Biochem. J. (1992) 288, 439–443 (Printed in Great Britain)

Uptake and accumulation of 1-methyl-4-phenylpyridinium by rat liver mitochondria measured using an ion-selective electrode

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The compound 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes selective destruction of nigrostriatal dopaminergic neurons in primates, giving rise to a condition resembling Parkinson's disease. The toxicity of MPTP is believed to be due to its metabolite 1-methyl-4-phenylpyridinium (MPP^+). MPP^+ is an inhibitor of mitochondrial respiration at the NADH–ubiquinone oxidoreductase site and this, together with its selective transport into dopaminergic nerve terminals, accounts for its neurotoxicity. In this paper an electrode selective for MPP^+ was developed and used to measure the rate of uptake and the steady-state accumulation of MPP^+ in rat liver mitochondria. The initial rates of MPP^+ uptake were not saturable, confirming previous work that the transport of MPP^+ is not carrier-mediated. The membrane potential of mitochondria respiring on succinate was decreased by MPP^+ and the steady-state accumulation ratio of MPP^+ did not come to equilibrium with the mitochondrial transmembrane potential gradient ($\Delta\psi$). The effect of the cation exchanger tetraphenylboron (5 μM) was to increase the initial rate of MPP^+ uptake by about 20-fold and the steady-state accumulation by about 2-fold. This suggests that there may be a mechanism of efflux of MPP^+ from mitochondria which allows MPP^+ to cycle across the membrane and thus decrease $\Delta\psi$. These data indicate that MPP^+ interacts with mitochondria independently of its inhibition of NADH–ubiquinone oxidoreductase, and these alternative interactions may be of relevance for its mechanism of neurotoxicity.

INTRODUCTION

The parkinsonian syndrome in primates produced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) appears to be dependent on its conversion by monoamine oxidase B (EC 1.4.3.4; MAO B) to its pyridinium derivative, 1-methyl-4-phenylpyridinium (MPP^+), by way of the dihydropyridinium intermediate 1-methyl-4-phenyl-2,3-dihydropyridine ($MPDP^+$) (Chiba *et al.*, 1984; Salach *et al.*, 1984; Castagnoli *et al.*, 1985). The cation MPP^+ is a substrate for the presynaptic dopaminergic re-uptake system (Javitch *et al.*, 1985) and it is further electrophoretically accumulated into synaptic mitochondria where it inhibits NADH–ubiquinone oxidoreductase (complex 1; EC 1.6.99.3) (Nicklas *et al.*, 1985).

Early studies indicated that the rate of MPP^+ uptake into mitochondria was apparently saturable, suggesting it to be carrier-mediated (Ramsay & Singer, 1986; Ramsay *et al.*, 1987). However, subsequently it was found that charged and uncharged structural analogues of MPP^+ were passively transported into the mitochondria in an energy-dependent manner (Hoppel *et al.*, 1987), suggesting that uptake was not carrier-mediated. This view was further supported by the demonstration that the tetraphenylboron anion (TPB^-) stimulated the transport of MPP^+ via an energy-dependent concentration process across the mitochondrial inner membrane, driven by the transmembrane potential gradient ($\Delta\psi$) (Aiuchi *et al.*, 1988; Heikkila *et al.*, 1990), and enhanced the inhibitory effect in mitochondria by facilitating access to a hydrophobic site on NADH–ubiquinone oxidoreductase (Ramsay *et al.*, 1989).

The method used previously for studying the uptake of MPP^+ into mitochondria has involved studies on the accumulation of the ^3H-labelled compound. This paper reports the development of an MPP^+-selective electrode system and its use in determining the initial rate of MPP^+ uptake and the extent of MPP^+ accumulation by mitochondria. The effects of the lipophilic anions carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and TPB^- on MPP^+ uptake and on the steady-state distribution of MPP^+ are discussed. Also, experiments are reported using [^{3}H]methyltriphenylphosphonium ([^{3}H]$TPMP^+$) and $^{86}Rb^+$ distributions to determine the effects on membrane potential as MPP^+ is accumulated into the mitochondrial matrix.

