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BRIEF COMMUNICATION

Detection of a Neurotoxic Quaternary Pyridinium Metabolite in the Liver of Haloperidol-Treated Rats

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ABLORDEPPEY, S. Y. AND R. F. BORNE. *Detection of a neurotoxic quaternary pyridinium metabolite in the liver of haloperidol-treated rats.* PHARMACOL BIOCHEM BEHAV 46(3) 739–744, 1993.—Various theories have been proposed in the past to explain the side effects associated with haloperidol treatment. In this study, we tested the hypothesis that in vivo biotransformation of haloperidol to a quaternary pyridinium metabolite might contribute to some of these effects. Administration of haloperidol (IP or by gavage) to male Wistar rats results in its biotransformation to a butyrophenone quaternary pyridinium metabolite similar to MPTP metabolism to MPP^+. The corresponding methyl quaternary pyridinium compound was not detected in the liver or brain of the rats used in this study. The proposed methyl quaternary pyridinium compound and the observed butyrophenone metabolite were tested for neurotoxicity in a frog model of MPTP action and were found to have neurotoxic effects, although the methyl quaternary pyridinium compound was much more toxic.

Haloperidol Quaternary metabolite Metabolite Neurotoxicity MPTP Parkinsonism
Drug-induced side effects Extrapyramidal side effects Tardive dyskinesia

HALOPERIDOL [Fig. 1 (I)] is a drug of choice in the management of psychoses, including schizophrenia and mania, and acts in part by blocking D_2 receptors in the mesolimbic system (2,19,20). The widespread use of haloperidol, however, is limited by extrapyramidal disturbances, including tardive dyskinesia and dystonia (6,11), that are associated with long-term use of the drug. The reason for the side effects is presumably due to the nonselective blockade of dopamine receptors in the nigrostriatum and the subsequent supersensitivity of the receptors (24). Since some of the side effects are long lasting or irreversible, it is difficult to explain all the observed extrapyramidal actions by the supersensitivity hypothesis alone. Another hypothesis is the conversion of haloperidol in vivo to toxic compounds. Although several metabolism studies have been done on haloperidol, the majority of these studies focused on the biotransformation of the butyrophenone moiety and the piperidine moiety was ignored (5,9,10). At the time this study began, for example, only one paper had reported the observation of the *N*-dealkylated metabolite, 4-(4′-chlorophenyl)-4-piperidinol (CPPO), in an in vitro metabolism study (14).

As previously demonstrated (13), *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a neurotoxin with a pharmacological profile resembling idiopathic Parkinsonism. The mechanism of action involves conversion of MPTP to 1-methyl-4-phenylpyridinium compound (MPP^+), which is actively transported to the mitochondria, where it inhibits NADH oxidation (7,8,16,17). Because permanently charged compounds often show neurotoxic effect in the CNS (12,18), we have explored the possibility that haloperidol can undergo *N*-dealkylation and dehydration, then *N*-methylation, and be similarly transformed to 1-methyl-4-(4′-chlorophenyl)pyridinium compound ($MCPP^+$) (Fig. 1). Haloperidol could also undergo a direct biotransformation to a quaternary pyridinium metabolite [*N*-(4′-fluorobutyrophenone)-4-(4-chlorophenyl) pyridinium compound ($BCPP^+$) (Fig. 1). Both potential quaternary metabolites could perhaps cause some of haloperidol's Parkinsonism-like actions. Thus, the current knowledge of

