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Structural dependence of the inhibition of mitochondrial respiration and of NADH oxidase by 1-methyl-4-phenylpyridinium (MPP^+) analogs and their energized accumulation by mitochondria

(NADH dehydrogenase/membrane transport of cations/environmental neurotoxins/Parkinsonism)

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ABSTRACT **Nineteen structural analogs of 1-methyl-4-phenylpyridinium (MPP^+) were studied for their capacity to inhibit the mitochondrial oxidation of NAD^+-linked substrates and the aerobic oxidation of NADH in inner membrane preparations from cardiac mitochondria. In the majority of cases, a good correlation was found between the two inhibition effects monitored. A few compounds were effective inhibitors of NADH oxidase but had only marginal effects on mitochondrial respiration. From studies of their accumulation by mitochondria, it appears likely that the latter compounds are not effectively concentrated by intact mitochondria by the electrical gradient and, in part for this reason, cannot reach sufficiently high concentrations at the appropriate binding site of NADH dehydrogenase. In addition, evidence is presented that the penetration of pyridinium analogs to the inhibition site in the NADH dehydrogenase complex may also be rate limiting. The data support the thesis that, for a substituted tetrahydropyridine to be acutely neurotoxic, its pyridinium oxidation product must be actively accumulated in the mitochondria and must inhibit NADH-ubiquinone oxidoreductase in its membrane environment.**

Studies over the past 5 years in our laboratories and by others have provided substantial support for the hypothesis that the expression of the neurotoxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and the Parkinsonism it produces involves the following steps, all of which occur in the central nervous system. The initial bioactivation of MPTP is catalyzed by monoamine oxidase (MAO), yielding 1-methyl-4-phenylpyridinium (MPP^+) as the ultimate oxidation product (1, 2). In the case of 2′-substituted neurotoxic analogs of MPTP, part or all of the bioactivation is mediated by MAO-A (3, 4). The MPP^+ is then concentrated by the dopamine reuptake system and reaches the nigrostriatal neurons (5), where it equilibrates across the mitochondrial inner membrane in response to the electrical gradient (6, 7). When the concentration of MPP^+ in the mitochondria reaches levels of several millimolar, the reoxidation of NADH dehydrogenase by coenzyme Q_{10} is blocked; mitochondrial oxidation of NAD^+-linked substrates and, hence, oxidative phosphorylation ceases (8–10), and the resulting depletion of ATP causes collapse of the membrane and death of the nigrostriatal neurons. This is a simplified presentation of current views of the main events. Additional steps and alternative mechanisms may be involved (cf. ref. 7).

The present paper concentrates on the terminal events leading to nigrostriatal cell death: the inhibition of NADH oxidation in mitochondria and in inverted inner membranes by MPP^+ analogs and the energy-dependent accumulation of some of these analogs by intact mitochondria. The experiments reported may shed light not only on the mechanism of site 1 inhibition of mitochondrial respiration by the bioactivation products of MPTP but also on the nature of the inhibition site that MPP^+ shares with rotenone, piericidin A, and barbiturates (10).

MATERIALS AND METHODS

Rat liver mitochondria were prepared from Sprague–Dawley rats by the mannitol procedure (11). Oxygen consumption was measured polarographically. Since inhibition of mitochondrial respiration by MPP^+ and its analogs is a function of both the concentration of inhibitor and the time of its preincubation with the mitochondria, IC_{50} or K_i values cannot be calculated in a simple manner. Hence, the following procedure was adopted. The mitochondria, buffer, and glutamate and malate were preincubated in the polarographic chamber at 25°C with several concentrations of the inhibitor for times varying from 1.5 to 9 min. At that time, ADP was added and the rate of O_2 consumption was determined at 25°C as described (9). The results were then calculated from a plot of 1/time (min) of preincubation with the inhibitor required to reach 50% inhibition of state 3 respiration against the concentration of the inhibitor. The slope of the resulting line is K^* in Table 1. In instances in which the inhibition at all practical concentrations was so small even at 6 min as to make quantitative comparison impractical, the results were expressed as $\ll\ll MPP^+$—i.e., far less effective than MPP^+.