MATERIALS AND METHODS

Preparation of mitochondria

Rat liver mitochondria were prepared from female Wistar rats by homogenization followed by differential centrifugation (Chappell & Hansford, 1972) in ice-cold medium containing 250 mM-sucrose, 5 mM-Tris and 1 mM-EGTA adjusted to pH 7.4 with HCl at 25 °C. The mitochondrial suspension, which usually contained about 50–70 mg of protein/ml, was stored on ice and used for experiments within 4 h of isolation. Mitochondrial protein concentration was determined by the Biuret method using BSA as the standard (Gornall *et al.*, 1949).

Measurement of mitochondrial matrix volume, membrane potential and respiration rate

The mitochondrial matrix volume was measured (see Brown & Brand, 1985) using [^{14}C]sucrose (20 nCi/ml) as an extramitochondrial marker and ^3H_2O (0.5 μCi/ml) to determine the total pellet volume. Matrix space was calculated by subtracting the [^{14}C]sucrose space from the ^3H_2O space. $\Delta\psi$ was calculated using two methods as follows. (i) By measuring the distribution of [^{3}H]$TPMP^+$, in the presence of unlabelled TPMP (5 μM),

Abbreviations used: MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; $MPDP^+$, 1-methyl-4-phenyl-2,3-dihydropyridine; MPP^+, 1-methyl-4-phenylpyridinium; $TPMP^+$, methyltriphenylphosphonium; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TPB^-, tetraphenylboron; $\Delta\psi$, transmembrane potential gradient.

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across the mitochondrial inner membrane (see Brown & Brand, 1985). A correction factor for the 60% non-specific intramitochondrial binding of $TPMP^+$ was used when calculating $\Delta\psi$ from the Nernst equation (Brown & Brand, 1985). (ii) By measuring the distribution of $^{86}Rb^+$, in the presence of valinomycin, across the mitochondrial inner membrane (see Jackson & Nicholls, 1986); for these experiments nigericin was omitted from the incubation to avoid uncoupling. For most experiments nigericin and high concentrations of KCl were present, clamping the pH gradient across the mitochondrial inner membrane close to zero (Brown & Brand, 1985). Therefore the pH component of the protonmotive force was negligible and the membrane potential was the sole component of the protonmotive force.

Respiration rates were measured using an oxygen electrode (Rank Brothers, Bottisham, Cambridge, U.K.) built into the base of a 3 ml airtight chamber which was thermostatted and rapidly stirred. Rates were calculated assuming 444 nmol of O/ml at 30 °C (see Reynafarje *et al.*, 1985).

Construction of an MPP^+-selective electrode

Accumulation of MPP^+ into mitochondria was measured using an MPP^+-sensitive electrode. This was constructed in a similar way to the $TPMP^+$ electrode system described by Kamo *et al.* (1979) by procedures widely used for lipophilic cation-selective electrodes as described by these authors. The MPP^+-sensitive membrane consisted of an ion-exchanger, TPB^-, incorporated into a PVC membrane. This was fixed, using tetrahydrofuran, to the end of a piece of PVC tubing. The membrane was made selective for MPP^+ by placing 10 mM-MPP^+ solution inside the tubing and then storing the membrane part of the tube overnight in 10 mM-MPP^+. Platinum wire soldered to the central wire of a coaxial cable was placed inside the PVC tubing and the electrode was mounted in a Perspex block equipped with an injection port along with a Ag/AgCl reference electrode (Reagecon Ltd., Shannon, Ireland). This allowed the electrode to be placed into a 3 ml standard thermostatted chamber giving an air-tight seal. The coaxial cable from the MPP^+ electrode and the Ag/AgCl reference electrode were connected to a Phillips digital (PW 9409) pH meter, which in turn was connected to a Phillips dual-pen (PM 8252) chart recorder.

Materials

[^{14}C]Sucrose, ^3H_2O, [^{3}H]TPMP bromide and ^{86}RbCl were obtained from Amersham International. MPP^+ (iodide salt) was from Research Biochemicals Inc. (Wayland, MA, U.S.A.). All other chemicals were from Sigma or BDH.