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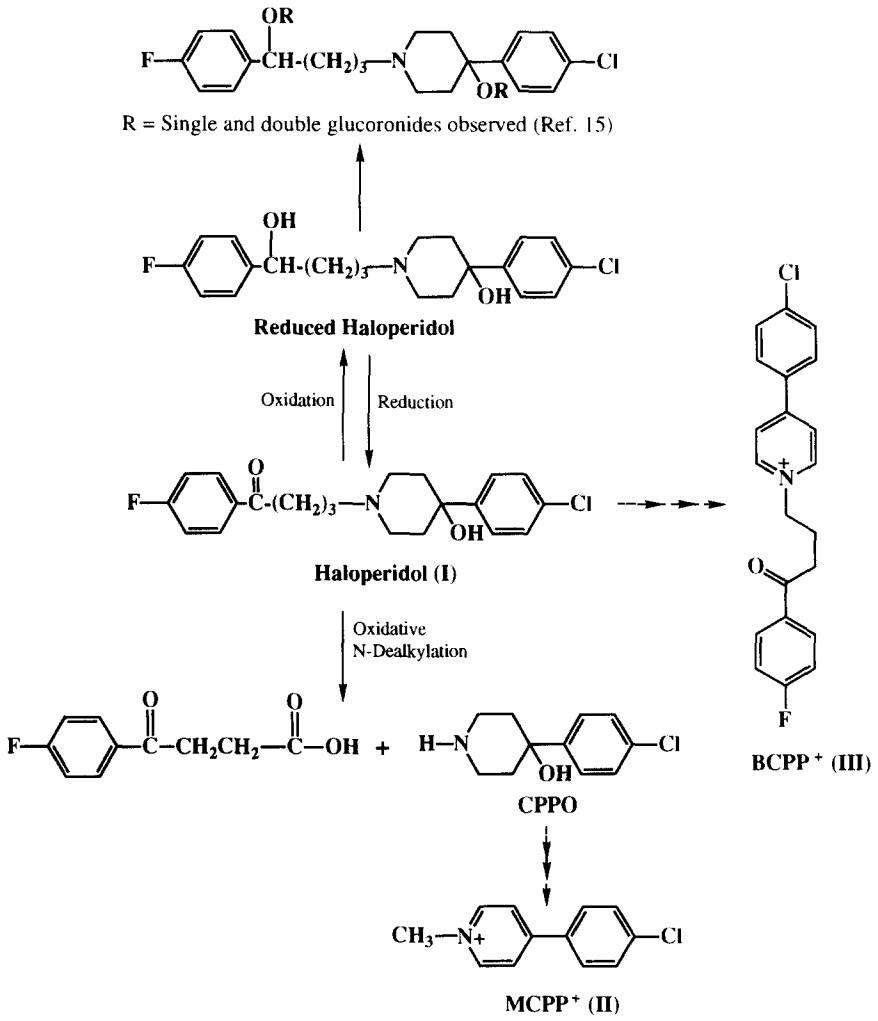

FIG. 1. Reported metabolic pathways for haloperidol (bold arrows) and the proposed pathways (dotted arrows) tested in this study.

MPTP metabolism, the mechanism of action of MPTP, and the structural and pharmacological similarities of MPTP and haloperidol prompted us to reexamine the metabolism of haloperidol and the consequences of that metabolism. The primary objective of the present study, therefore, was to look for the potential quaternary metabolites of haloperidol in the brain and liver of rats and to evaluate their effect on the quality of movement (QOM) in a frog model.

During the course of this study, the identification of $BCPP^+$ as a metabolite of haloperidol in rat brain and urine (22) and an in vitro study examining the metabolic pathway to the pyridinium compound (23) were reported.

METHOD

Chemicals

MPTP and 4-(4′-chlorophenyl)-4-piperidinol (CPPO) were available commercially from Aldrich Chemical Company, Milwaukee, WI. Haloperidol was obtained from Sigma Chemical Company, St. Louis, MO. Both $BCPP^+$ and 1-methyl-4-(4′-chlorophenyl)pyridinium compound ($MCPP^+$) were synthesized in our laboratories by standard synthetic procedures. The H-1 and C-13 NMR spectra (with APT) and LC/MS were consistent with the structure of $BCPP^+$. Tris buffer was prepared using 6.1 g of tris(hydroxymethyl)aminomethane and 11.2 g of KCl adjusted with 1.0 M HCl to pH 7.4 per liter of aqueous solution.

