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On the Mechanisms Underlying 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine Neurotoxicity: The Effect of Perinigral Infusion of 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine, its Metabolite and Their Analogs in The Rat[1]

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ABSTRACT

The discovery that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes parkinsonism in humans and other primates by selective destruction of substantia nigra dopaminergic neurons has spurred research to define the mechanisms underlying its toxicity. To avoid variables such as tissue distribution, extracerebral metabolism and blood-brain barrier permeability, the authors studied the neurochemical and morphologic effects of direct perinigral infusions of various concentrations of MPTP, its metabolites and analogs in the rat. MPTP, in the highest dose used, 1000 nmol, decreased dopamine and its metabolites in ipsilateral striatum by ~75%, whereas 3,3-dimethyl-MPTP (which is oxidized to 1,3,3-trimethyl-4-phenyl-2,3-dihydropyridinium cation but not to a pyridinium species) had no effect. The 2,2 and 3,3-dimethyl analogs of 1-methyl-4-phenyl-2,3-dihydropyridinium cation which also cannot be oxidized to pyridinium species, reduced striatal dopamine, suggesting that these compounds are toxic in their own right. 1-Methyl-4-phenylpyridinium cation (MPP^+) and its 4-(4-fluorophenyl) and 4-(2-pyridyl) analogs that have less negative reduction potentials than MPP^+, were most potent in decreasing striatal dopamine and metabolites, with MPP^+ being 5 to 10 times more effective than its two analogs and ~100 times more potent that MPTP and the two dimethyl 1-methyl-4-phenyl-2,3-dihydropyridinium cation analogs. These findings suggest that MPP^+ is ultimately responsible for MPTP toxicity but does not act *via* oxidant stress mechanisms. All compounds tested except MPTP produced large lesions around the infusion site, which nonselectively destroyed the entire substantia nigra and adjacent ascending noradrenergic and serotonergic pathways. MPTP produced smaller nonspecific lesions at the center of the infusion site but caused selective loss of dopamine neurons in distant regions of substantia nigra, suggesting that it alone, among the compounds tested, is selectively toxic. The lack of a similar selective effect by MPP^+ can be explained by its high local concentrations leading to severe local damage before significant diffusion can take place.

Systemic administration of MPTP causes acute and irreversible Parkinson-like illness in man and monkey. (See reviews by Langston, 1985; Snyder and D'Amato, 1986.) This finding stimulated research for possible toxic causes of Parkinson's disease. Because experimental MPTP toxicity is prevented by inhibitors of monoamine oxidase B (Heikkila *et al.*, 1984; Langston *et al.*, 1984), it is now believed that MPTP neurotoxicity is due to its enzymatic oxidation (fig. 1), initially yielding $MPDP^+$ (Chiba *et al.*, 1984, 1985; Fritz *et al.*, 1985), which is further oxidized to MPP^+ (Markey *et al.*, 1984). MPP^+ itself is toxic when injected directly into rodent brain (Bradbury *et al.*, 1985, 1986; Heikkila *et al.*, 1985c) or when added to dopaminergic neuronal explants in culture (Mytilineou *et al.*, 1985). MPP^+ is a structural relative of paraquat (fig. 1), a herbicide that is cytotoxic by virtue of "redox cycling" catalysis (*i.e.*, accepting single electrons from reductases and passing them directly to O_2, thereby short-circuiting respiration). This results in the production of superoxide and H_2O_2 in levels that exceed the protective antioxidant systems of the cell (oxidant stress). A similar mechanism of toxicity was originally proposed for MPP^+ (Johannessen *et al.*, 1985, 1986). More recently, it was

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ABBREVIATIONS: MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; $MPDP^+$, 1-methyl-4-phenyl-2,3-dihydropyridinium cation; MPP^+, 1-methyl-4-phenylpyridinium cation; $MPyP^+$, 1-methyl-4-(2-pyridyl)pyridinium cation; SN, substantia nigra; DA, dopamine; DOPAC, dihydroxyphenylacetic acid; HVA, homovanillic acid; NE, norepinephrine; 5-HT, 5-hydroxytryptamine (serotonin); 5-HIAA, 5-hydroxyindolacetic acid; EIMS, electron impact mass spectra; NMR, nuclear magnetic resonance.

