Biochemical Pharmacology, Vol. 28, pp. 3103–3107

0006-2952/79/1015-3103 $02.00/0

SPECIES DIFFERENCES IN THE RATE OF DISAPPEARANCE OF FENFLURAMINE AND ITS EFFECTS ON BRAIN SEROTONIN NEURONS*

LARRY R. STERANKA and ELAINE SANDERS-BUSH

Department of Pharmacology, Tennessee Neuropsychiatric Institute, Vanderbilt University School of Medicine, Nashville, TN, U.S.A.

(*Received* 16 *February* 1979; *accepted* 31 *March* 1979)

Abstract—A single injection of fenfluramine, the *m*-trifluoromethyl-*N*-ethyl derivative of amphetamine, produces long-term decreases in brain levels of 5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), tryptophan hydroxylase activity (TPH), and the synaptosomal uptake of [^{3}H]-5-HT in rats. In order to test the extent to which similar effects occur in mice, rats and mice were injected with various doses of fenfluramine and killed 2 weeks later for the determination of 5-HT and 5-HIAA in one half of the brain, 5-HT uptake in the hippocampus from the other half, and TPH in the remaining tissue. A dose of 20 mg/kg produced marked reductions in 5-HT and 5-HIAA in rats, while in mice significant reductions in both of these parameters were observed only after 80 mg/kg. TPH was diminished after 60 mg/kg in rats but not after any dose up to 80 mg/kg in mice. A 58 percent reduction in 5-HT uptake occurred after 40 mg/kg in rats and 80 mg/kg in mice. Complete recovery was evident by 2 months after the administration of fenfluramine to mice when, as indicated by previous reports, marked effects are still present in rats. These results demonstrate that mice are substantially less sensitive than rats to the long-term effects of fenfluramine on central 5-HT systems. In addition, the half-lives of fenfluramine and its active metabolite, norfenfluramine, were much shorter in mice than in rats. Thus, the rates of disappearance of fenfluramine and norfenfluramine are correlated with, and perhaps determine, the extent to which the administration of fenfluramine produces long-term, neurotoxic effects on central 5-HT mechanisms in rats and mice.

Fenfluramine, the *m*-trifluoromethyl-*N*-ethyl derivative of amphetamine, is an anorexigenic agent which is devoid of central stimulatory effects [1]. The administration of fenfluramine to rats causes pronounced decreases in brain levels of 5-hydroxytryptamine (5-HT) and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA), as well as decreases in the activity of the rate-limiting enzyme in 5-HT biosynthesis, tryptophan hydroxylase, and in the high-affinity synaptosomal uptake of 5-HT [2–5]. These effects are extremely long-lasting, having been observed as long as 2 months after a single injection of fenfluramine [25] and are similar to those produced by *p*-chloroamphetamine (PCA) in rats [5–8].

In mice, the effects of a single injection of PCA on 5-HT mechanisms are either absent or relatively short-lived [9, 10]. Recent data from our laboratory [11], demonstrating long-term decreases in brain levels of 5-HT and 5-HT-uptake capacity in mice up to 2 months after the continuous release of PCA from subcutaneously implanted ALZET Osmotic Minipumps, suggest that the relatively rapid rate of metabolism of PCA in mice [10] is responsible for their apparent insensitivity to the long-term effects of the drug.

The present experiments were designed to test the extent to which similar species differences might exist with respect to the long-term effects of fenfluramine.

* These investigations were supported in part by U.S. Public Health Service Grant MH-26463, Research Scientist Development Award MH-70898 to Elaine Sanders-Bush, and the Tennessee Department of Mental Health and Retardation.

