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Neurotoxicity and Neurobehavioral Effects of Manganese Phosphate/Sulfate Mixture in Male Sprague-Dawley Rats Following Subchronic Inhalation Exposure

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Résumé

Le méthylcyclopentadiényle manganèse tricarbonyle (MMT) est un dérivé organique de manganèse (Mn) utilisé comme antidétonant dans l'essence sans plomb au Canada. Plusieurs inquiétudes ont été soulevées quant à la neurotoxicité des principaux produits de combustion du MMT, à savoir: le phosphate de Mn, le sulfate de Mn et un mélange de sulfate/phosphate de Mn. L'objectif de cette recherche vise à évaluer et à comparer les concentrations tissulaires à la suite d'une exposition subchronique par inhalation à des particules d'un mélange de sulfate/phosphate de Mn chez des rats exposés à des concentrations faible, moyenne et élevée. Cette recherche vise aussi à évaluer la neuropathologie et les effets neurocomportementaux associés à l'exposition au mélange.

Ainsi, un groupe contrôle et trois groupes de 30 rats de Sprague-Dawley mâles ont été exposés dans des chambres d'inhalation, pendant 6 heures/jour, 5 jours/semaine durant 13 semaines consécutives. Les concentrations d'exposition étaient de l'ordre de 3000, 300, et 30 $\mu g/m^3$. Pour l'étude de la neuropathologie, le décompte des cellules neuronales a été réalisé au niveau du globus pallidus, du noyau caudé-putamen, et du cortex frontal. Quant au changement neurocomportemental, il a été évalué à l'aide de l'activité locomotrice et du tremblement. Pour ce faire, la moitié de chaque groupe des rats a été implantée avec des électrodes EMG dans le muscle de gastrocnémien de la patte arrière. L'évaluation du tremblement a été réalisée environ une heure après la dernière exposition pour une durée approximative de 5 minutes quand le rat était au repos, quand il marchait et quand il était en position verticale sur un écran grillagé de 39 cm.

À la toute fin de l'étude, l'activité locomotrice a été évaluée pendant 36 heures en utilisant un autotrack informatisé. Les rats ont ensuite été sacrifiés par exsanguination et les concentrations de Mn dans les tissus (foie, poumon, testicule et rein), dans le sang et dans le cerveau (noyau caudé- putamen, globus pallidus, bulbe olfactif, cortex frontal, et le cervelet) ont été déterminées par activation neutronique.

Des concentrations plus élevées de Mn ont été observées dans le sang, le rein, le poumon, le testicule et dans toutes les sections du cerveau chez le groupe le plus exposé. Le Mn dans le poumon et dans le bulbe olfactif était dose-dépendant. Qui plus est, le bulbe olfactif constitue la région du cerveau qui accumule davantage le Mn. Les paramètres biochimiques sanguins ont révélé aussi quelques différences significatives entre les groupes, en particulier pour la phosphatase alcaline, l'urée, et le chlorate.

L'activité locomotrice était significativement plus élevée chez les groupes exposés à 30 $\mu g/m^3$ et à 3000 $\mu g/m^3$. Chez ce dernier groupe, on observe aussi une perte significative de cellules neuronales dans les trois régions investiguées du cerveau. Par ailleurs, les résultats n'indiquent aucun signe de tremblement. En conclusion, l'exposition à un mélange de phosphate/sulfate de Mn entraîne des changements neuropathologiques au niveau du cerveau et des effets neurocomportementaux chez les rats.

Mots-clés: mélange de phosphate/sulfate de manganèse, rats Sprague-Dawley, exposition par inhalation, neuropathologie, effets neurocomportementaux

Abstract

Methylcyclopentadienyl manganese tricarbonyl (MMT) is an organic manganese (Mn) compound added to unleaded gasoline in Canada. The primary combustion products of MMT are Mn phosphate, Mn sulfate, and Mn phosphate /sulfate mixture. Concerns have been raised that the combustion products of MMT could be neurotoxic, even at low levels of exposure. The objective of this study is to assess and compare tissue concentration following subchronic inhalation exposure to a mixture of Mn phosphate/sulfate particles in rats exposed at low, intermediate and high levels. This research aims also to assess the neuropathology and neurobehavioral effects associated with exposure to the mixture.

A control group and three groups of 30 male Sprague-Dawley rats were exposed in inhalation chambers for a period of 13 weeks, 5 days per week, 6 hours a day. Exposure concentrations were 3000, 300, and 30 $\mu g/m^3$. The neuropathology aspect of this study was to count neuronal cells in globus pallidus, caudate putamen, and frontal cortex. Also the neurobehavioral change was measured by assessing locomotor activity and tremor among groups. In each group, half of the rats were implanted with chronic EMG electrodes in the gastrocnemius muscle of the hind lamb for the purpose of measuring tremor. Tremor assessment was conducted one hour after the last exposure by recording muscle EMG activity for approximately 5 minutes with implanted EMG electrode rats while resting in the cage, walking on the floor, and standing on a vertical 39 cm metal screen grid.

At the end, locomotor activity test was conducted for 36 hours using a computerized autotrack system. Rats were then sacrificed by exsanguinations, and Mn concentration in different tissues (liver, lung, testis, and kidney), blood and brain (caudate putamen, globus pallidus, olfactory bulb, frontal cortex, and cerebellum) were determined by neutron activation analysis. Increased manganese concentrations were observed in blood, kidney, lung, testis, and in all brain sections in the highest exposure group. Mn in the lung and in the olfactory bulb was dose-dependent. Our data indicate that the olfactory bulb accumulated more Mn than other brain regions following inhalation exposure. Biochemical profiles also revealed some significant differences in certain parameters, specifically alkaline phosphatase, urea, and chlorate.

Locomotor activity was significantly increased at 30 and 3000 $\mu g/m^3$. The neuronal cell loss was significantly different in all three areas of interest for rats exposed to the highest level of exposure (3000 $\mu g/m^3$). The result does not show any sign of tremor. In conclusion, exposure to Mn phosphate/sulfate mixture leads to onset the neuropathological changes in particular area of the brain and causes some neurobehavioral differences among the rats.

Key words: Manganese phosphate/sulfate mixture, Sprague-Dawley rats, inhalation exposure, neuropathology, neurobehavioral effect

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Abbreviations

ACGIH	American Conference of Governmental Industrial Hygienists
ATSDR	Agency for Toxic Substances and Disease Registry
EMG	Electromyography
EPA	Environmental Protection Agency
HSDB	Hazardous Substances Data Bank
INAA	Instrumental Neutron Activation Analysis
LOAEL	Lowest Observed Adverse Effect Level
MMT	Methylcyclopentadienyl Manganese Tricarbonyl
MRI	Magnetic Resonance Imaging
NOAEL	No Observed Adverse Effect Level
PCA	Portacaval Anastomosis
RfC	Reference Concentration
U.S.EPA	U.S. Environmental Protection Agency
WHO	World Health Organization

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CHAPTER I: Introduction

1.1 Manganese

Manganese (Mn) is a heavy metal that is an essential element in the diet of humans and animals. Mn is widely distributed in the environment and can be found in rock, soil, air, water, and food (WHO, 1999). Mn plays an important role for biological functions including bone mineralization, metabolic regulation, cellular protection from damaging by free radical species, and the formation of glycosaminoglycans. Mn can be found as a component of over 100 minerals and does not exist naturally in its pure form. It exists in various forms as silicates, carbonates, phosphates, sulfides, borates and oxides (ATSDR, 2000).

Mn compounds are produced from Mn ores and they have a variety of applications. Metallic Mn is used principally in steel production along with cast iron and superalloys to improve hardness, stiffness, and strength in the production of manganese-iron alloy (HSDB, 1998). Mn dioxide is commonly utilized in the production of dry-cell batteries, matches, fireworks, and glass-bonding materials (U. S. EPA, 1984). Mn chloride is employed as a precursor for other Mn compounds, as a catalyst in the chlorination of organic compounds, and in animal feed (HSDB, 1998). Mn sulfate is used primarily as a fertilizer, livestock supplement, ceramic, and fungicide. Potassium permanganate has been used as an oxidizing agent, disinfectant, antialgal agent, metal cleaning, water purifier, and a preservative for flowers and fruits (HSDB, 1998). Any of these uses may result in the release of Mn to the environment.

Mn compounds can exist as solids in the soil and as solutes or small particles in water. Most of the Mn salts are soluble in water except the phosphate and the carbonate (WHO, 1999). Mn exists in 11 oxidative states and the most common valences are +2, +4, and + 7 while the most stable and biologically important valence state is +2 (ATSDR, 2000). Due to low vapor pressure, inorganic Mn compounds exist in the particulate matter (NICNAS, 2003).

1.2 Sources and Level of Exposure

Food is the most significant source of Mn exposure in the general population. The total dietary Mn intake among individuals might vary depending upon nutritional intake. Most people have a daily intake with a reported range of 0.9 to 10 mg per day (Finley and Davis, 1999). Grains, tea, nuts, and green leafy vegetables contain the highest amounts of Mn. The daily intakes of 1 to 5 mg Mn from food have been estimated for humans to stay healthy. The Food and Nutrition Board has set an adequate intake level of Mn at 2.3 mg/day for men and 1.8 mg/day for women. The current recommendations for infants and children are 0.03 to 0.6 and 1.2 to 1.9 mg/day, respectively (Food and Nutrition Board, 2002). The tolerable upper intake level for Mn has been set at 11 mg/day, which is the highest level of daily intake that is likely to cause no adverse health effects in the general population (Food and Nutrition Board, 2002).

Mn represents about 0.1% of the earth's crust and it is a naturally occurring component of all soils in a range of 2 to 7000 mg/kg. Volcanic eruption and soil erosion can release Mn into the atmosphere (Schroeder et al., 1987). Mn exists naturally in surface and

underground water at low levels ranging from 0.4 to 10 mg/L. The primary sources for surface and underground water Mn contamination are industrial facilities discharge, landfill disposal sites, and soil leaching. Mn level in water can be as high as 2000 mg/L in areas close to industrial sources or waste disposal lands (ATSDR, 2000). Using potassium permanganate in drinking water treatment, industrial wastewater purification and odor abatement is another source of Mn release into water. The average Mn concentration in drinking water ranges from 0.02 to 0.15 mg/L (EPA, 2002).

In addition to natural existence, Mn can be introduced into the environment by human activity. The most important sources of Mn released into the air are industrial emissions associated with ferroalloy production, iron and steel foundries, power plant, coke oven, and dust from uncontrolled mining operation (Lioy, 1983). Average Mn in urban and rural areas without significant pollution sources is in the range of 0.01 to 0.007 $\mu g/m^3$ (WHO, 1999). U.S. EPA has established a mean Mn background concentration of 0.04 $\mu g/m^3$ for urban area. According to data summarized by EPA, 80 % of U.S. cities have Mn levels well below 0.1 $\mu g/m^3$, and only 4.7 % have levels above 0.3 $\mu g/m^3$. Higher ambient levels, up to 5 or even 10 $\mu g/m^3$ occur near industrial point sources, such as steel plants (EPA, 2002).

Another main source of inorganic Mn contamination in the urban environment is the combustion of methylcyclopentadienyl manganese tricarbonyl (MMT) a fuel additive used in unleaded automotive gasoline, particularly in areas with high traffic density (Joselow et al., 1978). The average atmospheric Mn concentrations measured in a high

traffic area in Montreal were 0.05 and 0.024 $\mu g/m^3$ for total and respirable (MnR) fractions respectively (Loranger and Zayed, 1997; Zayed et al., 1999c).

According to a recent study in Montreal, the average outdoor MnR concentration in the urban area (0.025 $\mu g/m^3$) was significantly different compared to the rural area (0.005 $\mu g/m^3$). Also, the indoor urban MnR concentration (0.017 $\mu g/m^3$) was significantly different from indoor concentration in rural areas (0.007 $\mu g/m^3$). This study has also suggested that a higher outdoor atmospheric Mn concentration leads to higher indoor concentration (Bolté et al., 2004). Besides, in specific environments, such as houses near highway, the atmospheric Mn concentration could exceed EPA's reference concentration (0.05 $\mu g/m^3$) (Zayed et al., 1999a).

Another source of exposure to Mn is at the workplaces. The occupational exposure to Mn is most likely occurring by inhalation of Mn fumes or dust. There is a potential for workers to be exposed to Mn in the industrial facilities. For instance, workers in the ferroalloy production sites, welding operations, and the dry battery manufacturing facilities are exposed to Mn concentrations ranging from 300 to 20000 $\mu g/m^3$. It has been reported that the workers in mining operation are exposed to much higher Mn concentrations, up to 45000 $\mu g/m^3$ (Gibbs et al., 1999). Mn levels in certain workplace environments such as garages are due to the use of MMT in gasoline. A study in Montreal showed that blue-collar workers are exposed to mean Mn levels of 0.04 $\mu g/m^3$, while garage mechanics are exposed to 0.43 $\mu g/m^3$ (Sierra et al., 1995).

1.3 Toxicokinetics

Mn absorption occurs primarily from the gastrointestinal tract after ingestion and from the alveolar lining after inhalation. The dermal absorption is not a considerable route for inorganic Mn since they cannot penetrate through the skin. The dermal pathway is most likely important for absorption of organic Mn compounds such as maneb, mancozeb, and MMT. Age, chemical species, dose, dietary condition, and route of exposure all have an effect on the extent and rate of Mn absorption and retention (Greger, 1999). Inhaled Mn may be absorbed more rapidly and to a greater extent than ingested Mn (Tjälve et al., 1996). The solubility of Mn species is another determinant of Mn absorption and distribution (Roels et al., 1997).

1.3.1 Absorption

The animal studies indicated that important determinants of Mn absorption are the route of exposure and the chemical form of Mn (Smith et al., 1995; Tjälve et al., 1996; Roels et al., 1997; Anderson et al., 1999). Under normal conditions, only about 3-5 % of ingested Mn is absorbed by the gastrointestinal tract in the human body (Finley et al., 1994). The absorption of dietary Mn appears to be controlled by biliary excretion, intestinal absorption, and intestinal elimination. Homeostatic mechanisms control the absorption of Mn from the gut. There are many other factors that have been found to affect Mn absorption, including dietary Mn level, dietary levels of various minerals, age and developmental state of the individual and iron status (Greger, 1999). Low-protein and iron-deficiency increase the absorption, whereas increased calcium and phosphorus levels decrease Mn absorption (Murphy et al., 1991).

The absorption of inhaled Mn is a function of particle size and its solubility. Particles that are deposited in the lower airway are mainly absorbed, while particles deposited in the upper airways may be moved by mucociliary transport to the throat, where they are swallowed and enter the stomach. Thus Mn may be absorbed both from the lungs and in the gastrointestinal tract following inhalation exposure (ATSDR, 2000).

Roels et al. (1997) measured Mn levels in blood following intranasal administration of 1.22 mg/kg as either $MnCl_2$ or MnO_2 in rats. $MnCl_2$, a soluble form, is quickly absorbed into the blood compared to the insoluble MnO_2. Blood Mn concentrations were increased by 68% and 41 % following $MnCl_2$ and MnO_2 exposure, respectively. Moreover, the soluble particles were more readily delivered to the brain than insoluble Mn compounds (Roels et al., 1997). Dorman et al. (2001) also reported that inhaled $MnSO_4$ was cleared from lung more rapidly than the less soluble phosphate or tetroxide compounds.

1.3.2 Distribution

The highest concentrations of Mn in the body with no excessive Mn exposure are found in the liver, pancreas, adrenal gland, and kidney. The lowest concentrations are observed in fat and bone (ATSDR, 2000). It has been suggested that tissues rich in mitochondria may accumulate higher levels of Mn (Kato, 1963). Tjälve et al. (1996) investigated the uptake of Mn in brain regions of rats following intranasal administration of 4 μg/kg. The whole body autoradiography of rats at different time points indicated that the olfactory bulb contained the highest concentration of Mn at 1, 3, and 7 days post-dosing (90, 69, and 47 %, respectively), while the value decreased to 16 % after 12 weeks. Significant

uptake of Mn by other brain regions was not observed until the third day, when the basal forebrain, cerebral cortex, hypothalamus, and striatum had 21, 2, 3, and 1 % of the labeled Mn, respectively. By contrast, liver and kidneys, each contained approximately 1 % of measured Mn at 1, 3, and 7 days with values decreasing consistently after 12 weeks.

The absorbed Mn exists in divalent and trivalent states in the plasma (Scheumamer and Cherian 1983). Both forms can enter the central nervous system via the blood-brain barrier and the blood-cerebrospinal fluid barrier. Divalent Mn can be transported to brain capillary endothelial cells and choroidal epithelial cells via undefined transporters (Takeda, 2003). Mn may also circumvent the blood-brain barrier and pass directly into the central nervous system via olfactory pathways (Tjälve et al., 1996; Frumkin and Solomon, 1997).

A large portion of divalent Mn may exist in non-protein-bound form as the free ion (May et al., 1997). This free ion can be transported into the brain via calcium channels (Narita et al., 1990) or through Na and Ca exchange pathways (Frame et al., 1991). Trivalent Mn is able to connect to transferrin and pass through the brain barrier via receptor-mediated endocytosis. Mn entering the brain can combine with metalloproteins such as glutamine, which are synthesized in astrocytes (Takeda et al., 2003).

Pharmacokinetic factors that may contribute to the increased efficiency of brain Mn delivery following inhalation include greater Mn absorption from the lungs and slower clearance of absorbed Mn from the circulation (Andersen et al., 1999). Moreover, inhalation exposure to soluble forms of Mn results in higher brain Mn concentration

compared with insoluble form of Mn (Dorman et al., 2001). Another study has shown that after intratracheal instillation, a surrogate for inhalation exposure, Mn concentrations were higher in brain for soluble $MnCl_2$ than for the insoluble MnO_2. Striatal Mn concentrations increased by 205% and 48% following $MnCl_2$ and MnO_2 administration, respectively (Roels et al., 1997).

Absorbed Mn is transported to other organs via the iron-binding protein transferrin, α2-macroglobulin, and albumin. Manganese readily crosses the blood-brain barrier, and is largely distributed to the central nervous system (Aschner et al., 1999). Following chronic exposure to high level of Mn in humans and other primates, the metal preferentially accumulates in the thalamic nuclei, substantia nigra, pallidum, and other brain regions that also accumulate iron (Barbeau et al., 1984). Under normal plasma concentrations, Mn enters the brain mainly across the cerebral capillaries. As the plasma concentration increases, entry of Mn across the choroids plexus becomes more important (Murphy et al., 1991).