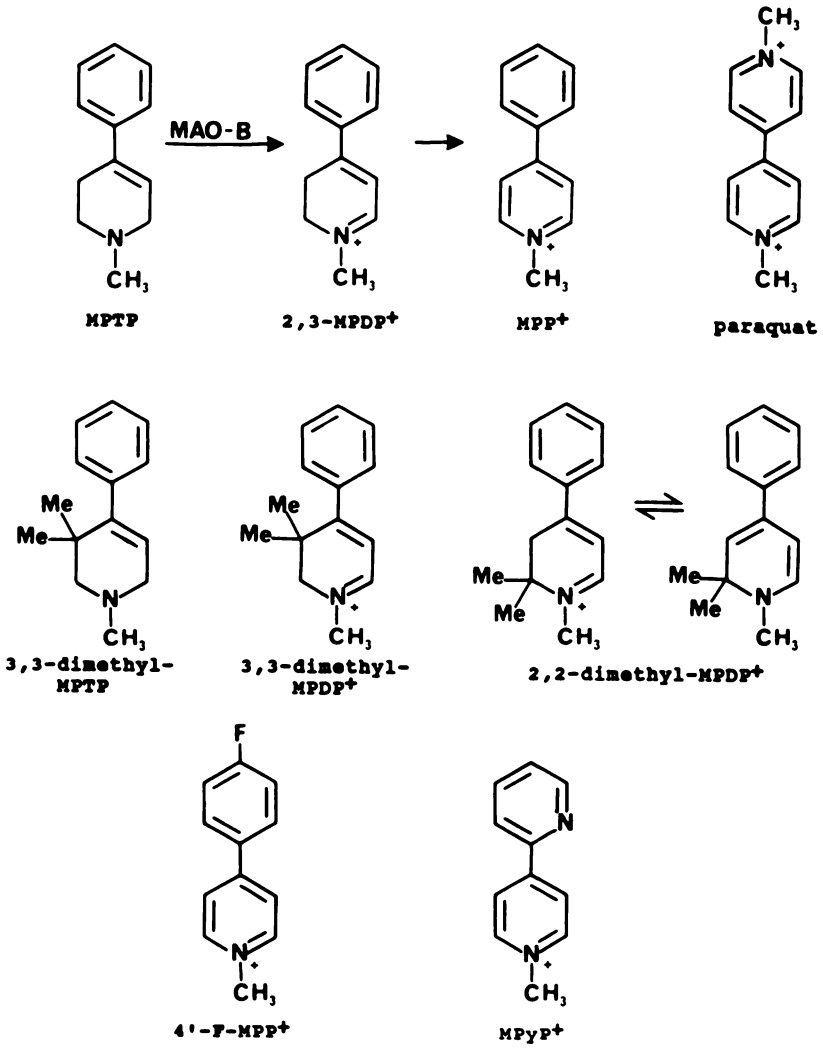


Fig. 1. Structural formulae of MPTP, its metabolites and analogs. MAO-B, monoamine oxidase B.

suggested that MPP^+ toxicity is due to its inhibition of mitochondrial respiration (Nicklas *et al.*, 1985; Vyas *et al.*, 1986). Another possible culprit is the electrophilic intermediate, $MPDP^+$, which might cause toxicity by acting as an alkylating agent of enzymes or other cellular components. $MPDP^+$ is indeed neurotoxic (Sun *et al.*, 1985), but it is unclear whether toxicity is due to its direct action or to its facile conversion to MPP^+. Although Javitch *et al.* (1985) proposed the selective uptake of MPP^+ by dopaminergic nigrostriatal neurons to explain the specific toxicity of MPTP, many of the basic mechanisms that underlie neurotoxicity after systemic MPTP administration remain unknown.

We have taken a chemical structure-activity approach to investigate these mechanisms by examining the neurotoxicity produced by the perinigral infusion of MPTP, its metabolite, MPP^+, and their analogs in the rat. We presented preliminary evidence that MPP^+, rather than $MPDP^+$, is the main toxin by showing that the toxicity of MPP^+ is at least 100 times greater than that of 3,3-dimethyl-$MPDP^+$ (Sayre *et al.*, 1986b). 3;3-Dimethyl-$MPDP^+$ is the geminal 3,3-dimethyl analog of $MPDP^+$ (fig. 1), which undergoes similar electrophilic and reduction-reoxidation chemistry as does $MPDP^+$, but, unlike the latter, cannot be oxidized to a pyridinium compound (Sayre *et al.*, 1986a). However, 3,3-dimethyl-$MPDP^+$ is "locked" in its electrophilic form and cannot exist in equilibrium with a neutral enamine, as is the case with $MPDP^+$. For this reason, we examined 2,2-dimethyl-$MPDP^+$, another $MPDP^+$ analog (fig. 1), which also cannot be oxidized to a pyridinium ion but which can exist in equilibrium with a neutral enamine (fig. 1). We also tested the neurotoxicity of 3,3-dimethyl-MPTP, which can be oxidized by monoamine oxidase B to 3,3-dimethyl-$MPDP^+$ (Riachi *et al.*, in press) but not further, and that of two MPP^+ analogs for which the corresponding MPTP analogs have been found to be nontoxic upon systemic administration to mice (Heikkila *et al.*, 1985b; L. M. Sayre, P. K. Arora, I. Irwin and J. W. Langston, unpublished). These two MPP^+ analogs, 4'-F-MPP^+ and $MPyP^+$ (Fig. 1), have lower oxidation potentials than MPP^+ (P. K. Arora, S. C. Feke, F. L. Urbach and L. M. Sayre, unpublished) and should thus be more neurotoxic if "redox cycling" is the primary mechanism responsible for the cytotoxic action of MPP^+.

We appreciate the biologic importance of the fact that MPTP causes profound and selective toxicity when given systemically in small quantities to primates, yet there are too many unknowns between the point of systemic MPTP administration and the action of its toxic metabolites(s) on the nigrostriatal system to permit valid mechanistic conclusions based on observed chemical structure-activity trends. We thus used the direct perinigral infusion method in an attempt to provide reliable chemical structure-neurotoxicity data on the various compounds without having to take into account their systemic

metabolism, their ability to traverse the blood-brain barrier and variations in their regional brain concentrations.

In this report, we present neurotoxicity data on MPTP, its metabolite and several analogs after direct infusion of these compounds into the rat SN. Neurotoxicity was assessed biochemically by measuring levels of DA and metabolites, DOPAC and HVA, in the ipsilateral striatum and NE and 5-HT and its metabolite, 5-HIAA, in the ipsilateral frontal cortex. Neurotoxicity was also assessed by microscopic examination of histologic sections of the toxin infusion sites in the SN. Although the rat is known to be resistant to the neurotoxic effects of systemic MPTP, we believe that this is mostly due to differences in the total body distribution and metabolism of MPTP (Kalaria *et al.*, 1987). This conclusion is supported by our present results showing that MPTP infusion into the rat SN causes relatively selective destruction of dopaminergic neurons without major damage to other neurons and glia in the vicinity of the infusion site.

Materials and Methods

Chemicals. MPTP·HCl, MPP^+ I^- and 3,3-dimethyl-$MPDP^+$ Br^- were synthesized as described before (Sayre *et al.*, 1986a,b). 3,3-Dimethyl-MPTP·HCl was prepared by addition of phenyllithium to the known 1,3,3-trimethyl-4-piperidone (Katvalyan and Mistryukov, 1968), followed by HCl-mediated dehydration by standard methods (Schmidle and Mansfield, 1956a.) This compound was recently synthesized using a similar strategy by Fries *et al.* (1986). 2.2-Dimethyl-$MPDP^+$ Br^- was prepared by a standard H_2O_2, trifluoroacetic anhydride, HBr oxidation sequence (Gessner *et al.*, 1984) from 2,2-dimethyl-MPTP; the latter was obtained by HCl-mediated dehydration of the known 1,2,2-trimethyl-4-phenyl-4-piperidinol (Nazarov and Makin, 1957). $MPyP^+$ methylsulfate was prepared from 2,4′-bipyridine, as described by Fischer and Summers (1980), and converted to $MPyP^+$ Cl^- by anion exchange. Addition of 4-fluorophenylmagnesium bromide to 1-methyl-4-piperidone (both from Aldrich Chemical Co., Milwaukee, WI), followed by HCl-mediated dehydration, according to standard methods (Schmidle and Mansfield, 1956a), gave 1-methyl-4-(4-fluorophenyl)-1,2,3,6-tetrahydropyridine. This compound was subjected to exhaustive dehydrogenation (N-demethylating) (Schmidle and Mansfield, 1956b) to 4-(4-fluorophenyl)pyridine, and the latter was remethylated with methyl iodide in acetone to give 4′-F-MPP^+ I^-.