MATERIALS AND METHODS

Experimental animals and drugs. Male Sprague-Dawley rats (200–220 g) and male albino mice (20–25 g) were obtained from Harlan Industries (Cumberland, IN) and housed five to six per cage with constant access to food and water. A 12 hr light–dark cycle and an ambient temperature of 68–72° were maintained in the animal quarters. Fenfluramine, (*m*-trifluoromethyl)-*N*-ethylamphetamine, was kindly furnished by A. H. Robins Research Laboratories (Richmond, VA). Norfenfluramine, the *N*-dealkylated derivative of fenfluramine, was a gift from Lilly Research Laboratories (Eli Lilly & Co., Indianapolis, IN). Drugs were injected intraperitoneally, except for the intravenous administration of fenfluramine (via tail vein), for a determination of its half-life in brain. Doses are expressed in terms of the hydrochloride salts.

Assay of 5-HT, 5-HIAA, and the activity of tryptophan hydroxylase in brain. 5-HT and 5-HIAA were assayed by fluorescence after derivatization with *o*-phthalaldehyde [12], according to the method of Curzon and Green [13]. Tryptophan hydroxylase activity was measured by a modification of the method of Gal and Patterson [14], as described in detail previously [15].

Synaptosomal uptake of 5-HT. A modification of the method of Snyder and Coyle [16] was used. Samples were homogenized in 100 vol. of 0.25 M sucrose in a Potter–Elvehjem tissue homogenizer. Homogenates were centrifuged at 1000 *g* for 10 min at 5°. The supernatant fluid containing synaptosomes and other cellular components was removed and gently mixed.

The incubation medium consisted of the following: 10 ml of Krebs–phosphate buffer (pH 6.8) containing 2 mg dextrose, 0.9 mg ascorbic acid, 0.2 mg ethylenediamine tetra-acetic acid, 0.12 mg pargyline hydrochloride and approximately 0.5 μCi of [^{3}H]-5-HT in a final concentration of 1×10^{-8} M. The tubes were kept in an ice bath until all additions were completed and then were placed in a Dubnoff metabolic shaker at 37°. Five minutes later, duplicate 0.2–ml aliquots of the crude synaptosomal preparation were added and samples were incubated for an additional 2.5 min. The samples were then immediately filtered through Millipore filters (AAWP02500) by suction. The filters were washed four times with 4 ml of saline, placed in 15 ml of ACS scintillation fluid (Amersham/Searle Corp., Arlington Heights, IL), and counted in a Nuclear Chicago Isocap/300 Liquid Scintillation System. Protein content of the crude synaptosomal preparations was determined by the method of Lowry *et al.* [17].

Brain levels of fenfluramine (FE) and norfenfluramine (NF). Brain levels of FE and its dealkylated metabolite, NF [18] were determined by a modification of the gas chromatographic assay used routinely in our laboratory for the determination of *p*-chloroamphetamine [19, 20]. Brains were homogenized in 10 ml of cold 0.6 N perchloric acid containing 10 μg of *m*-chloroamphetamine (MCA) as an internal standard. As described in detail by Sekerke *et al.* [19], FE, NF and MCA were extracted into benzene, reextracted into 0.1 N HCl, and finally extracted and concentrated into 500 μl cyclopentane. A 400–μl aliquot of the cyclopentane was transferred to a 1–ml Reactivial (Pierce Chemical Co., Rockford, IL) containing 5 μl trifluoroacetic acid (Pierce Chemical) and evaporated to dryness under vacuum. After the addition of 50 μl trifluoroacetic anhydride (Pierce Chemical) and 50 μl acetonitrile (Pierce Chemical), the vials were capped and the mixed samples were allowed to stand for 30 min at 60°. The solutions were evaporated to dryness under vacuum and the residues were dissolved in 5–10 μl cyclopentane containing 2% trifluoroacetic anhydride.

Analyses were performed on a Varian model 3700 gas chromatograph, which was equipped with a flame ionization detector, using a 200 cm by 2 mm inside diameter column containing 3% OV-17 on Gas-Chrom Q (Applied Science Laboratories, Inc., State College, PA.). The conditions were as follows: column temperature, 110°; injector and detector temperature, 150°; N_2 and H_2 flow rate, 30 cc/min; and O_2 flow rate, 150 cc/min. Under these conditions, the retention times of NF, FE and MCA were 4, 7 and 10 min respectively. Standard curves were determined for each experiment by adding known amounts of FE, NF and MCA to homogenates and determining the ratios of FE and NF peak heights/MCA peak heights.