1.3.3 Metabolism

Mn as a metallic element does not go undergo metabolic transformation. Mn has a potential to exist in a number of oxidation states in biological systems, and may transfer from Mn (II) to Mn (III) in the body (ATSDR, 2000). The α-globulin protein, ceruloplasmin, appears catalyze the alteration of Mn (II) to Mn (III) (Andersen et al., 1999). The high affinity of the iron-transporting protein transferrin for Mn (III) probably enhances this reaction. Besides, small fraction of absorbed Mn exists as the free ion,

which readily forms complexes with some organic and inorganic ligands, albumins, and proteins such as transferrin and α-macroglobulin (EPA, 2002). Mn also plays a role in enzymatic reactions with hydrolases, kinases, transferases, and decarboxylases (Keen et al., 1999; Lee, 2000).

1.3.4 Excretion

Several animal studies demonstrated that biliary/fecal excretion is the main route for Mn elimination (Thompson et al., 1982; Davis et al., 1993; Malecki et al., 1996). In addition, Mn might be excreted in the urine, pancreatic fluid, hair, sweat, and breast milk (WHO, 1981). Excretion of inorganic Mn from the body is rapid and does not require active processes. Most of inorganic Mn rapidly appears in the bile and feces, with a portion undergoing enterohepatic recirculation.

Blood levels of Mn are increased in patients with impaired Mn clearance due to liver dysfunction (Hauser et al., 1996). The biological half-time in blood ranges from about 12 days in healthy miners to approximately 40 days in healthy volunteers (Mahoney and Small, 1968). Experiments in animals indicated that the elimination of Mn from the brain is much slower compared with the whole body (Newland et al., 1989). Cotzias et al. (1968) demonstrated that the biological half-time for intravenously injected Mn is 37.5 days in healthy subjects, 15 days in healthy miners, and 28 days in subjects with chronic Mn exposure.

Occupational exposure to high concentrations of Mn as well as hepatic failure appears to alter Mn distribution in the central nervous system (Layrargues et al., 1995). People with impaired clearance of Mn resulting from liver dysfunction or portal systemic shunting show increase of Mn concentration in the brain. The average 70 kg man carries a total body burden of about 12 to 20 mg of Mn. Iron status (assessed as serum concentration of sodium ferritin) may possibly affect Mn excretion. The biological half-time of Mn for subjects with low ferritin was twice as of the rate determined for high ferritin status subjects (Finley, 1999).

1.4 Health Effects

Mn is an important constituent of normal diet for humans and animals. Thus, at low levels it has some beneficial effects while at higher levels of exposure via inhalation or ingestion, it can cause adverse health effects to pulmonary, reproductive and nervous systems (Komura and Sakamoto, 1991; Chang, 1996).

1.4.1 Beneficial Effects

Mn is a key element for normal physiological functioning in humans and all animal species (U.S. EPA, 1996). It plays an important role in bone mineralisation, protein and energy metabolism, metabolic regulation, cell protection from oxidative stress, and nervous system function (ATSDR, 2000). Mn is a regulatory and catalytic factor for enzymes including hydrolases, kinases, decarboxylases and transferases (Keen et al., 1999).

Animal studies have demonstrated that Mn deficiency associated with some physiological effects such as skeletal abnormalities, impaired growth, testicular degeneration, and impaired reproductive function (Strause, 1986). Rats with low Mn in their diet experienced more oxidation of mitochondrial membranes of the heart (Malecki and Greger, 1996), and ultrastructural changes in the retina (Gong and Amemiya, 1996). Mn deficiency has not been seen in the general population since human diet has relatively high content of Mn. According to a human study, Mn deficiency may decrease level of clotting proteins, serum cholesterol, retarded growth of hair and nails, and reddening of black hair (Friedman et al., 1987).

1.4.2 Toxic Effects

Mn dust in different forms can irritate lungs of humans and animals and result in an inflammatory response (Roels et al., 1987). There are significant adverse health effects induced by Mn particulate inhalation in workers such as impotence and loss of libido (ATSDR, 2000). The neurotoxic effects are primarily observed following chronic inhalation exposure to high Mn concentration (Mergler et al., 1994). Ingestion of contaminated well water (2 mg Mn/L) has also been reported to induce neurological effects (Kondakis et al., 1989). Since the inhaled Mn bypasses the typical excretory mechanisms in the gastrointestinal tract it probably accumulates more in the brain and is more available to the nervous system and other target organs. Therefore, inhaled Mn may be more toxic than ingested Mn (Mirowitz and Westrich, 1992; ATSDR, 2000).

1.4.2.1 Single Exposure

Studies in animals and humans indicate that the inorganic Mn compounds have very low acute toxicity by any route of exposure. Acute inhalation exposure to high concentrations of Mn dust (> 0.97 mg/m^3) can cause an inflammatory response in the lung, which can lead to impaired lung function in humans (Roels et al., 1987). This response however, is characteristic of nearly all inhalable particulate matter (EPA, 2002) and not specific to the Mn content of particles. Animal study has also revealed that acute inhalation exposure to Mn oxide at high concentrations (> 40 mg/m^3) can induce respiratory effects and increase susceptibility to pneumonia (Shiotsuka, 1984). The dermal pathway must also be considered during spillage of MMT in gasoline on the skin.

Following single oral exposure, LD_{50} ranged from 275 to 804 mg/kg body weight for Mn chloride in different rat strains. Also, LD_{50} from single exposure to Mn sulfate and Mn acetate in rats were 782 and 1082 mg/kg body weight, respectively (Singh and Junnarkar, 1991). Age possibly plays a role in susceptibility to acute Mn toxicity. In an animal study, it was found that Mn chloride induced greatest oral toxicity in the youngest than in the oldest (Kostial et al., 1978). Data on the irritant and contact sensitivity properties of Mn compounds are not available. The organic Mn compounds (MMT, mancozeb, and maneb) have been reported to be sensitizers in animals (Thomas et al., 1990).

1.4.2.2 Short-term Exposure

Short-term inhalation exposure in animal studies shows that the lungs and nervous system are the major target organs (Rodier, 1955). Inhalation exposure to 69 mg/m^3 Mn dioxide increased susceptibility to pneumonia (Maigetter et al., 1976). Subchronic inhalation studies in animals have yielded NOAELs or LOAELs values for systemic (0.7 to 3.9 mg/m^3), neurological (1.1 to 72 mg/m^3), and reproductive effects (61 mg/m^3). The range of exposure levels associated with these effects is too wide to define a threshold. It has been demonstrated that subchronic oral exposure of rats to $MnCl_2$ and $MnSO_4$ (> 22 mg/kg) might induce the neurological, reproductive, and systemic effects (Desole et al., 1994; Grant et al., 1997; Dorman et al., 2000). Systemic effects have been reported following subchronic oral exposure to Mn sulfate (40 mg/kg) and Mn chloride (11 mg/kg) including changes in blood cell counts, reduced liver weight, and decreased body weight in animals (Komura and Sakamoto, 1991).

1.4.2.3 Long-term Exposure

Available data from animal and epidemiological studies concerning the oral and the inhalation exposure have suggested that the nervous system is the most sensitive target for Mn (Mena et al., 1967; Wennberg et al., 1991). Chronic oral exposure to Mn (0.59 mg/L) in human has illustrated mild neurological signs (Kondakis et al., 1989). In contrast, animal studies have determined that the oral exposure to Mn chloride and sulfate may be associated with neurological (40 to 275 mg/kg) and reproductive effects (275 mg/kg) (Nachtman et al., 1986; Komura and Sakamoto, 1992). Chronic inhalation

exposure to high concentrations of Mn-containing dust can induce adverse neurological (0.027 to 5 mg/m^3), respiratory (0.18 to 3.6 mg/m^3), and reproductive (0.97 to 82.3 mg/m^3) effects in humans (Iregren, 1990; Roles, et al., 1992; Wu et al., 1996).

1.4.2.3.1 Neurotoxicity

Occupational studies of miners, industrial and agricultural workers have established that the lungs and brain are the primary target organs following chronic exposure to Mn. The nervous system is the critical target organ of Mn toxicity. Long-term exposure to Mn in occupational setting can result in a progressive neurological dysfunction, which can produce a disabling syndrome referred to as manganism. Progression of manganism is related to the dose and duration of exposure, as well as individual susceptibilities. The striatum and associated brain structures are important target sites of Mn neurotoxicity in humans (Calne et al., 1994).

The clinical symptoms of manganism occur in three stages. The initial stage is subjective and nonspecific such as anorexia, apathy, asthenia, headaches, bad temper, and weakness in the lower extremities. Gradually, an intermediate phase of developing neurological signs associated with speech disturbance, facial expression, alterations of gait, loss of balance, and fine tremor sets in. The final phase is characterized by persistent, often irreversible neurological deficits characterized by muscle rigidity, a staggering gait, dysphasia and a course intention tremor. Because some of these symptoms resemble those of Parkinson's disease, many investigators have used terms such as "Parkinsonism-like disease" (Rodier, 1955; Chang, 1996; Pal et al., 1999; Olanow et al., 2004).

Although, symptoms of manganism look like those of Parkinson's disease significant differences have been noted. The tremor and hypokinesia present in patients suffering from manganism are different from those seen in Parkinson's disease (Barbeau, 1984). Also psychiatric disturbances early in the disease such as "cock walk" and a propensity to fall backward, less frequent resting tremor, more dystonia, and failure to respond to dopaminomimetics are characteristics of manganism (Calne et al., 1994; Olanow et al., 2004).

There are some alterations in pathological findings between manganism and Parkinson's disease. Lesions in manganism are more distributed in the pallidum, the caudate nucleus, the putamen, and even the cortex. In contrast, in Parkinson's disease lesions are found in the substantia nigra and other pigmented areas (Calne et al., 1994). Using brain magnetic resonance imaging (MRI) techniques, researchers have demonstrated that manganism patients have elevated Mn concentrations in their basal ganglia, which has not been seen in patients with Parkinson's disease (Nagatomo et al., 1999). Mn can induce high signal intensity on T1-weighted MRI that is related to its chemical property. The abnormality of MRI signals has been reported in workers exposed to high concentrations of Mn (41.4%). However, measurement obtained with this technique is qualitative rather than quantitative (Kim, 2004).

More specific clinical signs of basal ganglia dysfunction characterize a slow or halting speech without tone or inflection, a dull and emotionless facial expression, altered gait, and fine tremor (Hochberg et al., 1996; Mergler and Baldwin, 1997). Severely affected

manganism patients also develop progressive, irreversible loss of dopaminergic neurons in globus pallidus and nigrostriatal pathway (Calne et al., 1994).

Animal studies also revealed that chronic Mn exposure by the oral route could develop muscular weakness and rigidity of the lower limbs. Oral exposure to Mn chloride in rodents increases spontaneous locomotor activity, rigid and unsteady gait (Chandra et al., 1983; Nachtman et al., 1986; Kristensson et al., 1986; Ali et al., 1995). The neurological effects have been reported at lower airborne Mn concentrations in humans than in animals (Newland and Weiss, 1992). Although, there are dissimilarities in the variety of neurological responses between animals and humans, it can be suggested that animal models, mainly rodents, are likely useful for determining quantitative dose-response relationships for neurological effects.

Occupational studies indicated that neurological symptoms were more significantly associated with exposure to airborne Mn between 0.2 to 5 mg /m^3 for more than 20 years (Crump and Rousseau, 1999; Gorell et al., 1999). The alteration of motor effects has been evidently attributed to an abnormal basal ganglia function (Huang et al., 1998). The neuropathological feature of these symptoms includes neuronal loss in basal ganglia (Jog et al., 1995).

1.4.2.3.2 Pulmonary toxicity

Inhalation of particulate Mn compounds such as Mn dioxide and Mn tetroxide leads to an inflammatory response in human lungs. The symptoms of lung irritation and injury

include bronchitis, cough, pneumonitis, and reduction in lung function (Roels et al., 1987; Abdel-Hamid, 1990; Akbar-Khanzadeh, 1993). Increases in respiratory symptoms, pneumonia and bronchitis have been reported among workers with occupational exposure and in people living near manganese alloy production facilities (WHO, 1999). One study has suggested an increase in prevalence of respiratory illnesses in school children residing near a point source of atmospheric Mn pollution (Nogawa et al., 1973). A higher incidence of pneumonia was observed among people living close to ferromanganese factory as well as factory worker (Tanaka, 1994). The increased susceptibility to respiratory infection might be related to the lung irritation and inflammation caused by inhaled particulate Mn.

1.4.2.3.3 Reproductive and Developmental Toxicity

Limited data are available on developmental toxicity of Mn following oral exposures. Several studies have reported developmental effects in animal models with orally administered Mn. These studies show a decrease in serum testosterone, reduced fetal body weight, and malformations in pups (Laskey et al., 1985; Szkmary et al., 1995; Dorman et al., 2000). Male mice exposed to elevated dietary Mn (< 20 mg/kg) also show decreased size of the testicles, seminal vesicles, and preputial glands, even without frank neurological signs such as tremor and ataxia (Gray and Laskey, 1980).

Some inhalation data from occupational studies revealed that male reproductive dysfunction is a major adverse health effect of Mn toxicity. The symptoms include loss of libido and impotence (U.S. EPA, 1996). Impotence, loss of libido and decreased number

of children have been associated with male workers exposed to Mn dust for many years (Laskey et al., 1985). A human study in an isolated island population with high Mn intake, found an apparent excess of fetal malformations (Kilburn, 1987). Impaired fertility has been observed in male workers exposed for 1-19 years to Mn dust at levels (0.97 mg/m^3) that did not produce any neurological effects (Lauwerys et al., 1985). No information was found regarding reproductive effect in women.

1.5 Mechanisms of Toxicity

The mechanism of Mn-induced adverse effects has been studied for decades, but it is not clearly established. Several hypotheses have been advanced to explain the relation between Mn exposure and its neurotoxicity. It is clear that the main portals of Mn absorption are the gastrointestinal tract and the lung (Aschner et al., 1991).

Absorbed Mn exists as divalent and trivalent Mn in plasma (Scheumamer and Cherian 1985). Both forms may distribute to the central nervous system via the blood-brain and the blood-cerebrospinal fluid barriers (Takeda, 2003). Mn may also circumvent the blood-brain barrier, and pass directly into the central nervous system via olfactory pathways (Tjälve et al. 1996; Frumkin and Solomon, 1997). The olfactory bulb in rats can play a significant role in the uptake of inhaled Mn and transport to brain (Tjälve et al., 1999). However, this route of Mn delivery to the brain is not clear in humans.

Divalent Mn can be transported into brain capillary endothelial cells and choroidal epithelial cells via undefined transporters. It also appears that a large portion of divalent Mn exists in

non-protein-bound forms as free ion (May et al., 1997). This free ion can be transported into the brain via calcium channels (Narita et al., 1990) or through Na and Ca exchange pathways (Frame et al., 1991). Trivalent Mn is able to bind to transferrin and be transported across the brain barrier via the receptor-mediated endocytosis (Takeda et al., 2003).

When Mn enters the brain, it can bind to Mn metalloproteins such as glutamine, which are synthesized in astrocytes. Several studies have documented that increased Mn concentrations in the brain may alter the level and metabolism of neurotransmitters such as dopamine, γ-aminobutyric acid, and glutamate (Erikson et al., 2003). Glutamate is the most common excitatory neurotransmitter, while γ-aminobutyric acid is the most abundant inhibitory neurotransmitter. These neurotransmitters are responsible for the control of motor behaviors (Carlsson, 1990). Astrocytes may play a key role in Mn-induced neurological effects in the brain (Hazell, 2002). Hazell (2002) suggested that the involvement of energy metabolism of mitochondria in astrocytes could cause the Mn-induced neuropathology.

Other studies have suggested that glutamate-mediated excitotoxicity may be responsible for the neurological effects of Mn in the brain (Brouillet et al., 1993). Hazell and Norenberg (1997) have studied the oxidant capacity of Mn in reduction of glutamate uptake. In a further study, they found that increased Mn concentrations in the basal ganglia could lead to oxidative damage in astrocytes, with a concomitant reduction of glutamate uptake (Hazell and Norenberg, 1998). Mn accumulation in the brain may therefore cause major changes in astrocytes, including: reduction of glutamate uptake,

reduction in mitochondria energy metabolism in astrocytes, increased excitotoxicity, and proliferation of astrocytes. These effects may play a key role in impaired astrocytic-neuronal interaction, which results in neuronal cell death (Hazell, 2002).

Overall, brain Mn deposition is region-selective, being predominant in the basal ganglia, which is involved in the control of movement and of some cognitive functions. Acute exposure to Mn is associated with an increase in dopamine neurotransmission, which is also manifested as hyperactivity. Nevertheless, long-term exposure results in a loss of dopamine in the brain, and the neuronal cell damage could be expressed as an increase in motor activity or variation in neurobehavioral effects (Bonilla, 1984; Nachtman et al., 1986).

1.6 Sensitive Population

Sensitive populations are defined as those that demonstrate an enhanced reaction to a chemical compared with other exposed population. Sensitivity may be attributed to many factors, for instance genetic, age, developmental stage, life style, and health status. Infants and children with developing organs as well as the elderly with declining organ function are expected to be more sensitive to Mn toxicity than healthy adults. Mn is excreted more rapidly in adults than in children (Fechter, 1999). Also there is evidence of increased sensitivity for neurotoxic effects following Mn exposure in neonatal rats compared to adult rats (Dorman et al., 2000).

U.S. EPA (1996) has identified other sensitive subpopulations for Mn. These are groups who may have greater potential for increased body burden due to increased absorption or different excretion mechanism. However, pregnant women, elderly, iron or calcium deficient individuals, and people with impaired liver function are considered as sensitive population (Davis and Elias, 1996).

Patients with chronic liver disease could be more vulnerable to the neurotoxic effects of Mn while they have a decrease in biliary excretion of Mn. In a study, portacaval anastomosis (PCA) rats not exposed to Mn, Mn content was increased in globus pallidus by 57% and in caudate putamen by 67% compared to a control group (Therrien et al., 1995). The relative influence of portal-systemic shunting and cholestasis on Mn accumulation and neuronal cell loss in the brain was determined in rats with chronic liver failure (Rose et al., 1999; Salehi et al., 2001). These studies indicated that compromised Mn clearance might influence Mn bioaccumulation and Mn neurotoxicity. It has been hypothesised that a relatively high exposure to Mn may be tolerated by normal animals and humans but may lead to an overload and neurological problems in those with hepatic dysfunction.

