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INHIBITION OF NADH OXIDATION BY PYRIDINE DERIVATIVES

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The neurotoxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, an impurity in an illicit drug, is expressed after its oxidation to 1-methyl-4-phenylpyridinium by monoamine oxidase. The pyridinium is concentrated by carrier-mediated transport into the mitochondria where it inhibits NADH dehydrogenase and, hence, ATP synthesis. Some structurally related compounds have been tested for their effect on the oxidation of NAD^+-linked substrates in intact mitochondria, and for the inhibition of the accumulation of the pyridinium into mitochondria and of NADH dehydrogenase activity in a membrane preparation. Some pyridine derivatives are more inhibitory to NADH dehydrogenase than is 1-methyl-4-phenylpyridinium but these are not concentrated into mitochondria by the uptake system. 4-Phenylpyridine, one of the most effective inhibitors, both occurs naturally and is an environmental pollutant.

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), an impurity in an illicit drug, causes Parkinsonian symptoms in primates. Substantial evidence has been reported that its neurotoxicity is expressed through oxidation to 1-methyl-4-phenylpyridinium (MPP^+) by monoamine oxidase (1-4), concentration in the nigrostriatal nerve terminals by the dopamine reuptake system (5), followed by carrier-mediated transported of the MPP^+ across the mitochondrial inner

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Abbreviations: **ETP**, an inverted inner membrane preparation from beef heart mitochondria; **MPTP**, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; **MPP^+**, 1-methyl-4-phenylpyridinium ion; **$MPDP^+$**, 1-methyl-4-phenyl-2,3-dihydropyridinium ion.

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membrane (6), where it inhibits NADH dehydrogenase (7-10) and, hence, ATP synthesis.

Several investigators (11,12) have suggested that Parkinsonism may have environmental causes and that pyridine compounds may be among the etiological agents. We have explored systematically the reaction of various pyridine derivatives on NADH dehydrogenase and their ability to enter the mitochondrial matrix where cell damage could be initiated. The biochemical assays used provide a relatively simple and reproducible means for screening compounds for potential neurotoxicity.

Measurement of mitochondrial respiration is the most straight-forward way to test compounds for their ability to inhibit energy production by mitochondria and, hence, cause energy depletion of the cell. However, MPP^+ and related compounds must first be transported across the mitochondrial inner membrane before their inhibitory effect on NADH dehydrogenase can be expressed. Thus, inhibition of respiration is the net result composed of the effects on these two separate steps which may be differently affected by alterations in the structure of the inhibitor. Various pyridinium compounds were screened for inhibition of mitochondrial respiration, and then their effects on uptake and on NADH dehydrogenase studied separately in order to assess which moeities of the inhibitor molecule were important for efficacy at each step.

MATERIALS AND METHODS

Rat liver mitochondria and ETP from beef heart were prepared as before (8). [^{3}H]Methyl-MPP^+ and [^{3}H]Methyl-MPTP were purchased from New England Nuclear (Boston, Massachusetts, U.S.A.). 4-Phenyl-1,2,3,6-tetrahydropyridine and 4-phenylpyridine were obtained from Aldrich Chemical Company. Synthesis of the other derivatives were as follows: 1-methyl-2-phenylpyridinium (13), 1-methyl-3-phenylpyridinium (14), 1,4-dimethylpyridinium (15), 1-methyl-4-t-butylpyridinium (16), 1,2-dimethyl-4-phenylpyridinium (17), and 2,3,4,9,9-pentahydro-2-methyl-1-H-indeno[2,1-C]pyridine (18). Because of the potential neurotoxicity of these compounds, containment procedures were followed and latex gloves worn in all experiments.

NADH dehydrogenase was assayed using O_2 as the electron acceptor and monitoring either O_2 consumption (polarographic assay) or NADH disappearance (spectrophotometric assay) (19). Uptake of [^{3}H]MPP^+ and [^{3}H]MPTP was measured as before (6). Mitochondrial respiration was measured polarographically in a Clark electrode at 25°C. The mitochondria (2 mg/ml) were incubated in a final volume of 2 ml of respiration medium (90 mM KCl, 20 mM Tris Cl, 10 mM K phosphate and 5 mM $MgCl_2$, pH 7.4 at 25°C), containing 5 mM glutamate and 2.5 mM malate, with or without inhibitor for 5 min (or other times as indicated in legends) before the addition of ADP (0.26 mM).

