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INHIBITION OF MITOCHONDRIAL NADH DEHYDROGENASE BY PYRIDINE DERIVATIVES AND ITS POSSIBLE RELATION TO EXPERIMENTAL AND IDIOPATHIC PARKINSONISM

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SUMMARY. 4-Phenyl-N-methylpyridinium (MPP^+), the oxidation product of the neurotoxic amine MPTP, is considerably more inhibitory to the oxidation of NAD^+-linked substrates in intact mitochondria in State 3 than is 4-phenylpyridine. On adding uncouplers, the inhibition by MPP^+ progressively diminishes, while the effect of 4-phenylpyridine remains. This is in accord with the fact that MPP^+ is rapidly concentrated in the mitochondria by an energy-dependent process, while 4-phenylpyridine seems to enter passively with the concentration gradient. Collapse of the electrical gradient after addition of uncouplers thus leaves the inhibiton by 4-phenylpyridine unaffected but causes efflux of MPP^+ from the mitochondria and a reversal of its inhibitory action. In isolated inner membranes the inhibition of NADH oxidation via the respiratory chain by 4-phenylpyridine is much greater than by MPP^+. MPTP and 4-phenyl-N-methylpyridinone also inhibit more than MPP^+, whereas N-methylpyridinium has relatively little effect. The block is not at the point of entry of electrons into the flavoprotein since the NADH-ferricyanide activity is not inhibited by MPP^+ at V_{max}.

INTRODUCTION. Parkinsonism is a degenerative disease caused by extensive destruction of nigrostriatal neurons and consequent depletion of the neurotransmitter dopamine. The neurological symptoms develop progressively and it has been usually estimated that the first clinical signs appear when an 80% decline in striatal dopamine level occurs as a result of comparable loss of nigrostriatal cells (1). It has been demonstrated that MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), the neurotoxic component of certain batches of illicit drugs, elicits parkinsonian symptoms in man and primates (2,3) and that this compound causes cell destruction in the substantia nigra, but only after metabolic processing by brain enzymes (4). The two types of monoamine oxidase, primarily the

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the B type, have been implicated in this bioactivation (5,6). MPTP is initially oxidized to the dihydropyridinium form and then more slowly to MPP^+, the 4-phenyl-N-methylpyridinium species. The latter has been suggested to be the compound directly responsible for neuronal destruction and it is concentrated in dopaminergic neurons by the dopamine reuptake system (7). Based on the inhibition of the oxidation of NAD^+-linked substrates in rat liver and brain mitochondria in State 3 by a moderate concentration (0.5 mM) of MPP^+, Nicklas et al. (8) postulated that when MPP^+ reaches the cell body of the neuron, it blocks mitochondrial NADH oxidation and thus prevents energy conservation, resulting in cell death. This idea presented two difficulties. First, at the MPP^+ levels found in the nigrostriatal cells of MPTP-treated mice (9) and monkeys (10) (~30 and ~50 μM, respectively), NADH oxidation is not inhibited in membrane preparations or in Complex I (11). Second, charged molecules are generally thought not to cross the inner membrane of mitochondria freely. Our recent paper (11) has resolved these problems by demonstrating the presence of a very active energy-dependent uptake system for MPP^+ in mitochondria, capable of concentrating MPP^+ inside the mitochondria at levels far higher than needed to block NADH oxidation.

We felt that it was timely to approach two closely related problems, the molecular mechanism of the inhibition of mitochondrial NADH dehydrogenase by MPP^+ and its possible relevance to idiopathic Parkinsonism. Langston's group (1,2) long ago called attention to the possibility that idiopathic Parkinsonism is the end result of the cumulative effect of xenobiotics. Snyder and D'Amato (12) called attention to the fact that, besides industrial wastes, certain foodstuffs contain substituted pyridines, such as 4-phenylpyridine, and reported that the latter compound causes moderate dopamine depletion in mice and also in cell cultures, implying that this type of compound might be a causative agent of the disease. We report here a comparative study of the effect of substituted pyridines on NADH oxidation in membranes and intact mitochondria.

MATERIALS AND METHODS. 4-Phenylpyridine was from the Aldrich Chemical Co., N-methylpyridine and 1-methyl-4-phenyl-2-pyridinone were kind gifts from Dr. N. Castagnoli. Other materials and methods were as before (5,6,11).