Electron transport particles (ETP) were isolated from beef heart mitochondria (12). NADH oxidase activity was measured spectrophotometrically at 30°C with O_2 as the terminal electron acceptor (10). Energy-dependent accumulation of pyridinium compounds was measured by using tritiated inhibitors as described (6).

Details of the synthesis of the MPP^+ analogs have been published elsewhere (4, 13).

RESULTS AND DISCUSSION

Table 1 compares the inhibition of NADH oxidation in inverted inner membrane preparations (ETP) from beef heart

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Abbreviations: ETP, electron transport particles (an inverted inner membrane preparation from mitochondria); Me-3-BzP^+ and Me-4-BP^+, 1-methyl-3-benzylpyridinium and 1-methyl-4-benzoylpyridinium, respectively; 1,1-Me_2-PTP^+, 1,1-dimethyl-4-phenyltetrahydropyridinium; MPP^+, 1-methyl-4-phenylpyridinium; 2′-$MeMPP^+$, 2′-$EtMPP^+$, 3′-$FMPP^+$, etc., 1-methyl-4-(2′-methylphenyl)pyridinium and 1-methyl-4-(2′-ethylphenyl)pyridinium, 1-methyl-4-(3′-fluorophenyl)pyridinium, etc.; $MPPyrimidinium^+$, 1-methyl-4-phenylpyrimidinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; TPB^-, tetraphenylboron anion.

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Table 1. Inhibition of mitochondrial respiration and NADH oxidation by MPP^+ analogs

	NADH oxidase activity		Mitochondrial respiration on NAD^+-linked substrates	
	IC_{50}, mM	Relative activity	K^*, $mM^{-1}\cdot min^{-1}$	Relative activity
MPP^+	4.0 ± 1.4	100	0.629	100
2′-$MeMPP^+$	4.1	98	0.547	87
2′-$EtMPP^+$	1.0	400	0.477	76
2′-$ClMPP^+$	0.7	571	0.448	71
2′-$OMeMPP^+$	1.2	330	1.05	167
3′-$MeMPP^+$	1.7	235	1.56	248
3′-$BrMPP^+$	2.3	174	0.631	100
3′-$ClMPP^+$	3.0	133	0.574	91
3′-$OMeMPP^+$	2.6	154	0.564	90
3′-$FMPP^+$	5.5	73	0.355	56
4′-$MeMPP^+$	0.4	1000	2.50	397
4′-$FMPP^+$	2.0	200	0.360	57
PPP^+	2.4	167	0.56	89
$MeCP^+$	4.8	83	0.626	100
Me-4-BP^+	3.0	133	0.16	25
Me-3-BzP^+	4.8	83	0.388	62
1,1-Me_2-PTP^+	3.0	133	≪MPP^+	
$MPPyrimidinium^+$	0.7	571	≪MPP^+	
$MePyrP^+$	7.4	54	0.095	15
DP^+	0.03	13300	29 → 105†	4600 → 16700

K^*, empirical expression relating inhibitor concentration to the time required to reach 50% inhibition of respiration, calculated from the slope of Dixon plots of [I] versus $1/t_{1/2}$. PPP^+, 1-propyl-4-phenylpyridinium; $MeCP^+$, 1-methyl-4-cyclohexylpyridinium; $MePyrP^+$, 1-methyl-4-(2-pyridinyl)pyridinium; DP^+, 1-dodecylpyridinium.
†Biphasic.

mitochondria by 18 MPP^+ analogs with their effect on the oxidation of glutamate plus malate (NAD^+-linked substrates) in mitochondria. Since the inhibition of mitochondrial respiration by MPP^+ and its analogs is time dependent, conventional kinetic expressions are inappropriate and, therefore, an empirical expression defined in the legend of Table 1 has been used.

