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ROLE OF *N*-METHYLTRANSFERASES IN THE NEUROTOXICITY ASSOCIATED WITH THE METABOLITES OF 1-METHYL-4-PHENYL-1,2,3,6-TETRAHYDROPYRIDINE (MPTP) AND OTHER 4-SUBSTITUTED PYRIDINES PRESENT IN THE ENVIRONMENT

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Abstract—Amine *N*-methyltransferases in the brains of humans, monkeys, mice, rabbits and rats, as well as two homogeneous enzymes isolated from rabbit liver, are capable of *N*-methylating 4-phenyl-1,2,3,6-tetrahydropyridine to 1-methyl-4-phenyltetrahydropyridine (MPTP), and 4-phenylpyridine to 1-methyl-4-phenylpyridinium ion (MPP^+). The product in each instance is a neurotoxin. The suggestion is offered that the known long half-life of methylpyridinium compounds in brain may be due to limitations in transport of such charged metabolites out of this tissue and to metabolic recycling of the desmethyl species by amine *N*-methyltransferases. The methylation of pyridines to quaternary amines is suggested as a means by which lipophilic compounds, having gained entrance to the cell, are converted to charged species that efflux much less readily.

A highly selective, irreversible neurotoxicity, responsible for a Parkinsonian syndrome in man and monkeys following the administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, **1**; Fig. 1) [1–4], results from the destruction of dopamine neurons in the substantia nigra. Since monoamine oxidase inhibitors block both the development of the syndrome and the conversion of MPTP to 1-methyl-4-phenylpyridinium ion (MPP^+, **2**; Fig. 1), it has been suggested that the quaternary pyridinium ion metabolite may play a pivotal role in the development of the Parkinsonian syndrome [5–10]. The sequence of events in this situation is parallel to a general concept in prodrug design formulated by Bodor and his colleagues [11, 12] who have pointed to a wide variety of lipophilic dihydropyridine prodrugs that are rapidly absorbed into the brain and then trapped there as quaternary pyridinium ions with remarkably long biological half-lives. Bodor was able to demonstrate that high concentrations of the desired therapeutic target drug could be attained

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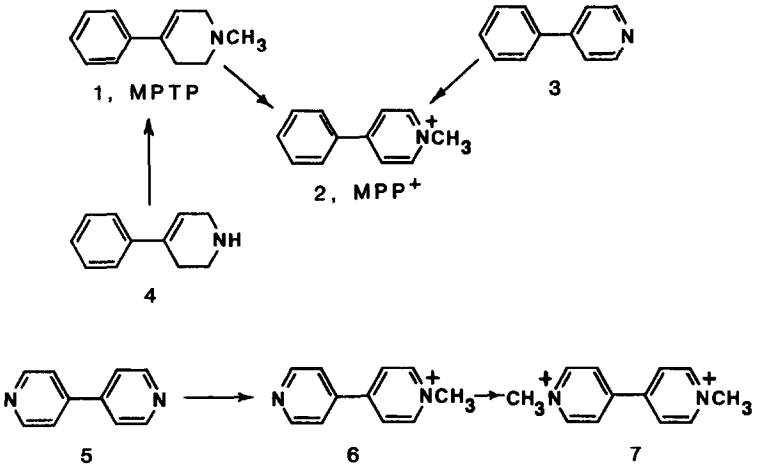

Fig. 1. Proposed metabolic transformation of 4-phenylpyridines and dipyridyl to bioactive pyridinium ions.

in brain despite significant oxidation of the dihydropyridine prodrug to the *N*-alkylpyridinium ion in both brain and liver.

Another means of converting lipophilic pyridines to quaternary pyridinium compounds that could be "trapped" in brain would involve methylation of the aromatic nitrogen. Extracts of liver and brain are known to contain enzymes catalyzing the methylation of primary and secondary amines [13] and the pyridine nitrogen of nicotine [14]. Recently, two amine *N*-methyltransferases of very broad substrate range have been prepared from rabbit liver in homogeneous form [15, 16]. With the availability of these characterized enzymes it became possible to evaluate both the methylation of 4-phenylpyridine (**3**) to the neurotoxin MPP^+, and the more general phenomenon of the conversion by methylation of environmental pyridines to toxic quaternary pyridinium compounds.