RESULTS AND DISCUSSION

Performance and application of the MPP^+-selective electrode

The response of the MPP^+-selective electrode to MPP^+ is shown in Fig. 1, where it is compared with that of a $TPMP^+$-selective electrode (Kamo *et al.*, 1979). The responses of both electrodes were linear with the logarithm of the cation concentration, with a slope of 58.5 mV per decade concentration down to a concentration of about 10^{-6} M for $TPMP^+$ and 10^{-5} M for MPP^+. Thus the MPP^+ electrode has a Nernstian response over much of its range and can be used to measure the uptake of MPP^+ by mitochondria. Controls were carried out to ensure that the electrodes did not respond to other compounds used here. During a run of experiments the tip of the electrode was stored overnight in 10 mM-MPP^+ to retain membrane sensitivity for MPP^+. After 3–4 days, drift of the electrode trace became a limiting factor and a constant baseline before MPP^+ calibration was not possible. In addition, the response time of the electrode decreased with use, eventually becoming too large to record accurately the initial rate of MPP^+ accumulation.

Contrary to an earlier report (Ramsay & Singer, 1986), the transport of MPP^+ into energized mitochondria was found to be linearly dependent on MPP^+ concentration. The development of an ion-selective electrode specific for MPP^+ provided a method

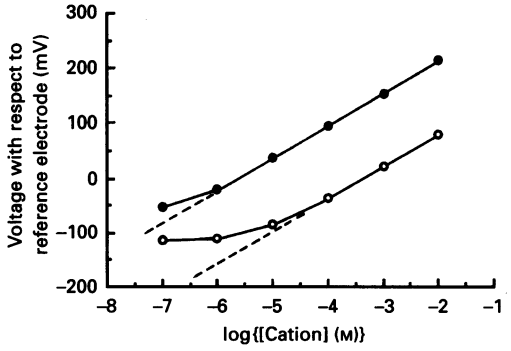

Fig. 1. Response of MPP^+ and $TPMP^+$ ion-selective electrodes

The MPP^+ (○)- or $TPMP^+$ (●)-sensitive electrodes were immersed in KCl (120 mM, pH 7.2) medium in a 3 ml system thermostatted at 30 °C, and the voltage of the ion-selective electrode was measured with respect to the Ag/AgCl reference electrode. The concentrations of the MPP^+ and $TPMP^+$ cations were varied by immersing the electrodes sequentially in media of greater cation concentrations and measuring the stable potential difference after 2 min. Data shown here are for one set of experiments for each electrode.