Comparison of the results of the two types of measurements shows a good correspondence in the inhibitory potencies of the various analogs tested, particularly if one considers that neither the IC_{50} value nor the K^* value is a simple linear function of the concentration of inhibitor but, rather, both are empirical expressions. There were, however, notable exceptions in which compounds were considerably more effective as inhibitors of NADH oxidation in purified, submitochondrial particles but were less effective in inhibiting mitochondrial respiration—e.g., 1,1-dimethyl-4-phenyltetrahydropyridinium, 1-methyl-4-phenylpyrimidinium, 1-methyl-4-(2′-ethylphenyl)pyridinium, 1-methyl-4-(2′-chlorophenyl)pyridinium, 1-methyl-4-(4′-fluorophenyl)pyridinium, 1-propyl-4-phenylpyridinium, and 1-methyl-4-benzoylpyridinium (1,1-Me_2-PTP^+, $MPPyrimidinium^+$, 2′-$EtMPP^+$, 2′-$ClMPP^+$, 4′-$FMPP^+$, PPP^+, and Me-4-BP^+, respectively). Possible explanations of this discrepancy are that these compounds may be only slowly accumulated by the mitochondria and/or that their accessibility to the inhibition site in intact mitochondria is restricted. Evidence is presented below that both of these factors may play a role. No compound had a significantly better relative activity in intact mitochondria than it did in ETP. Table 1 further shows a striking inhibition of both NADH oxidase activity and mitochondrial respiration by *N*-dodecylpyridinium. This potent effect is almost certainly due to the lipophilic properties of the compound carrying it into all the respiratory complexes rather than it being a selective inhibitor of site 1 oxidation, since succinate oxidation is also inhibited by this analog.

We have previously reported (9, 10) on the inhibition of NADH oxidation by other MPP^+ and MPTP analogs in inner membranes under conditions paralleling those in Table 1. Among the compounds tested, 4-phenylpyridine was always the most potent NADH oxidase inhibitor, but it had only a modest effect on respiration. This is almost certainly due to the fact that it is not actively accumulated in the mitochondria by the electrochemical gradient, since it is uncharged at neutral pH. In decreasing order of effectiveness, 4-phenyl-1,2,3,6-tetrahydropyridine > MPTP > 4-phenylpiperidine (all uncharged at neutral pH) >> MPP^+ > 1-methyl-2-phenylpyridinium = 1,2-dimethyl-4-phenyl-pyridinium were significantly inhibitory to NADH oxidase in ETP, whereas 1-methyl-3-phenylpyridinium, 1,2-dimethylpyridinium, and 1-methyl-4-*t*-butylpyridinium were not. These data (Table 1) add credence to the belief that there is significant structural specificity in the interaction of pyridine derivatives at the NADH dehydrogenase–CoQ junction.

To obtain evidence for the hypothesis that limited permeability across the inner membrane may account for the ineffectiveness of some MPP^+ analogs as inhibitors of NADH oxidation in intact mitochondria although they are effective in inverted membrane preparations, we measured the energy-dependent accumulation of MPP^+ analogs by mitochondria. Fig. 1 shows that the 2′-Me- and 2′-Et- analogs are accumulated to almost the same extent by mitochondria as is MPP^+, in line with the fact that they are almost as inhibitory to mitochondrial respiration as MPP^+ (Table 1). This agreement is particularly interesting, since 2′-$EtMPP^+$ is 4 times more effective an inhibitor of NADH oxidation in ETP than is MPP^+ itself. Clearly, in this instance, energized accumulation of the pyridinium compounds is a limiting factor. Similar experiments in Fig. 2 show that $MPPyrimidinium^+$ is concentrated by mitochondria only to a very small extent and thus probably never reaches the 0.7 mM intramitochondrial concentration needed to inhibit 50% of NADH oxidation in ETP (Table 1). This might explain why its inhibitory effect on intact mitochondrial respiration is very small. 4′-$MeMPP^+$ and particularly 4′-$FMPP^+$ are concentrated less effectively than MPP^+, in accord with their lesser inhibitory power in mitochondria than in inverted membranes. The correspondence between the effects shown in Fig. 2 and in Table 1 is by no means perfect in these instances. It is emphasized, however, that neither the accumulation nor the mitochondrial inhibition data are linear functions of the concentration of the