1.7 Public Health Concern

Mn toxicity is a well-recognized occupational hazard in human following exposure to high concentrations of respirable Mn dust. Respiratory symptoms such as cough, impaired pulmonary function, bronchitis, and pneumonitis (Roels et al., 1998) are related to respirable Mn. Although the respiratory effects are important, the greatest public health

concern is the neurotoxic effects such as manganism. Exposure to concentrations between 0.027 to 1 mg Mn/m^3 among miners and other industrial workers has been shown to provoke adverse respiratory, neurological, and reproductive effects (Iregren, 1990; Mergler et al., 1994; Lucchini et al., 1995).

The U.S. EPA has determined the potential health risks following exposure to Mn by setting an inhalation reference concentration. The RfC for chronic inhalation exposure was derived by using data from two epidemiological occupational studies (Roels et al., 1987; Roels et al., 1992). The significant effect was destruction of neurobehavioral function, as assessed by medical questionnaire, audio-verbal short-term memory, visual reaction time, eye-hand coordination, and hand steadiness. According to Roels et al. (1992), the LOAEL was identified to be 0.15 mg/m^3 and the RfC was calculated by dividing the LOAEL with a total uncertainty factor of 10^3, allowing 10 for each of the following factors: 1) potentially greater susceptibility of the elderly and children, 2) extrapolation of LOAEL to NOAEL, 3) for chronic duration. Then the result was divided by a factor of 3 for converting workday exposure (8 hr) to continuous exposure (24 hr), which yielded 0.05 $\mu g/m^3$ (U.S. EPA, 1996). This RfC is an estimate of a daily exposure to human population that is likely to be without a significant risk during lifetime.

Presently, the public health concern of airborne Mn is increased due to the replacement of lead with MMT. The use of MMT as a gasoline additive has led to an increase of inorganic Mn, mainly Mn phosphate/sulfate mixture, Mn phosphate, and Mn sulfate in

the atmosphere. Thus, increased level of Mn in the air could lead to potential health risks associated with chronic exposure.

Consequently, susceptible populations, such as children, individuals with chronic liver disease, people with sub-optimal Mn or iron intake, and those with other medical states (e.g., pre-parkinsonian state, aging) may have altered Mn metabolism and could be at greater risk for Mn toxicity (Pujol et al., 1993; Spahr et al., 1996; Malecki et al., 1999).

Although, human data are most useful for the assessment of potential health hazards such data concerning the long-term and low-level inhalation exposure to Mn species related to MMT combustion products are not available. Consequently, to provide adequate toxicological information on Mn species for risk assessment purposes, it is necessary to develop dose-response data using animal models.

1.8 Specific Concern Related to MMT

MMT ($C_9H_7MnO_3$) was developed by the Ethyl Corporation in the 1950s. MMT is an organometallic compound used as an antiknock agent in internal combustion engine fuels to increase the octane level of gasoline and therefore to improve the antiknock properties of the fuel (Davis, 1998). MMT has been used in the United States since 1976. Although in 1977, the adoption of the Clean Air Act limited the use of MMT to gasoline and U.S. EPA denied Ethyl's waiver for the use of MMT until October 1995, when a court decision allowed the company to offer it for sale to refiners for use in unleaded gasoline

and since that time MMT has been used freely (Wallace and Slonecker 1997, Landrigan, 2001).

The use of MMT in gasoline has been permitted in Canada since 1978 (Health and Welfare Canada, 1978). Its use increased steadily until it completely replaced tetraethyl lead in gasoline in 1990 (Hurley et al., 1992). Since 1997, trade of MMT was limited in Canada under the Mn-based Fuel Additive Act that effectively banned the importation of MMT into Canada. Eventually in 1998, the Government of Canada had removed restriction and continues to use it (Davis, 1998).

MMT is allowed to use in France (Ministre de L'Aménagement du Territoire et de L'Environnement, 1999), UK (British Standards Institute, 1999), China (China State Bureau of Quality and Technology Supervision, 2000), Russia (Ministry of Fuel and Energy of Russian Federation, 1997), and Argentina (Norma Argentina, 1999). In New Zealand, according to the Petroleum Products Specification Regulations 2002, automotive fuel must contain no more than 2.0 mg Mn/L.

Usually, 72 mg MMT/L is used in gasoline that corresponds to 18 mg Mn/L (Garrison et al., 1995). The maximum concentration of MMT allowed by the U.S. EPA is 8.3 mg Mn/L of gasoline. According to Health Canada, MMT is currently used in 80% of Canadian gasoline at various concentrations and the mean concentration is approximately 10 mg Mn/L (Wood and Egyed, 1995). However, Zayed and colleagues have found that

the MMT-added gasoline in Canada has a mean concentration of 6.5 mg Mn/L (Zayed et al., 1999c).

MMT is marketed as AK-33X (antiknock agent-33X) or HiTec 3062 (Jaques, 1987). It is a yellow, volatile liquid that has a very low vapor pressure at room temperature (0.05 mmHg @ 20°C) and is completely miscible in gasoline and very slightly soluble in water. MMT is extremely unstable in light and degrades rapidly in air; thus, exposure via gasoline exhausts is expected to be more to the oxidative products such as Mn oxides, phosphate, and sulfate rater than to the parent compound (Garrison et al., 1995; Ressler, 2000; Molders et al., 2001; NICNAS, 2003).

Generally, the combustion products of MMT depend on fuel composition, engine and catalytic converter thermodynamics. The actual portion of Mn emitted is also very variable and depends on various factors including, overall distance driven and driving condition (urban vs. highway) (Ardeleanu et al., 1999).

More recently, samples of particles emitted from several cars that has accumulated significant mileage on MMT-additive gasoline determined that there were three major Mn compounds including a mixture of Mn phosphate, $Mn_5(PO_4)_2[PO_3\text{-}(OH)]_2.4H_2O$, $MnSO_4.H_2O$, and Mn_3O_4 (Ressler et al., 2000; Molder et al., 2001). The ratio for each compound is dependent on the driving cycle and vehicle, however the percentage of combusted product stays relatively constant with the 80% of Mn phosphate and sulfate and less then 20 % of Mn oxide (Pfeifer et al., 2004).

It is also recognized that most of the emitted Mn in the form of phosphate/sulfate mixture in the tailpipe has particle sizes ranging between 0.2 and 10 μm (Zayed et al., 1994; Zayed et al., 1999b). More than 99 % of Mn particles are in the respirable fraction (< 5 μm) and 86 % are smaller than 1 μm (Ardeleanu et al., 1999; Zayed et al., 1999b). In conclusion, Mn particles emitted from MMT are more likely to reach the alveolar region. Many studies have established a positive relation between traffic density and Mn concentrations in the biotic and abiotic components of ecosystem (Loranger et al., 1996; Zayed et al., 2003). Ardeleanu suggested that motor vehicles would add 350 tons of Mn into the Canadian environment each year (Ardeleanu et al., 1999). The U.S. EPA (1994) estimated that if MMT used in gasoline increases by 100%, the level of Mn in ambient air will increase to 0.1 $\mu g/m^3$, way more than the RfC.

Nevertheless, exposure of the general population to Mn originating from MMT seems to be negligible compared to the contribution of the Mn from other industrial and natural sources (Loranger and Zayed, 1997). However, experience with lead in gasoline demonstrated that significant widespread exposure of the general population might occur by adding relatively small amounts of a substance to gasoline (Davis et al., 1996). To determine the risk posed to general population by inhaled Mn species, it is required to have more information about the potential toxicological hazards and the accepted levels of exposure.

1.9 Objectives

The main hypothesis in this study is that the exposure to Mn phosphate/sulfate mixture may result in different bioaccumulation, neuropathological, and neurobehavioral changes that could be dose-dependent. The objectives of this study are to evaluate the effects of Mn phosphate/sulfate mixture on Sprague-Dawley rats at low (30 $\mu g/m^3$), intermediate (300 $\mu g/m^3$) and high levels (3000 $\mu g/m^3$) on:

- the deposition and the bioaccumulation of Mn in different tissues and organs,
- the blood biochemical parameters,
- the neurobehavioral status (locomotor activity and muscular tremor), and
- the neuropathology effect in the brain.

The results of this study may correspond to another step forward to better understand the relationship between exposure and nervous system affects for Mn compounds resulting from the use of MMT in gasoline.

1.10 Special Consideration in this Study

To date, studies to evaluate the neurotoxic effects following inhalation exposure to Mn phosphate/sulfate mixture have not been undertaken. This mixture is the primary combustion product of MMT, receiving increased attention in relation to environmental contamination and public health. This study is unique in that it is the first study to investigate the adverse effects of the Mn phosphate/sulfate mixture following inhalation,

which is the main route of exposure associated with neurotoxicity of Mn in humans and animals.

In this study, equal weights of Mn phosphate and Mn sulfate were introduced into the inhalation chamber. However, the subsequent analysis of air samples by electron microscopy showed that 39 % of the particles were Mn sulfate and 61 % were Mn phosphate. This ratio is acceptable since it is near the ideal ratio of 1:1. Prior to the experiment, the molecular weight (MW) of each substance was taken into account to maximize the probability of obtaining a ratio of 50:50 for particles in the inhalation chamber. Since the MW was 728.65 and 169.03 for Mn phosphate and Mn sulfate respectively, representing a ratio of approximately 4:1, the inverse ratio was used for w/w (4 Mn sulfate / 1 Mn phosphate). Nevertheless, the proportion of particles for each Mn species was far away from the target of 1:1. In fact, more than 80 % of the particles were related to Mn sulfate compound. The details can be found in the report of Beaupré et al. (2004) in the Appendix of this thesis.

The exposure level of 3000 $\mu g/m^3$ was derived from the inhalation study of Coulston and Griffin (1977) that used 100 $\mu g/m^3$ of Mn compounds for 24 hr. Thus, 3000 $\mu g/m^3$ was derived by multiplying 100 $\mu g/m^3$ with 3 (24/8) and a correction factor of 10 in consideration of exposure duration. To help adequately evaluate the dose-response relationship in subchronic study, this level of exposure was divided by a factor of 10 sequentially, which yielded 300 and 30 $\mu g/m^3$.

Human exposure to high levels of Mn in mining and occupational exposure has been reported to induce neurobehavioral and neuropathological effects. Experimental studies in rodent and other animal species have been carried out to produce these effects. Rats seem to be a useful model to study neurotoxicity effects of Mn and its compounds (Russell, 1991).

2 CHAPTER II: Bioaccumulation and Locomotor Effects of Manganese Phosphate/Sulfate Mixture in Sprague-Dawley Rats Following Subchronic (90 Days) Inhalation Exposure

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Bioaccumulation and Locomotor Effects of Manganese Phosphate/Sulfate Mixture in Sprague-Dawley Rats Following Subchronic (90 Days) Inhalation Exposure

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Running title: Manganese bioaccumulation and locomotor activity

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2.1 Abstract

Methylcyclopentadienyl manganese tricarbonyl (MMT) is an organic manganese (Mn) compound added to unleaded gasoline in Canada. The primary combustion products of MMT are Mn phosphate, Mn sulfate, and a Mn phosphate /sulfate mixture. Concerns have been raised that the combustion products of MMT containing Mn could be neurotoxic, even at low levels of exposure. The objective of this study is to investigate exposure-response relationships for bioaccumulation and locomotor effects following subchronic inhalation exposure to a mixture of manganese phosphates/sulfate mixture. A control group and three groups of 30 male Sprague-Dawley rats were exposed in inhalation chambers for a period of 13 weeks, 5 days per week, 6 hours a day. Exposure concentrations were 3000, 300, and 30 $\mu g/m^3$. At the end of the exposure period, locomotor activity and resting time tests were conducted for 36 h using a computerized autotrack system. Rats were then sacrificed by exsanguination and Mn concentrations in different tissues (liver, lung, testis, and kidney), blood and brain (caudate putamen, globus pallidus, olfactory bulb, frontal cortex, and cerebellum) were determined by neutron activation analysis. Increased manganese concentrations were observed in blood, kidney, lung, testis, and in all brain sections in the highest exposure group. Mn in the lung and in the olfactory bulb was dose-dependent. Our data indicate that the olfactory bulb accumulated more Mn than other brain regions following inhalation exposure. Locomotor activity was increased at 30 and 3000 $\mu g/m^3$, but no difference was observed in resting time among the exposed groups.

At the end of the experiment, rats exposed to 300 and 3000 $\mu g/m^3$ exhibited significantly decreased body weight in comparison with the control group. Biochemical profiles also revealed some significant differences in certain parameters, specifically alkaline phosphatase, urea, and chlorate. This study is one of several ongoing studies in our laboratory that address the toxicity of different Mn species.

Keywords: Manganese phosphate/sulfate mixture, inhalation exposure, Sprague-Dawley rats, bioaccumulation, locomotor activity

2.2 Introduction

Methylcyclopentadienyl manganese tricarbonyl (MMT) is one of the main sources of inorganic manganese contamination in urban air, mainly in areas with high traffic density (Joselow et al. 1978). The main combustion products of MMT are essentially Mn-phosphate, Mn-sulfate, and Mn-phosphate/sulfate mixture (Zayed et al., 1999). Exposure to high concentrations of atmospheric Mn can lead to adverse health outcomes, notably respiratory and neurological effects. Exposure to concentrations >1 mg Mn/m^3 among miners and other industrial workers has been shown to persuade adverse respiratory, neurological, and reproductive effects (Iregren, 1999).

The clinical syndrome of manganese neurotoxicity (manganism) can be divided into an early phase characterized by obvious mood and behavior changes, and a later stage somewhat similar to Parkinson's disease that is characterized by dystonia and severe gait disorder (Pal et al., 1999). However, little is known about the potential health effects that may result from long-term low-level exposure of populations through ambient air. Certain subpopulations such as children and patients with chronic liver disease could be more susceptible to different levels of Mn contamination.

It is clear that the route of exposure can influence the distribution, metabolism, and potential for neurotoxicity of Mn-containing compounds (Roels et al., 1997). Inhalation exposure is more efficient than ingestion at transporting Mn to the brain. Pharmacokinetic factors that may contribute to the increased efficiency of brain Mn delivery following inhalation include greater Mn absorption from the lungs and slower

clearance of absorbed Mn from the circulation (Andersen et al., 1999). Moreover, inhalation exposure to soluble forms of Mn results in higher brain Mn concentration compared with insoluble form of Mn (Dorman et al., 2001). One study has shown that after intratracheal instillation, a surrogate for inhalation exposure, Mn concentrations were higher in brain following the administration of the soluble salt $MnCl_2$, than following the administration of the insoluble oxide MnO_2. Striatal Mn concentrations increased by 205% and 48% following $MnCl_2$ and MnO_2 administration, respectively (Roels et al., 1997).

The main brain target for Mn toxicity is the basal ganglia (caudate nucleus, globus pallidus, and putamen), which is involved in motricity. Disturbances of the basal ganglia can lead to unintentional contraction of the skeletal muscles, such as tremor and muscular rigidity, as in Parkinson's disease. Few studies have been conducted to describe the distribution of brain Mn following inhalation of different Mn species, the main route by which Mn intoxication occurs in workers. It seems likely that the neurotoxicity of inhaled manganese possibly be related to an uptake of this metal into the brain via olfactory neurons. The olfactory bulb in rats can make a significant role to uptake of inhaled manganese and then delivery to the brain (Tjälve et al., 1999). However, this route of delivery of manganese to the brain is not well clear in human. The primary objective of this study is to determine the effects of subchronic exposure to Mn phosphate/sulfate mixture on Mn tissues concentrations, and locomotor activity.

2.3 Materials and Methods

2.3.1 Chemicals

The manganese phosphate/sulfate mixture, a fine crystalline powder which includes $Mn_5(PO_4)_2(PO_3(OH))_24H_2O$ hureaulite mineral form, and manganese (II) sulfate monohydrate ($MnSO_4$. H_2O), were obtained from Alfa Aesar (Johnson Matthey Company), and combined in a 50:50 ratio. Whereas manganese sulfate is relatively water soluble, manganese phosphate is insoluble.

2.3.2 Animals

One hundred and twenty, 6 week-old male Sprague-Dawley rats weighting 125-150 g were purchased from Charles River Laboratories (St.Constant, Quebec, Canada) and marked on the tail for identification purposes. Rats were acclimated for approximately 2 weeks in HEPA filtered air. Animals were given NIH-07 chow *ad libitum* when they were not exposed. All experiments were undertaken with the consent of the animal ethics committee of the University of Montreal, and were conducted in accordance with the guidelines set out by the Canadian Council on Animal Care. Rats were individually housed in a polycarbonate cage with stainless steel wire lids under constant conditions of temperature and humidity, with 12:12 h day/night cycles. The subjects were randomly divided into 4 groups with 30 animals in each group for two different periods. Body weight and food and water consumption were measured weekly.

2.3.3 Inhalation Exposure

A control group and three groups of 30 male Sprague-Dawley rats each were respectively exposed to 3000, 300 and 30 $\mu g/m^3$ manganese phosphate/sulfate mixture. Exposure was conducted in two inhalation chambers with a total volume of 1 m^3 each (Hazelton Systems Company, Inc., Kalamazoo, MI) over 13 consecutive weeks, 5 days per week, 6 hr/day. The chamber received (HEPA) filtered air and was maintained at a constant temperature (22-25 °C) and relative humidity (25-40%) throughout the study. Mn aerosol was generated by a Fluidized Bed Aerosol Generator (Model 3400, TSI Inc., St. Paul, MN) with a flow rate of 200-250 L/min. Stainless steel ASME pressure tanks was used in order to obtain fine particles.

Concentrations were verified continuously using a Dust Track (Model 8520) aerosol monitor. In both chambers, air samples were collected on a daily basis to monitor Mn concentrations, using a sampling system consisting of Gilian pumps (Gilian Corp., West Caldwell, NJ) with standard 3-piece cassettes and 37-mm diameter filters. The filters were Teflon (manufactured for SKC Inc., Gelman Sciences, Ann Arbor, MI) 0.45 μm pores. Pumps were used at a constant flow rate of 1.5 L/min. The flow rate was calibrated each day with a Gilibrator (Gilian Corp., West Caldwell, NJ). The particle size distribution of manganese phosphate/ sulfate mixture was measured by using a six-stage Marple Personal Cascade impactor (Series 290). Rats were individually housed during the non-exposure period and were weighed weekly. Food and water were available *ad libitum* when the rats were not exposed.

2.3.4 Locomotor Activity Assessment

Four hours after the last Mn inhalation exposure, the rats were tested for motor activity. A computerized Auto-Track System (Columbus instruments, Ohio, U.S.A.) was used to measure locomotor activity for a period of 36 consecutive hours. This system is consisted of a 15 by 15 infrared beam array with an interbeam distance of 2.4 cm along the X and Y-axes. Data was collected every 0.1 second and the motor activity categorized as distance traveled by rats and resting time. The system was installed in a quiet isolated room with 12 h light / 12 h dark cycles.

