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Dithiocarbamate Pesticides Affect Glutamate Transport in Brain Synaptic Vesicles[1]

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ABSTRACT

Dithiocarbamate compounds are widely used agricultural fungicides that display low acute toxicity in mammals and that may become neurotoxic after prolonged exposure. Mancozeb, among other dithiocarbamates tested, proved to be the most potent (K_i= 0.27 μM) at noncompetitively inhibiting the in vitro ATP-dependent uptake of [^{3}H]glutamate in rat cortical vesicles. Furthermore, mancozeb partially (20%) inhibited the ATP-dependent uptake of [^{14}C]methylamine, used as an index for the vesicular transmembrane proton gradient (ΔpH), and evoked its efflux from organelles previously incubated with the ^{3}H-labeled marker. Meanwhile, the vesicular uptake of ^{36}chloride$^-$ anions whose concentrations regulate the transmembrane potential gradient ($\Delta\psi_{SV}$) was not impaired. The dithiocarbamate effects on the vesicular transport of [^{3}H]glutamate thus appeared to involve mainly the ΔpH gradient rather than the potential gradient. Dithiocarbamate metabolites, the potent neurotoxin carbon disulfide included, did not affect the uptake process, thus implying the relevance for inhibition of the persistence, if any, of parent compounds in the brain. The present novel and potent in vitro interferences of selected dithiocarbamate pesticides with the vesicular transport of glutamate, if representative of in vivo alterations, may play some role in the probably complex origin of dithiocarbamate neurotoxicity.

Dithiocarbamates are widely used chemicals that display high broad-spectrum activity against fungal plant diseases (Tomlin, 1994). Disulfiram, the thiuram disulfide of diethyldithiocarbamate (DDTC), also has clinical applications, having been used for almost 50 years in alcohol-aversion therapy (Brewer, 1993). Furthermore, dithiocarbamates are presently receiving attention as potential adjuncts to traditional oncological chemotherapy, due to their "immune restorative" effect, along with protection against the tissue toxicity of cisplatin treatment and the potentiation of tumoricidal therapies (for references see Cohen and Robins, 1990). Although dithiocarbamates are known to display low acute and chronic toxicities in human and experimental animals (Liesivuori and Savolainen, 1994), the extreme reactivity mainly related to their metal-chelating ability (Allain and Krari, 1991), and high affinity for –SH group containing proteins, underlies the wide range of their adverse effects. These include neurotoxicity (Miller, 1982), a sympathetic vascular-asthenic syndrome, antithyroid properties, skin sensitization, and teratogenesis (Hayes, 1991). Furthermore at low doses, DDTC provokes cytotoxicity, both in human cell lines of lymphoid origin (Cohen and Robins, 1990) and in serum-free dissociated mesencephalic-striatal cocultures (Soleo et al., 1996). We have recently shown that disulfiram and DDTC proved to differentially affect the vesicular transport and in vivo release of striatal dopamine and glutamate in rats (Vaccari et al., 1996, 1998). It was suggested that these effects were partially involved in disulfiram-provoked neurological symptoms (see Ellenhorn et al., 1997). In this study, we wanted to assess whether dithiocarbamate pesticides could affect the vesicular transport system for glutamate. This is a mechanism that participates in the important neuroprotective function of rapidly removing the otherwise potent excitotoxin glutamate from extracellular spaces when in excess of physiological concentrations (Choi, 1988; Meldrum and Garthwaite, 1990; Rothstein et al., 1996). It will be shown that selected dithiocarbamate pesticides affect the in vitro vesicular transport of glutamate.

Materials and Methods

Materials. Male Sprague-Dawley rats (250–300 g) were used. L-[2,3-^{3}H]Glutamic acid (specific activity, ≈48 Ci mmol^{-1}), [^{14}C]-methylamine (specific activity, 54 mCi mmol^{-1}), and ^{36}chloride (Cl) (specific activity, 89 μCi/ml) were purchased from Amersham Corp. (Little Chalfont, UK). Tetraethylthiuram disulfide (disulfiram), diethyldithiocarbamate sodium salt, carbon disulfide, $MnCl_2$ tetrahydrate, ethylenethiourea (2-imidazolidinethione), and $ZnSO_4$ were obtained from Sigma Chemical Co. (St. Louis, MO). Dithiocarbamate pesticides, all pure for analysis products, were obtained from Dr.