Statistical analyses. The results were analyzed by means of two-tailed *t*-tests for experiments involving single or orthogonal comparisons and Dunnett's tests for experiments involving multiple comparisons with a single control mean. The half-life values were calculated from estimates of the first order rate constants, which were derived from least squares best-fitting straight line analyses of the natural logarithms of the tissue values.

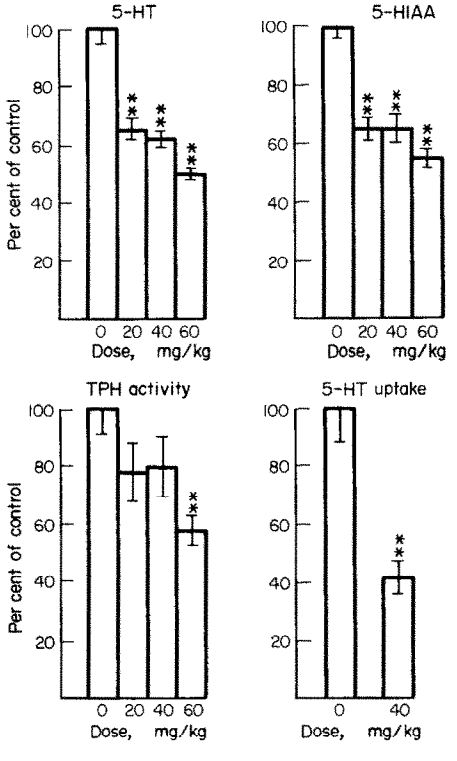

Fig. 1. Long-term effects of fenfluramine on 5-HT mechanisms in rats. Rats were injected i.p. with 0 (saline), 20, 40 or 60 mg/kg of fenfluramine and killed 2 weeks later for the determination of 5-HT and 5-HIAA in one half of the brain, 5-HT uptake in the hippocampus from the other half, and tryptophan hydroxylase (TPH) activity in the remaining tissue. The values shown represent the mean per cent of the saline control value ± S.E.M. of five to six animals. The mean ± S.E.M. of the control group was 0.58 ± 0.17 μg/g for 5-HT, 0.89 ± 0.03 μg/g for 5-HIAA, 1.71 ± 0.17 nmoles/mg/hr for TPH activity, and 0.39 ± 0.05 pmole/mg/2.5 min for 5-HT uptake. The double asterisk (**) indicates $P < 0.01$, compared to saline controls.

RESULTS

Long-term effects of fenfluramine on brain levels of 5-HT, 5-HIAA, tryptophan hydroxylase activity, and the synaptosomal uptake of 5-HT in rats. Rats were injected i.p. with 0(saline), 20, 40 or 60 mg/kg of fenfluramine and killed after 2 weeks. 5-HT and 5-HIAA were determined in one half of the brain, 5-HT uptake in the hippocampus from the other half, and tryptophan hydroxylase activitiy in the remaining tissue. As shown in Fig. 1, all three doses of fenfluramine produced significant reductions in the levels of 5-HT and 5-HIAA. The activity of tryptophan hydroxylase appeared to be less sensitive with a significant reduction only after the highest dose. Synaptosomal 5-HT uptake in hippocampus was markedly reduced after the intermediate dose of 40 mg/kg.

