TOXICOLOGY AND APPLIED PHARMACOLOGY 96, 347–359 (1988)

MPTP and MPTP Analogs Induced Cell Death in Cultured Rat Hepatocytes Involving the Formation of Pyridinium Metabolites

YOGENDRA SINGH, ERIC SWANSON, EDWARD SOKOLOSKI,* R. KRISHNAN KUTTY, AND GOPAL KRISHNA[1]

*Section on Drug Tissue Interaction, Laboratory of Chemical Pharmacology and *Laboratory of Chemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892*

Received March 21, 1988; accepted July 27, 1988

MPTP and MPTP Analogs Induced Cell Death in Cultured Rat Hepatocytes Involving the Formation of Pyridinium Metabolites. SINGH, Y., SWANSON, E., SOKOLOSKI, E., KUTTY, R. K., AND KRISHNA, G. (1988). *Toxicol. Appl. Pharmacol.* **96**, 347–359. MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) which has been shown to produce a Parkinson-like syndrome in humans and monkeys also causes cell death in cultures of rat hepatocytes. Treatment of cells with MPTP or its metabolite MPP^+ (1-methyl-4-phenyl pyridinium ion), resulted in leakage of lactic acid dehydrogenase and ^{14}C-labeled adenine nucleotides, as well as marked depletion of ATP and glutathione. Deprenyl, a specific inhibitor of monoamine oxidase-B, the enzyme catalyzing the oxidation of MPTP into MPP^+, blocked the lethal effect of MPTP, but gave no protection from MPP^+-induced cell death. The 4′-fluoro and 4′-chloro analogs of MPTP evoked toxicities similar to that of the parent compound, while *N*-butyl-PTP, 4′-amino-MPTP, and 2′-methyl-MPTP were relatively less toxic. *N*-Acetylamino-MPTP was found virtually nontoxic. The cell death produced by these analogs was also associated with leakage of [^{14}C]adenine nucleotides, which is an indicator of loss of ATP from cells. All these compounds except the *N*-acetylamino analog were converted to corresponding pyridinium metabolites by liver cells when analyzed by high-pressure liquid chromatography and plasma desorption mass spectrometry. MPTP and its analogs also served as substrates for rat liver mitochondrial monoamine oxidase to varying degrees. Toxicity of various analogs, with the noticeable exception of 2′-methyl-MPTP, was inhibited by deprenyl. These findings indicate that the conversion of MPTP and its analogs to corresponding pyridinium metabolites is essential for the expression of toxicity.

MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a by-product in the synthesis of a mepyridine analog, first used as a substitute for heroin, has been shown by several investigators to induce a Parkinson-like syndrome in humans (Langston *et al.*, 1983) and monkeys (Burns *et al.*, 1983). Several studies have indicated that MPTP is not neurotoxic by itself (Heikkila *et al.*, 1984; Cohen *et al.*, 1985) but instead is converted to a neurotoxic pyridinium compound by monoamine oxidase-B (MAO-B, EC 1.4.3.4) in the brain (Markey *et al.*, 1984; Chiba *et al.*, 1984; Irwin and Langston, 1985). Supporting this theory are the facts that dopamine-containing areas of the brain that are rich in MAO-B are highly susceptible to the neurotoxic effects of MPTP (Westlund *et al.*, 1985) and that these effects can be virtually prevented by treatment with

[1] To whom reprint request should be addressed: Bldg. 10, 8N-107, NIH, Bethesda, MD 20892.

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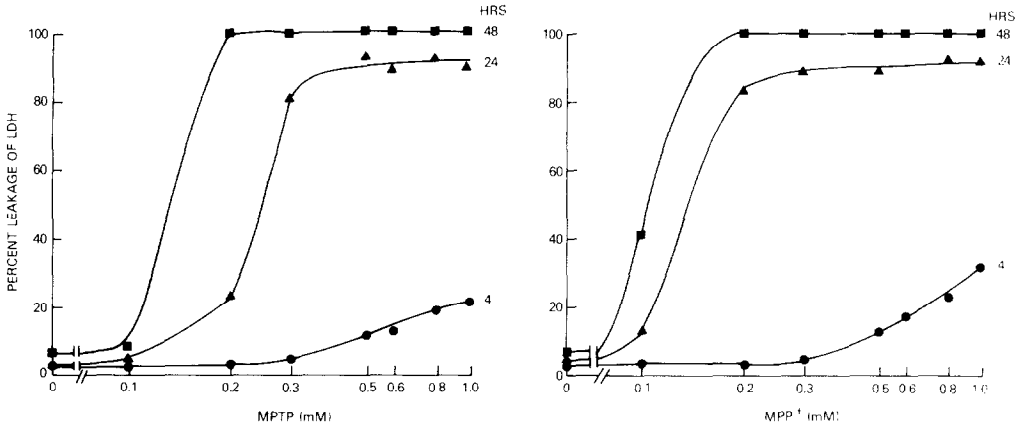


FIG. 1. Dose response of hepatocytes to MPTP and MPP^+ as measured by induced leakage of LDH in 4, 24, and 48 hr. The hepatocytes were incubated with varying concentrations of MPTP and MPP^+ (0.1–1 mM) and aliquots of media were collected after selected time intervals and tested for leakage in the manner described under Materials and Methods. Values are the means of four separate hepatocyte incubations. Standard errors of the mean were less than 5%.

specific MAO-B inhibitors such as deprenyl (Langston *et al.*, 1984; Heikkila *et al.*, 1984; Cohen *et al.*, 1985). It has been postulated that MPP^+ formed from MPTP in glial cells is released into the vicinity of dopamine-containing neurons, which actively take up MPP^+ through the dopamine uptake system (Snyder and D'Amato, 1986; D'Amato *et al.*, 1986). The mechanism by which these pyridinium compounds cause cell death after accumulation in the dopamine-containing neurons, however, is still not clear.