RESULTS AND DISCUSSION. Fig. 1 compares the effects of MPP^+ and 4-phenylpyridine on the oxidation of L-glutamate and L-malate in tightly coupled rat liver mitochondria. Both inhibitors were added at 0.5 mM concentration but, whereas MPP^+ is rapidly taken into the mitochondria under these conditions (11), reaching a concentration of about 20 mM in minutes, 4-phenylpyridine does not seem to be concentrated into the mitochondria by this mechanism. The conclusion that the system responsible for the energy-dependent uptake of MPP^+ in liver and brain mitochondria does not recognize 4-phenylpyridine as a substrate was based on experiments in which the uptake of 0.5 mM [^{3}H]-MPP^+ was unaffected by the presence of 1 mM 4-phenylpyridine (13). It appears from this that 4-phenylpyridine, being neutral, may diffuse passively into the mitochondria, rather than being actively concentrated by an energy-requiring process. This may explain why in mitochondria pre-incubated with equal concentrations of MPP^+ and 4-phenylpyridine malate and glutamate oxidation is more inhibited by MPP^+ than by 4-phenylpyridine, although in isolated membranes the latter compound is much more inhibitory (Figs. 2 and 3). It may also explain the progressive decline of inhibition by MPP^+ after addition of uncoupler, in contrast to the persistence of the inhibition by 4-phenylpyridine (Fig. 1). In experiments to be published elsewhere (13) we found that when the mitochondria are preloaded with MPP^+ in the presence of an energy source and an

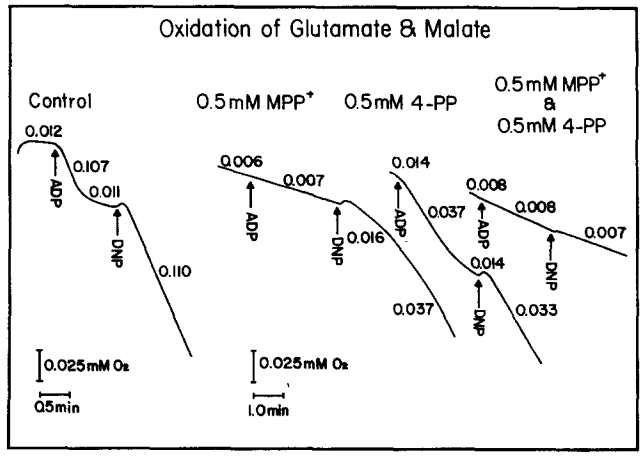

Fig. 1. Polarographic measurement of the respiration of rat liver mitochondria (1.9 mg/ml) at 25°. Malate, glutamate, ADP, and 2,4-dinitrophenol (DNP) were added to give 2.5, 5, 0.25, and 0.019 mM concentrations, respectively. Preincubation with MPP^+ or 4-phenylpyridine (4-PP) was for 5 min., in the presence of substrate.

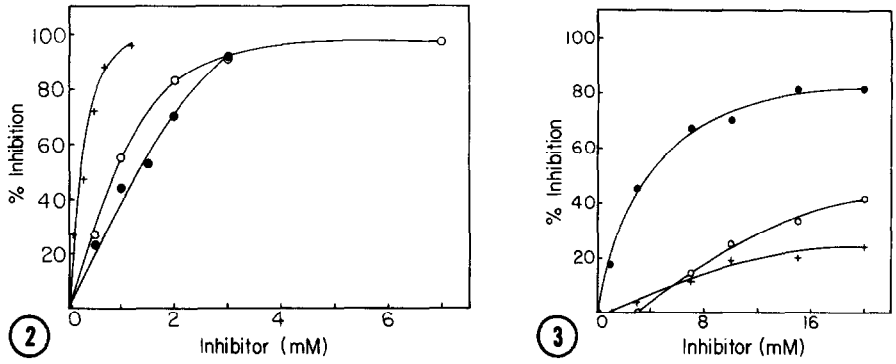

Fig. 2. Aliquots of 25 µl of a beef heart ETP suspension (2 µg/ml) were diluted to 0.25 ml with 0.25 M sucrose — 0.05 M potassium phosphate, pH 7.6, containing the indicated concentration of inhibitor and incubated for 5 min. at 25°. The tubes were rapidly chilled and aliquots of 25 µl were immediately assayed in a 1 ml reaction volume containing 0.25 M sucrose — 0.05 M phosphate, pH 7.6, 0.28 mM NADH, and the indicated amount of inhibitor by following the initial rate of disappearance of the 340 nm band of NADH at 25°. Symbols: X—X, 4 phenylpyridine; O—O, MPTP; ●—●, N-methyl-4-phenylpyridinone.