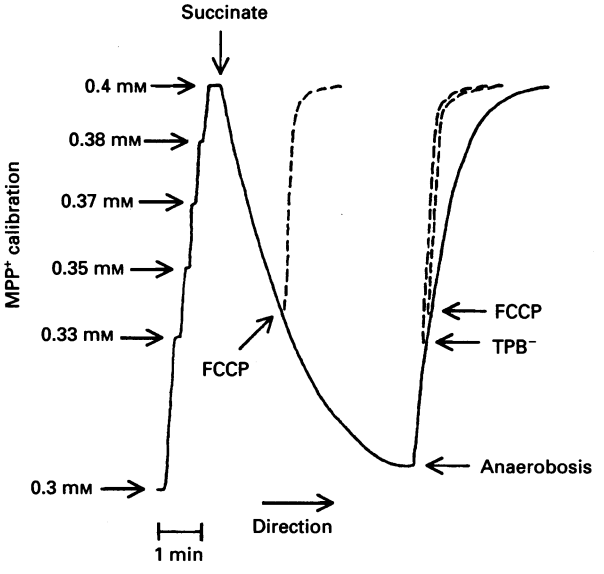

Fig. 2. Accumulation of MPP^+ by rat liver mitochondria

Mitochondria (2 mg/ml) were incubated in medium (0.12 M-KCl, 10 mM-Hepes, 1 mM-EGTA, pH 7.2) containing the NADH–ubiquinone oxidoreductase inhibitor rotenone (5 μM), the ATP synthase inhibitor oligomycin (1.25 μg/ml) and the ionophore nigericin (0.2 μg/ml) at 30 °C for 2 min in a 3 ml stirred chamber. The MPP^+ electrode was calibrated by sequential additions of MPP^+. Mitochondria were then energized by addition of succinate (10 mM; K^+ salt) and the initial rate of uptake of MPP^+ was measured. Uptake continued until a steady-state accumulation of MPP^+ was reached. When the system became anaerobic, all of the MPP^+ was released. In separate experiments, shown by broken lines, the efflux of MPP^+ was initiated by addition of FCCP (0.5 μM) prior to anaerobosis and was accelerated by addition of FCCP (0.5 μM) or TPB^- (10 μM) after anaerobosis.

Table 1. Steady-state distribution of MPP^+ and $TPMP^+$ between the matrix and the external medium

Conditions for accumulation into mitochondria are given in the legends to Figs. 2 and 3. The steady-state amount of MPP^+ inside the mitochondria and the concentration of MPP^+ outside were calculated from the uptake traces at a range of initial concentrations of MPP^+. In parallel experiments the mitochondrial volumes were calculated and used to determine the concentration of matrix MPP^+. In separate experiments the distribution of [^{3}H]$TPMP^+$ was determined as described in the Materials and methods section and this ratio was corrected for intramitochondrial binding of $TPMP^+$. Results are shown as mean values ± S.E.M. from experiments on at least three different mitochondrial preparations.

[MPP^+] outside initially (mM)	[MPP^+] outside as steady state (mM)	[MPP^+] inside as steady state (mM)	[$TPMP^+$] inside / [$TPMP^+$] outside at steady state
0.4	0.331 ± 0.003	49.0 ± 2.0	345.3 ± 9.5
0.8	0.696 ± 0.004	73.8 ± 2.4	224.0 ± 10.3
1.2	1.080 ± 0.000	85.5 ± 2.0	167.3 ± 6.9
1.6	1.452 ± 0.011	104.5 ± 7.9	151.4 ± 12.9
2.0	1.825 ± 0.026	125.0 ± 18.4	118.1 ± 3.8

for directly determining the time course of accumulation of the compound by mitochondria, thus allowing initial rates to be measured with greater accuracy. The rapid departure of the time course from initial-rate conditions (see Fig. 2) may account for the earlier report of saturation kinetics from experiments where the accumulation of MPP^+ was measured after a fixed time (Ramsay & Singer, 1986). A further advantage of the MPP^+ electrode is that it enables the effects of different compounds to be studied by serial additions in a single incubation. During the preparation of this paper the construction of a MPP^+-selective electrode which has similar properties to that described here was reported by Aiuchi *et al.* (1992).

Fig. 2 shows the results of a typical experiment in which the time-dependent uptake of MPP^+ into energized mitochondria was measured. The MPP^+ accumulation reached a steady state (see also Fig. 6). This steady-state distribution persisted until the mitochondrial suspension become anaerobic. At that stage $\Delta\psi$ was dissipated and the MPP^+ inside was released back into the external medium. In separate experiments, addition of the uncoupler FCCP prior to anaerobosis caused the immediate release of MPP^+ back into the external medium due to de-energization. The rate of MPP^+ efflux after the addition of FCCP was greater than during anaerobosis (Fig. 2), and addition of FCCP or TPB^- to an anaerobic suspension increased MPP^+ efflux from the mitochondrial matrix (Fig. 2). TPB^- forms a neutral ion-pair complex with lipophilic cations such as MPP^+, thus facilitating its membrane transport (Grinuis *et al.*, 1970; Ketterer *et al.*, 1971; Hunziker *et al.*, 1985) by lowering the activation energy for passage of MPP^+ through the lipid bilayer (Aiuchi *et al.*, 1988). The stimulation of MPP^+ efflux caused by FCCP relative to efflux on anaerobosis was presumably because it too is a lipophilic anion (Miyoshi & Fujita, 1987) which can form an ion pair with MPP^+ in the same manner as TPB^-.