Di Monte *et al.* (1986) have recently found that relatively high concentrations of MPTP and MPP^+ cause cell death in rat hepatocyte suspension through depletion of cell ATP, and that pargyline, an inhibitor of both forms of monoamine oxidase, was capable of preventing MPTP-induced cell death without affecting the toxicity of MPP^+. The uptake of MPP^+ by isolated mitochondrial preparations from brain and liver has been shown to cause inhibition of the oxidation of NADH-linked substrates (Nicklas *et al.*, 1985; Ramsay *et al.*, 1986). MPTP and MPP^+ produced a collapse in mitochondrial membrane potential (Krishna *et al.*, 1987) which would result in the inhibition of ATP synthesis. MPP^+ at higher concentration (1.5 mM) caused GSH depletion following inhibition of glutathione reductase by 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (Di Monte *et al.*, 1986b). Lowering of the intracellular glutathione content by treating cells with diethylmaleate failed to potentiate the toxicity of MPTP (Smith *et al.*, 1987).

In this study we have investigated the mechanism by which MPTP causes cell death in cultured rat hepatocytes. These cells are relatively easy to maintain *in vitro* and contain both types of monoamine oxidase (Gomez *et al.*, 1986), as well as several other enzyme systems that may play a role in MPTP-induced cell death, such as cytochrome *P*-450 and cytochrome *P*-450 reductase (Sinha *et al.*, 1986). We have also studied the effects of selective inhibition of MAO-B with deprenyl on cell death induced by MPTP, its metabolite, MPP^+, and a number of MPTP analogs. Moreover, we have examined their cell-killing effects at low concentrations over an extended period of 24–48 hr and correlated their toxic effects with depletion of cell ATP and GSH and release of radioactively labeled

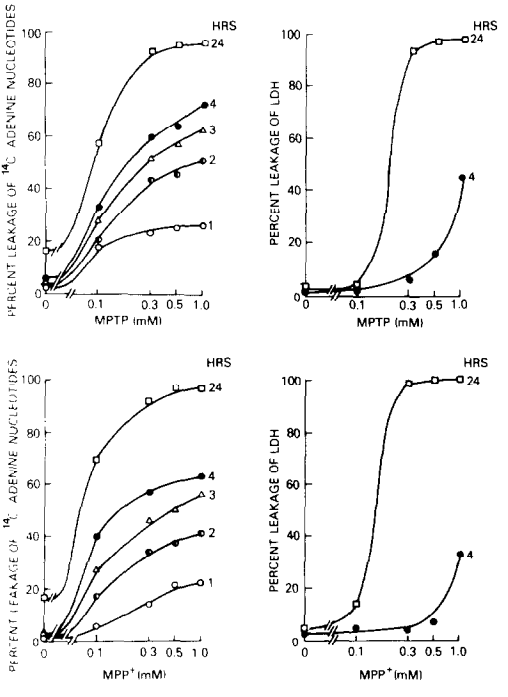


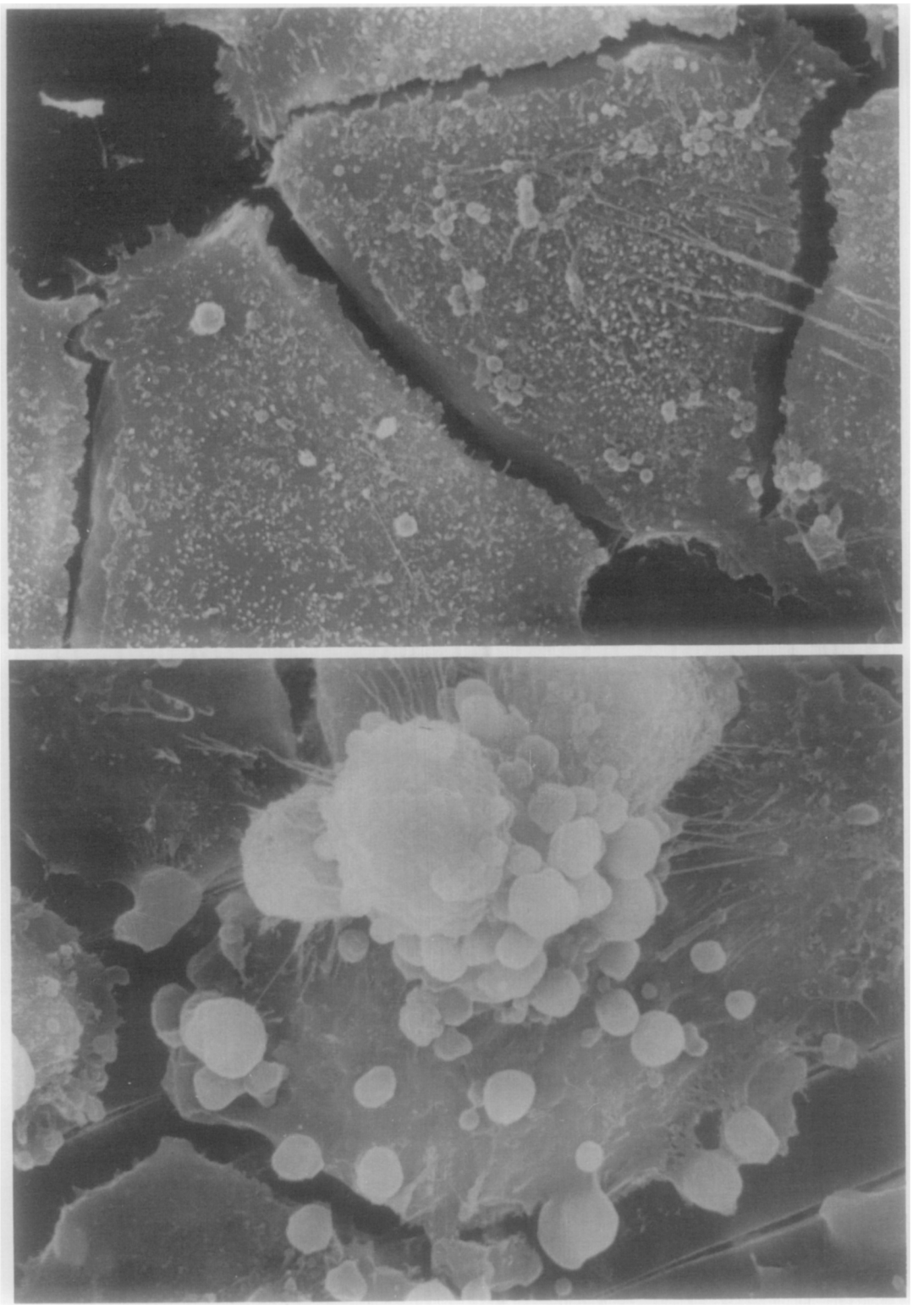
FIG. 2. Dose and time responses of hepatocytes to MPTP and MPP^+ as measured by induced leakage of [^{14}C]adenine nucleotides and LDH. The hepatocytes were incubated as above with varying concentrations of MPTP and MPP^+; aliquots of medium were taken after selected time intervals and tested for leakage as described under Materials and Methods. Values are the means of four separate hepatocyte incubations. Standard errors of the mean were less than 2.5 and 5% for [^{14}C]adenine nucleotide and LDH leakage, respectively.