Animals

Male Wistar rats (weighing 220–390 g), obtained from Charles River Breeding Laboratory, were used in the metabolism study. *Rana pipiens* frogs (weighing 10–35 g) were obtained from Lemberger, Oshkosh, WI and were used in the neurotoxicity evaluation.

Metabolism of CPPO

Two rats (approx. weights 350 g) were given CPPO intraperitoneally (IP) at 10 mg per rat daily for 7 successive days. Food and water were provided ad lib. CPPO solutions were prepared fresh daily in distilled water (1 ml) and a drop of glacial acetic acid. Four hours after the last dose, the rats were

anesthetized with ether, and the liver and brain were removed and washed with water. The tissues were then cut into small pieces, homogenized separately in Tris buffer (1 g tissue to 3 ml Tris buffer), centrifuged (12,500 × *g* for 10 min at 0°C), and the supernatants of each tissue pooled.

Metabolism of Haloperidol

Three rats (approx. 390 g) and a fourth rat (which provided the liver and brain tissues that served as negative control) were used in this study. Each rat had a different dose regimen. Food and water were provided ad lib. The first rat received haloperidol IP (5 mg/day for 3 successive days). The drug was dissolved in 8% aqueous ethanol and two drops of glacial acetic acid. The rat was sacrificed after ether anesthesia 24 h after the last dose. The liver was removed, homogenized in ice-cold Tris buffer (1 g tissue to 3 ml buffer) at 12,500 × *g* for 10 min at 0°C, and 5 ml of the supernatant was taken through the standard assay procedure. The second rat was given haloperidol IP at 5 mg/day for 4 successive days. The drug was dissolved in 8% aqueous ethanol and two drops of glacial acetic acid. The rat was anesthetized with ether and sacrificed 3 h after the last dose. The brain was removed and homogenized in ice-cold Tris buffer (1 g tissue to 3 ml buffer). The homogenate was then centrifuged at 12,500 × *g* for 10 min at 0°C and the supernatant was removed; 5 ml of the supernatant was subjected to the standard assay procedure. A third rat received haloperidol (7.5 mg/day for 3 successive days) dissolved in 5% dextrose solution by gavage. Twenty-four hours after the last dose, the rat was anesthetized, the brain removed, and treated as for the second rat.

In order to confirm the presence of $BCPP^+$ as a metabolic product, synthetic $BCPP^+$ was added to liver homogenate extracts and injected onto the HPLC. In addition, two mobile phases were used to elute the column (i.e., 45% and 50% methanol in appropriate phosphate buffers).

Equipment

High performance liquid chromatography (with ultraviolet detection) analyses were performed on a Shimadzu Corp. (Japan) solvent delivery module model LC-9A, a Shimadzu Corp. model SPD-6A variable UV spectrophotometric detector, a Rheodyne automatic injector with a valve position sensing switch model 7161 and Actuator position sensing switch model 7162 (Cotati, CA), and a Shimadzu Corporation Chromatopac integrator model CR 601. The analytes were separated on a Whatman Partisil 5 ODS-3 C-18 reverse-phase 12.5 cm × 4.7 mm (i.d.) octadecyl reversed-phase 5 μm particle size column (Whatman, Clifton, NJ) and a C-18 reversed-phase guard column (17 × 4 mm). A mobile phase of 45–55% MeOH in phosphate buffer (1.65 g dipotassium hydrogen phosphate, 2.1 g potassium dihydrogen phosphate, and 1.5–2 g *N,N*-dimethyloctylamine per liter of distilled water) at a flow rate of 1.0 ml/min. The UV detector was set at 302 nm to optimize for the pyridinium metabolites.