Uptake of MPP^+ by energized rat liver mitochondria

The MPP^+-selective electrode was used to measure the initial rates of MPP^+ uptake in experiments similar to those shown in Fig. 2. For each experiment it was necessary to calibrate the electrode with MPP^+ before energizing the mitochondria in order to calculate accurately the rate of uptake. Fig. 3(*a*) shows that the initial rate of uptake of MPP^+ was linearly dependent on the

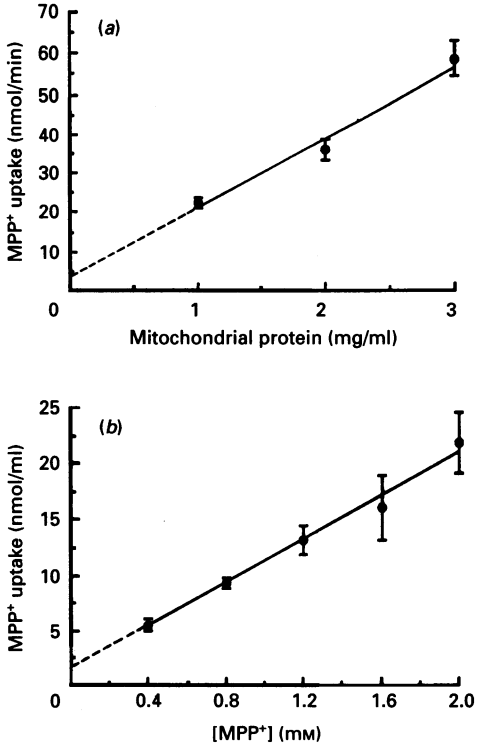

Fig. 3. Initial rate of uptake of MPP^+ by energized rat liver mitochondria

Mitochondria were incubated as described in the legend to Fig. 2. In (*a*), mitochondria (1, 2 or 3 mg/ml) were incubated and, following MPP^+ calibration up to a final concentration of 0.4 mM, uptake was initiated by succinate (10 mM; K^+ salt). The initial rate of uptake was calculated from a calibration curve and plotted against the concentration of mitochondria. In (*b*), mitochondria (2 mg/ml) were incubated with different concentrations of MPP^+ and, following calibration, uptake of MPP^+ was initiated by addition of succinate (10 mM; K^+ salt). Rates of initial uptake were calculated as previously described above. Results for (*a*) and (*b*) are expressed as mean values ± S.E.M. of duplicate determinations carried out on three separate preparations of mitochondria.

amount of mitochondrial protein. Thus, within the concentration range used in this work (2 mg of mitochondrial protein/ml), the response time of the electrode does not limit the measurement of MPP^+ uptake. From Fig. 3(*b*) it can be seen that the initial rate of MPP^+ uptake was a linear function of the extramitochondrial MPP^+ concentration. This demonstrated that the accumulation of MPP^+ occurred in a non-saturable energy-dependent manner. In both parts of Fig. 3 the lines did not pass through the origin, indicating some non-specific binding of MPP^+ to mitochondria. In Fig. 2 it can be seen that after anaerobosis the MPP^+ trace returned to approximately the same position as the baseline prior to addition of succinate, suggesting that the non-specific binding of MPP^+ seen in Fig. 3 was due to energized mitochondria.

Mitochondrial membrane potential and the steady-state distribution of MPP^+

The time courses of MPP^+ accumulation were determined and the concentrations in the matrix and the external solution were calculated when the plateau, steady-state distribution of MPP^+ was reached (see Figs. 2 and 6). In parallel experiments the mitochondrial volumes and the distribution of [^{3}H]TPMP between the matrix and the external medium were determined during this steady-state distribution of MPP^+. The mitochondrial volume under these conditions was 0.6 ± 0.1 μl/mg of protein (mean ± S.E.M.; $n = 16$) and was unaffected by concentrations of