adenine nucleotides prior to enzyme loss from the cell.

MATERIALS AND METHODS

MPTP-HCl, MPP^+I^-, 2'-methyl-MPTP, and L-deprenyl were obtained from Research Biochemicals, Inc., Wayland, Massachusetts. *N*-Butyl, 4'-fluoro, 4'-chloro, 4'-amino, and *N*-acetyl-4'-amino analogs of MPTP were synthesized and kindly supplied by Dr. S. Markey (Lyle and Anderson, 1966; Allen and Hamburys, 1966; Johannessen *et al.*, 1987). Hydrocortisone-21-succinate, dexamethasone, aminolevulinic acid, insulin, pyruvate, NADH, NADPH, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), ATP reagent, glutathione (oxidized and reduced), sodium azide, 2,2'-azinodi(3-ethylbenzthiozoline-6-sulfonic acid), horseradish peroxidase, and ATP were purchased from Sigma Chemical Co., St. Louis, Missouri. Collagenase was purchased from Cooper Biomedical, Malvern, Pennsylvania. Nu Serum was obtained from Collaborative Research, Inc., Lexington, Massachusetts. Williams' E medium and Earle's balanced salt solution were purchased from GIBCO Labs, Grand Island, New York. [^{14}C]Adenine hydrochloride (53 mCi/mmol) and 1-[*methyl*-^{3}H]MPTP (82.3 Ci/mmol) were obtained from New England Nuclear Corp., Boston, Massachusetts. Male Sprague–Dawley rats (200–250 g) were purchased from Taconic Farms, Germantown, New York. Hepatotytes were isolated using a collagenase perfusion technique (George *et al.*, 1982) and cultured on collagen-coated, 75-cm^2 petri dishes in Williams' E medium supplemented with 10% Nu Serum, antibiotics (100 μg/ml neomycin, 100 μg/ml streptomycin, and 100 U/ml penicillin), 20 mIU/ml insulin, 5 μM dexamethasone, 10 μM hydrocortisone, and 100 μM δ-aminolevulinic acid. Cells (2.5×10^6 in 5 ml of culture medium) were placed in a humidified incubator for 24 hr at 37°C under 95% air and 5% CO_2. At the end of the 24-hr culture period, the medium was replaced with fresh medium containing a known concentration of MPTP, an analog of MPTP, or MPP^+. In studies of the inhibition of MAO-B, a final concentration of 10 μM of deprenyl was added to each plate and preincubated for 2 hr; the medium was then removed and replaced with medium containing 10 μM deprenyl and the drug being tested. Conversion of MPTP to MPP^+ was studied by incubating cultured hepatocytes with different concentrations of unlabeled MPTP and [^{3}H]MPTP (1 μCi/ml, sp act., 82.3 Ci/mmol) for up to 24 hr. The medium was removed at various time intervals, plates were washed with Earle's balanced salt solution, and cells were solubilized in 2 ml of emulgen dissolved in phosphate buffer. The medium or cell extract (1 ml) was alkalinized by adding 100 μl of 1 N NaOH (pH 13) and was extracted with 2 ml of ethyl acetate to remove [^{3}H]MPTP. The aqueous phases (100 μl) containing MPP^+ and other metabolites were separated by HPLC at a flow rate of 1 or 2 ml/min by the method of Markey *et al.* (1984) on a 25-cm C_{18} Bondapak column using a solvent system consisting of 60% acetonitrile and 40% sodium acetate (100 mM) containing 0.1% triethylamine (pH 5.6), or a solvent system consisting of 80% acetonitrile and 20% ammonium acetate (100 mM) containing 0.1% triethylamine. The latter solvent system was used for quantitation of pyridinium compounds in the culture media without any ethyl acetate extraction.