All intermediates and final products were characterized by 200 MHz NMR. Final products were further characterized either by combustion analysis or by exact-mass (high-resolution) EIMS. Pertinent data concerning chemical purity are presented in the Appendix.

Animals. Adult male Wistar rats (about 250 g) were used in all experiments. Rats were housed 4 to 6 per cage under diurnal light conditions with unlimited access to food and water. Stereotaxic microinfusions of the neurotoxins were performed under chloral hydrate anesthesia (400 mg/kg). Details of this procedure have been previously reported (Harik *et al.*, 1982). In brief, a glass microelectrode (~25 μ tip diameter) attached to a Hamilton syringe was stereotaxically introduced into the SN (3 mm posterior and 2 mm lateral to the bregma and 7.5 mm below the dura). The neurotoxin, dissolved in 2 μl of isotonic saline, was slowly infused over a period of 5 min. Approximately equal numbers of right and left lesions were made. The rats were returned to their cages after recovering from anesthesia and kept for ~3 weeks before they were decapitated and their striata and samples of the dorsomedial frontal cerebral cortex quickly dissected and frozen for later assays of their DA, DOPAC, HVA, NE, 5-HT and 5-HIAA content by high-performance liquid chromatography with electrochemical detection. Results, in nanograms per gram wet weight of tissue, from the ipsilateral striatum and cerebral cortex of each rat were compared with those of the contralateral side, which acted as an internal control.

In selected rats, midbrains were fixed by immersion in 10% buffered formalin solution, dehydrated and embedded in paraffin. Twenty-micron sections were stained with either hematoxylin and eosin or with cresyl violet for morphologic assessment of the lesion. In another experiment, rats were given perinigral infusions of either 1000 nmol of MPTP ($n = 3$) or 4 nmol of MPP^+ ($n = 3$). Three weeks later, the rats were given pargyline (50 mg/kg i.p.) and killed by decapitation within a few hours. Their midbrains were frozen on dry ice, and 10-μ cryostat sections were alternately processed for catecholamine histofluorescence (de la Torre, 1980) and thionin staining.

Results

Neurochemical findings. The effects of perinigral infusion of various concentrations of MPTP, MPP^+ and their analogs on the levels of DA and its metabolites in the ipsilateral striatum are shown in table 1. MPTP, at a maximal dose of 1000 nmol, depletes striatal DA by ~75% and DOPAC and HVA by ~60%. 3,3-Dimethyl-MPTP, at a concentration as high as 1000 nmol, is ineffective in reducing DA and its metabolites in the striatum (table 1). Thus, 3,3-dimethyl-MPTP, which can be oxidized to 3,3-dimethyl-$MPDP^+$ by monoamine oxidase (Riachi *et al.*, in press) but which cannot be fully oxidized to a pyridinium species, is not toxic to SN dopaminergic neurons. However, 3,3-dimethyl-$MPDP^+$ is quite effective in reducing striatal DA and its metabolites, and 2,2-dimethyl-$MPDP^+$, another analog of $MPDP^+$, is even more toxic than 3,3-dimethyl-$MPDP^+$ (table 1).

Of the pyridinium species that were tested, MPP^+ is by far the most toxic. About 50% depletion of striatal DA is induced by the perinigral infusion of 4 nmol of MPP^+ (table 1). Both of its analogs, 4′-F-MPP^+ and $MPyP^+$, are about 10 times less effective.

The effects of the perinigral infusion of these toxins on the ascending noradrenergic and serotonergic axons that innervate the frontal cerebral cortex are shown in table 2. Both MPTP and 3,3-dimethyl-MPTP are not effective, even at the highest concentrations used, in depleting NE, 5-HT and 5-HIAA in the ipsilateral cerebral cortex. On the other hand, 3,3-dimethyl-$MPDP^+$, 2,2-dimethyl-$MPDP^+$ and all the pyridinium compounds are highly effective in decreasing cortical NE, 5-HT and 5-HIAA, especially at higher concentrations (table 2). These results indicate that only MPTP may be selective in its toxic effects on SN dopaminergic neurons, whereas all the positively charged compounds destroy dopaminergic neurons and ascending noradrenergic and serotonergic axons. These conclusions of the chemical findings are supported by the histopathologic observations discussed below.

Histopathologic findings. The perinigral infusion of these compounds produces a wide range of histopathologic effects. For comparative purposes, the effects of chemically equitoxic concentrations of the toxins are studied in detail. One hundred nanomoles of 4′-F-MPP^+ and $MPyP^+$ and 1000 nmol of 2,2-dimethyl-$MPDP^+$ and 3,3-dimethyl-$MPDP^+$ all produce large lesions in the midbrain that encompass and obliterate the SN. Twenty nanomoles of MPP^+ produce similar lesions in the midbrain that also obliterate the SN (fig. 2). Because the brains were examined 3 weeks after the perinigral infusions, the tissue reaction is already well developed and has produced a large cavity filled with debris and lipid-laden macrophages and surrounded by reactive gliosis (fig. 2).

In contradistinction to these nonspecific destructive lesions, 1000 nmol of MPTP causes a considerably smaller nonspecific

TABLE 1

Effect of unilateral neurotoxin infusion into the SN on striatal DA, DOPAC and HVA

Mean values ± S.E.M. of the number of observations in parentheses. ND, nondetectable. DM, dimethyl.

