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FREEZING ACTION OF A METABOLITE OF HALOPERIDOL IN FROGS

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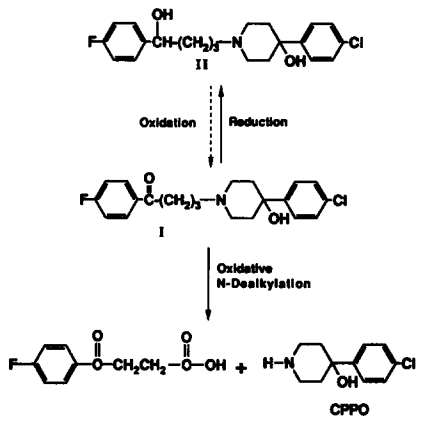
Abstract—*In vivo* metabolism studies led to the identification of a previously proposed metabolite of haloperidol, 4-(4′-chlorophenyl)-4-piperidinol (CPPO), in the liver of a haloperidol-treated rat. However, the secondary metabolites of CPPO that we have proposed were not observed in this study. Neurotoxicity studies in frogs, which have been used to detect *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) action, showed that CPPO did not mimic the neurotoxicity of MPTP but caused a delayed and persistent freezing action in *Rana pipiens* frogs. It is proposed that this action may contribute to some of the delayed side-effects associated with haloperidol therapy.

Haloperidol (I) is a potent butyrophenone drug widely used in the management of psychosis, especially in schizophrenia and mania. This action is partly due to its dopamine (D-2) receptor-blocking properties in the mesolimbic or the mesocortical region of the brain [1–3]. As is expected of all non-selective dopamine blocking antipsychotic drugs, haloperidol induces extrapyramidal disturbances due to its concomitant blockade of dopamine receptors in the substantia nigra [4]. Haloperidol, however, appears to induce a higher incidence of the extrapyramidal actions, including Parkinsonism-like side-effects, when compared with many other neuroleptic drugs [5]. In a search of reasons for the higher incidence of extrapyramidal actions associated with haloperidol, we [6] and others [7, 8] have shown that haloperidol is metabolized to a quaternary pyridinium compound with a potential to cause *N*-methyl - 4 - phenyl - 1,2,3,6 - tetrahydropyridine (MPTP§)-like actions.

Previous metabolism studies by Forsman and others [9–11] have shown that haloperidol undergoes oxidative N-dealkylation to inactive metabolites in humans and rats (Scheme 1). The N-dealkylated metabolite of haloperidol, 4-(4′-chlorophenyl)-4-piperidinol (CPPO), was reportedly observed in *in vitro* metabolism studies by Marcucci and others [12], but no data were shown. To our knowledge, CPPO has not been reported from *in vivo* studies, and if it is produced, its ultimate fate is unknown. Based on recent studies on the neurotoxin, MPTP [13], we have proposed that CPPO could undergo



Scheme 1. Primary metabolic pathways for haloperidol [9–11].

secondary metabolism to MPTP-like species, as shown in Scheme 2.

In a recent study, Bowen and his colleagues [14] reported that CPPO selectively binds to sigma receptors, while haloperidol and its reduced metabolite (II) non-selectively bind to both sigma and dopamine receptors. The current knowledge of sigma receptors and their functions is limited. However, available evidence appears to have implicated sigma receptors in the control of motor functions [15–18] either directly or indirectly through the regulation of dopamine neurons, which are directly involved in the control of motor functions.

We have synthesized the proposed potential metabolites of haloperidol and evaluated these metabolites in the frog model of MPTP action [19, 20]. We now report that while CPPO was observed in the liver of rats administered haloperidol, none of the proposed potential metabolites was observed in the *in vivo* model. More importantly, while CPPO may be devoid of antipsychotic activity as proposed by Forsman and others [9–11], it does