To study [^{14}C]adenine nucleotide leakage, 24-hr cultured hepatocytes were preincubated with [^{14}C]adenine (0.1 μCi/ml, 53 mCi/mmol) in Williams' E medium for 2 hr. At the end of the period, the medium was removed and cells were washed with Earle's balanced salt solution. Five milliliters of 10% Nu Serum-supplemented Wil-



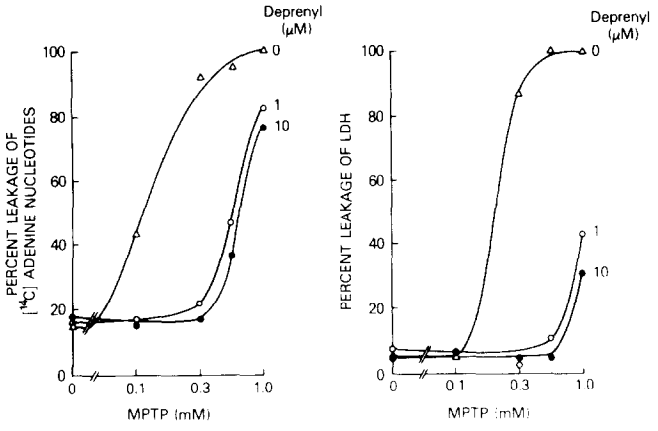


FIG. 4. Prevention of MPTP-induced cell death by deprenyl. Hepatocytes were preincubated with deprenyl (10 μM) in Williams' E medium along with [^{14}C]adenine for 2 hr. The medium was then replaced with fresh deprenyl-containing medium plus varying concentrations of MPTP (0.1–1 mM). At the end of 24 hr an aliquot was taken to determine leakage of [^{14}C]adenine nucleotides and LDH as described under Materials and Methods. Values are means of four separate hepatocyte incubations. Standard errors of the mean were less than 2.5 and 5% for [^{14}C]adenine nucleotide and LDH leakage, respectively.

liams' E medium containing a known concentration of the analog of MPTP being tested was then added to each dish. Aliquots of the medium were removed from each dish after selected intervals and radioactivities were determined in a Packard Tri-Carb 460 CD liquid scintillation counter to determine ^{14}C-labeled nucleotide leakage induced by the drug being tested.

Lactic acid dehydrogenase (LDH) leakage from cells was also determined with an aliquot of the medium using pyruvate as the substrate and NADH as the cofactor. The rate of change in absorbance of NADH at 340 nm was measured every 2 sec for 30 sec with an HP-8450 spectrophotometer (Shirhatti and Krishna, 1985). The results were expressed as percentage of total LDH. Total LDH (leaked into medium + remaining in cells) was determined after lysing the cells with 1% Triton X-100. One hundred percent leakage represents 100% cell death. Total glutathione in cell extracts was assayed spectrophotometrically by measuring the formation of *p*-nitrothiophenolate anion during the cyclic enzymatic reduction of GSSG and oxidation of GSH by DTNB (Tietze, 1969).

ATP was extracted from the cells with 2 ml of 0.6 N perchloric acid. After precipitation of perchloric acid with KOH, the amount of ATP in the supernatant fluid was determined with a DuPont luminescence biometer using a method based on the luciferin–luciferase reaction (Shirhatti and Krishna, 1985).

After incubation with MPTP or MPP^+, the cells were fixed for 15–30 min with 2.5% glutaraldehyde and then washed with 0.15 M sodium cacodylate buffer, pH 7.2 (300 mOSM/kg). Before the samples were dehydrated by treating them with a series of alcohol concentrations (Karnovsky, 1965), they were postfixed in 2% osmium tetraoxide and washed with buffer several times. The cells were subjected to critical point-drying and coated with a thin layer of gold–palladium. They were then examined with a JEOL JSM 35 scanning electron microscope (Anderson, 1951).

Californium-252 plasma desorption mass spectrometry (PDMS) was performed by the method of Pannell *et al.* (1985). Each medium containing a pyridinium compound was mixed with an equal volume of methanol and electrosprayed onto aluminized mylor film. Spectra were obtained with a Cf plasma desorption mass spectrometry system which has been described elsewhere (Sundqvist and Macfarlane, 1985). Californium-252 (10 μCi) was used as the primary ion source and a 45-cm flight tube

FIG. 3. Scanning electron micrograph of MPTP-treated hepatocytes. Freshly isolated hepatocytes were cultured for 2 hr in Williams' E medium and incubated with a fresh medium containing MPTP at 0.3 mM. (Top) Control cells (magnification ×3500); (bottom) cells treated with MPTP (300 μM) (magnification × 3500).