Neurotoxin Dose in Nanomoles	DA		DOPAC		HVA	
	Ipsilateral	Contralateral	Ipsilateral	Contralateral	Ipsilateral	Contralateral
	μg/g		μg/g		μg/g	
MPTP						
100 (2)	10.26	9.65	1.44	1.35	1.18	1.19
500 (4)	6.66 ± 1.82	9.73 ± 1.15	1.29 ± 0.38	1.46 ± 0.11	0.97 ± 0.20	1.13 ± 0.17
1000 (5)	2.53 ± 1.01	11.10 ± 1.32	0.49 ± 0.14	1.45 ± 0.12	0.34 ± 0.09	0.87 ± 0.06
3,3-DM-MPTP						
1000 (4)	6.98 ± 2.19	10.65 ± 0.73	0.97 ± 0.25	1.35 ± 0.06	0.51 ± 0.12	0.74 ± 0.04
3,3-DM-MPDP$^+$						
100 (5)	8.45 ± 1.79	10.81 ± 1.26	1.29 ± 0.13	1.53 ± 0.21	0.82 ± 0.06	0.90 ± 0.04
500 (3)	3.96 ± 1.81	11.66 ± 4.30	0.93 ± 0.19	1.91 ± 0.26	0.57 ± 0.07	1.04 ± 0.12
1000 (5)	0.53 ± 0.11	8.80 ± 0.82	0.12 ± 0.04	1.51 ± 0.08	0.08 ± 0.05	0.88 ± 0.06
2,2-DM-MPDP$^+$						
250 (4)	10.26 ± 1.23	9.79 ± 1.21	1.00 ± 0.12	1.03 ± 0.10	0.66 ± 0.09	0.69 ± 0.08
500 (4)	0.10 ± 0.03	10.00 ± 0.26	ND	1.51 ± 0.07	ND	0.70 ± 0.05
1000 (3)	0.05 ± 0.05	12.62 ± 0.55	0.03 ± 0.03	1.50 ± 0.15	0.11 ± 0.11	0.87 ± 0.03
MPP$^+$						
0.8 (4)	8.73 ± 0.54	10.13 ± 0.62	1.26 ± 0.10	1.84 ± 0.68	0.67 ± 0.09	0.70 ± 0.08
4 (4)	5.12 ± 1.74	13.02 ± 1.67	0.73 ± 0.27	1.23 ± 0.04	0.46 ± 0.11	0.91 ± 0.09
20 (3)	0.81 ± 0.78	8.10 ± 0.65	0.16 ± 0.13	1.57 ± 0.18	0.08 ± 0.08	0.75 ± 0.08
100 (3)	.06 ± 0.04	9.41 ± 1.47	0.34 ± 0.17	2.12 ± 0.57	0.02 ± 0.02	0.81 ± 0.01
4′-F-MPP$^+$						
10 (3)	9.36 ± 0.38	9.95 ± 0.10	1.38 ± 0.00	1.23 ± 0.09	0.75 ± 0.05	0.70 ± 0.05
100 (4)	0.90 ± 0.43	13.32 ± 2.26	0.16 ± 0.07	1.65 ± 0.20	0.15 ± 0.10	0.94 ± 0.07
MPyP$^+$						
10 (3)	8.97 ± 0.49	9.46 ± 0.36	1.14 ± 0.08	1.33 ± 0.14	0.61 ± 0.04	0.63 ± 0.02
100 (4)	0.68 ± 0.42	9.74 ± 0.36	0.16 ± 0.08	1.32 ± 0.09	0.06 ± 0.06	0.70 ± 0.04

TABLE 2

Effect of unilateral neurotoxin infusion into the SN on cortical NE, 5-HT and 5-HIAA

Mean values ± S.E.M. of the number of observations in parentheses. ND, nondetectable. DM, dimethyl.

Neurotoxin Dose in Nanomoles	NE		5-HT		5-HIAA	
	Ipsilateral	Contralateral	Ipsilateral	Contralateral	Ipsilateral	Contralateral
	ng/g		ng/g		ng/g	
MPTP						
100 (2)	173	179	228	229	233	235
500 (4)	136 ± 22	198 ± 14	277 ± 87	314 ± 32	187 ± 37	212 ± 6
1000 (4)	164 ± 17	214 ± 11	192 ± 80	326 ± 48	155 ± 34	255 ± 26
3,3-DM-MPTP						
1000 (4)	201 ± 25	210 ± 8	261 ± 46	228 ± 27	333 ± 39	310 ± 30
3,3-DM-MPDP$^+$						
100 (5)	123 ± 34	192 ± 22	308 ± 66	255 ± 7	284 ± 72	206 ± 12
500 (3)	67 ± 34	171 ± 18	254 ± 79	333 ± 62	279 ± 69	234 ± 15
1000 (5)	75 ± 20	226 ± 17	179 ± 30	429 ± 37	160 ± 28	300 ± 49
2,2-DM-MPDP$^+$						
250 (2)	329	323	266	372	135	167
500 (4)	3 ± 3	207 ± 14	43 ± 43	379 ± 24	86 ± 61	174 ± 16
1000 (3)	ND	210 ± 7	ND	311 ± 21	52 ± 26	224 ± 7
MPP$^+$						
0.8 (4)	145 ± 10	180 ± 5	345 ± 14	370 ± 37	283 ± 26	283 ± 26
4 (4)	176 ± 26	225 ± 12	272 ± 28	321 ± 27	266 ± 38	297 ± 30
20 (3)	48 ± 21	204 ± 13	178 ± 79	304 ± 55	147 ± 43	226 ± 37
100 (3)	ND	280	23	301	31	297
4′-F-MPP$^+$						
10 (2)	172	276	259	405	260	284
100 (4)	15 ± 11	194 ± 11	96 ± 39	308 ± 20	62 ± 24	169 ± 16
MPyP$^+$						
10 (3)	234	199	284 ± 53	231 ± 32	236 ± 20	208 ± 15
100 (4)	42 ± 19	208 ± 17	197 ± 75	316 ± 21	122 ± 47	191 ± 6

type of lesion at the site of the infusion center (fig. 3). Rats given infusions of 1000 nmol of MPTP show selective cell loss in the pars compacta of the SN in regions distant from the center of the MPTP infusion site, but without evidence of neuronal or glial destruction in the pars reticulata (fig. 3).