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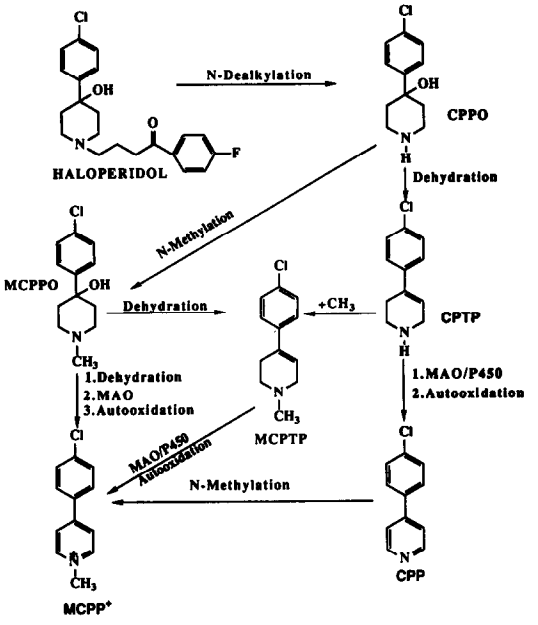
§ Abbreviations: MPTP, *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; CPTP, 4-(4′-chlorophenyl)-1,2,3,6-tetrahydropyridine; CPPO, 4-(4′-chlorophenyl)-4-piperidinol; MCPPO, *N*-methyl-4-(4′-chlorophenyl)-4-piperidinol; MCPTP, *N*-methyl-4-(4′-chlorophenyl)-1,2,3,6-tetrahydropyridine; CPP, 4-(4′-chlorophenyl)-pyridine; and MAO, monoamine oxidase.



Scheme 2. A proposed potential metabolic pathway for haloperidol.

induce a persistent freezing action in a frog model of MPTP action.

MATERIALS AND METHODS

Drugs. MPTP, CPPO, 4-(4′-chlorophenyl)-1,2,3,6-tetrahydropyridine hydrochloride salt (CPTP), and 4-phenyl-4-piperidinol (internal standard) were obtained from the Aldrich Chemical Co., Milwaukee, WI. Haloperidol was obtained from the Sigma Chemical Co., St. Louis, MO. *N*-Methyl-4-(4′ - chlorophenyl) - 4 - piperidinol (MCPPO), *N* - methyl - 4 - (4′ - chlorophenyl) - 1,2,3,6 - tetrahydropyridine (MCPTP) and the hydrochloride salt of 4-(4′-chlorophenyl)pyridine (CPP) were synthesized in our laboratories by standard synthetic procedures. All solutions used were prepared fresh daily for intraperitoneal injections using 8% aqueous ethanol. The injection volume in all cases was 0.5 mL. 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 the Charles River Breeding Laboratory, were used in the metabolism study. Frogs (*Rana pipiens*), weighing 10–35 g, were obtained from Lemberger, Oshkosh, WI, and were used in the neurotoxicity evaluation.

Metabolism of haloperidol. Three rats (approx. 390 g) were used in this study. Food and water were provided *ad lib.* One rat was given haloperidol intraperitoneally at 5 mg/day for 4 successive days. The drug was dissolved in distilled water and 2 drops of glacial acetic acid. The rat was anesthesized with ether and killed 3 hr after the last dose. The liver 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° and the supernatant was removed; 5 mL of the supernatant was subjected to the standard assay procedure.

A second 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 anesthesized, and the liver was removed and treated as above.

The third rat provided the liver tissue which served as the positive control. The positive control sample was prepared by spiking a 5-mL liver homogenate preparation with the synthetic standards at 10 μg/mL and at 20 μg/mL for internal standard.

Biological matrices. Liver homogenate preparations were obtained from rats killed after ether anesthesia. Fresh rat liver was removed and washed with water to remove blood and hair. The tissue was then cut into small pieces, homogenized with ice-cold 0.05 M Tris buffer (1 g tissue to 3 mL Tris buffer) in a Potter–Elvehjem tube, transferred to polycarbonate tubes, and centrifuged at 12,500 *g* for 10 min at 0°. The supernatant obtained was used fresh or stored at −20° until used.