2.3.5 Tissue Concentrations

Following locomotor activity assessment, rats were anesthetized in a glass-walled container containing a pledget wet with metofane, an inhalation anesthetic. As soon as the animals were anesthetized, they were sacrificed by exsanguinations and the following tissues and organs weighed and analyzed: liver, lung, kidneys, testis, and one hemisphere of the brain. (olfactory bulb, globus pallidus, caudate/putamen, frontal cortex, and cerebellum). Blood samples were taken from the abdominal aorta for chemical and biochemical analyses and determination of Mn concentrations.

2.3.6 Chemical Analysis

Manganese concentrations in blood, food, water, and tissues were measured by INAA (Kennedy, 1990) using a flue nuclear reactor and an EG&G ORTEC (model DSPec) digital gamma-ray spectrometer incorporating a high-resolution large-volume germanium detector. The potential interference from Fe was reduced by irradiating samples in a more thermalized neutron spectrum, verified and corrected for blood.

2.3.7 Biochemical Profile

Blood samples were collected from the abdominal aorta of anesthetized rats. Blood serum was obtained by centrifugation at 4°C for 15 minutes at 3000 g. The biochemical tests were performed using standard techniques for glucose, urea nitrogen, serum creatinine, bilirubin, transaminases (AST, ALT), and alkaline phosphatases (ALP).

2.3.8 Statistical Analysis

The Dunnet T3 test was used to compare among all pair treatment groups for mean concentrations of Mn in blood, kidney, liver, lung and testis. A similar analysis was conducted for Mn concentrations in different regions of the brain (cerebellum, frontal cortex, globus pallidus, caudate putamen, and olfactory bulb), motricity (distance travelled and resting time), the ten serum biochemical parameters, and body weight in each of the four treatment groups. Homogeneity of variances for the treatment groups was assessed using Levene's test. All analyses were performed using SPSS statistical software (version 11.0). The level of statistical significance was set at $P < 0.05$ for the set of 6 pairwise comparisons performed among the treatment groups using Dunett's test. Data are presented as group mean ± standard deviation (SD).

2.4 Results

2.4.1 Mn in the Inhalation Chamber

The average Mn concentrations obtained in this study were 34.8 ± 9.2, 290.8 ± 76.8, and 2841 ± 529 $\mu g/m^3$ for target concentrations of 30, 300, and 3000 $\mu g/m^3$, respectively. Based on the cascade impactor, 80% of the Mn phosphate /sulfate mixture particles in the inhalation chamber were smaller than 1.55 μm in aerodynamic diameter (Table 2.1). Overall mean daily chamber temperatures ranged from 22-25 °C, and relative humidity ranged from 25-40 %.

2.4.2 Tissue Concentrations

Tissue Mn concentrations are presented in Table 2. 2. Elevated lung manganese concentrations were observed following exposure to 30, 300, and 3000 $\mu g/m^3$ Mn. Mn accumulation in the lung increasing in a dose-dependent manner. Increased testis manganese concentrations were observed following inhalation exposure at 300 and 3000 $\mu g/m^3$. Mn concentrations were elevated in blood and kidney only in rats exposed to the highest level of Mn (3000 $\mu g/m^3$). Liver manganese concentrations were unaffected at all exposure levels.

Brain tissue manganese concentrations are shown in Table 2. 3. Manganese concentrations in olfactory bulb increased in a dose-dependent manner. In addition, Mn concentrations in the caudate putamen and globus pallidus were significantly increased following exposure to 300 and 3000 $\mu g/m^3$. Mn concentration in the cerebellum and frontal cortex were elevated only in rats exposed to the highest level (3000 $\mu g/m^3$). Mn concentration ranged from 0.56-0.83 μg/g in cerebellum, 0.61-1.56 μg/g in the globus

pallidus, 0.62-1.19 μg/g in the caudate putamen, 0.58-0.98 μg/g in the frontal cortex and 0.46-2.32 μg/g in the olfactory bulb. The highest average Mn concentrations in exposure animals were observed in the olfactory bulb, and the lowest in the cerebellum.

2.4.3 Body Weight

There was a significant reduction ($p < 0.05$) in body weight after 13 weeks at the two highest levels of exposure (Fig.2.1). No significant difference in food intake was observed among the different exposed groups. The average Mn concentrations in food and water were 100 μg/g and 0.00002 μg/L, respectively.

2.4.4 Locomotor Activity Assessment

Locomotor activity was assessed in term of the resting time (RT) and distance traveled (DT). After 36 hours, DT was significantly increased after exposure to 30 and 3000 $\mu g/m^3$ Mn, although there was no difference at 300 $\mu g/m^3$. No significant difference in RT was observed among the exposed groups (Table 2. 4).

2.4.5 Blood Biochemical Analysis

Table 2. 5 summarize the biochemical test results. Glucose, sodium, chlorate, urea, and alkaline phosphate levels were significantly different in the animals exposed to highest level of the Mn mixture.

2.5 Discussion

Different MMT combustion products are produced depending on fuel combustion and engine and catalytic converter thermodynamics. It is now well accepted that Mn is emitted from the tailpipe primarily as a mixture of manganese phosphate and manganese sulfate particles, with size ranging between 0.2 and 10 μm in aerodynamic diameters (Zayed et al., 1999a). In fact, in the present study, the size of 100% of the particles was < 10 μm, while 87% were < 3.5 μm.

Rats exposed to manganese in mixture form in our study had lower lung manganese concentrations compared with levels observed in rats exposed to either manganese sulfate (Dorman et al., 2001) or manganese phosphate (Vitarella et al., 2000, Normandin et al., 2002). These results suggest that the mixture is more rapidly cleared from the lung compared to other Mn compounds. The Mn phosphate/sulfate mixture is more water-soluble than Mn phosphate due to the water-solubility of Mn sulfate.

Accumulation of Mn phosphate/sulfate mixture in the lung is dose-dependent, which is similar to what was reported previously by Vitarela et al (2000) and Normandin et al (2002) after exposure to Mn phosphate. This characteristic is important since the rate of clearance from the lung is well known to influence metal delivery to the brain and other organs. In a study by Rhoads and Sanders (1985), a number of metal compounds that were more quickly cleared from the lungs were retained for longer periods at other sites in the body, where they could potentially exert their toxic effects.

A significant increase in blood manganese concentration for rats exposed to 3000 $\mu g/m^3$ was observed. This was not seen in rats exposed to either manganese phosphate (Normandin et al., 2002) or manganese sulfate (Dorman et al., 2001). In comparison with Mn phosphate, testis displayed also a different response after exposure to Mn mixture. Groups exposed to 300 and 3000 $\mu g/m^3$ had significantly higher testis manganese concentrations compared to the control group. This finding is in agreement with the results of Dorman et al. (2001), where animals were exposed to high levels of manganese sulfate. Exposure of laboratory animals to high levels of manganese has been associated with a variety of reproductive effects, including evident histopathologic and biochemical changes in the somniferous tubule (Chandra, 1971; Imam and Chandra, 1975; Murthy et al., 1980).

Studies conducted by Dorman and colleagues have confirmed that delivery of inhaled manganese to the brain is influenced by particle solubility. Moreover, animals exposed to high level of the soluble sulfate form had significantly higher brain manganese concentrations when compared with levels achieved following exposure to the insoluble tetroxide or phosphate forms (Dorman et al., 2001). Our data indicate a significant difference in brain manganese concentration in regions of interest such as the cerebellum, frontal cortex, globus pallidus, caudate putamen, and olfactory bulb in rats exposed to highest level (3000 $\mu g/m^3$) of Mn as compared to the reference group. Manganese concentration in the olfactory bulb was dose dependent. Also, Mn concentrations in the globus pallidus and caudate putamen were higher than those reported by Vitarella (2000)

following exposure to manganese phosphate (Normandin et al., 2002) and metallic manganese (St-Pierre et al., 2001).

Absorbed manganese is transported to other organs via the iron-binding protein transferrin, α2-macroglobulin, and albumin. Manganese readily crosses the blood-brain barrier, and is largely distributed to the central nervous system (Aschner et al., 1999). Following chronic exposure to high level of Mn in human and other primates, the metal preferentially accumulates in the thalamic nuclei, substantia nigra, pallidum, and other brain regions that also accumulate iron (Barbeau et al., 1976; Hill and Switzer, 1984). In normal plasma concentrations, Mn enters in the brain mainly across the cerebral capillaries. Since the Mn plasma concentration increases, entry of Mn across the choroids plexus becomes more important (Murphy et al., 1991). Intra-nasally administered Mn may circumvent the blood-brain barrier and then pass directly into the central nervous system via olfactory pathways (Tjälve et al., 1996), increasing its bioavailability to the brain (Frumkin and Solomon, 1997). A fraction of inhaled Mn is likely to reach cerebral target sites before hepatic clearance (Roels et al., 1997).

Our data show that Mn concentrations in the olfactory bulb and other brain regions were elevated. This is in agreement with Dorman et al (2001), who recently showed that an inhaled metal could be delivered directly to the brain via the olfactory nerve. These data indicate that following inhalation of manganese, manganese concentration achieved in the olfactory bulb is significantly higher than those observed in either the striatum or cerebellum, lending additional support to the direct olfactory transport theory. In the rat,

the olfactory bulb comprises a relatively large section of the central nervous system, and the nasal olfactory mucosa cover roughly 50% of the total nasal epithelium (Gross et al., 1982). In human, these structures are proportionately smaller than in rats. It has been suggested that if this route of brain delivery is operative in humans, then it might be less important in humans than in rats (Dorman et al., 2001). Nevertheless, our results show that Mn concentration in the olfactory bulb is at least 50 % higher than all other brain tissues when rats are exposed to 3000 $\mu g/m^3$. Moreover, after such exposure, tissue concentration for the olfactory bulb increased by 504%, in comparison to 255%, 191%, 169% and 148% for the globus pallidus, the caudate putamen, the frontal cortex and the cerebellum respectively.

The analysis of the biochemical parameters revealed some significant differences in sodium, glucose, chlorate, and urea in blood of rats exposed to highest level of the Mn mixture (3000 $\mu g/m^3$), leading to levels that can result in kidney failure (Murry et al., 1995). In our study, rats exposed to 300 and 3000 $\mu g/m^3$ also had higher levels of alkaline phosphate compared to the control group, which is suggestive of liver dysfunction (Murry et al., 1995). However, modification of AST, ALT, and total bilirubin, which are indicative of liver malfunction, was not apparent in this study.

Rats exposed to 300 and 3000 $\mu g\ Mn/m^3$ had exhibited a significant decrease in mean body weight compared to the control group. This finding is quite different from other results reported by our research group for metallic Mn (St-Pierre et al., 2001) and manganese phosphate (Normandin et al., 2002). However, other studies have reported

decreasing body weight following Mn exposure (Brenneman et al., 1999). There were no significant differences among groups in total mean food consumption.

Our data indicate a significant effect of Mn on locomotor activity. We observed that exposure led to a significant increase in distance traveled for the groups exposed to 30 and 3000 $\mu g/m^3$ as compared to the control group. However, there were no significant differences in resting time. The increase in locomotor activity appeared to be associated with increased Mn concentration in the brain. Similarly, in a study where rats were exposed to 0.1 or 5.0 mg/ml of $MnCl_2$ in drinking water for 8 months, a significant increase in spontaneous motor activity was noted in the first month. Nevertheless, during months 2 to 5 rats showed normal activity, with a significant decrease in motricity in months 6 to 8 (Bonilla, 1984). Rats exposed to Mn orally were significantly more active than controls in the empty open field, and also did not show habituation (Calabresi et al., 2001).

Brain Mn deposition is region-selective, being predominant in the basal ganglia, which is involved in the control of movement and of some cognitive functions. Other than the olfactory bulb, brain Mn deposition is mainly in the caudate putamen and globus pallidus, which corresponds to the onset of neurological outcomes. The differences in spontaneous motor activity can be explained by the alterations in dopamine transmission in Mn toxicity. Acute exposure to Mn is associated with an increase in dopamine neurotransmission, which is also manifested as hyperactivity. Nevertheless, long-term

exposure results in a loss of dopamine in the brain, and the neuronal cell damage could be expressed as an increase in motor activity (Bonilla, 1984; Nachtman et al., 1986).

Does chronic exposure to the combustion products of the organomanganese compound MMT used in gasoline may lead to toxic effects? The results of the present study represent another step towards understanding the contribution of these emissions to nervous system deficits.

2.6 Acknowledgments

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Table 2-1: Particles size distribution of Mn phosphate/sulfate mixture in the inhalation chamber

Particle Size (μm)	Percentage (%)
6.0 - 9.8	6.6 ± 2.7
3.5 - 6.0	6.2 ± 0.9
1.55 – 3.5	7.1 ± 0.1
0.93 – 1.55	12.7 ± 2.8
0.52 – 0.93	40.8 ± 5.1
< 0.52	17.3 ± 1.8
B-F[a]	9.3 ± 6.8

[a]B-F: backup filter

Table 2-2: Mean manganese concentrations (μg/g ± SD) in different tissues and blood following subchronic (90 days) inhalation exposure to a manganese phosphate/sulfate mixture

Tissue	Exposure Concentration			
	Control (n = 26)	30 μg/m^3 (n = 25)	300 μg/m^3 (n = 29)	3000 μg/m^3 (n = 29)
Blood	0.005±0.007	0.006±0.004	0.008±0.002	0.018±0.006[abc]
Kidney	1.01±0.13	1.03±0.20	1.06±0.12	1.37±0.15[abc]
Liver	2.22±0.35	2.23±0.35	2.27±0.38	2.32±0.42
Lung	0.17±0.03	0.42±0.08[a]	2.15±0.5[ab]	5.97±1.72[abc]
Testis	0.30±0.06	0.34±0.02	0.36±0.03[a]	0.42±0.02[abc]

[a] Significantly different from the control group ($p<0.05$)
[b] Significantly different from the 30 μg/m^3 group ($p<0.05$)
[c] Significantly different from the 300 μg/m^3 group ($p<0.05$)

Table 2-3: Mean manganese concentrations (μg/g ± SD) in brain regions after subchronic (90 days) inhalation exposure to manganese phosphate/sulfate mixture

Brain region	Exposure Concentration			
	Control (n = 26)	30 μg/m^3 (n = 25)	300 μg/m^3 (n =29)	3000 μg/m^3 (n = 29)
Cerebellum	0.56±0.15	0.57±0.04	0.60±0.04	0.83±0.07[abc]
Frontal Cortex	0.58±0.20	0.59±0.08	0.64±0.14	0.98±0.32[abc]
Globus Pallidus	0.61±0.15	0.63±0.04	0.81±0.08[ab]	1.56±0.40[abc]
Caudate Putamen	0.62 ±0.2	0.57±0.03	0.74±0.11[b]	1.19±0.11[abc]
Olfactory Bulb	0.46±0.07	0.61±0.05[a]	1.32±0.16[ab]	2.32±0.25[abc]

[a] Significantly different from the control group ($p<0.05$)
[b] Significantly different from the 30 μg/m^3 group ($p<0.05$)
[c] Significantly different from the 300 μg/m^3 group ($p<0.05$)

Table 2-4: Mean distance traveled and resting time over 36 hour period after subchronic (90 days) inhalation exposure to manganese phosphate/sulfate mixture

Locomotor activity	Exposure concentration			
	Control	30 $\mu g/m^3$	300 $\mu g/m^3$	3000 $\mu g/m^3$
	(n = 26)	(n = 25)	(n = 29)	(n = 29)
Distance traveled (m)	875±180	1206±94[a]	928±127	1398±104[ab]
Resting time (hr)	31.9±9	31.7±5	31.9±8	32.4±6

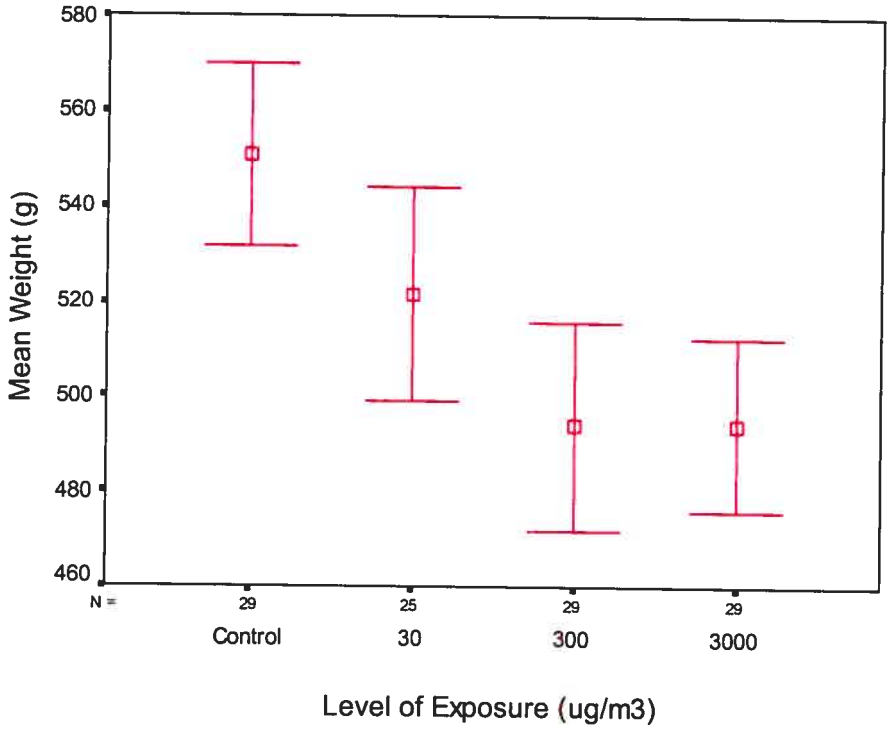
[a] Significantly different from the control group ($p<0.05$)
[b] Significantly different from the 300 $\mu g/m^3$ group ($p<0.05$)

Table 2-5: Biochemical parameters in serum following subchronic (90 days) inhalation exposure to a manganese phosphate/sulfate mixture