Standard Assay Procedure for Quaternary Compounds

Five milliliters of the brain or liver homogenate preparation spiked with or without internal standard were transferred to polycarbonate centrifuge tubes; 0.3 ml of SDS (10 mg/ml) was added to the mixture and shaken to mix well. After extraction with 2 × 5 ml of ethyl acetate, the organic phase was removed and filtered through a column of anhydrous sodium sulfate (2 g) to remove water and extraneous solid matter. The solution was dried with a stream of nitrogen in a lukewarm water bath and the residue was taken up in 100–200 μl of the mobile phase. The resulting solution (50–60 μl) was injected onto the HPLC/UV system with an automatic injector delivering 20 μl. Peak area ratios were used to estimate the amount of metabolites in the biological matrix. Having been shown not to be a metabolite of haloperidol, $MCPP^+$ subsequently served as the internal standard in the estimate of the amount of $BCPP^+$ in the liver homogenate preparations. Calibration samples were prepared from stock solutions (1 mg/ml) of standard synthetic $BCPP^+$. The amounts of analytes were calculated using the formula: $C_a = C_s \times PAR_b/IS_b \times IS_c/PAR_c$, where C_a = concentration of analyte, C_s = concentration of standard ($BCPP^+$), PAR = peak area ratio, IS = internal standard, and subscripts b and c refer to biological matrix and calibration sample, respectively.

Neurotoxicity Testing

Neurotoxicity testing was done by the method of Barbeau et al. (3,4) as recently modified (1). The quality of movement (QOM) was checked by placing the frogs on their back and observing the time required for the frogs to turn over (flip-over time or righting reflex). If the frogs failed to turn over after 5 s, they were considered to have lost their righting reflex. Animals in groups of four or five received successive daily doses of each drug equivalent to 0.19 mmol of MPTP per kg body weight (i.e., 33 mg/kg body weight of MPTP). MPTP served as the positive control, while the vehicle served as the negative control. The dose of 33 mg per kg body weight was selected to ensure toxic effects were observed in the frogs (3,4).

RESULTS

Standard Assay Procedure

Although there were variations in retention times, when a mobile phase of 45% methanol in phosphate buffer (with 2 g per liter of *N,N*-dimethyloctylamine) was used, the retention times of $MCPP^+$ and $BCPP^+$ were found to be 6.1 and 9.5, respectively. Using a mobile phase of 50% methanol, retention times were 4.4 and 5.6 min for $MCPP^+$ and $BCPP^+$, respectively. The method was moderately sensitive (10 ng/ml, lower limit of quantification). Extraction recoveries for $BCPP^+$ were: water (87%), brain (98%), and liver (110%). Applied to brain homogenate preparations, intra-assay precision was found to be good; mean of four peak area ratios ± SD = 0.756 ± 0.008 on 100-ng/ml samples. The coefficient of variation was 1.1%. The method was found to be linear over the concentration range of 10 ng/ml to 1 μg/ml ($y = 0.960x + 6.77$). No interference was observed between $BCPP^+$ and liver homogenate preparation extractives (Fig. 2).

Metabolism of CPPO

The target of the in vivo metabolism study of CPPO was to detect $MCPP^+$, a potential metabolite that is potentially very toxic. Two rats were injected IP with 10 mg per rat per day of CPPO for 7 successive days. Although CPPO was detected in the liver homogenate obtained from these rats, $MCPP^+$ was not detected in either brain or liver tissue (data not shown). As expected, the rats did not show any obvious abnormal movement or posture during the period of observation (7 days).

