg *p*-CA was still present at 9 and 14 days after injection, although the intensity of the Nissl stain and the argyrophilia was reduced, and there was a relative absence of perineuronal spaces. By 30 days after *p*-CA the Nissl and silver stain still revealed evidence of continuing degenerative changes (Fig. 2c). Some B-9 cells still demonstrated a clumping of Nissl material and an above-normal argyrophilia. Many of the cells still exhibited a shrunken and irregular appearance. In silver-stained sections there were a number of irregularly shaped, argyrophilic bodies, suggesting that at least some of the cells had become necrotic and undergone dissolution. Finally, from the first to the 30th day after *p*-CA there was an increased staining of glial cells and evidence of glial reactions (see arrow, Fig. 2c). In contrast to these observations in the B-9 area, there were no observable effects of 20 mg/kg *p*-CA on cells within the substantia nigra at 30 days after injection.

Some rats receiving 20 mg/kg *p*-CA were decapitated at 3 or 30 days after injection and their brains (minus olfactory bulbs and cerebellum) were assayed for serotonin content (*14*). Serotonin content of brain (Table 1) was decreased by 71 percent at 3 days and by 53 percent at 30 days after *p*-CA. This long-term depletion of serotonin suggests that the cells undergoing degenerative changes in the B-9 cell group were in fact serotonergic cells. Hence, *p*-CA appears to produce a highly selective neurotoxic effect, presumably on serotonergic cell bodies within the B-9 cell group of the ventral midbrain tegmentum. This neurotoxic effect of *p*-CA would explain the long-term decreases in serotonin content, tryptophan-5-hydroxylase activity, and serotonin uptake. The basis of the selective action of *p*-CA on the B-9 cell bodies is not clear at present, but could be due to a unique ability of these cells to convert *p*-CA to a toxic metabolite. This action of *p*-CA could be used to advantage in experiments that require a selective destruction of this cell group.

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References and Notes

1. A. Pletscher, W. P. Burkard, H. Bruderer, K. F. Gey, *Life Sci.* **2**, 828 (196
2. R. W. Fuller, C. W. Hines, J. Mills, *Biochem. Pharmacol.* **14**, 483 (1965); B. K. Koe and A. Weissman, *J. Pharmacol. Exp. Ther.* **154**, 499 (1966); E. Jequier, W. Lovenberg, A. Sjoerdsma, *Mol. Pharmacol.* **3**, 274 (1967); J. Duhault and C. Verdavainne, *Arch. Int. Pharmacodyn. Ther.* **170**, 276 (1967); E. Sanders-Bush and F. Sulser *J. Pharmacol. Exp. Ther.* **175**, 419 (197
3. E. Costa and A. Revuelta, *Neuropharmacology* **11**, 291 (197
4. E. Sanders-Bush, J. A. Bushing, F. Sulser, *Biochem. Pharmacol.* **21**, 1501 (1972).
5. E. Sanders-Bush, J. A. Bushing, F. Sulser, *Eur. J. Pharmacol.* **20**, 385 (1972).
6. R. W. Fuller and B. B. Molloy, *Adv. Biochem. Psychopharmacol.* **10**, 195 (1974).
7. E. Sanders-Bush, D. A. Gallager, F. Sulser, *ibid.*, p. 185; E. M. Gál, P. Christiansen, L. M. Yunger, *Int. J. Neuropharmacol.*, in press.
8. A. Carlsson, *J. Pharm. Pharmacol.* **22**, 729 (197
9. D. T. Wong, J. S. Horng, R. W. Fuller, *Biochem. Pharmacol.* **22**, 311 (1973).
10. H. G. Baumgarten, A. Björklund, A. F. Holstein, A. Nobin, *Z. Zellforsch.* **129**, 256 (1972); F. E. Bloom, S. Algeri, A. Groppetti, A. Revuelta, E. Costa, *Science* **166**, 1284 (1969).
11. J. S. De Olmos and W. R. Ingram, *Brain Res.* **33**, 523 (1971).
12. R. C. Fernstrom, *Stain Technol.* **33**, 175 (1958). Our modifications of this method consisted of the use of 1.0 percent parlodion in place of 0.5 percent celloidin; 0.55 percent cresylechtviolett (Grübler and Company) in place of the 0.5 percent solution; an additional wash in a mixture of xylene and alpha-turpineal (1 : 1) between the butanol and xylene steps; and elimination of the distilled water wash. These modifications increased the sensitivity of the Nissl stain for detecting the neurotoxic effects of *p*-CA described in this report.
13. R. P. Fink and L. Heimer, *Brain Res.* **4**, 369 (1967).
14. A. Dahlström and K. Fuxe, *Acta Physiol. Scand.* **64**, Suppl. 232, 1 (1964).
15. J. C. Hedreen and J. P. Chalmers, *Brain Res.* **47**, 1 (1972).
16. A. Heller and R. Y. Moore, *Adv. Pharmacol.* **6(A)**, 191 (1968).
17. F. E. Bloom, *ibid.*, p. 2
18. G. K. Aghajanian, F. E. Bloom, M. H. Sheard, *Brain Res.* **13**, 266 (1969); K. Fuxe and G. Johnsson, *Adv. Biochem. Psychopharmacol.* **10**, 1 (1974).
19. D. F. Bogdanski, A. Pletscher, B. B. Brodie, S. Udenfriend, *J. Pharmacol. Exp. Ther.* **117**, 82 (1956).
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