Biochemical Parameter	Exposure Concentration			
	Control (n = 26)	30 μg/m^3 (n = 25)	300 μg/m^3 (n = 29)	3000 μg/m^3 (n = 29)
Glucose (mmol/L)	11.6±2.3	9.8±0.97[a]	10.5±1.7	9.8±1.7[a]
Creatinine (μmol/L)	54.2±4.5	54.3±2.1	52.1±3	53±2.9
Sodium (mmol/L)	139.3±1.8	144.8±1.6[a]	143.1±3.1[a]	142.5±2.2[a]
Potassium (mmol/L)	5.1±0.4	4.9±1.1	5.3±1.1	5.8±0.9
Chlorate (mmol/L)	100.9±2.1	104.6±1.7[a]	104.1±1.8[a]	103.2±1.4[a]
Bilirubin (μmol/L)	3.5±0.9	4.0±0.6	3.9±0.7	3.7±1.2
Urea (mmol/L)	5.6±0.7	6.3±0.6[a]	5.7±0.6[b]	6.4±0.6[ac]
AST (U/L)[1]	77.8±21.2	88.4±18.5	70.5±17.3	72.9±17.2
ALT (U/L)[2]	43.3±8.2	53.8±15.5	48.6±7.3	51.8±15.5
Alkaline Phosphatase (U/L)	113.6±34.7	140.9±42	180.1±49.4[a]	183.8±51.3[a]

[1]Asparate aminotransferase.
[2]Alanine aminotransferase.
[a] Significantly different from the control group ($p<0.05$)
[b] Significantly different from the 30 μg/m^3 group ($p<0.05$)
[c] Significantly different from the 300 μg/m^3 group ($p<0.05$)

Figure 2-1: Mean body weight of rats following subchronic (90 days) inhalation exposure to a manganese phosphate/sulfate mixture



3 CHAPTER III: Neurotoxicity and Neurobehavioral Effects of Manganese Phosphate/Sulfate Mixture on Sprague-Dawley Rats

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Neurotoxicity and Neurobehavioral Effects of Manganese Phosphate/Sulfate Mixture on Sprague-Dawley Rats

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3.1 Abstract

Methylcyclopentadienyl manganese tricarbonyl (MMT) replaced tetraethyl lead in gasoline as an antiknock agent in 1976. The combustion of MMT leads to increased manganese concentrations in the atmosphere, and represents one of the main sources of human exposure to manganese. The nervous system is the major target of the toxicity of manganese and its compounds. The purpose of this study was to investigate the relative neuropathology and neurotoxicity of a manganese phosphate/sulfate mixture in Sprague-Dawley male rats following subchronic (90 days) inhalation exposure. Three groups of 30 rats were exposed to 30, 300 and 3000 $\mu g/m^3$ of the manganese phosphate/sulfate mixture in inhalation chambers for a period of 13 weeks, 5 days per week, 6 hours a day, along with an unexposed control group of the same size. In each group, half of the rats had EMG electrodes implanted in the gastrocnemius muscle of the hind lamb to measure tremor. At the end of exposure period, rats were sacrificed by exsanguinations and Mn concentrations in different tissues (liver, lung, testis, and kidney), blood and brain (caudate putamen, globus pallidus, olfactory bulb, frontal cortex, and cerebellum) were determined by neutron activation analysis. Neuropathology was assessed by counting neuronal cells in 2.5 mm^2 grids in the globus pallidus, caudate putamen, and frontal cortex. Neuronal cell loss was significantly different in all three areas of interest in rats in the high dose group (3000 $\mu g/m^3$). Neurobehavioral change was assessed by measuring locomotor activity and tremor among groups. Although there was no indication of tremor in any of the exposed groups, locomotor activity was significantly increased at 3000 $\mu g/m^3$. These results demonstrate that high levels of exposure to the manganese

phosphate/sulfate mixture results in neuropathological changes in areas of the brain that can lead to neurobehavioral effects. .

Keywords: Manganese phosphate/sulfate mixture, inhalation exposure, neuropathology, neurobehavioral effects

3.2 Introduction

Manganese (Mn) is a necessary element for many biological functions such as enzyme activation, and is a component of metalloenzymes. Mn deficiency in humans is characterized by skeletal abnormalities and seizure while excessive exposure has been associated with motor and psychological disturbances similar to Parkinson's disease, resulting in a condition called manganism (Pal et al., 1999). Manganism occurs mainly as a result of chronic inhalation of high levels of Mn (Mena et al., 1967). Clinical symptoms include the presence of generalized bradykinesia, rigidity, tremor and dystonia (Verity et al., 1999). Manganism is caused by an increase of Mn in the globus pallidus, caudate putamen, and substantia nigra regions of the brain. Less severe neurological symptoms occurring as a result of chronic inhalation exposure to Mn include deficits in short-term memory capacity, hand steadiness, reaction time and hand-eye coordination (Mergler et al., 1994).

Occupational studies have revealed neurological symptoms following exposure to airborne Mn at concentrations of 0.2 to 5 mg/m^3 for periods exceeding 20 years (Crump and Rousseau, 1999, Gorell et al., 1999). Alteration of motor effects following Mn exposure has been attributed to abnormal basal ganglia function (Huang et al., 1998). Neuropathological features of these symptoms include neuronal loss and polymicrocaviation in the basal ganglia (Jog et al., 1995). Although the mechanism of Mn neurotoxicity is not entirely clear, it has been suggested that its toxicity is associated with oxidative stress, which may play an important role in neuronal degeneration (Hamai

et al., 2004). Another hypothesis is that Mn may affect dopamine metabolism in the brain (Montes et al., 2001).

Although chronic occupational exposure to Mn at relatively high levels has been shown to cause manganism, little is known about the potential adverse effects of long-term lower-level exposures. The introduction of the anti-knock agent methylcyclopentadienyl manganese tricarbonyl (MMT) in gasoline leads to increased Mn concentration in the atmosphere. There is some evidence to suggest that inhalable Mn (<5 μm in diameter) is present at higher ambient concentrations in areas with higher traffic density (Loranger and Zayed, 1997, Bolté et al., 2004). The presence of different Mn species resulting from the combustion of MMT has raised the concerns about Mn neurotoxicity, particularly in children, the elderly (Weiss and Rehul, 1994) and in people with chronic liver disease (Malecki et al., 1999).

The purpose of this study is to assess neuropathology and neurotoxicity of Mn phosphate/sulfate mixture one of the combustion products of MMT in ambient air, following subchronic inhalation exposure in rats. Neurotoxicity and neuropathology effects were investigated by assessing locomotor activity, tremor, and counting neuronal cells in critical brain areas.

3.3 Materials and Methods

3.3.1 Chemicals

The manganese phosphate/sulfate mixture is an amalgamation of $Mn_5(PO_4)_2(PO_3(OH))_24H_2O$ (hureaulite mineral form) and manganese (II) sulfate monohydrate ($MnSO_4.\ H_2O$) obtained from Alfa Aesar (Johnson Matthey Company), and combined as 50 wt/50 wt. The chemistry of the mixture was described in a previous publication (Beaupré et al., 2004). Interestingly, Mn sulfate is relatively water soluble, and Mn phosphate is insoluble. All chemicals used in surgery, including anesthetic mixtures such as hypnorm-midazolan, buprenorphine (analgesic), and baytril (antibacterial solution), were obtained from the veterinary facility of the Institute of Animal Care and Use at the University of Montreal.

3.3.2 Animals

All experiments were approved by the animal ethics committee of the University of Montreal, and were carried out in accordance with the guidelines defined by the Canadian Council on Animal Care. Six-week old male Sprague-Dawley rats (n=120) were purchased from Charles River Laboratories (St.Constant, Quebec, Canada) and marked on the tail for identification purposes. Rats were acclimated for approximately 2 weeks in HEPA filtered air. Animals were given NIH-07 chow *ad libitum* when they were not exposed to Mn. Rats were individually housed in polycarbonate cages with stainless steel wire lids under constant conditions of temperature and humidity, with 12:12 hr day/night cycles. The animals were randomly divided into 4 groups with 30 animals in each group.

In each group, half of the rats were implanted with EMG electrodes. Body weight and food and water consumption were measured weekly.

3.3.3 Inhalation Exposure

Three groups of 30 male Sprague-Dawley rats each were exposed to 3000, 300 and 30 $\mu g/m^3$ manganese phosphate/sulfate mixture, respectively, along with an unexposed control group of the same size. Exposure occurred in two inhalation chambers, each with a total volume of 1 m^3 (Hazelton Systems Company, Inc., Kalamazoo, MI), for 13 consecutive weeks, 5 days per week, 6 hr/day. Further details are given in Salehi et al. (2003).

3.3.4 Electrode Implant Procedures

Half of the animals in each group were surgically implanted with chronic EMG electrodes in the gastrocnemius muscle in the left hind limb after 10 weeks exposure. Rats were anesthetized with a mixture of hypnorm-midazlan (0.27 ml/100g). A large area of the back of the neck and left hind limb were then shaved and sterilized with alcohol, and an electrode comprised of 3 fluorocarbon-insulated stainless steel wires attached to 3 strip header of a fiberglass mesh base implanted in that area (Gardiner et al., 1986). An approximately 3 cm cutaneous cut was made beside the dorsal midlane in the cervicothoracic section, and the fiberglass mesh base of electrode was located under the skin in the cervicothoracic region. On the left leg, the gastrocnemius muscle was opened and two of the three electrodes were inserted into the muscle. To prevent wire movement,

the ends of the wires were capped with quick-drying epoxy. The third electrode was left free under the skin to serve as a ground. At termination, opened skins were sutured and stapled with an auto clip. The analgesic (0.05 mg/kg) and antibacterial solution were administered subcutaneously immediately after surgery and 24 hours later.

3.3.5 Oxotremorine Administration

Oxotremorine, a Parkinson mimetic agent, was administrated in rats to induce gastrocnemius muscle tremor. Specifically, a 0.1 mg/kg dose of oxotremorine was injected intraperitonealy into the two implanted EMG electrodes rats. Tremor was observed five minutes after injection, and rapid muscle EMG activity was recorded.

3.3.6 Tremor Assessment

An assessment of tremor was conducted one hour after the last exposure by recording muscle EMG activity for approximately 5 minutes while the animals were resting in their cages, walking on the floor, and standing on a vertical 39 cm metal screen grid. A header connector was attached to each rat's electrode in order to transmit the EMG signals to an EMG amplifier (Honeywell, Denver, CO). An oscilloscope (Hewlett Packard, model 1741A, Palo Alto, CA) was used to observe the amplified signals, which were recorded on a stereo video cassette (A.R. Vetter, model SLV-585HF, Rebersburg, PA). The recorded signals were converted to a power spectrum by using Fourier transform program.

3.3.7 Neuropathological Assessment

The frozen left hemisphere of the brain was sectioned in a cryostat and adjacent 20 μm sections, 2.4-3.4 mm lateral according to the rat brain atlas of Paxinos and Watson (1982), were mounted on gelatinized slides. Slides were fixed in a solution of 0.4 M sodium cacodylate, 50% glutaraldehyde for one hour at 4°C. The slides were then placed in a buffer solution of one part 0.4 M sodium cacodylate and three parts water overnight at 4°C. Slides were then stained with Cresyl violet, dehydrated serially in 70, 95, and 100% ethanol, cleared in Xylen, and coverglassed with Permount. Slides were evaluated by light microscopy at a magnification of 40 x 10. In all cases, sections were chosen to ensure neuronal cell counts were carried out at the same brain level. Neuronal cell counts were obtained in the globus pallidus, frontal cortex, and caudate putamen in 2.5 mm^2 grids.

3.3.8 Statistical Analysis

Pairwise comparisons among the four experimental groups were performed using the Dunnet T3 test. Homogeneity of variances among the treatment groups was assessed using Levene's test. All analyses were performed using SPSS statistical software (version 11.5). The level of statistical significance was set at $P = 0.05$ for the set of 6 pairwise comparisons performed among the four treatment groups. Data are presented as group means ± standard deviation (SD).

3.4 Results

3.4.1 Mn Concentrations in the Inhalation Chamber

Mean Mn concentrations were observed to be 34.8 ± 9.2, 290.8 ± 76.8, and 2841 ± 529 $\mu g/m^3$ for the target concentrations of 30, 300, and 3000 $\mu g/m^3$, respectively. About 80% of the Mn phosphate/sulfate mixture particles in the inhalation chamber were smaller than 1.55 μm in aerodynamic diameter. The daily chamber temperatures ranged from 22 - 25 °C, and relative humidity ranged from 25 - 40 %.

3.4.2 Neuropathological Assessment

Neuropathological assessment was based on neuronal cell counts in the brain areas of interest, including the globus pallidus, frontal cortex, and caudate putamen. Neuronal cell counts were significantly decreased in the globus pallidus (51.23 ± 5.50) and caudate putamen (78.92 ± 5.07) in rats exposed to 3,000 $\mu g/m^3$, in comparison with unexposed controls (72.55 ± 3.37 and 93.90 ± 2.46 respectively) (Figure 3.1).

3.4.3 Tremor Assessment

Tremor was observed in rats within 5 minutes after injection of oxotremorine. Figure 3.2 shows the frequency of the EMG signals for observed tremor. Figure 3.3 shows a typical EMG signal for an exposed animal. The mean percentage of the maximum power of the EMG signal was calculated for visible tremor at frequencies between 0 to 50 Hz (Table 3. 1). More than 82 % of maximum power of the EMG signal was concentrated in the range

0 to 20 Hz. The mean percentage of maximum power of the EMG signal also was calculated when the test animals were resting in the cage, walking on the floor, and standing on a vertical grid. The maximum power of the EMG signal derived from oxotremorine injected rats did not correspond with that observed in rats treated with the Mn phosphate/sulfate mixture. The analysis data of EMG signal are presented in the tables 3.2, 3.3, and 3.4. The analysis of tremor signal caused by oxotremorine indicated that 29% of the maximum power of the EMG signal was observed at frequencies between 0 to 10 Hz, with 53% of the maximum power between 10 to 20 Hz. We did not observe a similar percentage of maximum power between 0 to 20 Hz in Mn treated rats.

3.5 Discussion

The extensive use of MMT in gasoline resulting in Mn emissions to the atmosphere represents a significant source of human exposure to inorganic Mn in urban areas with high traffic density (Zayed et al., 2001). Exposure to high concentrations of Mn for long periods of time can lead to a classic syndrome known as manganism. Manganism is characterized by a slow deterioration of neurological function that begins insidiously as non-specific symptoms and subclinical neurological signs. Dysfunction of the basal ganglia develops, characterized by alterations of gait, loss of balance, fine tremor, loss of facial expressions, and speech disturbances. Finally, a severe disabling Parkinson's-like clinical condition develops, characterized by muscle rigidity, a staggering fait, dysphasia, and a course intention tremor (Rodier, 1955). Occupational studies revealed neurobehavioral effects following chronic inhalation exposure to relatively low levels of Mn, including impaired performance of tasks that require coordinated, sequential, and alternating movements at a rapid speed (Mergler et al., 1994, Lucchini et al., 1999).

Few studies have assessed the neurotoxic effects associated with inhalation exposure to low level of Mn, particularly Mn species related to MMT combustion. Our neuropathological assessment revealed a significant decrease in the number of neuronal cells in the globus pallidus and caudate putamen at the highest level of exposure (3000 $\mu g/m^3$). This finding differs from previous reported by our research group for Mn phosphate (Normandin et al., 2002). This difference may be explained by the higher Mn brain concentrations following exposure to the Mn phosphate/sulfate mixture. In particular, Mn concentrations in the caudate putamen (1.19 ± 0.11) and globus pallidus (1.56 ± 0.40)

were significantly higher for the Mn phosphate/sulfate mixture (Salehi et al., 2003) in comparison with data obtained with Mn phosphate (Normandin et al., 2002) in the caudate putamen (1.06 ± 0.14) and globus pallidus (1.25 ± 0.23) and 0.86 ± 0.04 and 0.93 ± 0.06 respectively for metallic Mn (St-Pierre et al., 2001). Dorman et al. (2001) noted that delivery of inhaled Mn to the brain is influenced by particle solubility. Exposure to the soluble form of Mn compound may result in elevated brain concentrations, as compared to the insoluble form (Dorman et al., 2001).

Our observation of higher Mn concentrations in globus pallidus and caudate putamen and reduced neuronal cell counts in these areas of the brain are consistent with the loss of brain cells in patients with manganism (Nagatomo et al., 1999). The application of magnetic resonance imaging (MRI) techniques to patients with manganism demonstrated elevated Mn concentrations in the basal ganglia, particularly in the globus pallidus (Nagatomo et al., 1999). Severely affected patients also develop progressive irreversible loss of dopaminergic neurons in the globus pallidus and nigrostriatal pathway (Calne et al., 1994; Olanow, 2004).

Although the mechanism of Mn-induced neurological effects been studied for decades, it is still not fully understood. Several hypotheses have been advanced to explain the relation between Mn exposure and the loss of neuronal cells. The main portals of Mn absorption in plasma are the gastrointestinal tract and the lung (Aschner et al., 1991). Absorbed Mn exists as divalent and trivalent Mn in plasma (Scheumamer and Cherian 1985). Both forms may distribute to the central nervous system via the blood-brain and the blood-cerebrospinal fluid barriers (Takeda, 2003). Mn may also circumvent the blood-

brain barrier, and pass directly into the central nervous system via olfactory pathways (Tjälve et al., 1996, Frumkin and Solomon, 1997). Divalent Mn can be transported into brain capillary endothelial calls and choroidal epithelial cells via undefined transporters. It also appears that a large portion of divalent Mn exists in non-protein-bound as a free ion (May et al., 1997). This free ion can be transported into the brain via calcium channels (Narita et al., 1990) or Na and Ca exchanger (Frame et al., 1991). Trivalent Mn is able to bound to transferin and be transported across the brain barrier via the receptor-mediated endocytosis (Takeda et al., 2003). When Mn enters in the brain, it can bind to Mn metalloproteins such as glutamine, which are synthesized in astrocytes.

Several studies have documented that increased Mn concentrations in the brain may alter the level and metabolism of neurotransmitters such as dopamine, γ-aminobutyric acid, and glutamate (Erikson et al., 2003). Glutamate is the most common excitatory neurotransmitter, and γ-aminobutyric acid is the most abundant inhibitory neurotransmitter. These neurotransmitters are responsible for the control of motor behaviors (Carlsson, 1990). Astrocytes may play a key role in Mn-induced neurological effects in the brain (Hazell, 2002). Hazell (2002) suggested that the involvement of energy metabolism of mitochondria in astrocytes could cause the Mn-induced neuropathology. Other studies have suggested that glutamate-mediated excitotoxicty may be responsible for the neurological effects of Mn in the brain (Brouillet et al., 1993). Hazell and Norenberg (1997) have studied the oxidant capacity of Mn in reduction of glutamate uptake. In a further study, they found that increased Mn concentrations in the basal ganglia could lead to oxidative damage in astrocytes, with a concomitant reduction

of glutamate uptake (Hazell and Norenberg, 1998). Mn accumulation in the brain may therefore cause major changes in astrocytes, including: reduction of glutamate uptake, reduction in mitochondrial energy metabolism in astrocytes, increased excitoxicity, and proliferation of astrocytes. These effects may play a key role in impaired astrocytic-neuronal interaction, which resulting in neuronal cell death (Hazell, 2002).