RESULTS AND DISCUSSION

Figure 1 shows time courses for the expression of the inhibition of mitochondrial respiration by pyridinium derivatives. It is important to realize that this inhibition depends on the concentration of inhibitor in the matrix which in turn depends on the ability of the mitochondria to accumulate the inhibitor. The latter is both time dependent and concentration dependent (6) as is demonstrated by the curves for 0.5 mM MPP^+ and 0.05 mM MPP^+ in Fig. 1A. After 50 min incubation with 0.05 mM MPP^+, inhibition of mitochondrial respiration is complete. The matrix space in rat liver mitochondria is 0.6 µl/mg (6), so if even 50% of the MPP^+ present were taken up, a matrix concentration of 40 mM could be reached -- more than enough to give complete inhibition of NADH dehydrogenase (see Table 1).

The relative efficacy of the pyridinium derivatives tested in the inhibition of the oxidation of glutamate plus malate by rat liver mitochondria

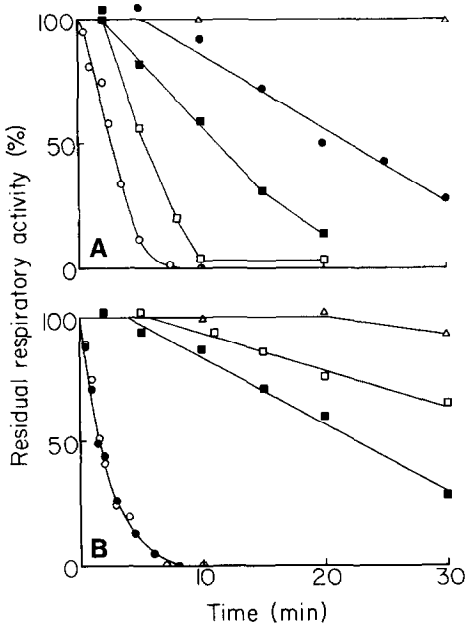

Figure 1. **Inhibition of mitochondrial respiration by pyridinium derivatives.** The procedure is described in the Experimental section. A. The protein concentration of the rat liver mitochondria was 1.9 mg/ml. The incubations contained no inhibitor (control, △); 0.5 mM MPP^+ (○); 50 µM MPP^+ (●); 0.5 mM 1-methyl-3-phenylpyridinium (□); 0.5 mM 1-methyl-2-phenylpyridinium (■). B. The protein concentration was 2.2 mg/ml. The incubations contained no inhibitor (control, △) or 0.5 mM inhibitor: MPP^+ (○); 1,2-dimethyl-4-phenylpyridinium (●); 1,4-dimethylpyridinium (□); 1-methyl-4-t-butylpyridinium (■). The increase in respiratory activity induced by the addition of ADP was expressed as a percentage of the control value.

TABLE 1. Inhibition of NADH oxidase activity in inner membrane preparations

Inhibitor	I_{50} (mM)
1-Methyl-4-phenylpyridinium (MPP^+)	7.2
1-Methyl-3-phenylpyridinium	>20
1-Methyl-2-phenylpyridinium	11
1,2-Dimethyl-4-phenylpyridinium	11
1,4-Dimethylpyridinium	not reached
1-Methyl-4-t-butylpyridinium	not reached
4-Phenylpyridine	1.3
$MPDP^+$	0.60
MPTP	0.92
4-Phenyl-1,2,3,6-tetrahydropyridine	1.1
2,3,4,9,9-Pentahydro-2-methyl-1-H-indeno[2,1-C]pyridine	0.75

NADH oxidase activity of beef heart ETP was measured spectrophotometrically at 30^0 in 0.25 M sucrose-0.05 M K phosphate, pH 7.6, except in the case of $MPDP^+$, for which O_2 uptake was measured polarographically. I_{50} is the concentration of inhibitor required to decrease the activity by 50% immediately after addition of the inhibitor.

is 1-methyl-4-phenylpyridinium = 1,2-dimethyl-4-phenylpyridinium > 1-methyl-3-phenylpyridinium > 1-methyl-2-phenylpyridinium > 1-methyl-4-t-butylpyridinium > 1,4-dimethylpyridinium. Thus, the position of the phenyl substituent on the pyrdine ring is of major importance. Further, substitution of a t-butyl or methyl group for the phenyl residue substantially lowers the effectiveness, but an additional methyl group in position 2 has no effect.