Long-term effects of fenfluramine on 5-HT parameters in mice. Mice were injected i.p. with 0(saline), 20, 40, 60 or 80 mg/kg of fenfluramine and killed 2 weeks later for the determination of 5-HT and 5-HIAA, tryptophan hydroxylase activity, and 5-HT uptake capacity (Fig. 2) as described above. In contrast to the marked reductions observed in rats after 20 mg/kg of fenflura-

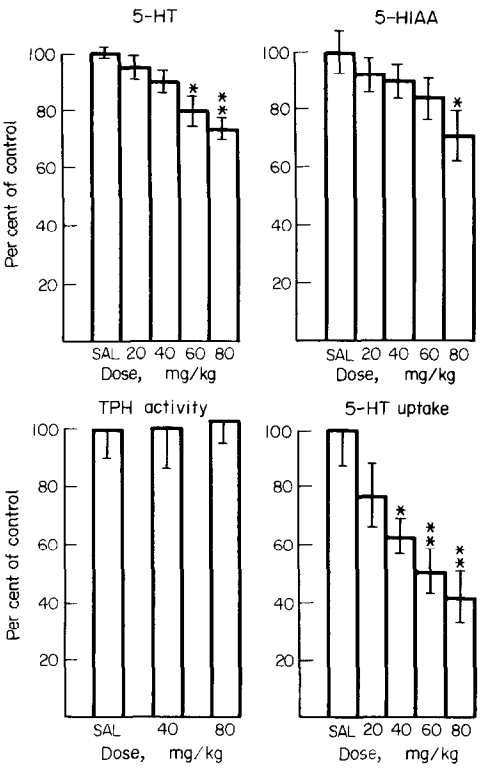

Fig. 2. Long-term effects of fenfluramine on 5-HT mechanisms in mice. Mice were injected i.p. with 0 (SAL), 20, 40, 60 or 80 mg/kg and killed 2 weeks later for the determination of 5-HT and 5-HIAA in one half of the brain, 5-HT uptake in the hippocampus from the other half, and TPH activity in the remaining tissue. The values shown represent the mean per cent of saline controls ± S.E.M. of five to six animals. The mean ± S.E.M. of the control group was 0.66 ± 0.01 μg/g for 5-HT, 0.38 ± 0.02 μg/g for 5-HIAA, 1.75 ± 0.18 nmoles/mg/hr for TPH activity, and 1.11 ± 0.15 pmoles/mg/2.5 min for 5-HT uptake. The asterisk (*) indicates $P < 0.05$, compared to saline controls. The double asterisk (**) indicates $P < 0.01$, compared to saline controls.

mine, brain levels of 5-HT and 5-HIAA were significantly reduced only after 60–80 and 80 mg/kg respectively. In addition, tryptophan hydroxylase activity was not changed even after the highest dose. In contrast to the relative insensitivity of these measures, hippocampal 5-HT uptake was markedly reduced after 40, 60 and 80 mg/kg. However, in mice, a dose of 80 mg/kg was required to reduce 5-HT uptake to 42 per cent of

Table 1. Effects of fenfluramine on brain levels of 5-HT and 5-HT uptake after 2 months in mice*

Group	5-HT (μg/g ± S.E.M.)	[^{3}H]-5-HT uptake (pmoles/mg/2.5 min)
Saline	0.83 ± 0.04	0.086 ± 0.005
Fenfluramine	0.77 ± 0.03	0.100 ± 0.008

* Mice were injected with either saline or 60 mg/kg of fenfluramine and killed 2 months later for the determination of 5-HT in one half of the brain and the uptake of [^{3}H]-5-HT by synaptosomes derived from the hippocampus from the other half. Values shown represent means ± S.E.M. of five to six animals.

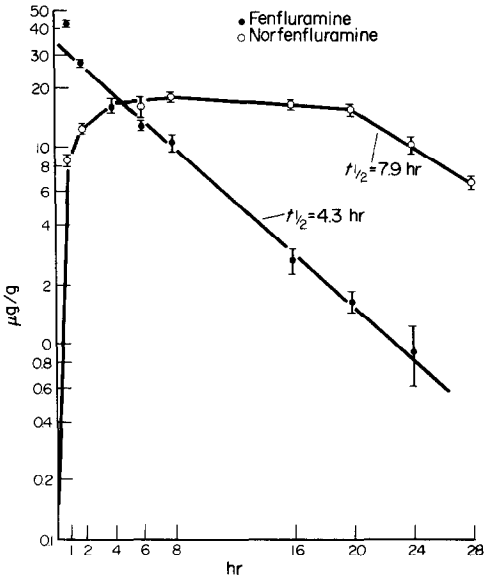

Fig. 3. Brain levels of fenfluramine and norfenfluramine at 1, 2, 4, 6, 8, 16, 20, 24 and 28 hr after the i.v. administration of 20 mg/kg of fenfluramine to rats. The values shown represent the means ± S.E.M. of four to five animals.

controls (Fig. 2), while this degree of reduction was produced by 40 mg/kg in rats (Fig. 1).