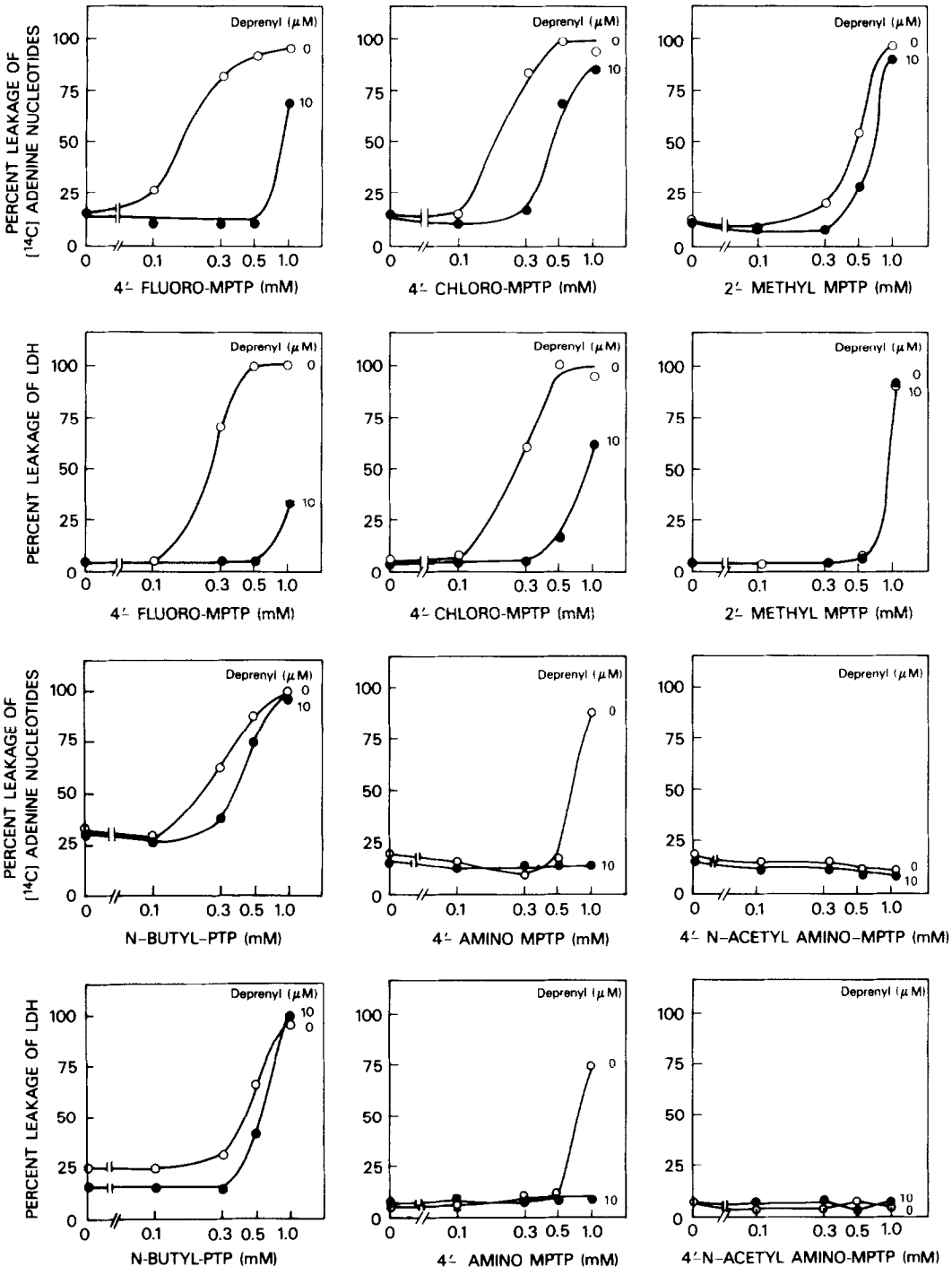


FIG. 5. Dose responses of hepatocytes to six MPTP analogs (4'-fluoro, 4'-chloro, 2'-methyl, *N*-butyl, 4'-amino, and *N*-acetylamino), measured in terms of induced [^{14}C]adenine leakage and induced leakage of LDH in 24 hr with and without deprenyl treatment. The hepatocytes were incubated with varying concentrations of the analog (0.1–1 mM); aliquots were taken after selected time intervals and tested for leakage as described under Materials and Methods. Values are the means of four separate hepatocyte incubations. Standard errors of the mean were less than 2.5 and 5% for [^{14}C]adenine nucleotide and LDH leakage, respectively.

TABLE 1

PLASMA DESORPTION MASS SPECTROMETRIC DETECTION OF MPP^+ ANALOGS FORMED FROM MPTP ANALOGS BY METABOLISM BY LIVER CELLS

MPTP analogs	HPLC ret. time (min)	MPP^+ analogs	Mol wt	Positive ion detected	HPLC ret. time (min)
4′-Fluoro-MPTP	5.0	4′-F MPP^+	188	188.0	3.0
4′-Chloro-MPTP	6.4	4′-Cl MPP^+	204[a]	204.1	3.2
			206[a]	206.1	
2′-Methyl-MPTP	8.5	2′-CH_3 MPP^+	184	184.1	3.1
4′-Amino-MPTP	3.1	4′-NH_2 MPP^+	185	185.1	2.6[b]
4′-Acetylamino-MPTP	2.9	4′-$NHCOCH_3$ MPP^+	277	N.D.	N.D.
N-Butyl-PTP	4.0	N-C_4H_7 PP^+	212	212.1	3.4[b]
MPTP	5.0	MPP^+	170	170.1	3.3[b]

Note. Various MPTP analogs were incubated for 24 hr with hepatocytes in 5 ml of Williams' E medium at 1 mM. The media containing metabolites were analyzed by plasma desorption mass spectrometry utilizing ^{252}Cf bombardment and by high-pressure liquid chromatography on a C_{18} Bondapak column using a solvent system consisting of 80% acetonitrile, 20% ammonium acetate (100 mM), and 0.1% triethylamine, pH 5.6, at a flow rate of 2 ml/min. The peaks were monitored at 254 and 280 nm for all compounds except for 4′-amino-MPTP, when monitoring was performed at 254 and 365 nm. N.D., not detected; Ret., retention.

[a] Chlorine isotope.

[b] Same retention time as authentic pyridinium analog.

was used with a 15 kV accelerating voltage to separate the ions.

Rat liver mitochondria were isolated from rat liver by 10 mM sodium phosphate homogenization in buffer, pH 7.4, containing 0.25 M sucrose and by differential centrifugation as described by Fleisher *et al.* (1979). A mitochondrial pellet was suspended (2 mg protein/ml) in 100 mM sodium phosphate buffer, pH 7.4, containing 3 mM sodium azide. MAO activity in liver mitochondria was measured using MPTP or its analogs as substrates and measuring its rate of formation of H_2O_2 as described by Szutowicz *et al.* (1984).

RESULTS

Both MPTP and MPP^+ were found to induce dose- and time-dependent cell death in rat hepatocytes as measured by leakage of LDH enzyme from the cells (Fig. 1). The dose response of cells to MPP^+ indicated that it is slightly more toxic to hepatocytes than MPTP at lower concentrations—TD50 at 24 hr has been estimated to be 0.15 mM for MPP^+ and about 0.24 mM for MPTP. MPTP and MPP^+ also induced dose- and time-dependent release of [^{14}C]adenine nucleotides (Fig. 2) which we have shown earlier to be a good representation of the loss of cellular ATP (Shirhatti and Krishna, 1985). Both MPTP and MPP^+ caused a proportionately greater leakage of [^{14}C]adenine nucleotides (60–75% at 1 mM after 4 hr) than of LDH (only 30–45% at the same concentration and time). Examination of hepatocytes with a scanning electron microscope (SEM) after 2 hr of incubation with 0.3 mM MPTP revealed a massive blebbing of the cell surface associated with the loss of microvilli, indicative of the serious damage done to the cells (Fig. 3).