Our previous assessment of locomotor activity showed an increase in motor activity for rats exposed to higher concentration of Mn phosphate/sulfate mixture (Salehi et al., 2003). The differences in spontaneous motor activity may be explained by the alterations in dopamine transmission associated with Mn toxicity. Acute exposure to Mn is associated with an increase in dopamine neurotransmission, which is manifested as hyperactivity. Nevertheless, long-term exposure to Mn results in a loss of dopamine in the brain, and the concomitant neuronal cell damage could be expressed as an increase in motor activity (Nachtman et al., 1986; Bonilla, 1984). Therefore, neuronal loss can be related to neurobehavioral changes, including motor function, and alterations in complex cognitive functions (Vettori et al., 1999).

The neurophatological changes following exposure to the Mn phosphate/sulfate mixture is inconsistent with previous results for Mn phosphate reported by our research group (Normandin et al., 2002). This difference can be explained by the hypothesis that astrocytic neurotoxicity following Mn exposure is related to the particular Mn species involved and their solubility (Erikson et al., 2003).

Tremor was assessed by electromyography (EMG) examination for 5 minutes on the last day of exposure by recording muscle EMG activity. At the beginning of the study, two implanted EMG electrodes rats were injected with oxotremorine and muscle EMG activity measured. Oxotremorine is an active metabolite of a cholinergic drug, which leads to tremor in rats with maximum frequency of 8-9 Hz (Slater and Dickinson, 1982). Tremor as result of oxotremorine injection appears in the form of a tremor involving the head and all limbs, similar to resting tremor in Parkinson's disease (Wilms et al., 1999). The maximum power of the EMG signal for oxotremorine injected rats was observed at 13.8 Hz, a finding close to that reported previously by other investigators in our research group (Normandin et al., 2002). This finding is also similar to the maximum power of the tremor signal caused by oxotremorin in rats observed at frequencies between 5 and 12 Hz (Nickel et al., 1997).

The analysis of tremor signal caused by oxotremorine indicated that 29% of the maximum power of the EMG signal was observed at frequencies between 0 to 10 Hz, with 53% of the maximum power between 10 to 20 Hz. Consequently, more then 82 % of maximum power of the EMG signals is found at frequencies between 0 to 20 Hz. Our data did not show a similar result of maximum power between 0 to 20 Hz in Mn treated rats.

In general, tremor caused by Mn is less common at lower levels of exposure and, when present, it tends to be postural or kinetic rather than resting as seen in Parkinson disease (Huang et al., 1993). Resting tremor in Parkinson disease is characterized with

degeneration of dopamine neurons in the substantial nigra (Hughes et al., 1992). In contrast, Mn-induced tremor is described by damage to the palladium and striatum (Wenning et al., 2000). An occupational study also indicated that workers exposed to more than 27 mg/m^3 of ambient Mn exhibit postural but not resting tremor (Huang et al., 1998). The tremor signal induced by Mn in humans occurs at frequencies less than 10 Hz (Sadek et al., 2003).

To date, few studies have investigated tremor as a result of Mn exposure in animal models; most of these studies have found no evidence of tremor following exposure to Mn (Normandin et al., 2002; Pearce et al., 1995; Olanow et al., 1996). However, adult rhesus monkeys have shown other neurological syndromes following intravenous injection of Mn chloride, including facial grimacing, bradykinesia, and rigidity, but not tremor (Olanow, 2004). The only animal study in which active tremor was observed involved monkeys subjected to 7 intravenous injections of 10 mg/kg of Mn chloride (Newland and Weiss, 1992). In general, the histopathological characteristics in resting tremor from Parkinson's disease and tremor caused by Mn are completely different. Further studies are needed to provide more information on the clinical, pharmacological, and pathological features of Mn tremor and Parkinson tremor.

More research is also needed to clarify the toxicity of inorganic Mn, which is emitted to the atmosphere as consequence of the increased usage of MMT in gasoline. The Mn phosphate/sulfate mixture considered in this paper is one of the main combustion products of MMT, which represents one of the first toxicological studies on this mixture.

Our results raise concerns about the potential for Mn neurotoxicity with increasing exposure due to higher traffic density. Subpopulations such as elderly in the early stages of neurodegenerative disease (Gwiazda et al., 2002) and people with liver disease (e.g., cirrhosis) (Montes et al., 2001) may be particularly susceptible to Mn neurotoxicity. Definition of the dose-response relationship for the manganese phosphate/sulfate mixture will be useful in establishing reference doses and human exposure guidelines for airborne manganese.

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Table 3-1: Mean percentage of the maximum power of EMG signals in oxotremorine injected rats

Frequency (Hz)	Percentage of maximum power of EMG signal
0-10	29.1
10-20	53.6
20-30	4.8
30-40	8.0
40-50	4.4

Table 3-2: Mean percentage of maximum power of EMG signal for rats in the walking position at different levels of Mn exposure

Frequency (Hz)	Percentage of maximum power of EMG signal			
	Control (n=11)	30 $\mu g/m^3$ (n=10)	300 $\mu g/m^3$ (n=12)	3000 $\mu g/m^3$ (n=11)
0-10	11.65 ± 4.81	10.59 ± 3.12	11.81 ± 4.23	12.59 ± 4.11
10-20	8.93 ± 3.77	9.44 ± 3.03	7.98 ± 3.21	11.67 ± 3.55
20-30	15.42 ± 3.59	15.88 ± 3.15	16.02 ± 3.62	17.22 ± 3.19
30-40	26.50 ± 5.11	28.22 ± 4.91	24.98 ± 6.13	28.41 ± 5.93
40-50	37.5 ± 7.98	35.87 ± 7.83	39.21 ± 7.88	30.11 ± 7.54

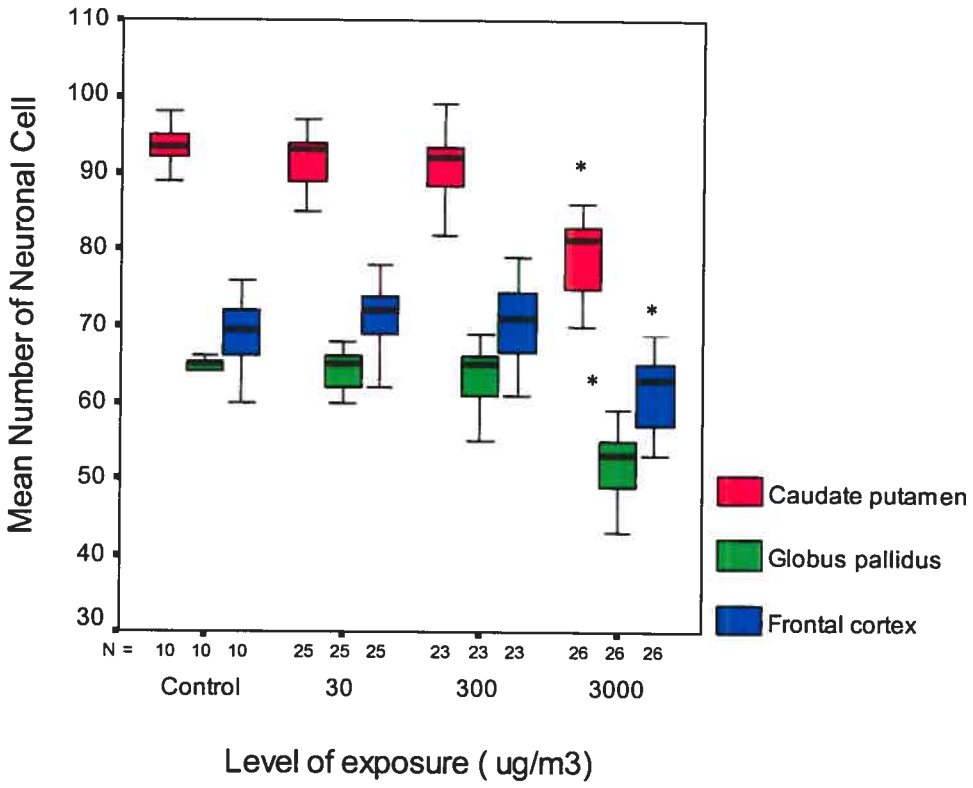
Table 3-3: Mean percentage of maximum power of EMG signal in rats in the resting position at different levels of Mn exposure

Frequency (Hz)	Percentage of maximum power of EMG signal			
	Control (n=11)	30 $\mu g/m^3$ (n=10)	300 $\mu g/m^3$ (n=12)	3000 $\mu g/m^3$ (n=11)
0-10	9.14 ± 4.88	8.26 ± 3.12	9.21 ± 3.43	11.58 ± 5.31
10-20	8.03 ± 3.27	7.44 ± 3.03	7.28 ± 3.51	8.67 ± 3.45
20-30	19.42 ± 8.59	18.98 ± 3.12	19.92 ± 5.62	19.21 ± 3.17
30-40	29.50 ± 5.52	31.22 ± 6.21	34.15 ± 7.13	36.41 ± 8.53
40-50	33.91 ± 7.98	34.1 ± 7.53	29.44 ± 7.68	24.13 ± 7.44

Table 3-4: Mean percentage of maximum power of EMG signal in rats when standing on a vertical grid for different levels of Mn exposure

Frequency (Hz)	Percentage of maximum power of EMG signal			
	Control (n=11)	30 $\mu g/m^3$ (n=10)	300 $\mu g/m^3$ (n=12)	3000 $\mu g/m^3$ (n=11)
0-10	21.65 ± 8.29	14.53 ± 5.18	11.88 ± 5.31	14.59 ± 6.22
10-20	10.94 ± 4.77	8.49 ± 3.53	7.08 ± 3.40	8.70 ± 3.45
20-30	11.52 ± 3.19	12.08 ± 3.90	11.92 ± 4.39	13.82 ± 3.17
30-40	23.04 ± 9.81	25.19 ± 8.91	25.93 ± 7.62	24.91 ± 6.37
40-50	32.85 ± 9.88	39.71 ± 9.87	43.19 ± 9.18	37.98 ± 8.53

Figure 3-1: Mean neuronal cell counts in the globus pallidus, caudate putamen, and frontal cortex of rats after subchronic inhalation exposure of Mn (± SD)



* Significantly different from the control group ($p<0.05$)

Figure 3-2: Tremor signals in oxotremorine injected rats. A shows a pattern of frequency (Hz) of EMG signals per time (ms). B represents rectified signals. C shows the power (volt) of frequency of signals.

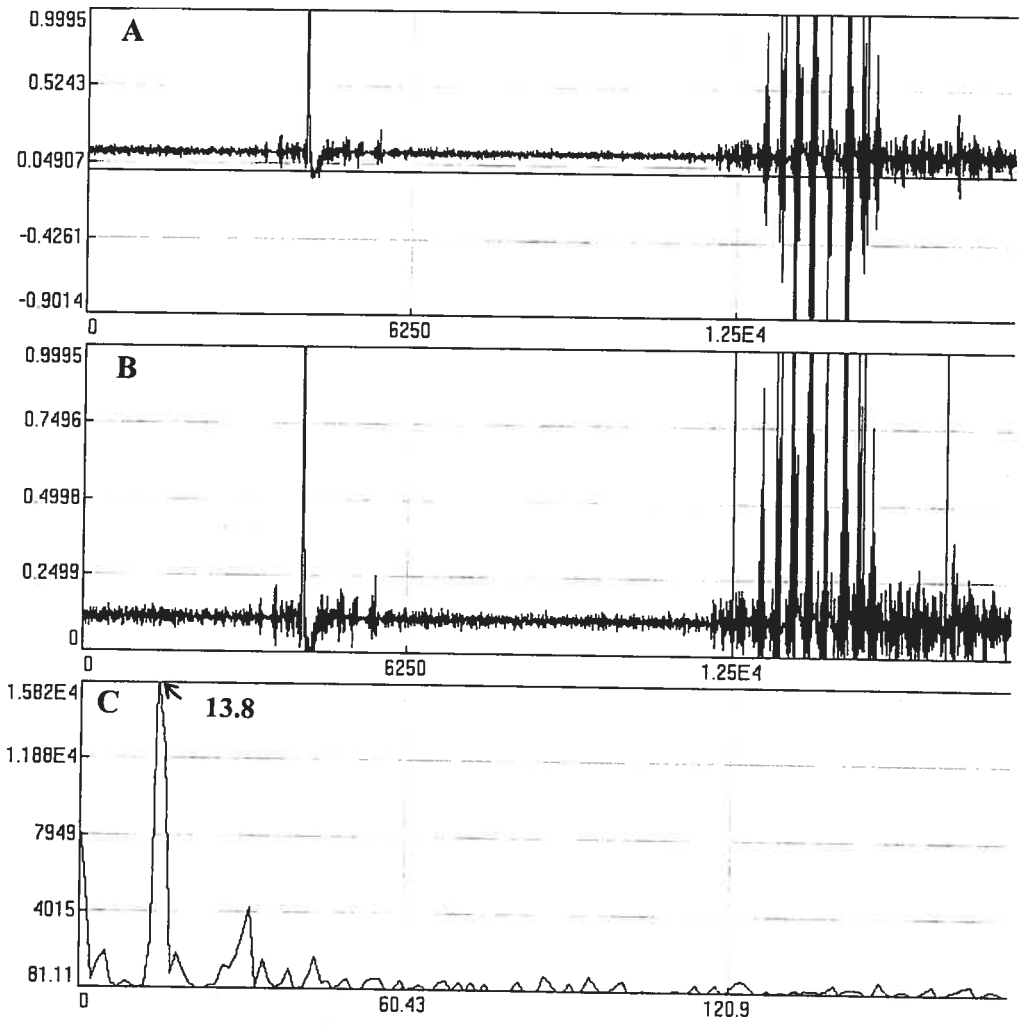
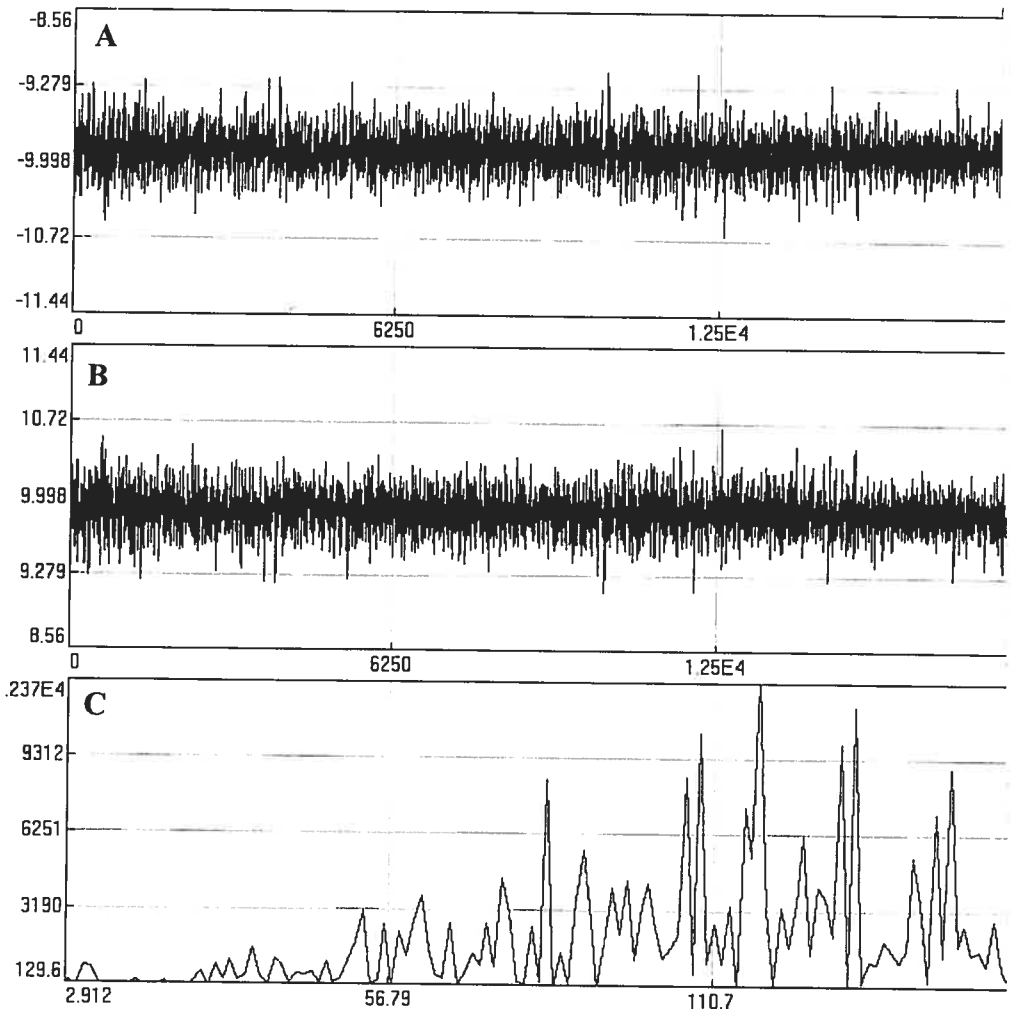


Figure 3-3: Typical EMG signals in rats exposed to Mn. A shows a pattern of frequency (Hz) of EMG signals per time (ms). B represents rectified signals. C shows the power (volt) of frequency of signals.



CHAPTER IV: Discussion

Discussion

The rate of Mn clearance from the lung has been recognized to influence the metal delivery to the brain and other organs. In a study by Rhoads and Sanders (1985), a number of metal compounds that were more quickly cleared from the lungs were retained for longer periods at other sites in the body, where they could potentially exert their toxic effects. In our study the rats that were exposed to a Mn mixture had lower lung manganese concentrations compared with the levels observed in rats exposed to either Mn sulfate (Dorman et al., 2001) or Mn phosphate (Vitarella et al., 2000, Normandin et al., 2002). In addition, blood Mn concentration was significantly increased at the highest level of exposure (3000 $\mu g/m^3$) that was not seen in rats exposed to either Mn phosphate (Normandin et al., 2002) or Mn sulfate (Dorman et al., 2001). These variations of Mn concentrations may likely illustrate that the Mn mixture is more rapidly cleared from the lung and readily absorbed into the blood as compared with other individual Mn compounds.