The active accumulation of the compounds into the mitochondrial matrix driven by the electrical gradient across the inner membrane (6) can be measured only if radioactively labelled compounds are available. Since they were not, we estimated the relative uptake of each by its inhibition of $[^3H]MPP^+$ uptake. The reliability of this procedure was tested by assessing the effectiveness of unlabeled 2 mM MPP^+ (Fig. 2, solid circles) on the uptake of 0.5 mM $[^3H]MPP^+$. Since the K_m of the uptake system is 5 mM (6), the net rate of total MPP^+

uptake increased (not shown), but the uptake of radiolabelled MPP^+ is slower by a factor of 0.73[1]. This inhibition was as expected on the basis of competition for the carrier. In contrast, 2 mM 1-methyl-2-phenylpyridinium was ineffective[2] and the 3-phenyl analogue was less inhibitory than MPP^+ itself, but 6 mM 1,2 dimethyl-4-phenylpyridinium inhibited MPP^+ uptake extensively. This may explain the impressive inhibition of mitochondrial respiration by the latter compound (Fig. 1), despite its higher I_{50} in Table 1. The inhibition of MPP^+ uptake by the other analogues (Fig. 2) is in line with their effect on the oxidation of glutamate and malate (Fig. 1).

The energy-dependent uptake process can concentrate positively charged inhibitors such as MPP^+ into the matrix by at least 40-fold (6), resulting in concentrations in excess of 20 mM. Thus, the high I_{50} values for the pyridinium compunds (Table 1) can be achieved in intact mitochondria. The concentration dependence of the inhibition by these compounds is not a simple titration effect. Fig. 3 shows that plateau values are reached above which no further inhibition is observed. A phenyl substituent in the 4 position seems to be critical for complete inhibition. When the phenyl group is in the 2 or 3 position, only about 50% inhibition is reached and when it is not present the maximum inhibition is even less (Fig. 3).

An additional methyl group on the pyridinium ring had little effect (MPP^+ versus 1,2-dimethyl-4-phenylpyridinium in Fig. 1 and Table 1). It was of interest, therefore, to examine the inhibitory effect of 4-phenylpyridine which lacks the 1-methyl group, and in addition, is uncharged. The I_{50} value (Table 1) shows that it is a much more effective inhibitor of NADH dehydrogenase, yet it is less inhibitory than MPP^+ to mitochondrial respiration (10). Thus, access to the NADH dehydrogenase inhibitory site must be limited. Lacking the positive charge, 4-phenylpyridine is not accumulated by the MPP^+ uptake system.

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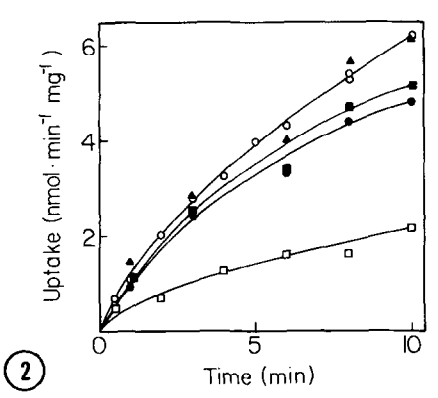
[1] For $[^3H]MPP^+$ alone, $v_1 = VK_m/(K_m+S)$, where K_m = 5 mM and S = 0.5 mM. For $[^3H]MPP^+$ plus unlabelled MPP^+, S = 2.5 mM, so $v_1/v_2 = 5.5/7.5 = 0.73$.

[2] For addition of 2 mM inhibitor to cause at least a 10% difference in the rate of uptake, the K_i must be lower than 18 mM, calculated on the basis of $v_2 = VK_m/[K_m(1+I/K_i)+S]$, where I = 2 mM inhibitor.