MATERIALS AND METHODS

4 - Phenyl - 1,2,3,6 - tetrahydropyridine hydrochloride, MPTP hydrochloride, 4-phenylpyridine, and 4,4′-dipyridyl hydrochloride were used as obtained from the Aldrich Chemical Co. [^{3}H-CH_3]-*S*-Adenosyl-L-methionine and [^{3}H-CH_3]-*N*-methyl-4-phenylpyridine were from New England Nuclear.

The two amine *N*-methyltransferases, A and B, were homogeneous preparations from frozen New Zealand rabbit liver (Pel Freeze Biologicals) [15, 16]. Rabbit, mouse and rat brains were removed from the animal and immediately homogenized in 3 vol. (w/v) of 10 mM potassium phosphate at pH 7.8. Frozen brain from a cynomolgus monkey (Charles River Primate Center) and a frozen coronal section of human brain* were maintained at −80° until homogenization in the same buffer. Each homogenate was centrifuged at 10,000 *g* and 100,000 *g* as described for the rabbit liver enzymes [16]. The resultant extract was dialyzed overnight against the same buffer and concentrated about 4-fold with an Amicon PM10 membrane. An extract of rabbit brain was subjected to DEAE-cellulose chromatography [16] and used for kinetic studies.

Enzyme activity of *N*-methyltransferases A and B, and of the partially purified rabbit brain extract, were assessed for 1 mM tryptamine and 1 mM 4-phenyltetrahydropyridine by a previously described procedure [16]. With 1 mM 4-phenylpyridine (**3**) and 1 mM 4,4′-dipyridyl (**5**) as acceptor substrates under otherwise standard conditions [16], the reaction was terminated with 0.5 ml of 0.5 M boric acid followed by addition of 50 μl of the ion-pair reagent, 0.1 M sodium dodecyl sulfate (SDS). The mixture was extracted with 3 ml of ethylacetate with vigorous shaking for 2 min. Then, 10 ml of Econofluor was added with a gentle swirling motion; the overall efficiency of the extraction was 89% when a commercially available sample of [^{3}H-CH_3]-labeled **2**, was used as standard. Observed radioactivity was corrected for background radioactivity, always less than 10% of the activity with all compounds, by using an identical incubation mixture free of exogenous methyl group acceptor.

Enzyme activity of each of the dialyzed brain extracts (Table 2) was measured by the formation of radioactive N-methylated product following incubation at 37° for 60 min of extract in a total volume of 200 μl containing 0.1 M Tris–HCl at pH 7.8, 34 μM [^{3}H-CH_3]-*S*-adenosyl-L-methionine (about 370,000 cpm) and 1 mM amine. With tryptamine or **4** as acceptors, the reaction was terminated by adding 0.5 ml of 0.5 M potassium borate at pH 9.5, followed by 6 ml of 3% isoamyl alcohol in toluene. After vigorous shaking for 1 min, a 4-ml aliquot of the organic phase was removed and evaporated under reduced pressure [13]. Each determination was corrected by subtracting radioactivity in a control sample (as high as 200 cpm) which was free of exogenous methyl group acceptor.

With 4-phenylpyridine as acceptor, brain extracts were incubated in a total volume of 0.5 ml containing 0.1 M Tris–HCl at pH 7.8, 34 μM [^{3}H-CH_3]-*S*-adenosyl-L-methionine (0.8 μCi) and 1 mM 4-phenylpyridine. Following incubation at 37° for 60 min, the reaction was stopped with 1 ml of 0.5 M boric acid. Control incubations contained enzyme subjected to 2 min in a boiling water bath. To each sample, 150 μl of 0.1 M SDS, followed by 8 ml ethyl acetate, were added and the resulting mixture was agitated with a vortex mixer. After brief centrifugation, the upper layer was taken to dryness under reduced pressure and the residue dissolved in 200 μl of water–acetonitrile (1:1). A 70-μl aliquot was subjected to analysis by HPLC, using a C-18 reversed phase column (Altex Ultrasphere-ODS 0.46 × 25 cm) at a flow rate of 1 ml/min with a mobile phase of 2 g KH_2PO_4, 0.5 g triethylamine, 150 ml water and 350 ml acetonitrile. Column fractions were collected every 15 sec directly into scintillation vials that were measured for radioactivity after addition of 10 ml Hydrofluor.