Fig. 3. Conditions were as in Fig. 2. Symbols: ●—●, MPP^+; O—O, NAD^+; X—X, N-methylpyridine.

uncoupler is then added, an efflux of MPP^+ occurs. In the case of 4-phenylpyridine the intramitochondrial concentration cannot exceed that prevailing outside and thus uncouplers have no effect and the inhibition of NADH dehydrogenase remains.

Figs. 2 and 3 compare the inhibition of NADH oxidation via the complete respiratory chain by substituted pyridines in ETP, an inner membrane preparation from beef heart. The data are based on spectrophotometric measurements of the initial rate of NADH oxidation, after preincubation of the particles with the appropriate inhibitor for 5 min. It is seen that 4-phenylpyridine was the most potent inhibitor, followed by MPTP, 1-methyl-4-phenyl-2-pyridinone, MPP^+, and N-methylpyridinium. Note that, in accord with our earlier data (11), 0.5 mM MPP^+ produces only slight inhibition, but nearly complete inhibition occurs at 10 mM or higher concentrations. This concentration is rapidly reached inside the mitochondria at cytoplasmic concentrations of 0.5 mM or less MPP^+ (11,13). Interestingly, NAD^+ itself, another substituted pyridinium compound, also inhibits NADH oxidation at very high concentrations (Fig. 2). However, the site of inhibition of NADH oxidation is not at the point of entry of pyridine-nucleotide generated reducing equivalents into the electron transport chain (i.e., the flavin), as has been suggested (8), since in the ferricyanide assay MPP^+ does not inhibit at V_{max}

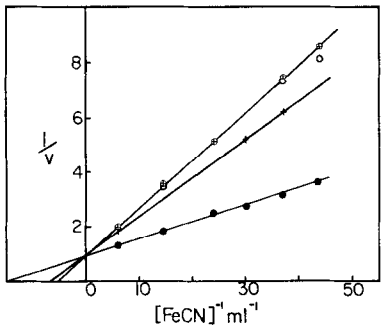

Fig. 4. Double reciprocal plot of NADH-ferricyanide activity. The ETP preparation used in the experiments of Figs. 2 and 3 was incubated for 5 min. at 30° in 0.04 M triethanolamine buffer, pH 7.8, the indicated amounts of 10^{-2} M ferricyanide, and inhibitors as follows: O—O, none; ⊕—⊕, 1 mM 4-phenylpyridine; ●—●, 5 mM MPP^+; X—X, 10 mM methylpyridine. Assay was initiated by adding NADH to 1.5 X 10^{-4} concentration. Abscissa, reciprocal concentration of 10^{-2} M ferricyanide; ordinate, reciprocal absorbance change at 420 nm per min. at 30°.

(Fig. 4). It has been known for many years that ferricyanide accepts electrons from the low potential Fe-S cluster 1 of NADH dehydrogenase (14,15). Thus, the inhibition site must be past the low potential Fe-S cluster, possibly at the junction of NADH dehydrogenase and Q, as in the case of rotenone, amytal, and piericidin A (16). Curiously, high concentrations of MPP^+ stimulate NADH oxidation in the ferricyanide assay at all ferricyanide concentrations used by decreasing the K_M for this oxidant. The mechanism of this effect is not clear but it seems possible that the dehydrogenase, by virtue of its known affinity for hydrophobic molecules, may bind the aromatic part of MPP^+, exposing the positively charged nitrogen, which might then attract the ferricyanide anion, resulting in increased frequency of successful interaction and thus a lowering of the apparent K_M. The much lesser stimulation by N-methylpyridinium, which lacks the hydrophobic binding part and the lack of effect of 4-phenylpyridine (Fig. 4) which is uncharged at the pH of these experiments are in accord with this speculation.