The metabolism of MPTP to MPP^+ was demonstrated by incubating the hepatocytes with [^3H]MPTP (100 μM) and assaying the medium and cell extract by HPLC at selected time intervals. After incubation for 4 hr, more than 50% of the radioactively labeled MPTP had been converted into metabolites, of which MPP^+ comprised 30–40%. When the cells were pretreated with 10 μM deprenyl,

a selective MAO-B inhibitor, however, the formation of [^{3}H]MPP$^+$ was markedly reduced, although formation of two other as yet unidentified metabolites were increased (data not shown).

The leakage of adenine nucleotides and LDH induced by MPTP was significantly less in hepatocytes preincubated with 1 and 10 μM of deprenyl (Fig. 4). The protective potential of deprenyl was such that it completely blocked LDH leakage caused by 0.3 mM MPTP for at least 24 hr, although it could not prevent entirely the toxicity induced by 1 mM MPTP. Toxicity induced by MPP$^+$, however, was not affected by deprenyl at any time or at any concentration of MPTP (data not shown).

The effects of various substitutions within the MPTP molecule, as measured by hepatocyte cell death, are shown in Fig. 5. 4′-fluoro- and 4′-chloro-MPTP were found to induce cell death in a pattern similar to that of MPTP, while *N*-butyl-PTP and 2′-methyl-, 4′-amino-, and *N*-acetylamino-MPTP appeared to be less toxic. The toxicities induced by *N*-butyl-PTP, 4′-amino-MPTP, 4′-fluoro-MPTP, and 4′-chloro-MPTP were greatly reduced by deprenyl treatment, as was the toxicity of MPTP itself (Fig. 4), though the toxic effects (as measured by LDH leakage) of 2′-methyl-MPTP were virtually unaffected by the MAO-B inhibition. In addition, it was found through plasma desorption mass spectrometry and high-pressure liquid chromatography that all analogs except the 4′-*N*-acetylamino analog were converted into corresponding pyridinium compounds (Table 1). Moreover, deprenyl inhibited formation of pyridinium metabolites to varying degrees (Table 2). This was further confirmed by measurement of general MAO activity using MPTP and its analogs as substrates before and after inhibition by deprenyl (Table 3).

We have also monitored the loss of cell ATP from hepatocytes incubated with varying concentrations of MPTP and MPP$^+$. Figure 6 shows that MPP$^+$ appears to be more

TABLE 2

EFFECT OF INHIBITION OF MAO-B BY DEPRENYL ON THE CONVERSION OF MPTP AND ITS ANALOGS TO CORRESPONDING PYRIDINIUM METABOLITES IN RAT HEPATOCYTES

MPTP analog	Percentage inhibition of formation of pyridinium metabolite by 10 μM deprenyl	
	4 hr	24 hr
MPTP	86.3	91.3
4′-F-MPTP	82.1	83.1
4′-Cl-MPTP	69.7	64.8
2′-CH_3-MPTP	79.3	74.6
4′-NH_2-MPTP	—	75.0
4′-$NHCOCH_3$-MPTP	N.D.	N.D.
4-C_4H_7-MPTP	22.2	24.7

Note. Various MPTP analogs were incubated for 24 hr with hepatocytes in 5 ml of Williams' E medium at 1 mM. In cases of deprenyl treatment, the hepatocytes were first pretreated with 10 μM L-deprenyl for 2 hr before addition of MPTP analogs. The media containing the pyridinium metabolites was analyzed by HPLC as indicated in Table 1. N.D., not detected.

effective than MPTP at depleting cell ATP; cells treated with 1 mM MPP$^+$ had lost most of their ATP within 2 hr, while cells treated with 1 mM MPTP were able to last for 4 hr before the same extent of ATP depletion occurred. MPTP and MPP$^+$ also induced dose- and time-dependent loss of cell glutathione. Since glutathione is present in hepatocytes predominantly in its reduced form, GSH, this glutathione loss effectively represents a depletion of GSH in liver cells. MPTP appears to be more effective than MPP$^+$ in causing glutathione depletion, although the maximum loss induced was only 80%. No increases in levels of extracellular GSH or GSSG were found in the surrounding medium, however, thus indicating that the loss of glutathione was not caused simply by leakage of GSSG, which would have resulted in an increase in GSSG in the medium.

Figure 7 summarizes the sequence of events occurring in the hepatocytes after

TABLE 3

EFFECT OF DEPRENYL ON RAT LIVER MITOCHONDRIAL MAO USING MPTP AND ITS ANALOGS AS SUBSTRATES

Substrate	MAO activity (nmol of H_2O_2/mg protein/hr)		Percentage inhibition
	Minus deprenyl	Plus deprenyl (100 μM)	
MPTP	41	0	100
4'-F-MPTP	33	6	88
4'-Cl-MPTP	20	10	50
2'-CH_3-MPTP	100	17	83
4'-NH_2-MPTP	8	0	100
4'-$NHCOCH_3$-MPTP	—[a]	—[a]	—
N-C_4H_7-PTP	8	9	0

Note. Rat liver mitochondria (0.2 mg protein) were isolated by differential centrifugation and incubated with or without 1 mM MPTP or an analog in a 0.7-ml volume of buffer (pH 7.4) consisting of 100 mM sodium phosphate and sodium azide (3 mM) in the absence or presence of 100 μM L-deprenyl. The incubation was performed at 37°C in a shaking water bath for 1 hr. The enzyme reaction was stopped by addition of 0.7 ml of sodium phosphate buffer (pH 4.0) containing 1.8 mM 2,2'-azinodi(3-ethylbenzthiozoline 6-sulfonic acid) and 5 U of horseradish peroxidase. After 15 sec, 0.375 ml of 0.75 M HCl containing 5% sodium dodecyl sulfate was added and absorption at 414 nm was determined. Standard solutions of 2.5–20 nmol of H_2O_2 in phosphate buffer containing 3 mM sodium azide were used to generate standard curves. H_2O_2 formation in the absence of substrate has been subtracted for calculation of MAO activity. The values shown are means of four separate determinations.