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ABBREVIATIONS: DDTC, diethyldithiocarbamic acid; $\Delta\psi_{sv}$, potential gradient; ΔpH, proton gradient; Cl, chloride.

Ehrenstorfer GmbH (Augsburg, Germany). All compounds were freshly dissolved in the assay medium or in dimethyl sulfoxide. Control samples contained, in the latter case, an equal volume (2 μl) of dimethyl sulfoxide.

Preparation of Brain Synaptic Vesicles. Synaptic vesicles for the different assays were prepared from the entire cortex (at least 1 g of tissue) according to the Kish and Ueda (1989) procedure. Briefly, tissues were homogenized (1:10, w/v) with a Teflon-glass homogenizer in a solution containing 0.32 M sucrose, 0.5 mM calcium acetate, 1 mM magnesium acetate, and 1 mM $NaHCO_3$. The homogenates were spun for 15 min at 12,000*g* (4°C, Sorvall SS-34 rotor, Du Pont Company, Newton, CT). The resulting pellets were gently resuspended in 20 vol of ice-cold lysing solution (6 mM Tris-maleate, pH 8.1) for 45 min, and then centrifuged for 15 min at 43,000*g*. Supernatants were then spun for 55 min at 200,000*g* (Beckman 50 TI rotor; Beckman Instruments, Fullerton, CA). The final pellets were resuspended in a solution of 0.32 M sucrose, 1 mM $NaHCO_3$, and 1 mM dithiothreitol. The crude synaptic vesicles were stored at −70°C and routinely used within 3 days of their preparation. We had preliminarily confirmed the finding by Kish and Ueda (1989) that freezing the vesicles for 2 weeks provoked no appreciable loss of glutamate uptake activity.

Assay of Vesicular Uptake and Release of [^{3}H]Glutamate. In glutamate uptake experiments (Kish and Ueda, 1989), duplicate aliquots (40−50 μg) of cortical vesicular proteins were preincubated in 80 μl of medium (0.25 M sucrose, 4 mM $MgSO_4$, 5 mM Tris-maleate, pH 7.4, 4 mM KCl, and 2 mM potassium aspartate) for 5 min at 30°C in the absence or presence of test compounds. After preincubation, the uptake was initiated by the addition of a mixture (final concentration 50 μM) of unlabeled and [^{3}H]glutamate and 2 mM of ATP (neutralized with Tris base). Following incubation at 30°C for 10 min, the uptake was stopped by the addition of 2 ml of ice-cold 0.15 M KCl and immediate filtration through glass-fiber GF/F filters (previously soaked for 1 h in a 1% polyethyleneimine solution). Test tubes were rinsed with 2 ml of KCl solution three more times, and the filters washed an additional four times with the same solution. The values of radioactivity residual in vesicles incubated over ice (blanks) were subtracted from corresponding samples at 30°C.

For efflux experiments, at the end of the preincubation period cortical vesicles were incubated with 50 μM unlabeled plus [^{3}H]glutamate and 2 mM Tris-ATP for 5 min, the time when test compounds were added to the vesicle suspension. Samples were thereafter filtered at various times of incubation.

Vesicular Uptake and Efflux of [^{14}C]Methylamine. The ATP-dependent uptake of [^{14}C]methylamine into cortical vesicles was measured as a putative index of the transmembrane ΔpH to which the accumulation is proportional (Tabb et al., 1992). The preincubation medium contained 0.14 M potassium gluconate instead of sucrose to maintain iso-osmotic conditions, plus 20 mM HEPES (pH 7.4), 4 mM $MgSO_4$, 4 mM KCl, and 80 to 100 μg of vesicle proteins. Vesicles were preincubated for 1 h at 4°C in the above buffer, then test compounds were added where requested, and soon thereafter the uptake in a final volume of 100 μl was initiated by the addition of a mixture (20 μl) of 50 μM [^{14}C]methylamine plus 2 mM Tris-ATP (pH 7.2). After 5 min of incubation at 30°C, the uptake was stopped with 2 ml of ice-cold 0.15 M KCl, and immediate filtration through GF/F filters was carried out. T-tubes were then washed with 2 ml of KCl solution five more times. The radioactivity values measured in vesicles incubated at 4°C (blanks) were subtracted from the 30°C samples. For efflux experiments, 50 μM [^{14}C]methylamine plus 2 mM Tris-ATP was added to preincubated vesicle at time 0, when the incubation reaction at 30°C was started in order to fill the organelles with the marker. Mancozeb (0.25 and 25 μM) was then added at time 2.5 min, and the incubation was continued for various periods of time, after which the samples were filtered and residual radioactivity in vesicles measured.