With **4** as acceptor, the product, MPTP, was identified by the same method used for 4-phenylpyridine except that the reaction was stopped with 1 ml of 0.5 M potassium borate and extracted with two portions, 3 ml each, of ethyl acetate before chromatography using the same HPLC system.

Protein was determined with bicinchoninic acid [17] using bovine serum albumin (Pentex) as standard.

RESULTS

With either homogeneous amine *N*-methyltransferase A or B from rabbit liver, tryptamine, 4-phenyltetrahydropyridine, 4-phenylpyridine and 4,4′-dipyridyl were all effective substrates (Table 1). Since methylation of these compounds can occur, the question arose as to whether brain had this capacity. Although there are reports [13, 14] of *N*-methyltransferase activity toward tryptamine and nicotine in brain extracts, the actual amount of enzyme appears to be quite low, in part because of the presence of an inhibitor common to methyl-

* Brain was obtained from an otherwise normal, 25-year-old, woman auto accident victim and had been donated to the Brain Bank of the National Institute of Mental Health maintained at St. Elizabeth's Hospital, Washington, DC.

Table 1. Apparent kinetic constants for *N*-methyltransferase activity from rabbit

Substrate	Brain preparation		Transferase A		Transferase B	
	K'_m (mM)	V_{max}*	K'_m (mM)	V_{max}*	K'_m (mM)	V_{max}*
Tryptamine†	0.3	6.4	0.3	830	0.1	920
4-Phenyltetrahydropyridine†	0.8	2.0	0.2	550	0.3	52
4-Phenylpyridine‡	0.3	0.71	0.4	860	0.7	92
4,4'-Dipyridyl‡			0.3	161	4.6	52

* Expressed in pmoles·min^{-1}·(mg protein)$^{-1}$.
† At pH 8.5.
‡ At pH 7.8.

transferases [18], and is generally not easily measurable prior to dialysis. When tested with rat and mouse tissue, commercially obtained frozen brains appeared to be half as active as fresh preparations from the NIH colony. Because fresh tissue was not readily obtainable, frozen samples of human and monkey brain had to be used. With dialyzed and concentrated preparations from human, monkey, mouse, rabbit and rat brain, *N*-methyltransferase activity was observed with a primary aliphatic amine (tryptamine), a secondary amine (**4**) and a heterocyclic amine (**3**) (Table 2). Because of the reservations expressed with regard to frozen tissue, the absolute values of activity for each brain extract should be considered only as minimal estimates. Apparent kinetic constants have been measured with rabbit brain enzyme that has been subjected to chromatography on DEAE-cellulose [15, 16]. Similar K_m values were obtained for the homogenous liver transferases and for the relatively impure brain preparations (Table 1).

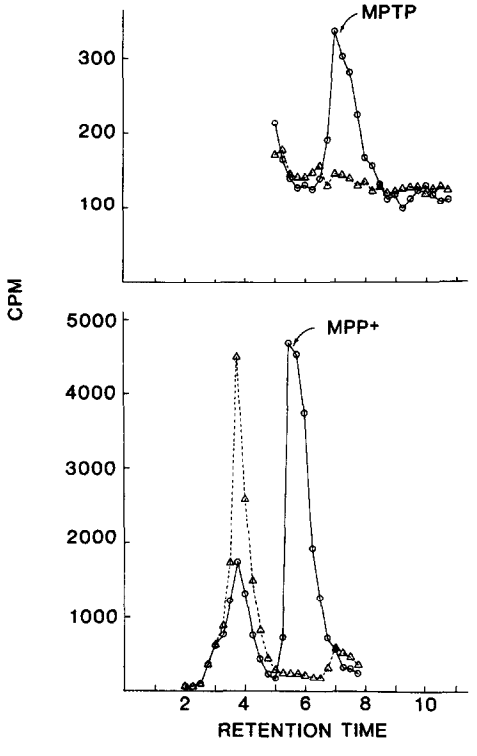

Fig. 2. [^{3}H]-HPLC radiochromatograms showing the formation of the neurotoxins MPTP and MPP$^+$ by brain extracts. (A) Top panel: formation of MPTP from 4-phenyl-1,2,3,6-tetrahydropyridine using a preparation from monkey brain (B) Bottom panel: formation of MPP$^+$ from 4-phenylpyridine using a mouse brain preparation. Key: (—○—) chromatogram obtained from the incubation of the test sample, and (--△--) chromatogram from the incubation using identical conditions but with heat-inactivated enzyme.