These histopathologic findings did not clarify two important questions. First, is MPTP toxicity restricted specifically to dopaminergic neurons of the SN, or is it also nonspecific? The second question concerns the relatively nonspecific but marked toxicity of MPP$^+$. Does MPP$^+$ have specific toxic effects on

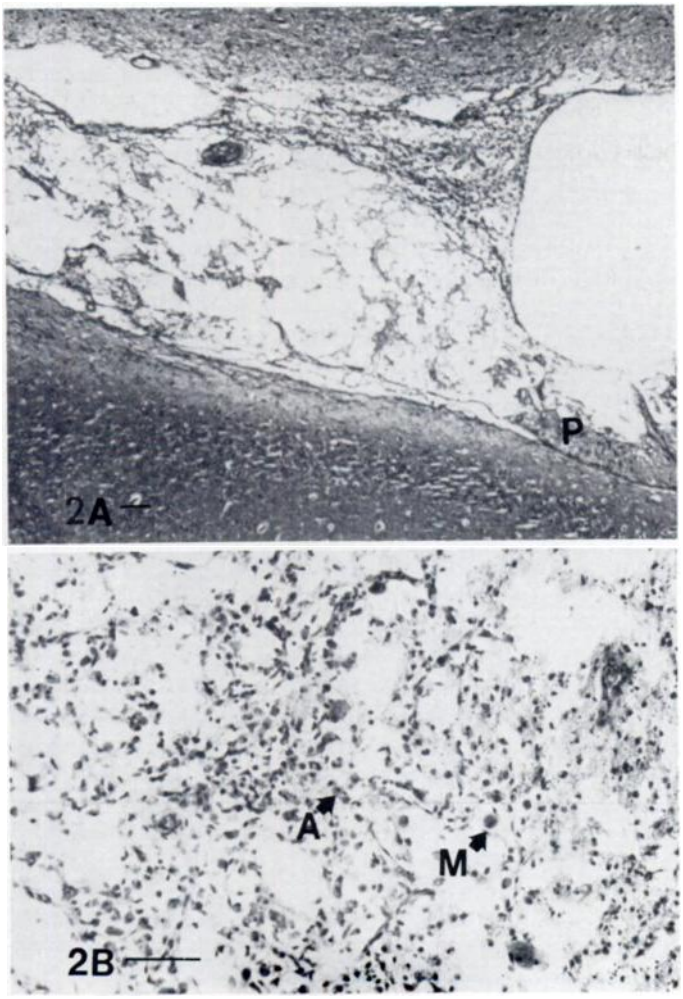


Fig. 2. Effects of infusion of 20 nmol of MPP^+ into the SN. Panel A: low-power view showing cystic cavity filled with debris. The destruction has extended ventrally to include the cerebral peduncle (P). The midline is to the right. Bar = 100 μ. Panel B: higher power view of cystic cavity showing reactive astrocytes (A) and macrophages (M). Bar = 100 μ. Paraffin sections are stained with cresyl violet.

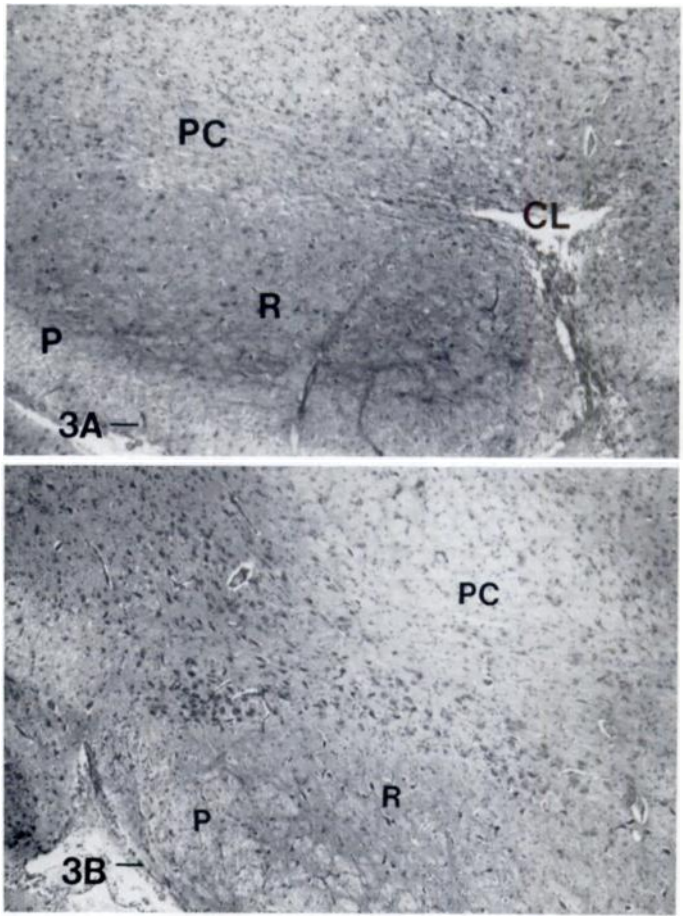
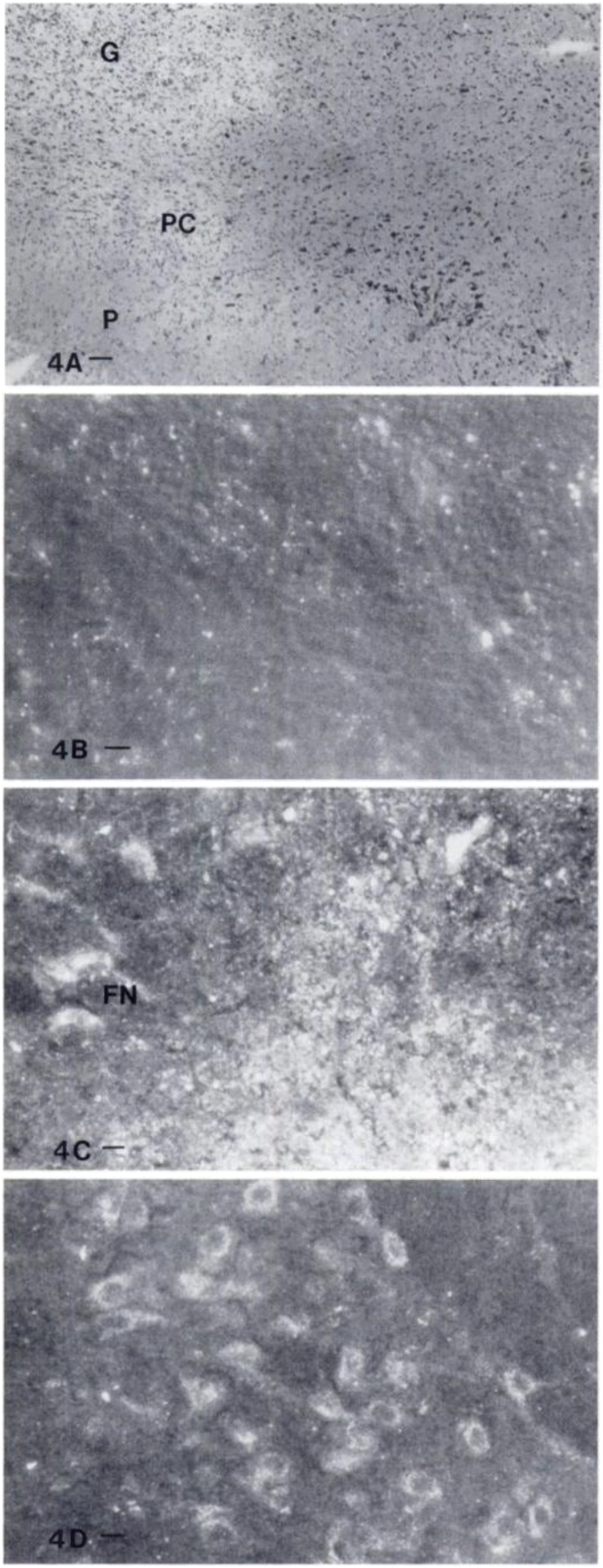