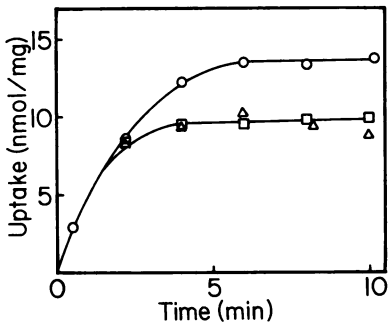

FIG. 1. Energy-dependent accumulation of MPP^+ analogs by mitochondria. The uptake of labeled compounds and intramitochondrial space were measured and the results calculated as in Fig. 1 of Ramsay and Singer (6). The concentration of rat liver mitochondria was 12 mg/ml, the temperature was 25°C, and the concentration of each pyridinium compound was 0.5 mM. The specific activities in cpm/nmol were as follows: MPP^+, 403; 2′-$MeMPP^+$, 60.3; 2′-$EtMPP^+$, 97.6. The results are corrected for non-energy-dependent accumulation observed in the presence of the uncoupling agent, 2,4-dinitrophenol (60 μm). ○, MPP^+; △, 2′-$MeMPP^+$; □, 2′-$EtMPP^+$.

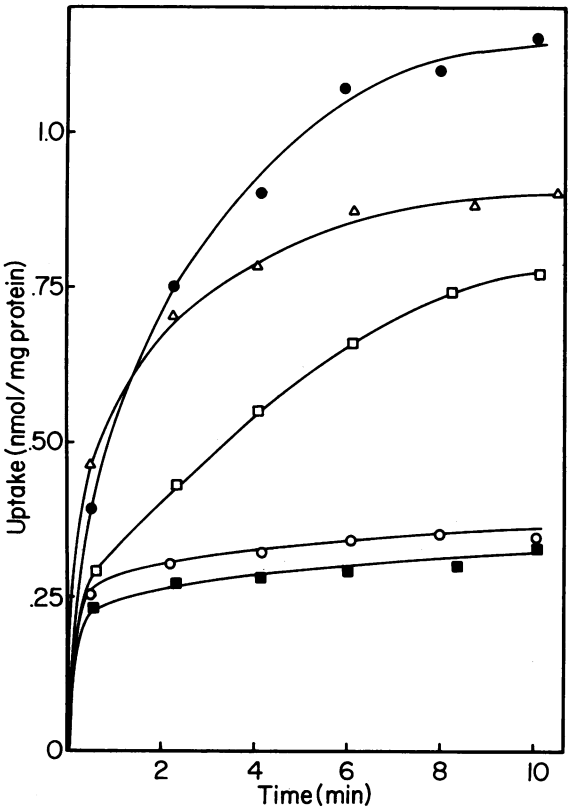

FIG. 2. Energy-dependent accumulation of labeled pyridinium compounds by rat liver mitochondria. Conditions were as in Fig. 1, except that the concentration of labeled compounds used as substrates for accumulation was 50 μM. The specific activity (cpm/nmol) of each analog is given in parenthesis. ●, [^{3}H]MPP$^+$ (262); △, [^{14}C]4′-MeMPP$^+$ (101); □, [^{14}C]1,1-Me$_2$-PTP$^+$ (165); ○, [^{14}C]-4′-FMPP$^+$ (60.4); ■, [^{14}C]4′-MPPyrimidinium$^+$ (115).

inhibitor. Thus, while they are reasonable indices of the effectiveness of different compounds as inhibitors of mitochondrial respiration or the degree to which they are accumulated at a given (50 μM) external concentration, the results from these two techniques permit only qualitative or semiquantitative comparison with each other. There is nevertheless a discrepancy in the behavior of 1,1-Me$_2$-PTP$^+$ in Fig. 2. This compound is appreciably accumulated by the mitochondria, although not nearly as well as MPP$^+$. It is marginally more effective than MPP$^+$ as an inhibitor of NADH oxidation in ETP, but its inhibitory effect on mitochondria is too low to permit quantitative comparison with MPP$^+$ (Table 1). This would suggest that additional factors may limit the effectiveness of such compounds as inhibitors of mitochondrial NADH oxidation.