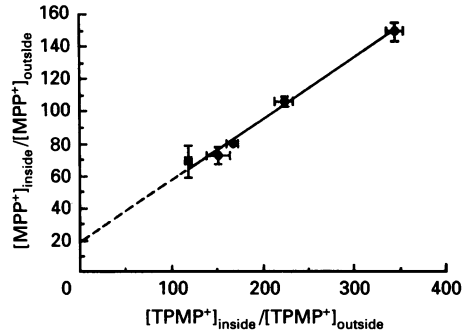

Fig. 4. Accumulation ratio of MPP^+ plotted against that of $TPMP^+$ at different initial concentrations of MPP^+

These data are taken from Table 1. Results are shown as the mean values ± S.E.M. from at least three determinations from three different mitochondrial preparations.

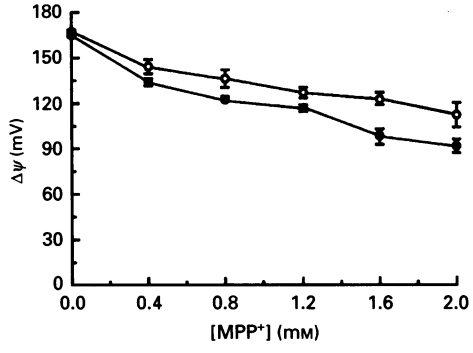

Fig. 5. Mitochondrial membrane potential at different concentrations of MPP^+

Conditions for measurement of $\Delta\psi$ by [^{3}H]TPMP distribution (○) are described in the Materials and methods section. For $^{86}Rb^+$ distribution (●), mitochondria were incubated in a different medium containing 0.25 M-sucrose, 5 mM-Hepes, 1 mM-EGTA, pH 7.2, and 0.2 μM-valinomycin. The suspensions were incubated for the same time taken for the MPP^+ uptake to come to a steady state, as seen in Figs. 2 and 6 (about 5 min). Initial MPP^+ concentrations ranged between 0 and 2 mM and results shown are the mean values ± S.E.M. from duplicate determinations from four separate mitochondrial preparations.

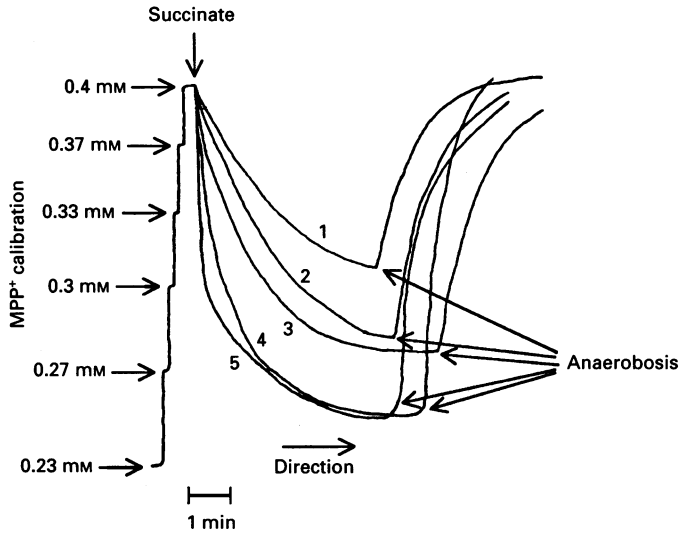

Fig. 6. Effect of TPB^- on the accumulation of MPP^+ by rat liver mitochondria

Mitochondria were incubated in medium at 30 °C as described in the legend to Fig. 2. The MPP^+ (0.4 mM) calibration was made and amounts of TPB^- were added. After 5 min of incubation, MPP^+ uptake was initiated by energizing the mitochondria with succinate (10 mM; K^+ salt). 1, MPP^+ alone; 2, MPP^+ plus 0.17 μM-TPB^-; 3, MPP^+ plus 0.33 μM-TPB^+; 4, MPP^+ plus 1.7 μM-TPB^-; 5, MPP^+ plus 5 μM-TPB^-.