GC/NPD method for non-quaternary compounds. The gas chromatography (with nitrogen and phosphorus detector), GC/NPD, method was developed on a Hewlett–Packard 5890A capillary GC equipped with a 30 m × 0.25 mm J & W DB-17 fused silica capillary column and a nitrogen phosphorus selective detector (NPD). Screw-cap polycarbonate tubes (15 mL) containing a mixture of 1 mL of liver homogenate preparation and 4 mL Tris buffer were spiked with analytes and internal standard (4-phenyl-4-piperidinol), basified with 0.4 mL sodium hydroxide (2.5 M), and extracted three times with 4 mL of 80% ethyl acetate in hexane. The tubes were centrifuged at 3000 rpm for 10 min; then the organic layers were removed and filtered through a column of 3 g of anhydrous sodium sulfate. The solution was warmed in lukewarm water and the solvent was removed with a stream of nitrogen. The residue was taken up in 100 μL of 50% acetic anhydride in ethyl acetate, heated on a steam-bath for 5 min and allowed to cool to room temperature. The reaction mixture was dried with a stream of nitrogen, the residue was taken up in 100 μL of ethyl acetate and 1 μL was injected onto the GC/NPD system. The system was run under temperature programming conditions as follows: initial time = 5 min; initial temperature = 220°; final temperature = 260°; final time = 17 min; rate = 5°/min; helium carrier gas flow rate = 1 mL/min; hydrogen = 26 psi; air = 50 psi; purge vent flow rate = 5 mL/min; split vent flow rate = 20 mL/min; injector temperature = 250°; and detector temperature = 300°.

Neurotoxicity testing. The frogs were acclimatized for at least 3 days in a basin containing a small amount of water and seaweeds before the first injection. The water was cooled with crushed ice from time to time and was changed about every other day. There was a rest platform in each basin. Four or five frogs were kept in each basin. Before

Table 1. Retention times and the extraction recoveries of potential non-quaternary metabolites of haloperidol, CPPO, MCPTP, CPTP, CPP and internal standard from liver homogenate preparations as determined by GC/NPD

Compound*	Retention time (min)	% Recovery
MCPTP	7.1	75.8
CPP	7.3	78.9
Internal standard	16.4	86.0
CPTP	19.8	82.9
CPPO	24.4	72.2

* Samples of liver homogenates containing analytes (2 μg/mL) run through the standard assay procedure.

Table 2. Precision for replicate liver homogenate preparations spiked with standards as determined by GC/NPD

Sample No.*	Mean peak area ratio (analyte/IS)			
	CPPO	MCPTP	CPTP	CPP
1	0.76	1.14	0.84	1.07
2	0.76	1.07	0.82	1.04
3	0.73	1.23	0.83	1.14
Mean	0.75	1.15	0.83	1.08
Standard deviation	0.01	0.06	0.01	0.04
Coeff. of variation (%)	1.3	5.2	1.2	3.7

* Samples of liver homogenates containing analytes (2 μg/mL) run through the standard assay procedure.

the daily injections, the frogs were inspected for changes in color and the quality of their movements according to a modified procedure of Barbeau *et al.* [19, 20]. 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 sec, they were considered to have lost their righting reflex. Animals received successive daily doses of each drug equivalent to 0.19 mmol MPTP/kg body weight (i.e. 33 mg/kg bodt wt of MPTP). MPTP served as the positive control, while the vehicle (8% aqueous ethanol and 2 drops of acetic acid) served as the negative control. The dose of 33 mg/kg body weight was selected to ensure that the toxic effects were observed in the frogs [19, 20]. Frogs were evaluated in groups of four or five and flip-over times were recorded when at least 75% of the frogs had the same flip-over time. The quality of movement was scored from 1 to 4 as follows: no apparent effect = 0; weak but not rigid, rights self before 2 sec = 1; weak but not rigid and rights self between 2 and 4 sec = 2; completely lost righting reflex, flip-over more than 5 sec, if at all = 3; frozen in a tonic and extended posture = 4. Darkening (MEL) in the frogs was scored qualitatively as follows: no color change = 0; mildly dark or 50% turned dark = 1; all very intensely dark = 2.