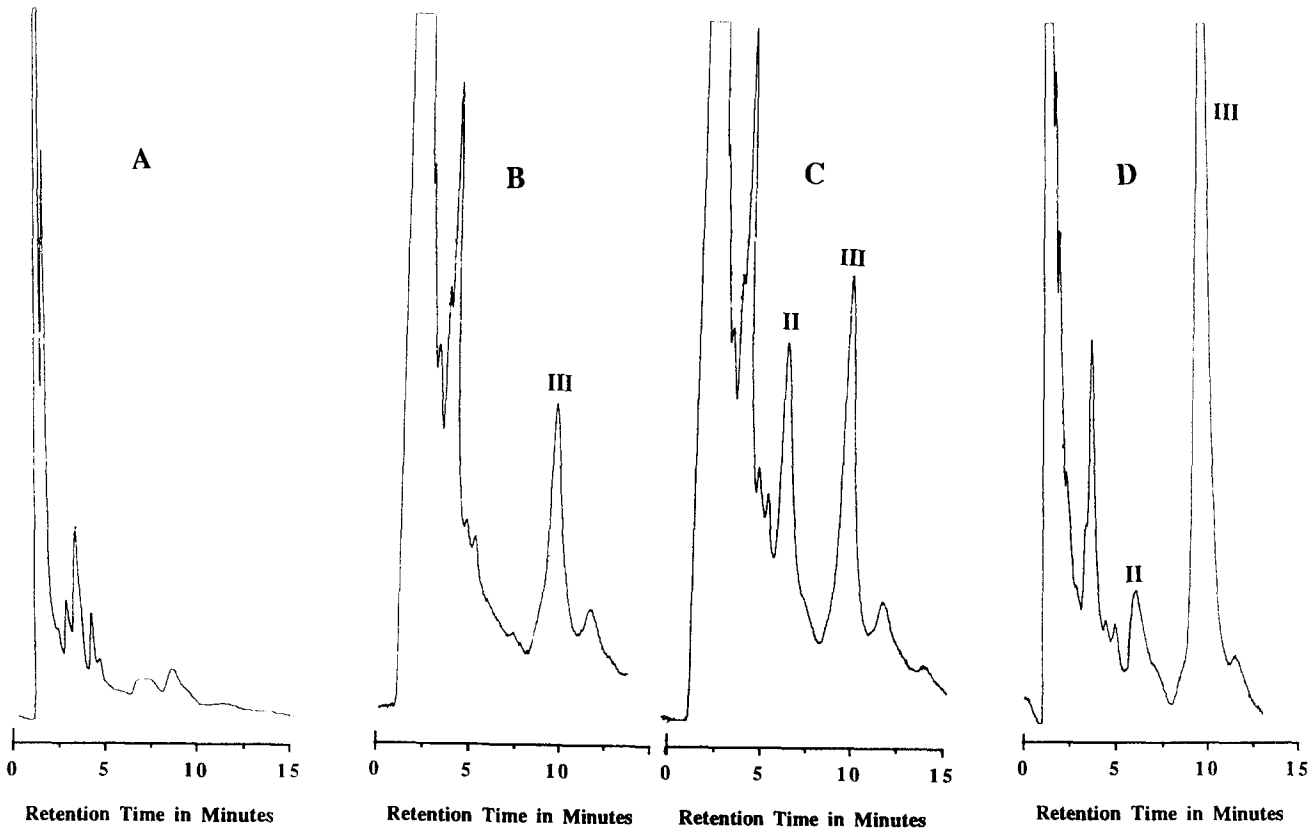

FIG. 2. Chromatograms of ethyl acetate extracts of liver homogenate preparations. (A) Blank liver homogenate extract obtained from an untreated rat (negative control). (B) Liver homogenate extract obtained from a haloperidol-treated rat without internal standard. (C) Liver homogenate extract obtained from a haloperidol-treated rat spiked with internal standard (500 ng of $MCPP^+$, II) and taken through the extraction procedure. (D) Liver homogenate extract as in (C) but spiked with synthetic metabolie [200 ng of $BCPP^+$ (III) was added directly to the liver extract]. x-axes = retention time in minutes; $MCPP^+$ (II, 6.1) and $BCPP^+$ (III, 9.5).