MPP^+ up to at least 2 mM. Table 1 shows the distribution ratios of $TPMP^+$ and MPP^+ for different initial concentrations of MPP^+. It can be seen from Fig. 4 that there was a linear relationship between the distribution ratios of $TPMP^+$ and MPP^+, but the line did not go through the origin, again suggesting some non-specific binding of MPP^+ to energized mitochondria. The linear relationship between the accumulation ratios of MPP^+ and $TPMP^+$ indicate that the steady-state distribution of MPP^+ was largely determined by, and had reached a steady state with respect to, $\Delta\psi$. However, the slope of the plot of the MPP^+ accumulation ratio against that of $TPMP^+$ (about 0.37) indicates that the distribution of MPP^+ did not equilibrate with $\Delta\psi$.

In addition, Fig. 4 indicates that the accumulation of MPP^+ decreased the $\Delta\psi$. In Fig. 5 it can be seen more clearly that $\Delta\psi$ measured by [^{3}H]$TPMP^+$ distribution across the mitochondrial membrane was decreased by increasing the initial concentration of MPP^+. Furthermore, this decrease in $\Delta\psi$ was maintained when $\Delta\psi$ was measured by the distribution of $^{86}Rb^+$ in the presence of valinomycin, indicating that the decrease in $\Delta\psi$ caused by MPP^+ was not an artefact due to nigericin or of MPP^+ interfering with the distribution of [^{3}H]$TPMP^+$. Intriguingly, it was shown that this decrease in $\Delta\psi$ was not paralleled by a large change in respiration rates (the respiration rate was 19.3 ± 0.8 nmol of O/min per mg at zero MPP^+ and 23 ± 3.4 nmol of O/min per mg at 2 mM-MPP^+; $n = 3$). In Fig. 6 it is shown that increasing the concentration of TPB^- to 5 μM increased the rate of uptake of MPP^+ by about 20-fold, and also increased the steady-state accumulation of MPP^+. This accumulation saturates at high concentrations of TPB^-, and at this point the accumulation ratio for MPP^+ was approximately twice that prior to addition of TPB^-. This indicates that the MPP^+ distribution was closer to equilibrium with $\Delta\psi$.

In previous work on the uptake of MPP^+ into mitochondria (Ramsay *et al.*, 1989), the authors speculated that this compound was concentrated across the membrane in response to $\Delta\psi$ but that a Nernstian equilibrium was not reached. The data presented above suggest that, after initial uptake by the mitochondria, MPP^+ accumulation and efflux reach a steady state which is not at equilibrium with $\Delta\psi$. At this steady-state point $\Delta\psi$ was decreased according to the amount of MPP^+ accumulated into the matrix.

The mechanism of this decrease of $\Delta\psi$ by MPP^+, which is presumably unrelated to its inhibition of NADH–ubiquinone oxidoreductase (as in these experiments the mitochondria were energized by succinate), is unclear. However, the evidence that MPP^+ does not equilibrate with $\Delta\psi$ suggests that there may be an electroneutral efflux of MPP^+, allowing cycling of MPP^+ across the mitochondrial inner membrane and thus dissipation of $\Delta\psi$.

In summary, an electrode specific for MPP^+ was developed which allowed the initial rates of uptake of MPP^+ by mitochondria and its steady-state distribution across the mitochondrial inner membrane to be measured. These results confirmed that the initial rate of uptake of MPP^+ was not saturable, suggesting that the uptake of MPP^+ is not carrier-mediated. The steady-state distribution of MPP^+ did not equilibrate with $\Delta\psi$, and in fact decreased the $\Delta\psi$ of mitochondria respiring on

succinate. These data suggest that the interactions of MPP^+ with mitochondria are more complicated than previously thought, and elucidation of the mechanism by which MPP^+ decreases $\Delta\psi$ may be of relevance for the understanding of idiopathic Parkinson's disease.

We are grateful to Dr. Martin D. Brand, Department of Biochemistry, University of Cambridge, for his initial suggestions on the construction of an electrode specific for MPP^+. In addition, we acknowledge the support provided to this work by Bioresearch Ireland.

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Received 2 April 1992/15 June 1992; accepted 18 June 1992