Fig. 3. Effects of infusion of 1000 nmol of MPTP into the SN. Panel A: SN infused with MPTP. The small cavity lesion around the center of the infusion site (CL) is seen at the right of the photograph. There is diffuse loss of neurons throughout the pars compacta (PC). The cerebral peduncle is at the bottom of the micrograph (P); the midline is to the right. R denotes pars reticulata of SN. Bar = 100 μ. Panel B: contralateral SN, same section. The midline is to the left. Intact neurons are seen in the pars compacta. Symbols are as in Panel A. Bar = 100 μ. Paraffin sections are stained with cresyl violet.

dopaminergic neurons of the SN, and are such specific effects masked or overshadowed by the nonspecific destruction induced by the high concentrations of MPP^+ at the infusion site? The answers to these questions were gleaned from histofluorescence studies. MPTP (1000 nmol) causes relatively small nonspecific lesions but widespread absence of catecholamine-fluorescent neurons in the infused SN (fig. 4). DA neurons of the contralateral SN were unaffected. Adjacent sections stained with thionin revealed that neurons and glia in the pars reticulata of the ipsilateral SN were also unaffected by MPTP except at the center of the infusion site. On the other hand, when 4 nmol of MPP^+ were infused, neurons with normal catecholamine histofluorescence were observed literally at the edge of the nonspecific tissue destruction (fig. 4). These findings support out contention that MPP^+ produces severe nonselective destruction, with no evidence of a specific effect on DA neurons even in regions beyond the center of the infusion site, where the MPP^+ concentration is expected to be relatively low.

Discussion

Our results highlight several important aspects of MPTP neurotoxicity. First, MPTP seems to be relatively selective for dopaminergic neurons of the rat SN when given in high concentrations. Such selective toxicity is similar to that of 6-hydroxydopamine (Ungerstedt, 1968) except that MPTP causes a larger degree of nonspecific damage at the center of the infusion site. Beyond the lesion center, dopaminergic neurons are primarily destroyed, whereas other neurons and glia are not affected (figs. 3 and 4). It is known that MPTP, when given systemically to rats, is not toxic to brain dopaminergic neurons, probably because it does not attain sufficiently high levels in the rat brain. One explanation for this may be the exceptionally high activity of monoamine oxidase B in endothelial cells of the rat brain capillaries, which would constitute an effective biochemical blood-brain barrier (Kalaria *et al.*, 1987). The discrepancy between our results and those of Chiueh *et al.* (1984), who found that intranigral infusions of MPTP were ineffective at decreasing striatal DA, is probably due to the fact that they only infused 10 μg of MPTP into the SN for 5 consecutive days, which is much less than the 1000-nmol amount that we found to be effective. We suspect that the rapid clearance of MPTP from the infusion site, which occurs concomitantly with its oxidation to the toxic culprit, is one explanation for the high dose of MPTP that is required to produce



neurotoxicity. Another finding that corroborates the relative specificity of MPTP neurotoxicity to DA neurons is the lack of appreciable effect of perinigral MPTP infusions on ascending noradrenergic and serotonergic axons that innervate the cerebral cortex (table 2).

In contrast to MPTP, 3,3-dimethyl-MPTP did not show evidence of toxicity at the highest dose that we used (1000 nmol). The fact that the latter can be oxidized only to the $MPDP^+$ stage (Riachi *et al.*, in press), whereas MPTP undergoes full oxidation to MPP^+, further supports the hypothesis that MPP^+ is the main culprit involved in MPTP toxicity. Our finding that 3,3-dimethyl-$MPDP^+$ is neurotoxic whereas 3,3-dimethyl-MPTP is not, may be due, at least in part, to the rapid clearance of the latter before its oxidation in a fashion similar to that described above for MPTP. Another explanation for the lack of apparent 3,3-dimethyl-MPTP toxicity may be the much higher K_m for its oxidation by monoamine oxidase than that of MPTP (Riachi *et al.*, in press).

In contradistinction to the relatively selective MPTP effect on DA neurons of the rat SN, we find that two analogs of $MPDP^+$, 3,3-dimethyl-$MPDP^+$ and 2,2-dimethyl-$MPDP^+$, as well as MPP^+ and its two analogs, 4′-F-MPP^+ and $MPyP^+$, are nonselectively toxic to all cellular elements in the vicinity of the infusion site. This is evident not only from the histopathologic observations but also from the results of the neurochemical assays (table 2), which indicate destruction of the ascending noradrenergic and serotonergic tracts in close proximity to the SN.