Evidence for this concept was obtained in following up a recent report (14) that the tetraphenylboron anion (TPB$^-$ accelerates and potentiates the inhibition of mitochondrial respiration by MPP$^+$. Earlier studies (15–17) had demonstrated that TPB$^-$ facilitates the movement of cations across membranes. This was also thought (14) to be the mechanism underlying the effect of TPB$^-$ in potentiating the effectiveness of MPP$^+$ as an inhibitor. Fig. 3 shows that 10 μm TPB$^-$ dramatically decreases the time required to block the ADP-stimulated oxidation of malate plus glutamate in liver mitochondria. The inhibitory effect of MPP$^+$ is virtually instantaneously expressed even at 50 μM external [MPP$^+$], a concentration at which even after 30 min only partial (80%) inhibition is observed in the absence of TPB$^-$. Fig. 4 demonstrates that while 10 μM TPB$^-$ indeed increases both the rate and the extent of the accumulation of MPP$^+$ in the

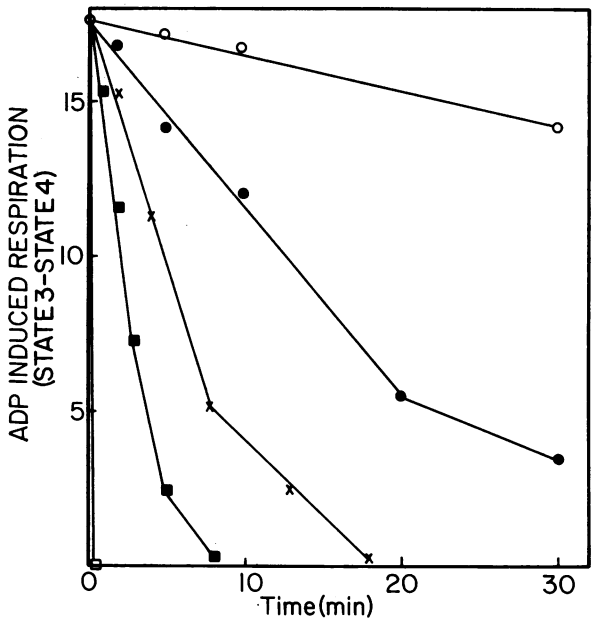

FIG. 3. Time course of the development of inhibition of state 3 respiration with MPP$^+$ and the effect of TPB$^-$ thereon. Rat liver mitochondria were incubated in state 4 at 1 mg of protein per ml in the presence of various concentrations of MPP$^+$ with glutamate and malate and buffer at 25°C as described (9). At the time indicated, ADP was added to 0.25 mM and the rate of O_2 consumption was recorded. Ordinate represents the increased respiration induced by ADP. ○, No MPP$^+$; ●, 50 μM MPP$^+$; ×, 200 μM MPP$^+$; ■, 500 μM MPP$^+$; □, 10 μM TPB$^-$ plus 50–500 μM MPP$^+$. TPB$^-$ (10 μM) alone has no effect on the control rate of respiration (data not shown).

mitochondria, this effect is not as great as its action on mitochondrial respiration (Fig. 3). In fact, as we have reported elsewhere (18), at external concentrations of 50–500 μM MPP$^+$, 10 μM TPB$^-$ increases the intramitochondrial MPP$^+$ concentration by the energy-dependent accumulation only 2.6-fold at apparent equilibrium. Higher concentrations of TPB$^-$ had no additional effect (18). This is clearly insufficient to account for the dramatic effect demonstrated in Fig. 3. This led us to examine the effect of TPB$^-$ on the inhibition