Metabolism of Haloperidol

This study was initiated essentially to find if $BCPP^+$ is formed in the liver or brain of haloperidol-treated rats. It was also expected to confirm the absence of $MCPP^+$ as a metabolite of haloperidol based on CPPO metabolism. Three different dose regimens were used, since there is no pharmacokinetic information on the formation of the quaternary metabolite. The first rat was given 5 mg of haloperidol (IP) daily for 3 successive days. The liver homogenate obtained from this rat 24 h after the last dose was taken through the standard assay procedure. Typical chromatograms of liver homogenate extracts obtained from an untreated rat (negative control) and a haloperidol-treated rat without internal standard are shown in Fig. 2 (A and B, respectively). The chromatogram of liver homogenate extract obtained from the haloperidol-treated rat showed the presence of a peak at retention time (Rt) of 9.5 min, which corresponded to the retention time of the peak of synthetic $BCPP^+$ (Fig, 2B). Figure 2C shows the chromatogram of a liver homogenate preparation that was spiked with internal standard and taken through the extraction procedure. When synthetic $BCPP^+$ was added to the liver homogenate extract in Fig. 2C, the resulting chromatogram showed the peak of synthetic $BCPP^+$ coeluted with this peak (Fig. 2D). Similar chromatograms of liver homogenate extracts eluted with a mobile phase of 50% methanol showed the presence of peaks with retention times 5.6 and 4.4, respectively, for $BCPP^+$ and $MCPP^+$. Again, synthetic $BCPP^+$ coeluted with the biotransformation product of haloperidol (data not shown).

To estimate the amount of $BCPP^+$ in the liver homogenate preparations, $MCPP^+$ was added to liver homogenate preparations and taken through the standard assay procedure. Assuming absolute recovery, the metabolite was estimated to be 5.9 (SEM = 0.4, n = 3) μg per whole liver or 480 ng per g of liver tissue. (Estimate was based on three liver homogenate preparations.) The brain homogenate preparation obtained from the second rat treated with a total of 20 mg of haloperidol over 4 days was treated as the liver homogenate above. The chromatogram showed the presence of $BCPP^+$, which was estimated to be 160 ng per whole brain or 140 ng per g of brain tissue. The brain homogenate preparation of the third rat that received a total of 22.5 mg of haloperidol by gavage over 3 days also showed the presence of $BCPP^+$. The amount of this metabolite was estimated to be 230 ng per whole brain or 160 ng per g of brain tissue.

Neurotoxicity Testing

MPTP served as the positive control in this study and turned all the frogs uniformly dark within 24 h after the first injection and killed all of them within 48 h. Barbeau et al.

(3,4) have shown that MPP^+ causes MPTP-like effects in frogs, but is much more toxic than MPTP. In contrast to MPTP, however, MPP^+ was reported to lighten the skin coloration. In this study, $MCPP^+$ killed the frogs within 24 h at equivalent molar dose of MPTP. When the dose was reduced by 75%, the frogs lost their righting reflexes within 24 h and died within 72 h. However, $MCPP^+$, like MPP^+, did not turn the frogs dark. $BCPP^+$, on the other hand, caused the frogs to lose their righting reflexes within 24 h and turned them moderately dark in 96 h at equivalent molar dose. Thus, the actions of $BCPP^+$ appear to mimic the actions of MPTP, but are less toxic than MPTP. None of the frogs survived the 8-day testing period. Thus, the results showed that $MCPP^+$ was the most toxic of the proposed metabolites, being rapidly and uniformly lethal to the frogs. The vehicle had no effect on either QOM or skin color of the frogs.

DISCUSSION

As recently reported, haloperidol undergoes *N*-dealkylation in vivo to form CPPO, which causes a persistent and freezing action in *Rana pipiens* (1). The ultimate fate of CPPO, however, remains unknown. We have proposed that just as MPTP is ultimately transformed into MPP^+, CPPO may undergo dehydration and *N*-methylation, and subsequently be transformed to the chloro analog of MPP^+ (i.e., $MCPP^+$). However, in vivo studies involving the treatment of two rats with CPPO (10 mg/day/rat for 7 successive days) failed to show the presence of the predicted $MCPP^+$ in the brain or liver of CPPO-treated rats. As expected, none of the rats showed any signs of motor impairment at this high dosage as observed by their free movement. Thus, $MCPP^+$ is not a major metabolite of haloperidol, since CPPO would appear to be the most viable route through which haloperidol could be converted to $MCPP^+$.