Additional groups of mice were killed 2 months after the i.p. administration of saline or 60 mg/kg of fenfluramine for the determination of 5-HT in one half of the brain and synaptosomal uptake of 5-HT in the hippocampus from the other half. As shown in Table 1, complete recovery of 5-HT and, surprisingly, of hippocampal 5-HT uptake was evident by 2 months.

Brain levels of fenfluramine and norfenfluramine at various times after the administration of fenfluramine to rats and mice. Rats and mice were injected i.v. with either saline or 20 mg/kg of fenfluramine and killed after 1, 2, 4, 6, 8, 16, 20 or 24 hr for the determination of brain levels of fenfluramine and norfenfluramine. Fenfluramine disappeared from rat brain (Fig. 3) in a monoexponential manner from 2 through 24 hr with a half-life ($T\frac{1}{2}$) of 4.3 hr. The brain level of norfenfluramine increased to a peak of 15–18 μg/g within 4 hr and remained within this range through 20 hr. The decline of norfenfluramine levels between 20 and 28 hr appeared to follow a monoexponential function with a $T\frac{1}{2}$ of approximately 7.9 hr. Apparently the rate of formation of norfenfluramine relative to its rate of disappearance had become negligible at these late times.

In mice (Fig. 4), the rate of disappearance of both compounds was faster with $T\frac{1}{2}$ values of 2.5 and 3.8 hr (estimated from the last three points) for fenfluramine and norfenfluramine respectively.

DISCUSSION

The present results show that mice are substantially less sensitive than rats to the long-term effects of a single dose of fenfluramine on central serotonergic parameters. Similar species differences have been demonstrated for PCA [9–11]. A dose of 20 mg/kg of fenfluramine produced 35 per cent reductions in the

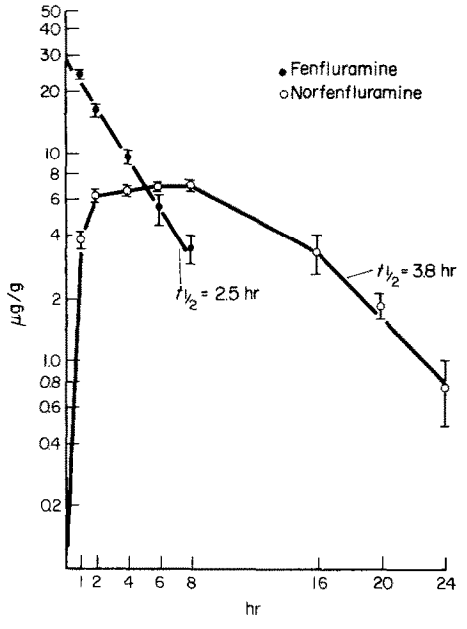

Fig. 4. Brain levels of fenfluramine and norfenfluramine at 1, 2, 4, 6, 8, 16, 20 and 24 hr after the i.v. administration of 20 mg/kg of fenfluramine to mice. The values shown represent the means ± S.E.M. of four to five animals.

brain levels of 5-HT and 5-HIAA after 2 weeks in rats, but no significant effects in mice. Although these data might suggest a qualitative difference between rats and mice with respect to their sensitivity to the neurotoxic effects of fenfluramine, extensive dose–response studies indicate that the species difference is a quantitative one. Thus, it was possible to produce significant decreases in 5-HT and 5-HIAA in mice; however, a dose of 80 mg/kg of fenfluramine (LD ≃ 35%) was required. Tryptophan hydroxylase activity was decreased significantly only after 60 mg/kg in rats and not after any of the doses used in mice, including the 80 mg/kg dose. These data clearly indicate that mice are markedly less sensitive than rats to the long-term, neurotoxic effects of fenfluramine.