The identity of the methylation products was verified by HPLC-radio chromatograms following incubation of brain extracts with methyl-labeled adenosylmethionine and acceptor. This is shown for monkey and mouse brain (Fig. 2), although each of the other brain extracts gave the same qualitative result. 4-Phenylpyridine yielded a metabolite peak at 5.6 min that was identical to that produced by a synthetic reference standard of MPP$^+$; this chromatographic peak was not detected when heat-inactivated enzyme was used (Fig. 2B). The methylated product from 4-phenyl-1,2,3,6-tetrahydropyridine, shown for a monkey brain preparation in Fig. 2A, yielded a peak at 7.2 min that was identical to that obtained with a reference standard of MPTP.

DISCUSSION

Clearly, each of the tested pyridine derivatives is capable of being methylated by enzymes present in brain. The ratio of activity for 4-phenylpyridine to tryptamine appears to fall into two groups with human (15:1), monkey (17:1) and mouse (21:1) brain constituting one and rat (2:1) and rabbit (2:1) brain the other. The species with a high ratio of activity for these substrates are, of course, those with known high susceptibility to the neurotoxic effects of MPTP, a relationship that is probably only coincidental but worth noting.

Although the metabolism of MPTP is complex, one of the major metabolic pathways is alpha-oxidation leading to a 2-pyridone and the formation of 4-phenyl-1,2,3,6-tetrahydropyridine [19, 20]. When radiolabeled MPTP was administered to monkeys, the brain levels of radioactivity were found to drop slowly over a 20-day period with only slightly faster clearances found for other test animals (half-life: monkey > guinea pig = mouse > rat) [21]. The long half-life seen in monkey has been attributed to slow

Table 2. *N*-Methyltransferase activity in extracts of brain

Source	Δ'-Methyltransferase activity					
	Tryptamine		Substrate 4-Phenyltetrahydropyridine		4-Phenylpyridine	
	[pmoles·hr^{-1}·(mg protein)$^{-1}$]	[pmoles·hr^{-1}·(g tissue)$^{-1}$]	[pmoles·hr^{-1}·(mg protein)$^{-1}$]	[pmoles·hr^{-1}·(g tissue)$^{-1}$]	[pmoles·hr^{-1}·(mg protein)$^{-1}$]	[pmoles·hr^{-1}·(g tissue)$^{-1}$]
Human*	0.23	3.4	0.17	2.5	3.7	53
Monkey*	0.30	2.3	2.9	22	3.6	40
Mouse	1.2	14	4.3	51	23	290
Rabbit	7.8	130	5.4	89	17	280
Rat	1.3	15	6.8	83	2.7	33

* Prepared from frozen tissue.

egress of the *N*-methyl-4-phenylpyridium ion species from brain, but the recycling of N-demethylated products by amine *N*-methyltransferases may also contribute to the unexpectedly long residence in this tissue. This transformation may account as well for the *in vitro* neurotoxicity that has been reported for 4-phenyl-1,2,3,6-tetrahydropyridine which was at least as active [22], if not more active [23], than MPTP despite belief that *N*-methyl-4-phenylpyridinium ion is the primary toxic agent [5–10]. If the pyridinium ion *is* the primary agent, the N-methylation of 4-phenylpyridine in brain, observed here with brain extracts, may account for the neurotoxicity of 4-phenylpyridine that had been reported [24].

The relationship between methylation and toxicity is also emphasized by methyltransferase activity with 4,4'-dipyridyl (**5**) to yield the monomethyl precursor (**6**) of paraquat (**7**). The two compounds are equally toxic when administered orally and yield pulmonary hemorrhage in acute tests [25]. Since 4,4'-dipyridyl and 4-phenylpyridine are both present in tobacco smoke, the formation of quaternary pyridinium ion metabolites by the *N*-methyltransferases may be linked to the lung damage that is associated with tobacco use.