Vesicular Uptake of $^{36}Cl^-$. The ATP-dependent influx of ^{36}Cl to cortical vesicles was measured with the same procedure used for the glutamate uptake assay. Briefly, duplicate aliquots (80–100 μl) of vesicular proteins were preincubated for 5 min at 30°C in 100 μl of "glutamate" medium, in the absence or presence of test compounds. The uptake was then started by the addition of 0.8 μCi of $^{36}Cl^-$ and 2 mM Tris-ATP. Following incubation at 30°C for 10 min, the uptake was stopped and samples were washed as for the glutamate uptake samples. Binding of $^{36}Cl^-$ to GF/F filters (presoaked in polyethyleneimine solution) approached 50% of total radioactivity measured (186 ± 17 cpm versus 377 ± 37 cpm, respectively; $n = 6$).

Statistical Analyses. The statistical analysis was performed with one-way analysis of variance followed by Newman-Keul's test for multiple comparisons, or with the Student's t test for grouped data. Kinetic parameters for competition experiments were calculated with the Biosoft RADLIG v0.4 and WINZYME programs (Biosoft, Ferguson, MO).

Results

Effects of Dithiocarbamates on ATP-Dependent [^{3}H]Glutamate Uptake in Cortical Vesicles. Dithiocarbamate pesticides inhibited the energy-dependent uptake of [^{3}H]glutamate to cortical vesicles with widely ranging (from nanomolar to millimolar) K_i values (Table 1). Mancozeb proved to be the most potent compound, with $K_i = 0.27$ μM, and metham and DDTC the weakest ones, with $K_i > 1000$ μM. Mancozeb displayed a clear noncompetitive-type inhibition (Fig.1) with unchanged Michaelis-Menten constant (K_m) and increased V_{max} values. Kinetic parameters were as follows. No mancozeb (controls) and with mancozeb 0.25, 2.5, and 25 μM: K_m (μM) = 209 ± 12; 227 ± 37; 259 ± 7, and 209 ± 5, respectively; V_{max} (pmol mg^{-1} protein; min^{-1}) = 266 ± 14; 236 ± 24; 189 ± 15*, and 11 ± 3**, respectively. Present values were mean ± S.E. from $n = 3$ to 5 experiments performed in duplicate (*$P < .05$; **$P < .01$ versus controls).

Effects of Mancozeb on Vesicular Content of [^{3}H]Glutamate. To ascertain whether the present effects of mancozeb as a representative of dithiocarbamate compounds could reflect on the vesicular content of amino acid, vesicles

TABLE 1

Inhibitory activity of dithiocarbamate pesticides, their metal components, and metabolites on ATP-dependent uptake of glutamate in cortical vesicles

Duplicate aliquots of rat cortical vesicles were preincubated for 5 min at 30C in the absence or presence of increasing concentrations of competitors; thereafter, 50 μM unlabeled + [^{3}H]glutamate and ATP was added, and the incubation was continued for 10 min. Data are presented as mean ± S.E. from n individual experiments. Affinity (K_i) values were calculated with the Biosoft RADLIG v.4 program.

Compound	Ionic substituent	K_i (μM)	n
Mancozeb	Mn, Zn	0.27 ± 0.02	4
Disulfiram		4.21 ± 0.5	5
Thiram		4.98 ± 0.16	3
Ferbam	Fe	8.07 ± 0.11	3
Maneb	Mn	11.9 ± 0.17	3
Nabam	Na	13.1 ± 0.07	3
Ziram	Zn	18.5 ± 0.11	3
Propineb	Zn	60.1 ± 0.22	3
Zineb	Zn	165 ± 0.33	3
Metham	Na	>1000	2
DDTC		>1000	2
Carbon disulfide		>1000	3
Ethylenethiourea		>1000	2
$MnCl_2$		>1000	2
$ZnSO_4$		>1000	2
Chlorpropham[a]		>1000	2

[a]A plant growth regulator, carbamate herbicide.