Groups exposed to 300 and 3000 $\mu g/m^3$ had significantly higher testis Mn concentrations compared to the control group. This finding is similar to the results of Dorman et al. (2001), where animals were exposed to high levels of Mn sulfate. In contrast, testis displayed a different response after exposure to Mn phosphate. Rats exposed to 300 and 3000 $\mu g/m^3$ Mn had exhibited a significant decrease in mean body weight compared to the control group. The analysis of the biochemical parameters revealed some significant differences in sodium, glucose, chlorate, and urea in blood of rats exposed to the highest level of the Mn mixture (3000 $\mu g/m^3$), leading to levels that can result in kidney failure

(Murry et al., 1995). In our study, rats exposed to 300 and 3000 $\mu g/m^3$ also had higher levels of alkaline phosphate compared to the control group, which is an indication of liver dysfunction (Murry et al., 1995). These findings are quite different from the results that have been reported by our research group for metallic Mn (St-Pierre et al., 2001) and Mn phosphate (Normandin et al., 2002). In the other hand, exposure to Mn phosphate/sulfate mixtures can be possibly more associated with systemic and reproductive adverse effects.

Dorman and colleagues have confirmed that delivery of inhaled Mn to the brain is influenced by particle solubility. Moreover, animals exposed to high level of the soluble sulfate form had significantly higher brain Mn concentrations compared with the insoluble tetroxide or phosphate forms (Dorman et al., 2001). Our data indicate a significant difference in brain Mn concentration in globus pallidus, caudate putamen, and olfactory bulb in rats exposed to the highest level (3000 $\mu g/m^3$). Mn concentrations in the globus pallidus and caudate putamen were higher than those reported by Vitarella (2000) following exposure to Mn phosphate (Normandin et al., 2002) and metallic manganese (St-Pierre et al., 2001). Hence, higher brain Mn accumulation can be corresponded to the either higher solubility or more effectiveness of the mixture compared to the individual compounds.

Our data show that the Mn concentrations in the olfactory bulb were at least 50 % higher than other brain regions at the highest level of exposure (3000 $\mu g/m^3$). Mn concentration in the olfactory bulb increased by 504 %, in comparison to 255 %, 191 %, 169 % and 148 % for the globus pallidus, caudate putamen, frontal cortex, and the cerebellum,

respectively. This is in agreement with Dorman et al. (2001), who recently showed that an inhaled metal could be delivered directly to the brain via the olfactory nerve. In the rat, the olfactory bulb represents a relatively large section of the central nervous system, and the nasal olfactory mucosa cover roughly 50% of the total nasal epithelium (Gross et al., 1982). This region of the brain is relatively smaller in human, therefore Mn delivery to the human brain via this region might be less significant (Dorman et al., 2001).

The locomotor activity assessment showed a significant increase in distance traveled for the groups exposed to 30 and 3000 $\mu g/m^3$ compared with the control group. The increased activity in rats exposed to 30 $\mu g/m^3$ did not seem to be accurate, because during the evaluation period there was a construction activity at upper floor that made too much noise and vibration. Therefore, the increased activity may not be accounted as a result of Mn exposure. There were also no significant differences in resting time among rats.

The increase in locomotor activity appears to be associated with increased Mn concentration in the brain. Since brain Mn deposition is region-selective, largely in the caudate putamen and globus pallidus, corresponding to the onset of neurological outcomes. In this study, Mn concentrations in the caudate putamen and globus pallidus were significantly higher for the Mn phosphate/sulfate mixture (Salehi et al., 2003) in comparison with the data obtained with Mn phosphate (Normandin et al., 2002) and the metallic Mn (St-Pierre et al., 2001).

Similarly, the neuropathological assessment revealed a significant decrease in the number of neuronal cells in the globus pallidus and caudate putamen at the highest level of exposure (3000 $\mu g/m^3$). Our results are different from previously reported data by our research group for Mn phosphate (Normandin et al., 2002).

It has been suggested that Mn accumulation in the brain may cause major changes in astrocytes, including reduction of glutamate uptake, reduction in mitochondrial energy metabolism in astrocytes, increased excitoxicity, and proliferation of astrocytes. These effects may play a key role in impaired astrocytic- neuronal interaction, which resulting in neuronal cells death (Hazell, 2002). Consequently, the neuronal cells loss can be associated to the neurobehavioral changes including locomotor activity.

Our observation of higher Mn concentrations in globus pallidus and caudate putamen and reduced neuronal cell counts in these areas of the brain are consistent with the loss of brain cells in patients with manganism (Nagatomo et al., 1999). The neurophatological changes following exposure to the Mn phosphate/sulfate mixture is inconsistent with previous results for Mn phosphate reported by our research group (Normandin et al., 2002). This difference can be explained by the hypothesis that astrocytic neurotoxicity following Mn exposure is related to the particular Mn species involved and their solubility (Erikson et al., 2003).

Tremor as a result of oxotremorine injection appears in the form of a tremor involving the head and all limbs, similar to resting tremor in Parkinson's disease (Wilms et al., 1999).

In this study, the maximum power of the EMG signals following oxotremorine injection was observed at the frequency of 13.8 Hz, which is close to the data reported previously by other investigators in our research group (Normandin et al., 2002). This finding is also similar to the maximum power of the tremor signals caused by oxotremorine in rats, which has been observed at the frequencies between 5 and 12 Hz by Nickel et al. (1997). The analysis of tremor signals caused by oxotremorine indicated that more then 82 % of maximum power of the EMG signals was found at frequencies between 0 to 20 Hz. Our data did not show a similar result of maximum power between 0 to 20 Hz in Mn exposed rats.

The type of tremor caused by Mn tends to be postural or kinetic rather than resting as seen in Parkinson's disease (Huang et al., 1993). Resting tremor in Parkinson's disease is characterized with degeneration of dopamine neurons in the substantial nigra (Hughes et al., 1992). In contrast, Mn-induced tremor is described by damage to the basal ganglia (Wenning et al., 2000). An occupational study also indicated that workers exposed to more than 27 mg/m^3 of ambient Mn exhibited postural but not resting tremor (Huang et al., 1998). The tremor signal induced by Mn in humans occurs at frequencies less than 10 Hz (Sadek et al., 2003).

To date, few studies have investigated tremor as a result of Mn exposure in animal models. Most of these studies have found no evidence of tremor following exposure to Mn (Pearce et al., 1995; Olanow et al., 1996; Normandin et al., 2002). The only animal study in which active tremor was observed involved monkeys subjected to 7 intravenous

injections of 10 mg/kg of Mn chloride (Newland and Weiss, 1992). Further well design animals studies are needed to provide more information on pathological, and clinical features of Mn tremor.

Overall, one of the limitations in this study was the lack of a very constant Mn concentration during exposure period. This inconsistency can be caused by variation in temperature and humidity in the inhalation chamber that in turn has some effects on particles generator. However, Mn concentrations obtained in this study were 34.8 ± 9.2, 290.8 ± 76.8, and 2841 ± 529 $\mu g/m^3$ for target concentrations of 30, 300, and 3000 $\mu g/m^3$, respectively. Another limitation in this study was the use of the young male rats instead of female or old rats. One of the goals of this study was to investigate the neurobiological and neurobehavioral alteration due to Mn exposure, which could be different in relation to the gender and age.

Finally, to extend the results obtained in this study to human and characterize the potential health risks following inhalation exposure to Mn phosphate/sulfate mixture, extrapolation can be made using the traditional approach. According to our data, the lowest observable adverse effect level (increase in locomotor activity) is 30 $\mu g/m^3$. This would correspond to 3 $\mu g/kg\text{-}day$ (30 $\mu g/m^3$ x 0.22 m^3/day x 6 h/24 h /0.55 kg). To calculate the dose, the following assumptions were made: (a) the rats inhaled 0.22 m^3/day of air and (b) the average body weight was 0.55 kg (Calabrese et al., 1991).

The human-equivalent LOAEL ($LOAEL_{HEC}$) would be 10.5 $\mu g/m^3$ (3 $\mu g/kg$-day x 70 kg / 20 m^3/day). For this calculation a breathing rate of 20 m^3/day and the average body weight of 70 kg were assumed. Human reference concentration can be calculated by dividing the $LOAEL_{HEC}$ by uncertainty factors (UF) for subchronic to chronic extrapolation (=10), animal to human extrapolation (=10), and LOAEL to NOAEL extrapolation (=10). Therefore, RfC can be derived as 0.01 $\mu g/m^3$ (RfC = LOAEL/UF; RfC = 10.5 $\mu g/m^3$/1000) that is different from RfC established by U.S. EPA (1996).

However, the U.S. EPA RfC has been established for total Mn without any consideration of Mn species. In fact, the results obtained with Mn phosphate by our research group, conducted with the same animal model (Normandin et al., 2002), showed that the level of 3000 $\mu g/m^3$ should be considered as LOAEL and 1.05 $\mu g/m^3$ can be derived as RfC from exposure to Mn phosphate (3000 $\mu g/m^3$ x 0.22 m^3/day x 6 h/24h / 0.55 kg = 300 $\mu g/kg$-day; 300 $\mu g/kg$-day x 70 kg/ 20 m^3/day = 1050/1000). Thus, the difference between Mn phosphate and Mn phosphate/sulfate mixture shows the essential role of chemical form in term of toxicity assessment.

Additional consideration of the Mn uptake by other routes and sources would be needed to refine the current estimates of RfC. This is particularly relevant because their relative importance might be different between animals and humans. In this context, contribution of food and water to the background level of Mn in the body, dermal exposure as well as ingestion by rats of skin-borne Mn (due to self-cleaning process) might be important. A

comprehensive evaluation of these processes in rats and humans should lead to the refinement of the current estimation of the RfC for Mn.

CHAPTER V: Conclusion

Conclusion

The Mn exposure has been an important issue in the last decades as a result of increased usage of MMT in gasoline. The present study is unique in that it is the first study to investigate the neurotoxic effects of Mn phosphate/sulfate mixture in rats. The results of this study indicate that exposure to this mixture leads to the onset of neuropathological changes in particular areas of the brain and causes some neurobehavioral differences among the rats. These changes are exclusive for this mixture and have not been seen with other Mn species used in our research group. Therefore, the results of this study imply that one should consider all different Mn species for assessing the potential health risk associated with the use of MMT in gasoline.

Our results raise concerns about the potential for Mn neurotoxicity with increasing exposure due to higher traffic density. Subpopulations such as elderly in the early stages of neurodegenerative disease and people with liver disease (e.g., cirrhosis) may be particularly susceptible to Mn neurotoxicity. Further studies are needed to investigate the neurotoxicity effect of Mn phosphate/sulfate mixture in female, young, and old animals to improve our ability to establish a RfC to adequately prevent potential risk of Mn exposure in general population.

CHAPTER VI: BIBLIOGRAPHY

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APPENDIX: Physical and Chemical Characterization of Mn Phosphate/sulfate Mixture Used in a Inhalation Toxicology Study

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Physical and Chemical Characterization of Mn Phosphate/Sulfate Mixture Used in an Inhalation Toxicology Study

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The use of methylcyclopentadienyl manganese tricarbonyl (MMT) in unleaded gasoline has given rise to numerous debates on the potential public health risk associated with manganese emissions. In fact, combustion products are mainly Mn phosphate, Mn sulfate, and Mn phosphate/sulfate mixture. Our research group did several inhalation studies in order to assess the toxicity of each Mn species. The objective of this study is to determine the physical and the chemical characteristics of a mixture of Mn phosphate/sulfate used in one of these inhalation toxicology studies. First, the mixture was analyzed by X-ray diffraction in order to obtain the specific peak of Mn phosphate and Mn sulfate. These peaks were used as reference. Second, samples of the mixture were collected on filters in the inhalation chamber at a concentration level of 3000 $\mu g/m^3$. They were analyzed by scanning electron microscopy (SEM), analytical transmission electron microscopy (ATEM), and x-ray energy-dispersive spectrometry (EDS) to show their size, morphology, and chemical composition. Results indicate that 33% of the particles were found to be agglomerated, while free particles accounted for 44% for Mn phosphate and 23% for Mn sulfate.

The methylcyclopentadienyl manganese tricarbonyl (MMT: $C_9H_7MnO_3$) is an organic form of manganese. MMT is a fuel additive developed in the 1950s to increase the octane level of gasoline and to improve the antiknock properties of the fuel (Lynam et al., 1990). It was introduced in Canada in 1976 and its use has increased since it completely replaced tetraethyl lead in gasoline in the 1990s (Royal Society of Canada, 1986). In 1997, a law (C-29) was adopted by the federal government of Canada, to ban both the interprovincial trade and the importation for commercial purposes of manganese-based substances, including MMT. However, the government reworded this law in July 1998 so that manganese-based fuel additives were not included in the restriction (Zayed et al., 1999). Nevertheless, the existing scientific knowledge related to the use of MMT in gasoline has not addressed the specific question of health risk related to environmental chronic exposure. The magnitude of the risk due to Mn emitted from the combustion of MMT remains uncertain (Zayed, 2001).

Several studies have been done in different occupational environments that have shown that exposure to high atmospheric Mn concentrations may have significant effects on human health (U.S. EPA, 1997). Other studies have shown a relationship between Mn exposure and neurological disorders (Mergler et al., 1994; Roels et al., 1987; Wennberg et al., 1992), such as those similar to Parkinson's disease (Barbeau, 1984; Zayed et al., 1990). However, the extrapolation of these results to chronic exposure at low concentrations of Mn remains difficult. Exposure of the general population is a concern because Mn exposure from MMT would occur over the course of a lifetime, rather than during one's working years (Lyznicki et al., 1999). If adverse effects do occur, it is likely that they will be subtle and difficult to detect (Zayed, 2001; U.S. EPA, 1997). This is why

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inhalation toxicology studies are needed with respect to the different Mn species emitted from automobiles using MMT.

A few years ago, a preliminary car exhaust study (Ardeleanu et al., 1999) provided qualitative data on the elementary composition of particles collected from a tailpipe. It showed that most of the particles consisted of Mn-O with phosphorus (P) and sulfur (S) and, in some cases, also contained other elements such as aluminum (Al), chromium (Cr), or iron (Fe). More recently, the physical and the chemical characteristics of the Mn compounds emitted from vehicles using gasoline-containing MMT were determined (Zayed et al., 1999). Particles emitted from the tailpipe were trapped and characterized by scanning electron microscopy (SEM) and x-ray energy-dispersive spectrometry (EDS) and by analytical transmission electron microscopy (ATEM). Results led to the conclusion that Mn is emitted from the tailpipe primarily as a mixture of Mn phosphate and Mn sulfate with sizes ranging between 0.2 and 10 μm. On average, more than 99% of the particles were in the respirable fraction (<5 μm) and 86% were less than 1 μm in size. These results are in agreement with those of Ressler et al. (2000) obtained by x-ray absorption near-edge structure (XANES) spectroscopy. They showed that the chemical forms of Mn-containing particulates emitted from MMT-added gasoline engines are Mn tetraoxide (Mn_3O_4), Mn sulfate ($MnSO_4 \cdot H_2O$), and a divalent Mn phosphate ($Mn_5(PO_4)_2[PO_3(OH)]_2 \cdot 4H_2O$).

Three subchronic inhalation studies were undertaken by our research group using an animal model in order to assess the toxicity of each of the three major Mn species emitted from MMT combustion, namely, Mn phosphate, Mn sulfate, and Mn phosphate/sulfate mixture (Salehi et al., 2003; Normandin et al., in press; Normandin et al., 2002). One of the critical issues related to this last species is the percentage of Mn phosphate and Mn sulfate obtained in the inhalation chamber. The aim of this study is to verify the proportion of each of them.

METHODS

A 50/50 mixture was obtained by weighting the same quantity of Mn phosphate (hureaulite: $[Mn_5(PO_4)_2(PO_3(OH))_2 \cdot 4H_2O]$) and Mn sulfate monohydrate ($MnSO_4 \cdot H_2O$) and then mixing automatically during 24 h. These two Mn species were obtained from Alfa Aesar (a Johnson Matthey Company, Ward Hill, MA).

One sample of each substance was collected and analyzed by x-ray diffraction in order to obtain the specific peaks of each substance. These peaks were used as reference.

Sampling

The inhalation chamber is a rectangular stainless-steel chamber (131 cm long, 65 cm wide, and 125 cm deep) with a total volume of 1 m^3 (Hazelton Company, Inc., Kalamazoo, MI). Air entering the chamber was filtered through a HEPA filter. The Mn aerosol particles were supplied to the chamber using a fluidized-bed aerosol generator (TSI, Inc., St.-Paul, MN). Before entering the chamber, Mn aerosol particles were delivered through a stainless-steel ASME pressure tank (Mc Master-Carr, NJ) for the settling of larger Mn aerosol particles. The concentration in the chamber was monitored continuously using a Dust Track (TSI, Inc., St.-Paul, MN) aerosol monitor. Five samples of the mixture were also collected in the inhalation chamber at a concentration level of 3000 $\mu g/m^3$ using a Gilian pump (Gilian Corp., Caldwell, NJ) with a standard 3-piece cassette and 37-mm-diameter Teflon filters (SKC, Inc., Eightyfour, PA) with 0.45 μm pore size. The pumps were calibrated at 1.5 L/min with a Gilibrator (Gilian Corp., Caldwell, NJ), and sampling lasted over 6 h/day during 5 consecutive days (one filter per day). To establish the particle size of the particles and to compare the results with the size of the Mn phosphate and the Mn sulfate particles measured in other studies, air samples were collected using a six-stage Marpel personal cascade impactor.

Scanning Electron Microscopy and X-Ray Energy Dispersive Spectrometry

Residual particles on filters were observed and analyzed by scanning electron microscopy (SEM, Jeol 840) using secondary electron images (SEI). The chemical composition of the particles was determined with an x-ray energy-dispersive spectrometry (EDS) detector coupled to the SEM and operated in the windowless mode, allowing the detection of light elements such as carbon (C) and oxygen (O) as well as all the heavier elements.

The working distance used was optimal for x-ray detection (39 mm). The acquisition time used was long enough to allow a clear differentiation between the two kinds of particles that were present on the filters. For a particle to be considered as a phosphate or a sulfate, the sulfur or phosphorus peaks needed to be statistically significant. To achieve this, the Rose criterion was used, which states that the change in signal (P or S peaks) has to exceed the background noise by a factor of 5 (Goldstein et al., 1992). To avoid charging of the sample by the incident electron beam and a possible destruction of the filters during the analysis, a thin layer (~15 nm) of a conductive coating of carbon was deposited onto the surface of the filters.