To what extent do these studies support the notion (8) that nigrostriatal cell death in susceptible MPTP-treated animals is triggered by inhibition of the respiratory chain -linked NADH dehydrogenase? Obviously, extensive inhibition of this enzyme leads to virtual cessation of ATP synthesis and, hence, neuronal destruction. The data presented show that at sufficiently high concentrations MPP^+ does inhibit the dehydrogenase almost completely. The uptake mechanism which we

have described (11) accumulates sufficient MPP^+ into the mitochondria to reach these levels, even at low steady-state concentrations of MPP^+ in the cytoplasm. MPTP itself, though considerably more inhibitory to NADH dehydrogenase than MPP^+, is not expected to reach the dopaminergic neurons to a significant extent, because it is rapidly oxidized by glia cells, is not taken up by the synaptic dopamine reuptake system (7), and is not actively concentrated by the mitochondrial system responsible for concentrating MPP^+ (13). Although compatible with the hypothesis that MPP^+ is the neurotoxic agent arising from MPTP and that NADH dehydrogenase is its ultimate target, these observations leave several unresolved questions, such as the highly selective toxicity of the oxidation products of MPTP for certain dopaminergic neurons. Although plausible explanations can be provided, for the moment they remain conjectural.

To the extent that these data point to the ultimate cause of the neurotoxicity of MPTP, they might also be taken to support the suggestions (12) that substituted pyridines, such as 4-phenylpyridine, contribute to the etiology of idiopathic Parkinsonism. 4-Phenylpyridine, a constituent of certain spices (12), is much more inhibitory to NADH dehydrogenase in membranes than is MPP^+, though less inhibitory in intact mitochondria (Fig. 1). This is so because 4-phenylpyridine is not concentrated by the pumping mechanism responsible for MPP^+ accumulation in mitochondria (13). Other structurally related xenobiotics may be even more inhibitory to NADH dehydrogenase. If electrically neutral, at intracellular pH, like 4-phenylpyridine, they would readily cross the blood-brain barrier, then diffuse with the concentration gradient into the mitochondria, resulting in low levels of the compound in the mitochondria and the inhibition of a fraction of the NADH dehydrogenase molecules present. If, by virtue of its hydrophobic nature, 4-phenylpyridine were to accumulate in the inner membrane, over a period of time its effect on the enzyme would also be cumulative. Depending on the frequency of exposure of the person to the xenobiotic, this may eventually lead to significant neuronal loss. Added to the natural decline in the number of nigrostriatal cells with age, the result may be a sufficient decrease in dopamine production for the appearance of overt neurological symptoms.

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REFERENCES

1. Calne, D.B., and Langston, J.W. (1983) Lancet 2, 1457-1459.
2. Langston, J.W., Ballard, P., Tetrud, J.W., and Irwin, I. (1983) Science, 219, 979-980.
3. Burns, R.S., Chiueh, C.C., Markey, S.P., Ebert, M.H.E., Jacobowitz, D.M., and Kopin, I.J. (1983) Proc. Nat. Acad. Sci. U.S. 80, 4546-4550.
4. Chiba, K., Trevor, A., and Castagnoli, N., Jr., (1984) Biochem. Biophys. Res. Communs. 120, 574-578.
5. Salach, J.I., Singer, T.P., Castagnoli, N., Jr., and Trevor, A. (1984) Biochem. Biophys. Res. Communs. 125, 831-835.
6. Singer, T.P., Salach, J.I., and Crabtree, D. (1985) Biochem. Biophys. Res. Communs. 127, 707-712.
7. Javitch, J.A., D'Amato, R.J., Strittmatter, S.M., and Snyder, S.H. (1984) Proc. Nat. Acad. Sci. U.S. 82, 2173-2177.
8. Nicklas, W.J., Vyas, I., and Heikkila, R.E. (1985) Life Sci. 36, 2503-2508.
9. Shinka, T., and Castagnoli, N., Jr., to be published.
10. Langston, J.W., Irwin, I., Langston, E.B., and Forno, L.S. (1984) Neurosci. Letts. 48, 87-92.
11. Ramsay, R.R., Salach, J.I., and Singer, T.P. Biochem. Biophys. Res. Commun., in press.
12. Snyder, S.H. and D'Amato, R.J. (1985) Nature (London) 317, 198-199.
13. Ramsay, R.R., and Singer, T.P., to be published.
14. Beinert, H., Palmer, G., Cremona, T., and Singer, T.P. (1965) J. Biol. Chem. 240, 475-480.
15. Singer, T.P., Gutman, M., and Massey V. (1973) in Iron-Sulfur Proteins, Vol. 1, Lowenberg, W. (ed.), Academic Press, New York, p. 225-300.
16. Gutman, M., Singer, T.P., and Casida, J.E. (1970) J. Biol. Chem. 245, 1992-1997.