[a] Below limit of detection.

treatment with either MPTP or MPP^+ (1 mM). In this series of experiments, all parameters were measured with the same population of cells. ATP depletion is followed by leakage of [^{14}C]adenine nucleotides after treatment with MPTP or MPP^+ (1 mM). Depletion of GSH occurs after ATP depletion has occurred—the enzyme leakage starts only after 80% of ATP has been depleted from the cells. This supports the view that loss of cell ATP leads to irreversible damage as represented by leakage of cytoplasmic enzymes.

DISCUSSION

Incubation of rat hepatocytes with MPTP caused dose- and time-dependent cell death as evidenced by leakage of LDH (Fig. 1). This toxic effect of MPTP was mimicked by its metabolite, MPP^+. Since conversion of MPTP to MPP^+ was blocked by deprenyl, which inhibits MAO-B without inhibiting MAO-A, it is assumed that MAO-B is involved in the metabolism of MPTP to MPP^+ in liver cells. Since deprenyl completely protects cells from the lethal effects of low concentrations of MPTP and partially protects the cells from higher concentrations (Fig. 4), it is likely that MPP^+ is the toxic metabolite of MPTP involved in cell death. MPP^+ has also been reported by several investigators to be the toxic metabolite involved in MPTP-induced Parkinson's disease (Irwin and Langston, 1985). Di Monte *et al.* (1986a) have reported earlier that MPP^+ may be the toxic metabolite in liver cells as well, and this study has confirmed their findings. Liver cells, however, also appear to metabolize MPTP to metabolites other than MPP^+, and inhibition of the monoamine oxidase pathway with deprenyl increases formation of two unknown metabolites. The reason for the increase is not clear at present.

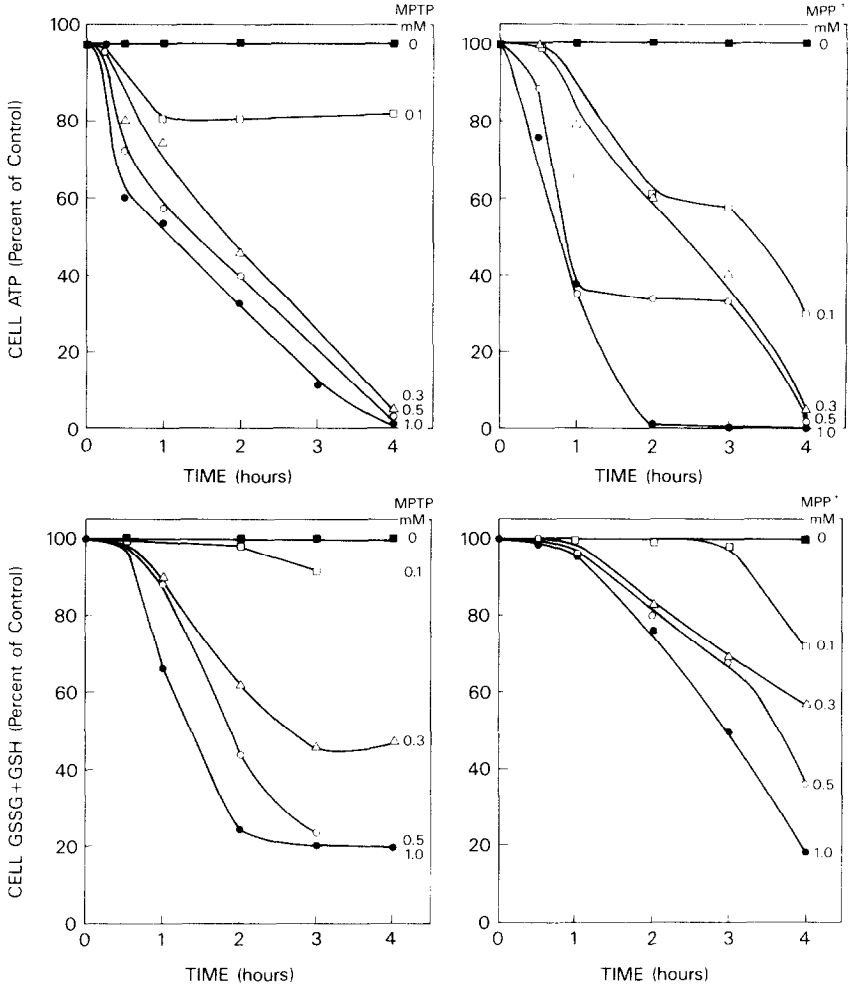


FIG. 6. Dose- and time-dependent graphs of depletion of ATP and loss of GSH caused by treatment of hepatocytes with MPTP and MPP^+. The hepatocytes were incubated with varying concentrations of MPTP and MPP^+ (0.1–1 mM) and aliquots were taken after selected intervals to be tested in the manner described under Materials and Methods. Values are the means of four separate hepatocyte incubations. Standard error of the mean was less than 5 and 6% for ATP and GSH, respectively.