RESULTS AND DISCUSSION

MPTP was assayed previously by Shih and Markey [21, 22] through derivatization with isobutyl chloroformate and analysis of the resulting carbamate by gas chromatography and mass spectrometry (GC/MS). Apart from the problems encountered by Markey and his colleague in analyzing trace amounts of MPTP by capillary column, the derivation method employed will lead to the formation of the same end product for some of the proposed metabolites and makes it difficult to estimate the amount of metabolites that may be formed. Thus, we avoided the use of similar methods in the analysis of the proposed metabolites of haloperidol in our study. Of the derivatization methods discussed by VandenHeuvel and Zacchei [23], we were unable to find one that was consistent with the GC/NPD available to us. After several trials, derivatization using acetic anhydride with sodium acetate or without a catalyst proved to be appropriate choices.

The method was found to be moderately sensitive (50 ng/mL detection limit) and allowed for recoveries of between 72 and 86% for all compounds used (Table 1). Based on three replicate assays, the precision of the method was found to be satisfactory (coefficients of variation are recorded in Table 2).

Figure 1 displays the gas chromatography with a nitrogen selective detector (GC/NPD) chromatograms of 80% ethyl acetate/hexane extracts of liver homogenate preparations. Panel A is a chromatogram of a blank extract of liver homogenate preparation from an untreated rat; panel B is the chromatogram of a liver homogenate preparation from a haloperidol-treated rat that received 5 mg/day of haloperidol for 4 successive days and was killed 3 hr after the last dose; and panel C is the chromatogram of a liver homogenate preparation spiked with authentic synthetic samples of the proposed metabolites. An internal standard was added to the liver homogenate preparations in panels B and C. The peak eluting at 24.4 min corresponding to the N-dealkylated product was estimated to be 0.98 μg/g of liver tissue, assuming a quantitative recovery. This peak, and that of the authentic standard (CPPO) have the same retention time of 12.3 min when there is no derivatization and at an isocratic temperature of 220°. When another rat was given haloperidol by gavage (rather than intraperitoneally) at 7.5 mg/day for 3 successive days and treated similarly 24 hr after the last dose, the liver homogenate preparation was found not to contain CPPO. We have detected a quaternary pyridinium metabolite of haloperidol in the liver of this rat [6]. Thus, the fact that CPPO was not detected suggests that either it is rapidly excreted from the liver or it is easily converted to other metabolites not evaluated in this study. For example, since haloperidol undergoes glucuronidation on the hydroxy group at position 4 of the piperidine ring [24], a similar conjugation may be a feasible pathway for CPPO. No attempt was made in this study to detect CPPO in the brain. However, previous studies