The two *N*-methyltransferases may also prove useful to the medicinal chemist in the design of new prodrugs of the pyridinium ion class that are to be directed to the brain. Just as Bodor and coworkers have demonstrated that dihydropyridines can serve as prodrugs to pyridinium ions active in the CNS by using an oxidation biotransformation [11, 12], simple pyridines, quinolines and the like could serve as prodrugs to the corresponding target quaternary compounds, using a pathway in which amine *N*-methyltransferases participate.

REFERENCES

1. G. C. Davis, A. C. Williams, S. P. Markey, M. H. Ebert, E. D. Caine, C. M. Reichert and I. J. Kopin, *Psychiat. Res.* **1**, 249 (1979).
2. J. W. Langston, P. Ballard, J. W. Tetrud and I. Irwin, *Science* **219**, 979 (1983).
3. R. S. Burns, C. C. Chiueh, S. P. Markey, M. H. Ebert, D. M. Jacobowitz and I. J. Kopin, *Proc. natn. Acad. Sci. U.S.A.* **80**, 4546 (1983).
4. J. W. Langston, L. S. Forno, C. S. Rebert and I. Irwin, *Brain Res.* **292**, 390 (1984).
5. R. E. Heikkila, L. Manzino, F. S. Cabbat and R. C. Duvoisin, *Nature, Lond.* **311**, 467 (1984).
6. J. W. Langston, I. Irwin, E. B. Langston and L. S. Forno, *Science* **225**, 1480 (1984).
7. S. P. Markey, J. N. Johannessen, C. C. Chiueh, R. S. Burns and M. A. Herkenham, *Nature, Lond.* **311**, 464 (1984).
8. J. A. Javitch and S. H. Synder, *Eur. J. Pharmac.* **106**, 455 (1985).
9. W. Gessner, A. Broosi, R. J. Shen and C. W. Abell, *Fedn Eur. Biochem. Soc. Lett.* **183**, 345 (1985).
10. K. Chiba, A. Trevor and N. Castagnoli, Jr., *Biochem. biophys. Res. Commun.* **120**, 547 (1984).
11. N. Bodor and J. W. Simpkins, *Science* **221**, 65 (1983).
12. N. Bodor, *Meth. Enzym.* **112**, 381 (1985).
13. J. M. Saavedra, J. T. Coyle and J. Axelrod, *J. Neurochem.* **20**, 743 (1973).
14. K. C. Cundy, C. S. Godin and P. A. Crooks, *Biochem. Pharmac.* **34**, 281 (1985).

15. S. S. Ansher and W. B. Jakoby, *J. biol. Chem.* **261**, 3996 (1986).
16. S. S. Ansher and W. B. Jakoby, *Meth. Enzym.*, in press.
17. P. K. Smith, R. I. Crohn, G. T. Hermann, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson and B. C. Klink, *Analyt. Chem.* **150**, 76 (1985).
18. E. S. Lyon, P. McPhie and W. B. Jakoby, *Biochem. biophys. Res. Commun.* **108**, 846 (1982).
19. J. K. Baker, R. F. Borne, W. M. Davis and I. W. Waters, *Biochem. biophys. Res. Commun.* **125**, 484 (1984).
20. J. Weissman, A. Trevor, K. Chiba, L. A. Peterson, P. Caldera, N. Castagnoli, Jr. and T. Baillie, *J. med. Chem.* **28**, 997 (1985).
21. J. N. Johannessen, C. C. Chiueh, R. S. Burns and S. P. Markey, *Life Sci.* **36**, 219 (1985).
22. R. E. Heikkila, S. K. Youngster, L. Manzino, F. S. Cabbat and R. C. Duvoisin, *J. Neurochem.* **44**, 310 (1985).
23. D. Cavalla, M. Hadjiconstantinou, H. E. Laird and N. H. Neff, *Neuropharmacology* **24**, 585 (1985).
24. S. H. Synder and R. J. D'Amato, *Nature, Lond.* **317**, 198 (1985).
25. D. F. Croce and R. D. Kimbrough, *J. Toxic. environ. Hlth* **10**, 363 (1982).