The toxicity of MPP^+ is one to two orders of magnitude higher than that of both $MPDP^+$ analogs (table 1), a finding that is consistent with MPP^+, the ultimate four-electron oxidation product of MPTP, being the major culprit in MPTP neurotoxicity rather than the initial monoamine oxidase B-generated two-electron oxidation product $MPDP^+$. Because, $MPDP^+$ is easily converted to MPP^+ through redox disproportionation or adventitious oxidation reactions, the neurotoxicity of $MPDP^+$ itself cannot be unambiguously assessed. We have addressed this question through the use of the 2,2- and 3,3-dimethyl analogs, which retain the electrophilic properties of $MPDP^+$ but cannot be further oxidized. The apparent greater toxicity of 2,2- compared with 3,3-dimethyl-$MPDP^+$ could arise from the ability of the former to exist in equilibrium with a neutral enamine (fig. 1), thereby facilitating its passage to its toxic site of action.

The factors responsible for the finding that the 4′-F-MPP^+ and $MPyP^+$ analogs of MPP^+ are about 10 times less toxic than MPP^+ are less clear. This result is compatible with the finding that MPTP analogs that would yield these two MPP^+ analogs upon oxidation exhibit little or no toxicity when administered sytemically to mice (Heikkila *et al.*, 1985b; L. M.

Fig. 4. Panel A: SN infused with 1000nmol of MPTP. The infusion site is marked by gliosis at the upper left of the micrograph (G). There is marked loss of neurons in the lateral pars compacta (PC). (Compare fig. 2B.) The midline is to the right; P = cerebral peduncle. Bar = 100 μ. Frozen section is stained with thionin. Panel B: section adjacent to that illustrated in Panel A, but processed for catecholamine histofluorescence, as in Panels C and D. There is granular fluorescence in the background, but no intraneuronal fluorescence. (Compare with Panel D.) Bar = 10 μ. Panel C: SN infused with 4 nmol of MPP^+. Nonspecific fluorescence is seen at the edge of the infusion site (right lower half of the micrograph). Fluorescent neurons are seen literally at the edge of the infusion site (FN). Bar = 20 μ. Panel D: contralateral SN, same animal as in Panel C; abundant intraneuronal fluorescence is present. Bar = 20 μ.

Sayre, P. K. Arora, I. Irwin and J. W. Langston, unpublished). However, the low sensitivity of mice to MPTP means that even small reductions in activity could result in the failure to observe evidence of neurotoxicity. Such structural changes in the MPTP molecule could decrease neurotoxicity by retarding monoamine oxidase activity or by deleteriously affecting any of several factors that influence the delivery of the ultimate toxin to its site of action. For example, 4′-F-MPTP is metabolized by monoamine oxidase B at ~50% of the MPTP rate (Heikkila *et al.*, 1985a). Nonetheless, the fact that 4′-F-MPP$^+$ and MPyP$^+$ are less toxic than MPP$^+$ does not support the redox cycling hypothesis of MPP$^+$ toxicity because these analogs are more easily reduced (P. K. Arora, S. C. Feke, F. L. Urbach and L. M. Sayre, unpublished). In addition, a structure-activity study on the ability of a large series of pyridinium compounds, including MPP$^+$ and paraquat, to be reduced physiologically indicates that redox cycling exhibits a cutoff at a potential very close to that of paraquat and very far from that of MPP$^+$ (J. L. Blumer, D. M. Frank, P. A. Arora and L. M. Sayre, unpublished). A very low redox cycling activity of MPP$^+$ compared with paraquat was previously reported (Sinha *et al.*, 1986). Although MPP$^+$ and paraquat are both cytotoxic, the mechanisms responsible for cell death appear to be distinct, with only paraquat exhibiting characteristics of oxidant stress (Di Monte *et al.*, 1986b).

Irrespective of the fact that MPDP$^+$ and MPP$^+$ analogs that were used here are less toxic than MPP$^+$, the question still remains as to what mechanism is responsible for their relatively potent toxic effects. If redox cycling by MPP$^+$ is not involved, the most likely mechanism underlying cytotoxicity is inhibition of mitochondrial respiration (Nicklas *et al.*, 1985; Vyas *et al.*, 1986). Even though the intrinsic inhibitory potency of MPP$^+$ on electron transport is relatively low (Ramsay *et al.*, 1986b), MPP$^+$ seems to be concentrated within mitochondria by an energy-dependent process (Ramsay *et al.*, 1986a, c; Ramsay and Singer, 1986). Our preliminary studies (P. K. Arora, L. M. Sayre and C. L. Hoppel, unpublished) indicate that 2,2- and 3,3-dimethyl-MPDP$^+$, as well as a number of MPTP and MPP$^+$ analogs, exhibit inhibitory activity comparable to that of MPP$^+$ on isolated electron-transport particles and on intact mitochondria. Thus, inhibition of mitochondrial respiration, which leads to ATP depletion and eventual cell death (Heikkila *et al.*, 1985d; Di Monte *et al.*, 1986a), could theoretically rationalize the cytotoxic effects of all the compounds reported in the current study.

It is curious that, in contrast to MPTP, MPP$^+$ (and other charged analogs) exhibited nonspecific tissue destruction at their infusion site, even at low concentrations. Because MPTP toxicity is assumed to be due to its metabolism to MPP$^+$, one might have expected MPP$^+$ to induce some selective destruction of DA neurons at locations distant front the center of the infusion site, where its concentration would be low and comparable to that resulting from brain MPTP metabolism. This is definitely not the case. One possible explanation for our findings is that MPP$^+$ is highly reactive and poorly cleared from brain tissue. It is likely that the specific toxicity of MPTP at DA neurons arises from the selective uptake of a rather low concentration of MPP$^+$ that is produced over a relatively long period of time, a situation that would not exist in the acute MPP$^+$ infusion protocol that we used.

Appendix

Analytical Data on Target Drugs and Key Intermediates

Melting points were determined on a Thomas Hoover capillary melting point apparatus and are uncorrected. ^{1}H-NMR spectra were recorded on a Varian XL-200 spectrometer, and data are reported as parts per million downfield from tetramethylsilane for $CDCl_3$ as solvent and from sodium 2,2-dimethyl-2-silapentane-5-sulfonate for D_2O as solvent. All target compounds were shown to be >95% pure by the complete absence of unaccountable ^{1}H-NMR absorptions at 200 MHz and ran as one spot on thin-layer chromatography (silica gel or C_{18} reverse-phase plates). High-resolution EIMS were recorded on an AEI-MS3076 instrument. Combustion analyses were performed by Galbraith Laboratories (Knoxville, TN).