An HPLC method was developed to detect the presence of $BCPP^+$ in liver and brain homogenates. Encouraged by the lack of biotransformation of CPPO to $MCPP^+$, we utilized $MCPP^+$ as the internal standard. The wavelength of HPLC detection was selected to be 302 nm (which is the approximate λ maxima of both $MCPP^+$ and $BCPP^+$) to optimize for $MCPP^+$ and $BCPP^+$ and exclude most of the other, less conjugated, metabolites previously reported (15).

Figure 2 shows typical chromatograms of ethyl acetate extracts of liver homogenate preparations obtained from the first haloperidol-treated rat (5 mg per day for 3 successive days) and from an untreated rat. From the results in Fig.2B, it is clear that $MCPP^+$ is not a metabolite of haloperidol in the rat liver, supporting the previous results that $MCPP^+$ was not a metabolite of CPPO. Assuming an absolute recovery of $BCPP^+$, the amount of the metabolite was found to be 5.9 μg per whole liver or 480 ng per g of liver tissue. $BCPP^+$ was also detected in the brain (230 ng in whole brain or 160 ng per g of brain tissue was obtained in the brain of the rat that received a total of 22.5 mg of haloperidol by *gavage* over 3 days). Furthermore, 160 ng (or 140 ng per g of brain tissue) of $BCPP^+$ was found in the brain of the haloperidol-treated rat that received a total of 20 mg of haloperidol *IP* over a period of 4 days. The number of rats used in this study places a limitation on the quantitative nature of these results. Nevertheless, the results support the recent report (22) that $BCPP^+$ is indeed found in the brain of haloperidol-treated rats. Thus, irrespective of oral or IP administration, $BCPP^+$ is found in the brain of haloperidol-treated rats. Because of the relatively high dosing regimen used (clinical dose is in the order of 0.5–2 mg/kg), we are unable to suggest whether $BCPP^+$ is a normal metabolic product or appears when the normal metabolic pathways are saturated by excess of haloperidol. Nevertheless, since a relatively large amount of $BCPP^+$ is made in the liver and is capable of entering the brain ($BCPP^+$ extracts readily into organic solvents such as chloroform or ethyl acetate), it suggests that relatively high concentrations of this metabolite can enter and accumulate in the brain with long-term use. Because $BCPP^+$, like other permanently charged pyridinium compounds (12,16,18), has been shown to have a dopamine-depleting capacity (22) and is a tenth as active as MPP^+ in the dopamine-depleting action, it could contribute to some of the extrapyramidal actions of haloperidol. In fact, depletion of dopamine in the brain over time can aid the development of supersensitivity of dopamine receptors, which has been suggested as the reason for the development of tardive dyskinesia (24).

Neurotoxicological evaluation was carried out in a frog model of MPTP action that has proved to be useful in producing clinical and biochemical changes similar to those seen in Parkinson's disease (3,4). In fact, MPTP's capacity to deplete dopamine in the brain of frogs has been shown to correlate very well with the frog's ability to turn over when placed on its back (righting reflex). As part of this study, we tested the capacity of $MCPP^+$ and $BCPP^+$ to induce MPTP-like action in the frog model developed previously. From these results, the action of $MCPP^+$ is similar to the action of MPP^+, while $BCPP^+$ shows similar neurotoxic actions as MPTP but is less toxic. Taken together, the results of this study and the previous report (22) suggest that haloperidol's in vivo transformation to biologically active species, and the potential liability to accumulate in the brain where it can reduce dopamine levels, may lend support to the theory that metabolites of haloperidol may contribute to some of the long-term extrapyramidal Parkinsonism-like side effects associated with haloperidol therapy.

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