Di Monte *et al.* (1986a) have recently shown that both MPTP and MPP^+ in millimolar concentrations markedly deplete cell ATP, and that ATP depletion caused by MPTP could be reduced through treatment of the cells with pargyline, an inhibitor of both MAO-A and MAO-B, although depletion caused by MPP^+ remained unaffected. We have found that submillimolar concentrations of MPTP and MPP^+ (Fig. 6) cause 40–60% decreases in cellular ATP within 2 hr, at a time when LDH leakage had not even begun to occur. Only after 80–90% of cell ATP had been depleted was leakage of LDH found (Fig. 7). It thus appears, in accordance with the results of Di Monte *et al.*, that ATP depletion precedes cell death in hepatocytes and that the specific effect of MPTP may be on mitochondrial ATP synthesis. However, cell glutathione was also found to be depleted

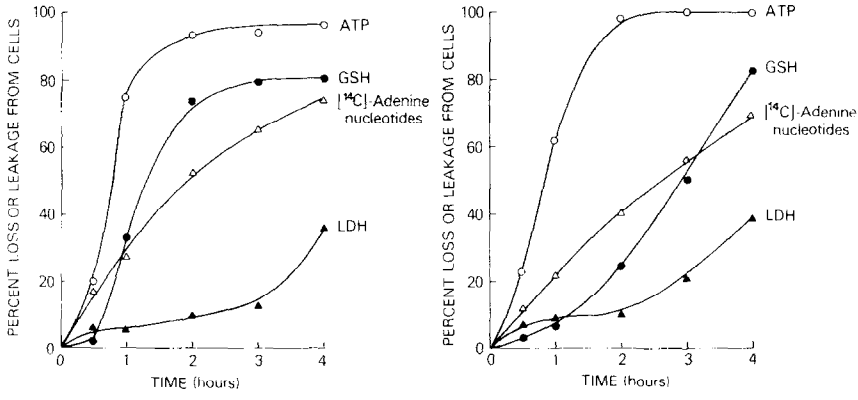


FIG. 7. Sequence of events during MPTP- and MPP^+-induced cell death. Hepatocytes were incubated with MPTP or MPP^+ (1 mM) in Williams' E medium. The medium was removed at selected times and leakage of [^{14}C]adenine and LDH was determined. The cells were lysed and GSH and ATP were isolated and assayed as described under Materials and Methods. (Left) The effects of MPTP; (right) the effects of MPP^+. Values are the means of four separate hepatocyte incubations. Standard error of the mean was less than 2.5 and 5% for [^{14}C]adenine nucleotide and LDH leakage, respectively. Standard error of the mean was less than 5 and 6% for ATP and GSH, respectively.

in response to MPTP and MPP^+, and this as well as other losses of protective molecules also may play a role in MPTP toxicity. Even though Di Monte *et al.* have shown that high concentrations of MPP^+ deplete GSH in hepatocyte suspension only after inhibition of glutathione reductase by BCNU, they did not demonstrate loss of GSH with either MPTP or MPP^+ in the absence of the inhibitor. The mechanism by which cell GSH is lost, however, is still not understood, and whether a similar loss of ATP and GSH occurs in MPTP-induced neuronal cell death remains to be investigated.

We have also studied the capabilities of a number of MPTP analogs in inducing cell death in cultured hepatocytes. *N*-Acetyl-4′-amino-MPTP was found to be virtually nontoxic, while *N*-butyl-PTP, 4′-amino-MPTP, and 2′-methyl-MPTP were generally less toxic than MPTP, and 4′-chloro- and 4′-fluoro-MPTP exhibited toxicities similar to that of their parent. All those analogs which were toxic to the cells, also caused leakage of [^{14}C]-adenine nucleotide prior to cell death (Fig. 5). We have shown earlier (Shirhatti and Krishna, 1985) that leakage of adenine nucleotides really represents loss of cell ATP. This implies that those analogs of MPTP which are toxic also cause loss of cell ATP. Thus, the mechanism of cell death by MPTP analogs may be the same as that by MPTP. All of the analogs except for *N*-acetylamino-MPTP were found through plasma desorption mass spectrometry and high-pressure liquid chromatography to have been metabolized into corresponding pyridinium compounds. The pretreatment of cells with deprenyl was found to reduce the toxicities and metabolism to pyridinium compounds of the *N*-butyl, 4′-amino, 4′-fluoro, and 4′-chloro analogs while having little effect on the toxicities of 2′-methyl-MPTP. This seems to suggest that *N*-butyl-PTP, 4′-chloro-MPTP, and 4′-fluoro-MPTP are substrates of MAO-B, while 2′-methyl-MPTP is possibly also metabolized by a different enzyme. MPTP and its analogs served as substrates to varying degrees for rat liver mitochondrial MAO and the reaction was inhibited to varying degrees by deprenyl (Table 3). Rat liver mitochondria appear to metabolize 2′-methyl-MPTP more rapidly than MPTP, but deprenyl appears to be less effective in inhibiting the conversion of 2′-

methyl-MPTP than MPTP. This suggests involvement of both MAO-A and MAO-B in the conversion of 2′-methyl-MPTP to 2′-methyl-MPP^+. In support of this view, Youngster *et al.* (1987) have recently shown that 2′-methyl-MPTP is possibly a substrate for MAO-A as well. Potencies of the various corresponding pyridinium compounds, however, may differ markedly from each other.

In summary, we have shown that both MPTP and MPP^+ induce marked depletion of cell ATP and cell glutathione, which precede cell death, and that conversion of MPTP and its analogs to corresponding pyridinium metabolites by MAO-B or another factor is essential in inducing cell death in rat hepatocytes.

ACKNOWLEDGMENT

We thank Dr. Victor Ferrans, Chief of Ultrastructure Section, Pathology Branch, NHLBI, for the valuable help and advice provided and for the use of his facility in obtaining scanning electron microscopic pictures of the liver cell samples.

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