1-Methyl-4-(4-fluorophenyl)-1,2,3,6-tetrahydropyridine. ^{1}H-NMR ($CDCl_3$) δ 2.41 (s, 3H, N-C*H*$_3$), 2.5 to 2.65 (m, 4H, C$_2$-*H* and C$_3$-*H*), 3.10 (m, 2H, C$_6$-*H*), 6.03 (m, 1H, C$_5$-*H*), 6.85 to 7.5 (m, 4H, Ar *H*); m.p. 176–178°C.

1-Methyl-4-(4-fluorophenyl)pyridinium iodide (4′-F-MPP$^+$ I$^-$). ^{1}H-NMR (D_2O) δ 4.36 (s, 3H, N-C*H*$_3$), 7.36 [dd, 2H, J = 8.1 and 8.1 Hz, 3′ Ar *H* (ortho to F)], 7.96 [dd, 2H, J = 5.2 and 8.1 Hz, 2′ Ar *H* (meta to F)], 8.27 and 8.75 (each d, 2H, J = 6.4 Hz, 2 and 3 Ar *H*); m.p. 178–179°C.

1-Methyl-4-(2-pyridyl)pyridinium chloride (MPyP$^+$ Cl$^-$). ^{1}H-NMR (D_2O) δ 4.54 (s, 3H, N-C*H*$_3$), 8.56 and 9.03 (each d, 2H, J = 6.4 Hz, 2 and 3 Ar *H*), 8.02 (t, 1H), 8.36 to 8.50 (m, 2H), 8.93 (d, 1H). EIMS (70 eV) m/z (M$^+$, 0.1%) calculated 171.0923, observed 171.0929; [(M-CH$_3$)$^+$, 100%] calculated for $C_{10}H_8N_2$ 156.0688, observed 156.0703; m.p. 195–196°C.

1,3,3-Trimethyl-4-phenyl-1,2,3,6-tetrahydropyridine HCl (3,3-dimethyl-MPTP). ^{1}H-NMR (free base in $CDCl_3$) δ 1.07 (s, 6H, C$_3$-C*H*$_3$), 2.34 (s, 2H, C$_2$-*H*), 2.36 (s, 3H, N-C*H*$_3$), 3.02 (d, 2H, J = 2.5 Hz, C$_6$-*H*), 5.42 (t, 1H, J = 2.5 Hz, C$_5$-*H*), 7.14 to 7.36 (m, 5H, Ar *H*). EIMS (40 eV) m/z (M$^+$) calculated 201.1519, observed 201.1556; m.p. 258°C (dec).

1,3,3-Trimethyl-4-phenyl-2,3-dihydropyridinium bromide (3,3-dimethyl-MPDP$^+$). ^{1}H-NMR ($CDCl_3$) δ 1.38 (s, 6H, C$_3$-C*H*$_3$), 3.88 (s, 2H, C$_2$-*H*), 4.07 (s, 3H, N-C*H*$_3$), 6.53 and 9.52 (each d, 1H, J = 5.0 Hz, C$_5$- and C$_6$-*H*), 7.23 to 7.66 (m, 5H, Ar *H*). EIMS (70 eV) m/z (M$^+$, 6.7%) calculated 200.1440, observed 200.1458; [(M-CH$_4$)$^+$, 100%] calculated for $C_{13}H_{14}N$ 184.1127, observed 184.1138; m.p. 204–205°C. Analysis: Calculated for $C_{14}H_{18}NBr$: C, 60.00; H, 6.42; N, 5.00. Found: C, 59.99; H, 6.52; N, 4.99.

1,2,2-Trimethyl-4-phenyl-1,2,3,6-tetrahydropyridine HCl (2,2-dimethyl-MPTP). This was obtained along with the isomer 1,6,6-trimethyl-4-phenyl-1,2,3,6-tetrahydropyridine from the dehydration of 1,2,2-trimethyl-4-phenyl-4-piperidinol and could be obtained in pure form by $NaBH_4$ reduction of 2,2-dimethyl-MPDP$^+$: ^{1}H-NMR (free base in $CDCl_3$) δ 1.20 (s, 6H, C$_2$-C*H*$_3$), 2.42 (s, 3H, N-C*H*$_3$), 2.50 (s, 2H, C$_3$-*H*), 3.30 (m, 2H, C$_6$-*H*), 5.97 (m, 1H, C$_5$-*H*), 7.20 to 7.48 (m, 5H, Ar *H*). EIMS (20 eV) m/z (M$^+$, 36%) calculated 201.1519, observed 201.1521; [(M-CH$_3$)$^+$, 95%] calculated for $C_{13}H_{16}N$ 186.1284, observed 186.1320; m.p. 171–172°C. Analysis: Calculated for $C_{14}H_{20}NCl \cdot 1/6\ H_2O$: C, 69.84; H, 8.51; N, 5.82. Found: C, 69.95; H, 8.56; N, 5.73.

1,2,2-Trimethyl-4-phenyl-2,3-dihydropyridinium bromide (2,2-dimethyl-MPDP$^+$). ^{1}H-NMR ($CDCl_3$) δ 1.62 (s, 6H, C$_2$-C*H*$_3$), 3.33 (s, 2H, C$_3$-*H*), 3.88 (s, 3H, N-C*H*$_3$), 7.03 and 9.60 (each d, 1H, J = 5.0 Hz, C$_5$ and C$_6$-*H*), 7.2 to 7.8 (m, 5H, Ar *H*). EIMS (70 eV) m/z (M$^+$) calculated 200.1440, observed 200.1259; [(M-H)$^+$, enamine] calculated 199.1362, observed 199.1391; [M-CH$_4$)$^+$, 100%] calculated 184.1127, observed 184.1238; m.p. 194–195°C. Analysis: Calculated for $C_{14}H_{18}NBr$: C, 60.00; H, 6.42; N, 5.00. Found: C, 59.72; H, 6.56; N, 4.94.

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