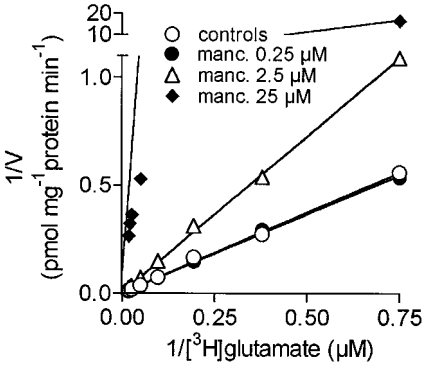

Fig. 1. Representative kinetic profile for the noncompetitive interaction of the dithiocarbamate fungicide mancozeb on ATP-dependent [^{3}H]glutamate uptake in rat cortical vesicles. Kinetic parameters were presented in *Results*.

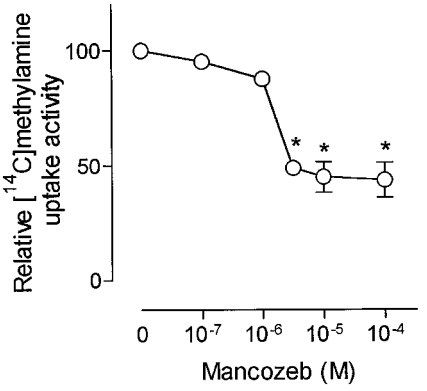

Fig. 3. Concentration-dependent effects of mancozeb on ATP-dependent [^{14}C]methylamine uptake in cortical vesicles. Data are presented as mean ± S.E. from four individual experiments performed in duplicate, and are expressed as percentage changes from controls (no test compounds added), where the uptake activity was 84.5 ± 5.5 pmol mg^{-1} protein. *$P < .01$ versus controls, Newman-Keul's test. Vesicles were preincubated (1 h at 4°C), then increasing concentrations of test compounds were added right before 50 μM [^{14}C]methylamine and ATP was added, and the incubation was run for 5 min at 30°C. The inhibition of the uptake process by mancozeb is assumed to reflect the collapse of the transmembrane ΔpH.

were previously incubated with [^{14}C]glutamate. As with disulfiram (Vaccari et al., 1998), the addition of mancozeb (25 μM) slightly (19%) but significantly decreased the vesicular content of [^{3}H]glutamate after 5 min of contact with the organelles (Fig. 2). This effect, however, was reverted by prolonging the exposure to the chemical by up to 25 min, when the amount of residual [^{3}H]glutamate in vesicles increased by 33% compared with controls.

Effects on ATP-Dependent [^{14}C]Methylamine Uptake and Efflux in Cortical Vesicles. The energy-dependent uptake of [^{14}C]methylamine represents an index for the transmembrane ΔpH to which it is proportional (Tabb et al., 1992). Mancozeb partially (≈50%) inhibited in a concentration-dependent manner the vesicular [^{14}C]methylamine uptake, with the highest inhibition being attained with a concentration of 10 μM (Fig. 3). There was also a time-dependent, mancozeb-provoked decrease (40–75%) in the vesicular content of methylamine (Fig. 2).

Effects on Vesicular Uptake of $^{36}Cl^-$. The vesicular uptake of [^{3}H]glutamate strongly depends on the Cl ion content in the incubation medium (Naito and Ueda, 1985; Wolosker et al., 1996). The incubation of cortical vesicles with $^{36}Cl^-$ in the absence or presence of up to 100 μM concentrations of mancozeb did not affect the uptake activity that, in controls, was 3.3 ± 0.5 pmol mg^{-1} protein.

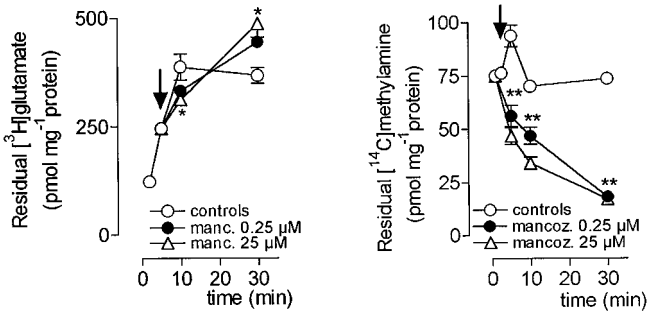

Fig. 2. Time course for the mancozeb-evoked efflux of [^{3}H]glutamate (left panel) and [^{14}C]methylamine (right panel) in cortical vesicles. Test compounds were added at 5 or 2.5 min (vertical arrows) to vesicles preincubated with [^{3}H]glutamate or [^{14}C]methylamine plus ATP, respectively, and the incubation was run for different time periods, after which the samples were filtered.