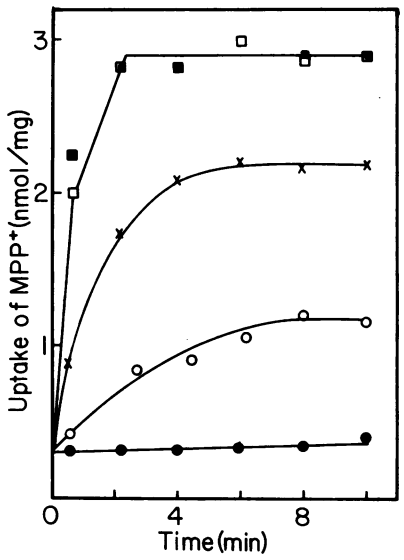

FIG. 4. Effect of TPB$^-$ on the accumulation of [^{3}H]MPP$^+$ by mitochondria. Accumulation was measured as in Fig. 1 at 25°C, with 50 μM [^{3}H]MPP$^+$ (specific activity, 286 cpm/nmol) in all samples. ○, MPP$^+$ alone; ●, MPP$^+$ plus 66 μM dinitrophenol; ×, MPP$^+$ plus 2 μM TPB$^-$; □, MPP$^+$ plus 5 μM TPB$^-$; ■, MPP$^+$ plus 10 μM TPB$^-$.

Table 2. Effect of TPB^- on inhibition of NADH oxidase activity by MPP^+ or 4-phenylpyridine

Inhibitor	Concentration, mM	Inhibition, % Without TPB^-	Inhibition, % With 10 μM TPB^-
MPP^+	0.5	0	34
	1.0	15	60
4-Phenylpyridine	0.2	48	48
	0.5	93	94
	1.0	100	100

The NADH oxidase activity of ETP (0.02 mg/ml) was measured spectrophotometrically at 340 nm and 30°C in 50 mM NaP_i (pH 7.5) in the presence of the inhibitor after preincubation with the inhibitor for 5 min at 0.8 mg of protein per ml.

of NADH oxidation by MPP^+ in ETP (18), since in this system the increased concentration resulting from mitochondrial accumulation is eliminated. TPB^- potentiated the inhibition by MPP^+, particularly at low concentrations of the inhibitor (Table 2). At 1 mM MPP^+ alone, 15% inhibition was observed but in the presence of TPB^- the inhibition was 60%. Together with its ability to enhance accumulation by the mitochondria, this effect could well account for the extraordinary effect of TPB^- demonstrated in Fig. 3.

It was also noted that the effectiveness of 4-phenylpyridine in blocking NADH oxidation in ETP was not affected by TPB^- (18). We have shown (19) that 4-phenylpyridine is a far more potent inhibitor of NADH oxidation in ETP or in complex I than is MPP^+. Neutral pyridines may reach the hydrophobic inhibition site much more readily than charged compounds. Perhaps, by forming a neutral ion pair, TPB^- circumvents the barrier to the access of charged compounds, rendering MPP^+ and its congeners more effective inhibitors once inside the matrix. More extensive data and a discussion of the mechanism of the TPB^- effect have been reported elsewhere (ref. 18; R.E.H., J. Hwang, S. Ofori, H. M. Geller, and W.J.N., unpublished data).