A similar quantitative difference was evident with regard to the effects of fenfluramine on the uptake of [^{3}H]-5-HT by synaptosomes derived from hippocampus. A 58 per cent reduction occurred after the administration of 40 mg/kg of fenfluramine to rats and 80 mg/kg to mice. However, although fenfluramine was clearly less potent in mice, it was possible to demonstrate a marked, long-term reduction in the synaptosomal uptake of 5-HT in hippocampus. Of course, the apparent extreme sensitivity of this measure may primarily reflect the fact that the synaptosomes were obtained from hippocampus, which is among the brain regions most sensitive to the long-term effects of PCA in rats [5] and mice [11], as well as of fenfluramine in rats [25]. It is possible that additional regional studies of the levels of 5-HT, 5-HIAA, and tryptophan hydroxylase activity would reveal similar reductions in these parameters 2 weeks after fenfluramine administration to mice.

Interestingly, the levels of 5-HT in whole brain and 5-HT uptake capacity in hippocampus were completely recovered within 2 months after the administration of fenfluramine to mice. Although previous investigators have reported significant recovery of all four parameters of 5-HT function between 2 weeks and 2 months after the administration of fenfluramine to rats [2, 25] marked reductions are still present in certain brain regions, including hippocampus, at 2 months [25]. Thus, the complete recovery observed in mice in the present study, in particular the recovery of hippocampal 5-HT uptake, is in marked contrast to the comparatively less dramatic recovery found in rats. These data suggest that the rate at which the recovery process occurs is comparatively faster in mice.

Consistent with previous reports [2, 18], norfenfluramine was present in rat brain as a major metabolite of fenfluramine, and appeared to disappear from brain at a slower rate than the parent compound. The present results demonstrate a similar pattern in mice. More interesting was the observation that the half-lives of fenfluramine ($T\frac{1}{2}$ = 2.5 hr vs $T\frac{1}{2}$ = 4.3) and norfenfluramine ($T\frac{1}{2}$ = 3.8 hr vs $T\frac{1}{2}$ = 7.9 hr) were much shorter in mice than in rats. Thus, the rate of disappearance of fenfluramine and its active metabolite, norfenfluramine, is correlated with, and perhaps even determines the extent to which the administration of fenfluramine produces long-term neurotoxic effects on central 5-HT mechanisms in rats and mice.

A similar hypothesis has been formulated to explain the species differences in the long-term effects of PCA [10, 11, 21, 22]. Thus, in the rat, a species in which the major pathway for the metabolism of amphetamine involves *p*-hydroxylation [23], the addition of the chlorine atom in the para position of the aromatic ring results in a marked increase in half-life and the occurrence of neurotoxic activity. In mice, in which the deamination and *p*-hydroxylation of amphetamine are of equal importance, the substitution of a chlorine atom in the para position results in only a slight increase in half-life without neurotoxic activity. However, the continuous release of PCA from subcutaneously implanted ALZET Osmotic Minipumps for a period of 3 days produced a decrease in brain levels of 5-HT which lasted for at least 4 weeks [11]. This finding further supports the hypothesis that the insensitivity of mice to the neurotoxic effects of PCA is related to its relatively short half-life in this species. These results thus indicate that PCA-induced neurotoxicity is dependent upon a relatively long duration of exposure to the drug, and suggest that long-term effects of PCA in rats are due to a reduced rate of metabolism resulting from a blockade of *p*-hydroxylation rather than to the presence of the chlorine atom *per se*. Studies with *m*-chloroamphetamine agree with this interpretation since this compound is toxic to rats only after pretreatment with desmethylimipramine, which prevents its rapid *p*-hydroxylation [22].