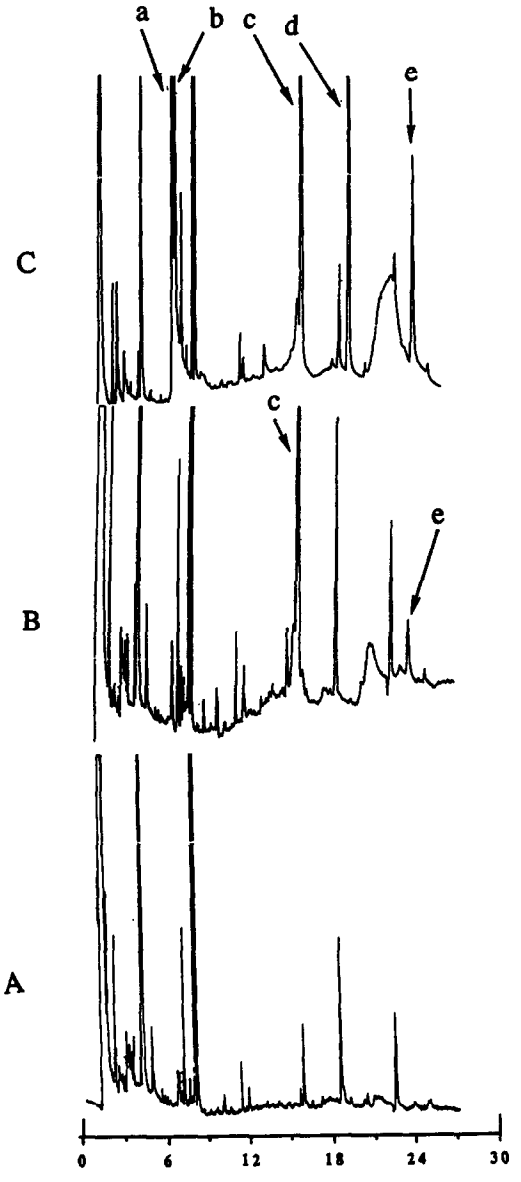


Fig. 1. GC/NPD chromatograms of extracts of liver homogenate preparations. Panel A is an extract of a liver homogenate preparation from an untreated rat (negative control); panel B is the chromatogram of the extract of a liver homogenate preparation from a haloperidol-treated rat; and panel C is the chromatogram of a liver homogenate preparation spiked with authentic synthetic standards [a = MCPTP (Rt = 7.1), b = CPP (Rt = 7.3), c = internal standard (Rt = 16.4), d = CPTP (Rt = 19.8), and e = CPPO (Rt = 24.4)]. Rt = retention time in minutes.

indicated that haloperidol is rapidly taken-up in the brain and undergoes N-dealkylation, although detection of CPPO was not reported [25, 26]. Thus, in spite of the relatively low levels of CPPO detected in the liver, if N-dealkylation occurred in the brain, it can be expected that CPPO might accumulate to fairly high levels. As far as we are aware, although various reports predicted CPPO as a metabolite of haloperidol, only Marcucci *et al.* [12] mentioned observing it in an *in vitro* study of haloperidol metabolism.

To test the hypothesis that a metabolite of haloperidol might be responsible for some of the side-effects associated with haloperidol, we evaluated the neurotoxic potential of CPPO and its proposed potential metabolites in the frog model of MPTP action. The compounds were tested according to a modified procedure developed by Barbeau and others [19, 20]. In this assay, *Rana pipiens* frogs were injected i.p. with an aqueous alcoholic solution of the proposed metabolites using MPTP and haloperidol as the positive controls and the vehicle as the negative control. The results of this study are recorded in Table 3, and the quality of movement of the frogs is graphically depicted in Fig. 2.

In the development of the frog model of MPTP action, Barbeau and others have assumed that MPTP induced darkening in the frogs as part of MPTP's neurotoxicity [19, 20]. Thus, it was expected that MPTP would turn the frogs dark as part of its neurotoxic action. What was unexpected, however, was the intense darkening that occurred in the frogs receiving haloperidol treatment (Table 3). In an attempt to explain this action, we proposed a dopamine receptor blocking mechanism as follows: Melanocyte stimulating hormone (MSH) stimulates melanin synthesis and dispersion that results in darkening of the skin [27–29]. Because of this, we have speculated that the darkening action noted for haloperidol is a result of blockade of dopamine receptors in the hypothalamus [6]. The hypothalamus produces melanocyte stimulating hormone inhibiting factor (MIF) upon stimulation by dopamine; hence, dopamine antagonists are expected to inhibit MIF secretion. Because MIF inhibits MSH production, dopamine antagonists should increase MSH production and hence darkening. This theory is consistent with the effect of dopamine antagonists on prolactin production [29].

MPTP-treated and haloperidol-treated frogs turned dark within 24 hr after the first injection, whereas CPPO-treated frogs maintained their normal skin color throughout the 8-day period of observation. Also, MPTP-treated frogs lost their righting reflex within 24 hr, but the haloperidol-treated frogs maintained a flip-over time of about 1 sec and died at the equivalent dose within the first 48 hr. When the haloperidol dose was reduced to half of the original one, all the frogs survived the 8-day testing period. At the original dose as well as the half-dose, haloperidol turned all the frogs intensely dark within 24 and 48 hr, respectively. However, haloperidol failed to produce any significant motor impairment in the frogs at the half-dose throughout the entire testing period. Clearly, haloperidol has an effect on frog melanocytes at doses that cause little or no motor impairment. Since CPPO-treated frogs remained active up to 72 hr after the first dose, and MPTP-treated frogs lost their righting reflex in 24 hr and died within 48 hr, CPPO did not mimic either the neurotoxicity of MPTP or the pharmacology of haloperidol. However, 96 hr after the administration of the first dose and within 24 hr after the third dose, CPPO caused a persistent freezing action on the CPPO-treated frogs. The frogs lost their righting