Discussion

In this study, evidence is provided for the first time that highly reactive dithiocarbamate pesticides can also act at the synaptic vesicular level, because several of them, such as mancozeb, thiram, and ferbam, potently (in the nanomolar to low-micromolar concentration range) inhibited [^{3}H]glutamate uptake into cortical vesicles (Table 1). The vesicular uptake of glutamate, the major excitatory neurotransmitter in the mammalian brain, is mainly temperature, ATP, and Cl dependent (Naito and Ueda, 1985; Cidon and Sihra, 1989; Tabb et al., 1992; Wolosker et al., 1996). A phosphocreatine-dependent and ATP- and Cl-independent glutamate uptake has also been recently demonstrated (Xu et al., 1996). The driving force for glutamate uptake into synaptic vesicles is given by a transmembrane electrochemical proton gradient ($\Delta\mu_{H^+}$) formed by a vacuolar H^+-ATPase. The H^+ protons pumped into the vesicle generate the ΔpH, acidic inside, and a potential gradient ($\Delta\psi_{sv}$), positive inside (Naito and Ueda, 1985; Cidon and Sihra, 1989; Shioi et al., 1989). The relative proportions of both potentials vary greatly, depending on the concentrations of the permeating anion Cl (Cl^-). At low Cl^- concentrations, $\Delta\psi_{sv}$ greatly predominates over ΔpH, whereas the latter increases with increasing Cl^- concentrations (Maycox et al., 1988). Both $\Delta\psi_{sv}$, which controls the affinity for glutamate, and ΔpH are important for accumulating and retaining glutamate in the vesicles (Wolosker et al., 1996). It is important to note that glutamate accumulation can also occur in the *absence* of ATP, thanks to $\Delta\psi_{sv}$, which is established along with the preexisting ΔpH (see Tabb et al., 1992).

The dithiocarbamate mancozeb proved to partially inhibit the ATP-dependent vesicular uptake of [^{14}C]methylamine (Fig. 3), and to evoke its efflux from the organelles (Fig. 2). Methylamine is a hydrophilic weak base that can permeate biological membranes only in the deprotonated, uncharged form, and undergoes accumulation into synaptic vesicles proportionally to the ΔpH (Johnson, 1988; Tabb et al., 1992). Thus, mancozeb partially attenuated the transmembrane

ΔpH, an effect probably contributing to the dithiocarbamate-provoked inhibition of glutamate uptake. A similar outcome appeared to occur with disulfiram but not with its reduction metabolite DDTC (Vaccari et al., 1998). An additional cause for dithiocarbamate effects on glutamate uptake might be the inhibition of vesicle-related Mg^{2+}-ATPase activity, similar to what has been shown to occur with disulfiram in synaptosomal (Mamatha and Nagendra, 1994) and chromaffin granular membranes (Schlichter et al., 1975).

Among the metal substituents in dithiocarbamate molecules (Table 1), zinc did not appear to be crucial for the inhibition of uptake, because it was present both in mancozeb and zineb molecules, the latter displaying a more than 600-fold lower affinity compared with mancozeb. Furthermore, $ZnSO_4$ also poorly inhibited the uptake process (Table 1). Although $MnCl_2$ did not affect [^{3}H]glutamate uptake, the manganous component of mancozeb (a coordination product of zinc ion and maneb, containing 20% manganese and 2.5% zinc; Tomlin 1994) seemed to have some inhibitory relevance, because maneb (manganese ethylenebis dithiocarbamate) fairly potently impaired the uptake process (Table 1). Mancozeb also modestly decreased the glutamate content of vesicles previously incubated with [^{3}H]glutamate, shortly (5 min) after its addition to the incubation medium (Fig. 2). However, after 25 min of contact with the pesticide, there was a clear tendency of residual [^{3}H]glutamate to increase (+33%), compared with time-matched controls. The early, inhibitory component might be explained by the mancozeb-provoked attenuation of ΔpH, and the later stimulatory component, with the consequent prevalence of $\Delta\psi_{sv}$ over the steadily depressed transmembrane ΔpH. In fact, residual [^{14}C]methylamine in vesicles in the presence of mancozeb was still less than in controls 25 min after the addition of the compound (Fig. 2). Extravesicular Cl^- normally enters the vesicle through an ATP-dependent, halide-sensitive transporter and a Cl channel (Hartinger and Jahn, 1993), thus neutralizing the charge of intraorganelle protons, facilitating the further transport of H^+, and resulting finally in increased ΔpH and declining $\Delta\psi_{sv}$ (Tabb et al., 1992; Wolosker et al., 1996). The finding that mancozeb dissipated ΔpH in the absence of any effect on the vesicular influx of $^{36}Cl^-$ after 10 min of incubation is in line with previous findings (Wolosker et al., 1996), and further supports the putative prevalence of $\Delta\psi_{sv}$ as a driving force for the uptake in the later times of exposure to the dithiocarbamate.