Applying these techniques to resolve the discrepancy observed with 1,1-Me_2-PTP^+ (Table 1), Fig. 5 demonstrates the action of 10 μm TPB^- on the ADP-induced respiration rate (state 3 − state 4) with this inhibitor in the presence and absence of TPB^-. Fig. 5 shows that without TPB^-, 50% inhibition is not reached even at 20 mM external concentration of 1,1-Me_2-PTP^+, almost 2 orders of magnitude higher than that required to block mitochondrial respiration. When 10 μM TPB^- is present, 50% inhibition is reached at 0.06 mM 1,1-Me_2-PTP^+. This major increase in efficacy cannot be

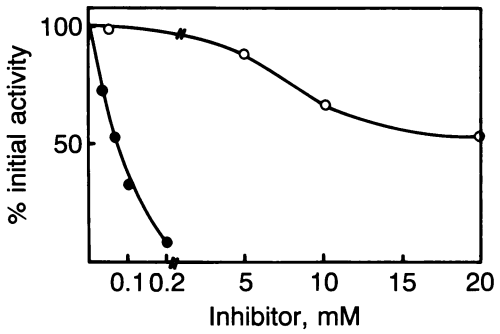

FIG. 5. Inhibition of malate and glutamate oxidation by rat liver mitochondria by 1,1-Me_2-PTP^+ with (●) and without (○) 10 μM TPB^-. Experimental conditions were as in Fig. 3, except as follows: the mitochondria were incubated with glutamate and malate for 2 min at 25°C; then the pyridinium compound was added at the indicated concentrations; at 3 min, 10 μM TPB^- was added and, at 8 min from the start, 0.25 mM ADP was added. Abscissa denotes the ratio of observed state 3 respiration/control state 3 respiration.

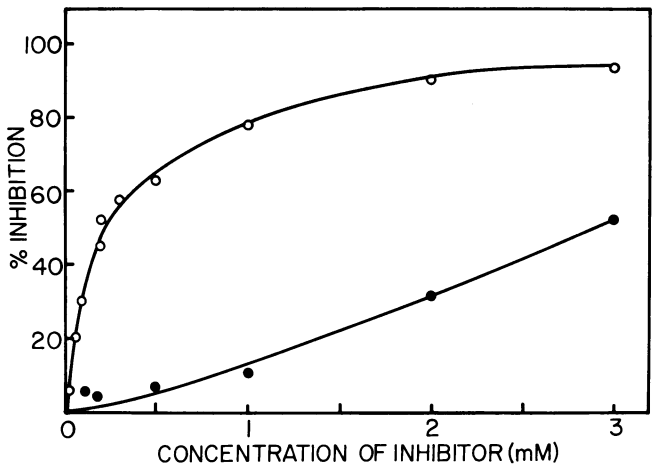

FIG. 6. Effect of TPB^- on inhibition of NADH oxidation in ETP by 1,1-Me_2-PTP^+. The NADH oxidase activity of ETP (0.02 mg/ml) was measured spectrophotometrically at 340 nm and 30°C in 50 mM NaP_i (pH 7.5) in the presence of the inhibitor after preincubation with the inhibitor for 5 min at 0.8 mg of protein per ml. ○, With 10 μM TPB^-; ●, without TPB^-.

accounted for solely by the increase in the energy-dependent accumulation of the inhibitor in the matrix (data not shown). Thus, increased accessibility of the positively charged inhibitor to the hydrophobic binding site in the presence of 10 μM TPB^- is likely to be a factor (Fig. 6).

On the basis of the data presented, we suggest the following model to account for the effect of MPP^+ analogs on mitochondrial respiration. For a pyridinium compound to be effective in blocking mitochondrial respiration and, hence, ATP synthesis, it must be positively charged at intracellular pH so that it is accumulated in the mitochondria as a result of the electrical gradient across the membrane. Once inside the matrix, it must reach the hydrophobic site of inhibition [where barbiturates, rotenone, and piericidins also block respiration (7, 19)], to which charged molecules penetrate only slowly. Comparison of the effectiveness of pyridinium compounds as inhibitors in ETP and in intact mitochondria, in the presence and absence of TPB^-, suggests that access to this site is even more restricted in intact mitochondria than in inverted membrane preparations, such as ETP and complex I. Thus, experiments with ETP or complex I reveal only the minimum requirement for the accessibility of a charged inhibitor to the "rotenone site." In intact mitochondria, additional restrictions may exist.

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