Table 3. Effects of potential metabolites of haloperidol on the quality of the righting reflex and skin coloration in *Rana pipiens*

Compounds	Observations (RR*/QOM†/Mel‡) 24 hr	48 hr	96 hr	192 hr	% Survived (8 days)
(I)	1/1/2	—	—	—	0
(I) (1/2D§)	1/1/0	1/1/2	1/2/2	2/2/2	100
CPPO	1/0/0	1/0/0	5/4/0	5/4/0	75
MCPTP	1/0/0	4/2/1	5/3/1	5/3/1	100
CPTP	2/2/0	4/2/0	5/3/0‖	5/3/0	100
MCPPO	1/0/0	1/0/0	1/1/0	1/1/0	100
CPP	5/3/0	5/3/0	5/3/0	5/3/0	0
MPTP	5/3/2	—	—	—	0
Vehicle	1/0/0	1/0/0	1/0/0	1/0/0	100

* RR = Flip-over time in seconds (frogs with flip-over times indicated as 5 sec could not turn over even after 20 sec without an external stimulus)/QOM = Quality of movement/ Mel = Effect on melanocytes. Frogs were evaluated in groups of four or five and flip-over times were recorded when 75% or more frogs showed the same flip-over time.

† The quality of movement was scored from 1 to 4 as follows. No apparent effect = 0; weak but not rigid, rights self before 2 sec = 1; weak but not rigid and rights self between 2 and 4 sec = 2; completely lost righting reflex = 3; frozen in a tonic and extended posture = 4.

‡ Darkening (MEL) in skin coloration in the frogs was scored qualitatively as follows: no color chage = 0; mildly dark or 50% turned dark = 1; all very intensely dark = 2.

§ Received half-dose.

‖ Observation at 120 hr.

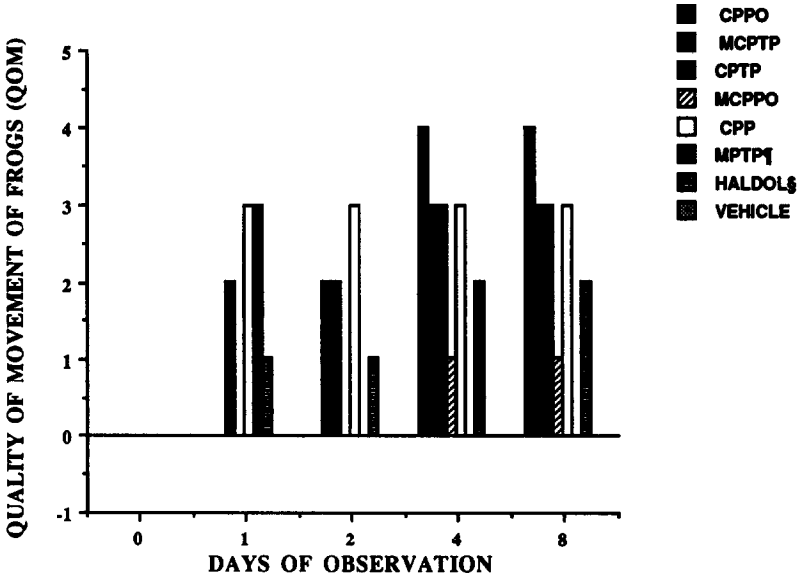


Fig. 2. Effects of potential metabolites of haloperidol on the quality of the righting reflex in *Rana pipiens* frogs. Frogs were evaluated in groups of four or five, and flip-over times were recorded when 75% or more frogs showed the same flip-over time. The quality of movement was scored from 1 to 4 as follows: no apparent effect = 0; weak but not rigid, rights self before 2 sec = 1; weak but not rigid and rights self between 2 and 4 sec = 2; completely lost righting reflex = 3; frozen in tonic and extended posture = 4. Key: (§) half-dose and (¶) all frogs died within the first 48 hr.