In the present experiments, vesicles were exposed to mancozeb over long (25–30 min) incubation times, although physiologically relevant effects on transport processes were expected to occur during the early phase of incubation. This choice was justified by the lack of information in the literature about the persistence of dithiocarbamates in brain tissues. Due to their recognized ability to chelate metals, highly lipophilic (dithiocarbamate-metal) complexes are, indeed, retained in the central nervous system for a long time (Oskarsson and Land, 1985), which makes it reasonable to suspect that even in an in vivo situation the synaptic vesicles may be exposed to these pesticides for a long period, as well as to their neurotoxic metabolites. A major role in the ethiogenesis of dithiocarbamate toxicity has been ascribed to carbon disulfide and ethylenethiourea metabolite/degradation products (Rainey, 1977; Chernoff et al., 1979). In the present experiments both compounds were unable to affect the vesicular uptake of [^{3}H]glutamate (Table 1). This would first suggest that severity in the impairment of the uptake process strictly depended on the persistence in the brain of the parent dithiocarbamate molecule. Secondly, it would suggest that the purported role of carbon disulfide in causing dithiocarbamate neurotoxicity (Rainey, 1977) does not involve the vesicular, glutamate transport process.

The present results do not allow us, of course, to draw sound toxicological implications. It is, however, tempting to speculate that the dithiocarbamate-provoked in vitro inhibition of the vesicular [^{3}H]glutamate uptake, if representative of in vivo effects, might be reflected in an increase of extracellular levels of the excitatory amino acid. The homeostatic maintenance of extracellular levels of glutamate by the glial and neuronal reuptake and storage mechanisms is indeed required to avoid excitotoxicity (Rothstein et al., 1996; Obrenovitch and Urenjak, 1997). However, it is highly controversial how much extracellular glutamate is needed to kill neurons in vivo. The chronic inhibition of glutamate transport in tissue cultures has been purported to represent a model of slow neurotoxicity (Rothstein et al., 1993; Okazaki et al., 1996; Velasco et al., 1996), and glutamate uptake inhibitors have been successfully used in in vivo studies for potentiating glutamate toxicity (McBean and Roberts, 1985). Nevertheless, the inhibition of glutamate uptake by itself does not seem to be enough to damage neurons (Massieu et al., 1995), and high extracellular glutamate levels do not consistently correlate with, nor necessarily produce, neuronal dysfunction or death in vivo (Obrenovitch and Urenjak, 1997).

In spite of their generally acknowledged low toxicity, dithiocarbamates are known to provoke a wide range of neurobehavioral effects, including ataxia, hindlimb paralysis, hemiparesis, convulsions, behavioral abnormalities, and neuropathological changes in the brain (see Miller, 1982 for references). Additionally, mancozeb, maneb, and propineb are teratogenic in rodents (Larsson et al., 1976; Chernoff et al., 1979). Finally, maneb and, more generally, dithiocarbamates, have been reported to induce a permanent extrapyramidal syndrome resembling Parkinsonism (Hoogenraad, 1988; Ferraz et al., 1988; Meco et al., 1994). Disulfiram (Antabuse) intoxication, as well as its chronic use in alcohol aversion therapy, has long been known to provoke several neurological symptoms for which a glutamatergic and dopaminergic contribution cannot be excluded (Vaccari et al., 1996, 1998).

A rough extrapolation of the effective in vitro concentrations (0.25–25 μM) of mancozeb would yield corresponding in vivo doses (0.6–68 mg/kg) far (14- to 1360-fold) exceeding the acceptable daily intake in humans, which is set at 50 μg/kg (Rohm and Haas Company, 1995). Furthermore, because absorption is expected to be significantly less than 100% efficient, and excretion will rather rapidly remove some of the parent compound (Liesivuori and Savolainen, 1994), the projected concentrations will, in fact, be far from being achieved in vivo after a single administration. Nevertheless, the prolonged exposure to dithiocarbamates and the putative chronic increase in extracellular levels of glutamate because of the impairment of the neuroprotective uptake systems that clear glutamate from the synaptic clef might well result in some excitotoxicity.

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