The present results add additional support to this view. Thus, the substitution of a trifluoromethyl group in the meta position of the aromatic ring of amphetamine results in a marked increase in the half-life of the compound in rats and confers neurotoxic properties to the compound. In mice, the increase in half-life relative to that of the parent compound is considerably less than in rats, and the compound is only weakly neurotoxic.

Taken together, these results suggest that, in general, the extent to which a ring-substituted, halogenated derivative of amphetamine will produce neurotoxic effects in any given species is determined by the extent to which the substitution results in an impaired metabolism and increased half-life of the compound in brain. Interestingly, long-term, neurotoxic changes in striatal dopamine metabolism are found following the continuous administration of amphetamine itself [24, 25], again suggesting that time of exposure is the prepotent variable.

Acknowledgements—The authors wish to thank Ms. Donita Frazier for skillful technical assistance.

REFERENCES

1. J. A. Douarec and C. Neveu, in *International Symposium on Amphetamine and Related Compounds* (Eds.) E. Costa and S. Garattini, p. 75. Raven Press, New York (1970).
2. B. V. Clineschmidt, A. G. Zacchei, J. A. Totaro, A. B. Pflueger, J. C. McGuffin and T. I. Wishousky, *Ann. N.Y. Acad. Sci.* **305**, 222 (1978).
3. J. A. Harvey and S. E. McMaster, *Psychopharmac. Commun.* **1**, 217 (1975).
4. R. W. Fuller, H. D. Snoddy and S. K. Hemrick, *Proc. Soc. exp. Biol. Med.* **157**, 202 (1978).
5. E. Sanders-Bush, J. Bushing and F. Sulser, *J. Pharmac. exp. Ther.* **192**, 33 (1975).
6. E. Sanders-Bush, J. Bushing and F. Sulser *Eur. J. Pharmac.* **20**, 385 (1972).
7. R. W. Fuller and H. D. Snoddy, *Neuropharmacology* **13**, 85 (1974).
9. E. M. Gal, P. A. Christiansen and L. M. Yunger, *Neuropharmacology* **14**, 31 (1975).
9. A. Pletscher, G. Bartholini, H. Bruderer, W. P. Burkard and K. F. Gey, *J. Pharmac. exp. Ther.* **145**, 344 (1964).
10. K. W. Miller, E. Sanders-Bush and J. V. Dingell, *Biochem. Pharmac.* **20**, 500 (1971).
11. L. R. Steranka and E. Sanders-Bush, *Biochem. Pharmac.* **27**, 2033 (1978).
12. R. P. Maickel and F. P. Miller, *Analyt. Chem.* **38**, 1937 (1966).
13. G. Curzon and A. R. Green, *Br. J. Pharmac.* **39**, 653 (1970).
14. E. M. Gal and K. Patterson, *Analyt. Biochem.* **52**, 625 (1973).
15. L. R. Steranka, R. O. Bessent and E. Sanders-Bush, *Commun. Psychopharmac.* **1**, 447 (1977).
16. S. H. Snyder and J. T. Coyle, *J. Pharmac. exp. Ther.* **165**, 78 (1969).
17. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
18. R. B. Bruce and N. R. Maynard, *J. pharm. Sci.* **57**, 1173 (1968).
19. H. Sekerke, H. Smith, J. Bushing and E. Sanders-Bush, *J. Pharmac. exp. Ther.* **193**, 835 (1975).
20. L. R. Steranka and E. Sanders-Bush, *J. Pharmac. exp. Ther.* **206**, 460 (1978).
21. R. W. Fuller and C. W. Hines, *J. pharm. Sci.* **56**, 302 (1967).
22. R. W. Fuller, R. J. Schaffer, B. W. Roush and B. B. Molloy, *Biochem. Pharmac.* **21**, 1413 (1972).
23. L. G. Dring, R. L. Smith and R. T. Williams, *Biochem. J.* **116**, 425 (1970).
24. G. Ellison, M. S. Eison, H. S. Huberman, F. Daniel, *Science* **201**, 276 (1978).
25. L. R. Steranka and E. Sanders-Bush, *Neuropharmacology*, in press.