reflexes and remained in an extended, tonic, and rigid posture up to 96 hr after the original observation, when the experiment was concluded. This observation suggests that haloperidol was not metabolized significantly to CPPO in the frog. Since the other proposed immediate metabolites of CPPO do not produce a similar freezing action in the frog (Table 3), the reason for the considerable delay (4 days)

before the onset of the CPPO freezing action is therefore open to speculation. Tardive dyskinesia and related side-effects of haloperidol have a delayed onset of action (although on a more extended time scale) and thus may appear similar with respect to the delayed onset of action observed after CPPO treatment. Haloperidol, CPPO and reduced haloperidol have all been shown to bind to sigma receptors [14]. However, only CPPO was selective for this receptor binding. Because sigma receptors have been implicated in the control of motor behavior [15–18], it is tempting to suggest that the freezing action of CPPO may be related in some way to sigma receptor binding. It is also conceivable that CPPO may act as an agonist at the sigma receptor, while haloperidol and reduced haloperidol may act as antagonists; this could explain the distinctiveness of the freezing action of CPPO. It should be noted, however, that two rats which received CPPO at a daily dose of 10 mg/day for 7 successive days failed to show any freezing action.

To test the possibility that metabolites of CPPO might contribute to the freezing action observed above, four potential metabolites of CPPO (MCPPO, MCPTP, CPTP and CPP) were taken through the frog assay. The N-methylated product of CPPO (MCPPO), the most plausible metabolite, not only failed to match the freezing action of CPPO but also did not produce any of the motor effects associated with CPPO. The fact that a simple N-methylation attenuated the freezing action of CPPO so completely (there was little or no difference between MCPPO-treated frogs and the negative controls, Table 3, and Fig. 2) suggests that there is a unique steric requirement for binding at the receptors involved in the freezing action. While MPTP turned the frogs dark and caused them to lose their righting reflexes, its *para*-chlorinated analogue, MCPTP, showed only mild actions (Table 3, and Fig. 2). This observation is consistent with previous reports that MCPTP is a poor substrate for monoamine oxidase-B (MAO-B) [30], MAO-B being the enzyme responsible for the bioactivation of MPTP to MPP^+. MCPTP also failed to mimic the action of MPTP by which it produces persistent depletion of striatal dopamine and its metabolites in mouse brains 7 days after the administration of four daily doses [31]. Because similar *para*-chloro substitution in some inhibitors of dopamine uptake did not appreciably reduce affinity for the dopamine uptake carrier [32], it has been speculated that insufficient formation of $MCPP^+$ may account for the inability of MCPTP to cause MPTP-like effects on striatal dopamine in mice [32]. Indeed, we have shown that 4′-chlorophenyl-1-methylpiridinium ion ($MCPP^+$) is extremely toxic, being rapidly and uniformly lethal in the frog model of MPTP action [6]. The other potential metabolites of CPPO, CPTP and CPP, showed significant neurotoxic effects in the frog (Table 3) but their time of onset and the intensity of the effects were very different from those of CPPO. Thus, the possibility that a metabolite of CPPO may be responsible for the freezing action has not been supported, and CPPO itself must be responsible for the freezing action.

In conclusion, we confirmed that haloperidol is metabolized *in vivo* to the metabolite CPPO, as previously proposed on the basis of *in vitro* data. This N-dealkylated product did not appear to undergo metabolic transformation to MPTP-like compounds, but did produce a unique freezing action in frogs. The proposed immediate metabolites of CPPO did not produce a similar freezing action in the frog, suggesting that metabolites of CPPO may not be responsible for its freezing action. Because there was a delay in the onset of the freezing action of CPPO, we have suggested that it may be involved in some of the delayed side-effects associated with human haloperidol therapy.

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