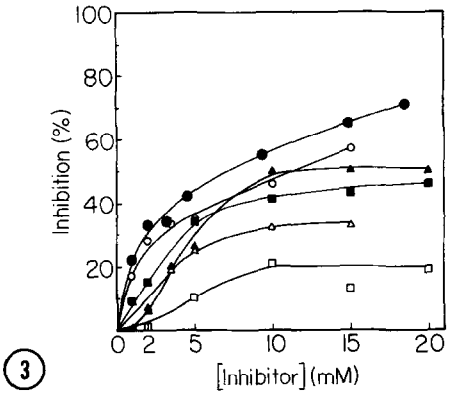


Figure 2. **Inhibition of the active uptake of [^{3}H]MPP$^+$ into rat liver mitochondria by some pyridinium derivatives.** The uptake was measured as described in the Experimental section. Mitochondria (12.0 mg/ml) were incubated with 0.5 mM [^{3}H]MPP$^+$ (0.20 Ci mol^{-1}) in the absence or presence of antimycin (10 uM) and rotenone (10 ug/ml) to obtain the difference, representing the energy-dependent (active) uptake. Non-radiolabelled derivatives added immediately before the [^{3}H]MPP$^+$ were: none (○); 2 mM MPP$^+$ (●); 2 mM 1-methyl-3-phenylpyridinium (■); 2 mM 1-methyl-2-phenylpyridinium (▲); or 6 mM 1,2-dimethyl-4-phenylpyridinium (□).

Figure 3. **Inhibition of NADH oxidase activity by MPP$^+$ analogues.** ETP (0.8 mg/ml) were incubated for 5 min at 25°C in a final vol of 0.2 ml 0.25 M sucrose 50 mM K phosphate, pH 7.6, with or without inhibitor. A sample (25 ul) of the incubation mixture was assayed in the same buffer in the presence of 0.28 mM NADH and the same concentration of inhibitor as present in the incubation. The inhibitors were: MPP$^+$, (●); 1-methyl-3-phenylpyridinium, (■); 1-methyl-2-phenylpyridinium, (▲); 1,2-dimethyl-4-phenylpyridinium, (○); 1,4-dimethylpyridinium, (□); 1-methyl-4-t-butylpyridinium, (△).

MPTP and its analogues all show I_{50} values of around 1 mM (Table 1) and are thus much more inhibitory to NADH dehydrogenase than is MPP$^+$. However, none of these di- or tetrahydropyridine derivatives nor 4-phenylpyridine affect MPP$^+$ uptake by the mitochondrial carrier nor is [^{3}H]MPTP accumulated by mitochondria (data not shown). Hence, their penetration into the matrix, to the extent it takes place, involves passive diffusion. Thus, despite the lower I_{50} values, these compounds are less likely to have direct, acute *in vivo* effects, because they are not concentrated in the vicinity of the dehydrogenase.

To the extent that the parkinsonian symptoms elicited by MPTP are a reliable model for idiopathic Parkinsonism, elucidation of the enzymic steps leading from the entry of MPTP into the brain to selective neuronal destruction may offer a unique opportunity to identify environmental agents responsible for the disease. The measurement of mitochondrial respiration, of the activity of

NADH dehydrogenase in membrane preparations, and of effects on [^{3}H]MPP^+ uptake into mitochondria, as used in this study, are all reasonably simple procedures, readily duplicated in most biochemical laboratories. Theoretically, a suspect compound should be tested in all three systems. For example, in the case of 4-phenylpyridine, the inhibition of mitochondrial respiration is only partial and the compound is not concentrated by the mitochondrial MPP^+ carrier, but its effect on the dehydrogenase is potent even at low concentrations.

Among the compounds tested in this study, only 4-phenylpyridine is a known industrial pollutant and a constituent of spices (20). Whether repeated or continuous exposure to this and related compounds, which readily cross the blood-brain barrier and the mitochondrial inner membrane, might not progressively accelerate the normal rate of decline in nigrostriatal cell population with age and thus bring about premature appearance of dopamine deficiency, is worth considering.

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