The particles of size down to about 0.5 μm were analyzed using SEM; the smaller particles could not be analyzed in the SEM because of the lack of resolution and the small number of x-ray counts originating from such small particles.

Analytical Transmission Electron Microscopy

Analytical transmission electron microscopy (ATEM) was used in order to analyze particles smaller than 0.5 μm, which could not be analyzed in the SEM. A Philips 300-kV microscope (model CM30) was used for this purpose. To prepare samples for ATEM, the filters were soaked in methanol and ultrasonic agitation was used to release the particles from the filters. Three millimeter Cu grids covered with a Formvar thin foil and a conductive coating of carbon were used to retrieve the particles from the methanol solution and employed as ATEM samples. The chemical composition was determined using an EDS x-ray system attached to the microscope, similar to the one used in

SEM. Since the resolution of the ATEM is much higher than that of SEM and because the EDS detector is much closer to the sample, it was possible to precisely analyze the very small particles. For the statistic validation of the data, the same criterion was used as for the SEM analysis (Rose criterion).

X-Ray Diffraction

A Philips X'pert spectrometer was used to acquire the x-ray diffraction spectrums. This method (Cullity, 1978) consists of an x-ray source on the left side and an x-ray detector on the right side while the sample is located in the center. The source and the detector are rotating around the sample in a symmetrical way. Since the sample is made of powder, the crystals are randomly oriented, allowing the diffraction of all the atomic planes. When there is diffraction, a larger amount of x-rays is detected, creating a peak on the spectrum at the specific angle where the diffraction occurs. Each peak on the spectrum corresponds to an atomic plane. The position of a peak on the spectrum can be converted to the d spacing of the atomic plane with the Bragg formula:

$$\lambda = 2d \sin \theta$$

where λ is the wavelength of the x-rays, which is known, and θ is the position of the peak on the spectrum, which can be measured. The d spacing of an atomic plane is the distance between the atomic planes of the same type. When the d spacing of the atomic planes is known, they can be identified by their Miller indices. By comparing the results with a database of known compounds, the sample analyzed can be identified.

RESULTS

The size distribution of the Mn phosphate/sulfate mixture found in the inhalation chamber (Table 1) shows that about 68% of the particles are less than 0.93 μm in size while 87% are less than 3.5 μm. These percentages are similar to those obtained with Mn phosphate or Mn sulfate used alone in our two other inhalation toxicology studies.

X-Ray Diffraction

Three powder samples were analyzed. The first one was the Mn sulfate (Figure 1), which shows a specific peak at 25.5° on the 2 theta axis with the highest intensity (100%). As for the Mn phosphate (Figure 2), we observe two specific peaks, one with the highest intensity (100%) at 28° on the 2 theta axis and the other at 29.5° on the 2 theta axis with an intensity of 85%. Figure 3 shows the results of the mixture (Mn phosphate/sulfate mixture) used in our inhalation toxicology studies. We see the specific peak of Mn sulfate (100%) at 25.5° on the 2 theta axis and only one peak of the Mn phosphate at 28° on the 2 theta axis with less intensity (85%) than before (100%). The other peak of Mn phosphate at 29.5° on the 2 theta axis has a very low intensity (near 38%).

SEM/EDS Characterization

Five filters were analyzed for residual particles as collected in the inhalation chamber. Hundreds of particles were analyzed and characterized on the filters. Figure 4 shows an SEM micrograph of one field. EDS analyses (Figures 5 to 12) show that the elements present in all the particles were Mn, P, S, and O. The presence of oxygen suggests that we obtained Mn sulfate and Mn phosphate. Carbon was also detected due to the coating.

Table 2 shows that 266 particles were analyzed. Thirty three percent (33%) of the particles were found to be agglomerated, while free particles totaled 118 for Mn phosphate and 60 for Mn sulfate (see example in Figure 13). An average of 61% and 39% of the manganese-containing particles were related to these Mn chemical forms, respectively.

The spectrum shown in Figure 14 was acquired on a 4 mm^2 region of the filter with an acquisition time of 600 s. It shows the global chemical composition of the filter. In addition to the P, S, Mn, and O peaks, which are specific to the phosphate and the sulfate powder particles; the spectrum also contains peaks of C from the coating and F from the Teflon filter. The P peak is more intense than the S peak. The ratio of the intensity P/S, calculated after background subtraction, is 3.8.

TABLE 1
Size distribution of Mn sulfate, Mn phosphate, and Mn phosphate/sulfate mixture in the inhalation chamber

Particle size (μm)	Mn sulfate (%)	Mn phosphate (%)	Mn phosphate/ sulfate mixture (%)
6.0–9.8	2.8 ± 0.2	3.6 ± 1.0	6.6 ± 2.7
3.5–6.0	3.5 ± 0.9	8.5 ± 2.3	6.2 ± 0.9
1.55–3.5	11.0 ± 2.8	8.3 ± 2.6	7.1 ± 0.1
0.93–1.55	35.2 ± 9.8	13.2 ± 1.9	12.7 ± 2.8
0.52–0.93	23.1 ± 7.9	39.2 ± 4.5	40.8 ± 5.1
<0.52	15.1 ± 4.6	18.4 ± 3.6	17.3 ± 1.8
B-F[a]	9.4 ± 0.6	8.9 ± 7.6	9.3 ± 6.8

[a]B-F, backup filter.

TABLE 2
Number of particles observed in different classes

		Free particles		Agglomerated particles	
Filters	Total analyzed	Mn phosphate	Mn sulfate	Mn phosphate	Mn sulfate
1	52	17	4	16	15
2	54	28	15	6	5
3	53	24	15	7	7
4	56	20	11	13	12
5	51	29	15	3	4
Total	266	118	60	45	43
%		44	23	33	

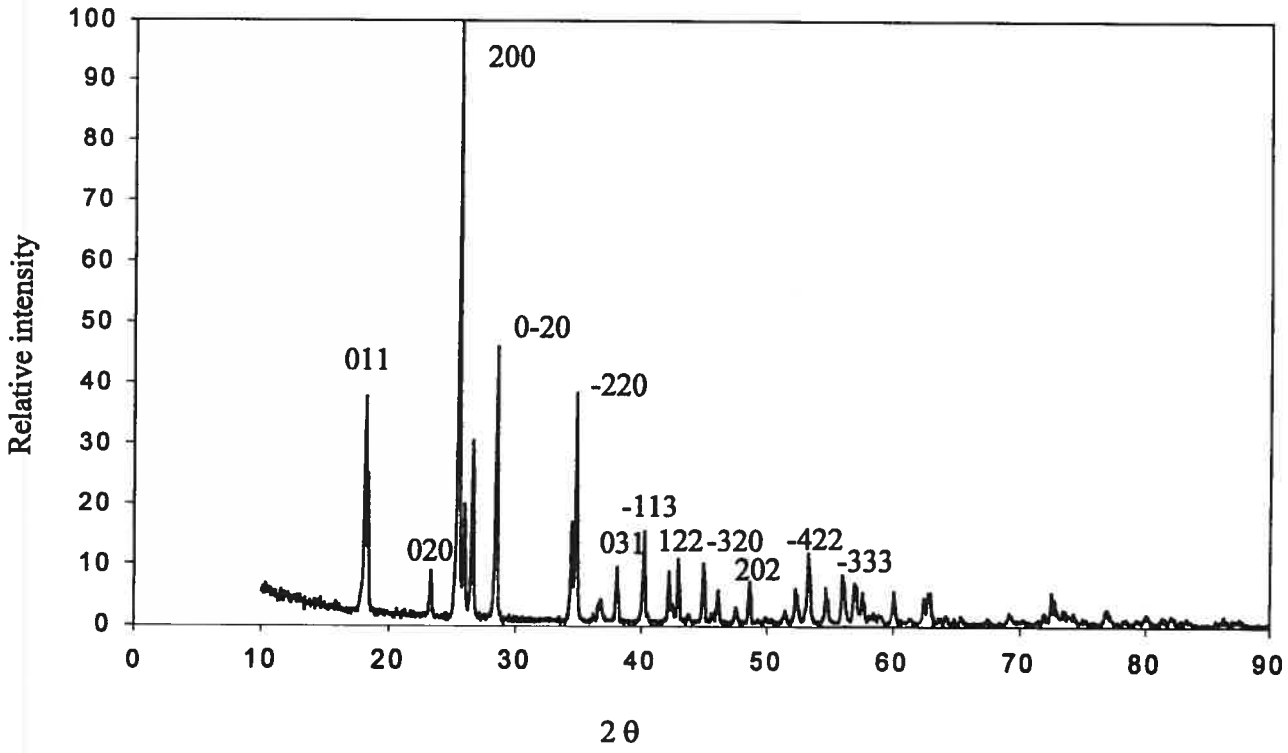

FIG. 1. X-ray diffraction pattern of manganese sulfate powder. The numbers are the Miller indices of the diffracting planes.

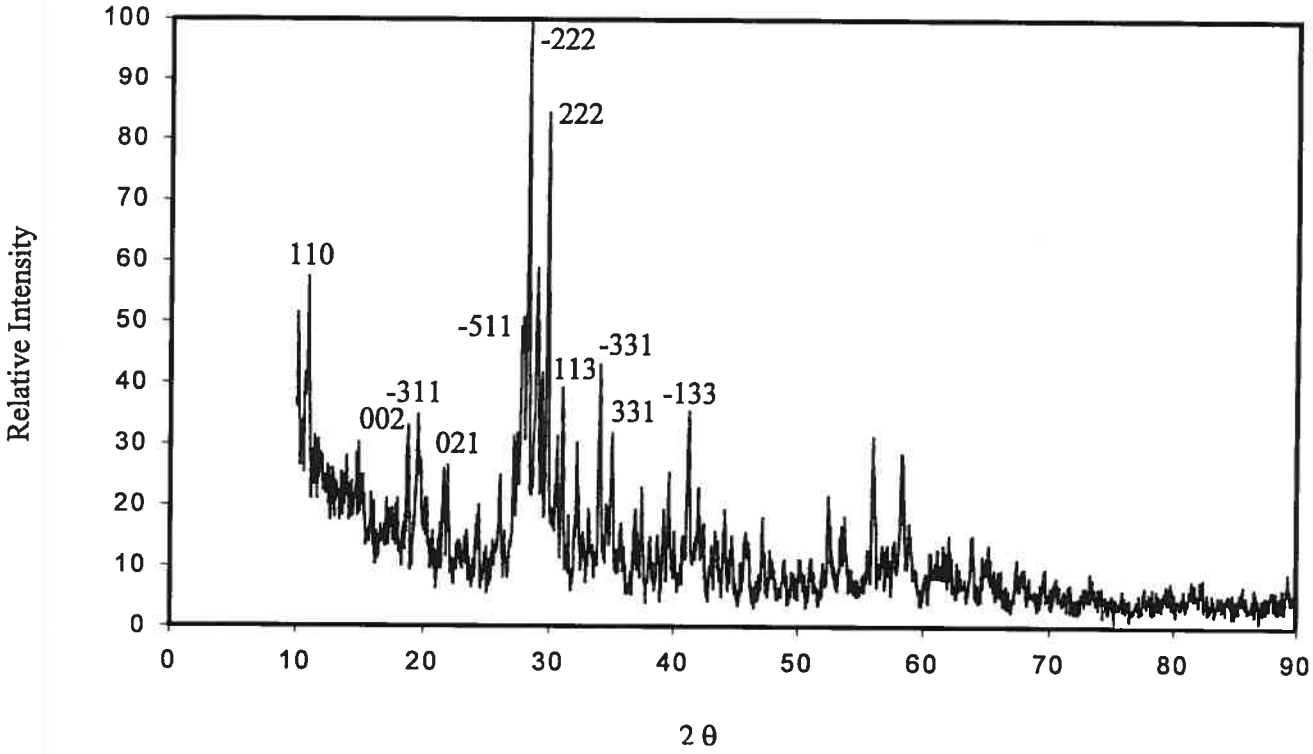

FIG. 2. X-ray diffraction pattern of manganese phosphate powder. The numbers are the Miller indices of the diffracting planes.

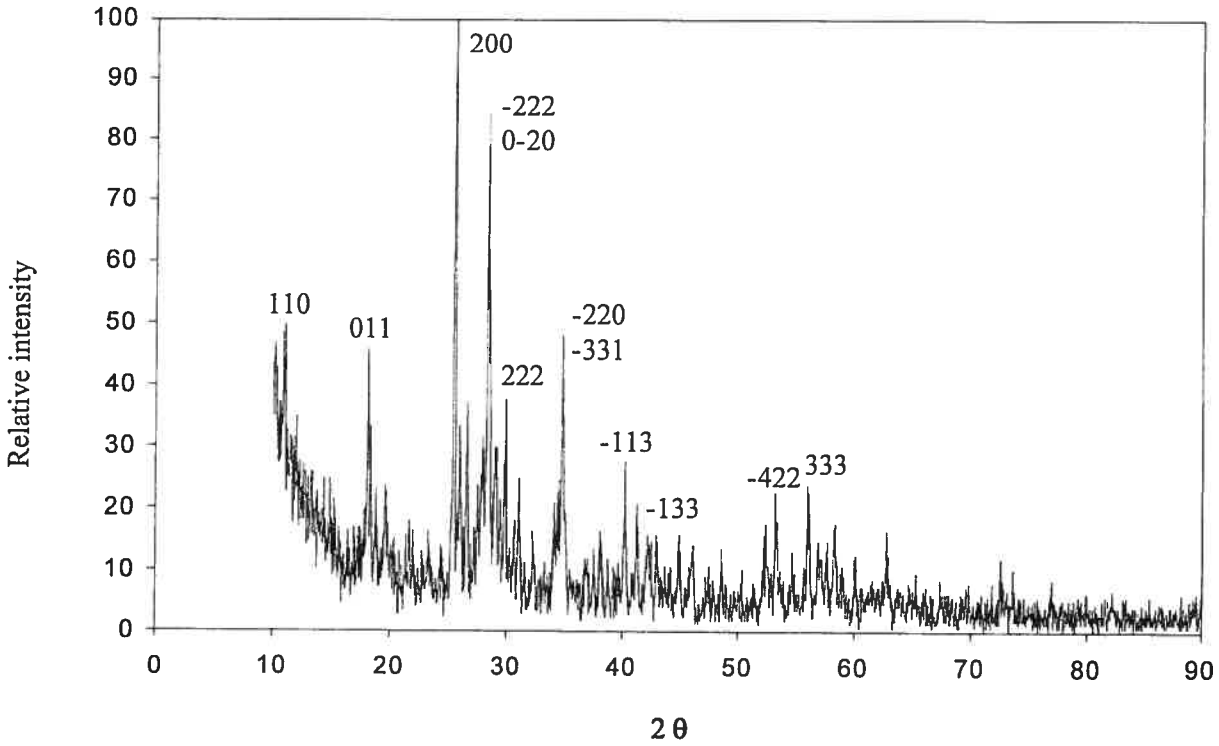

FIG. 3. X-ray diffraction pattern of the manganese sulfate/phosphate mixture. The numbers are the Miller indices of the diffracting planes.

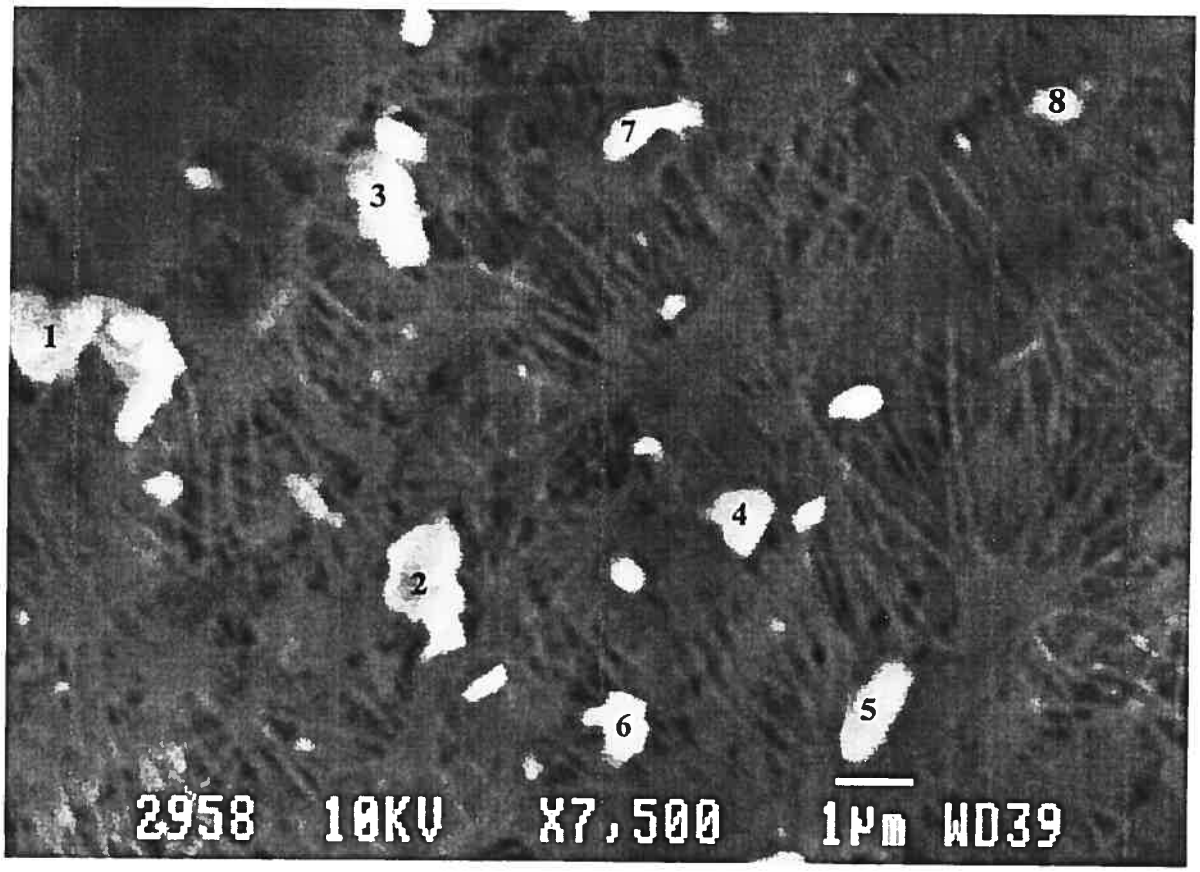

FIG. 4. SEM micrograph showing particles on the filter.

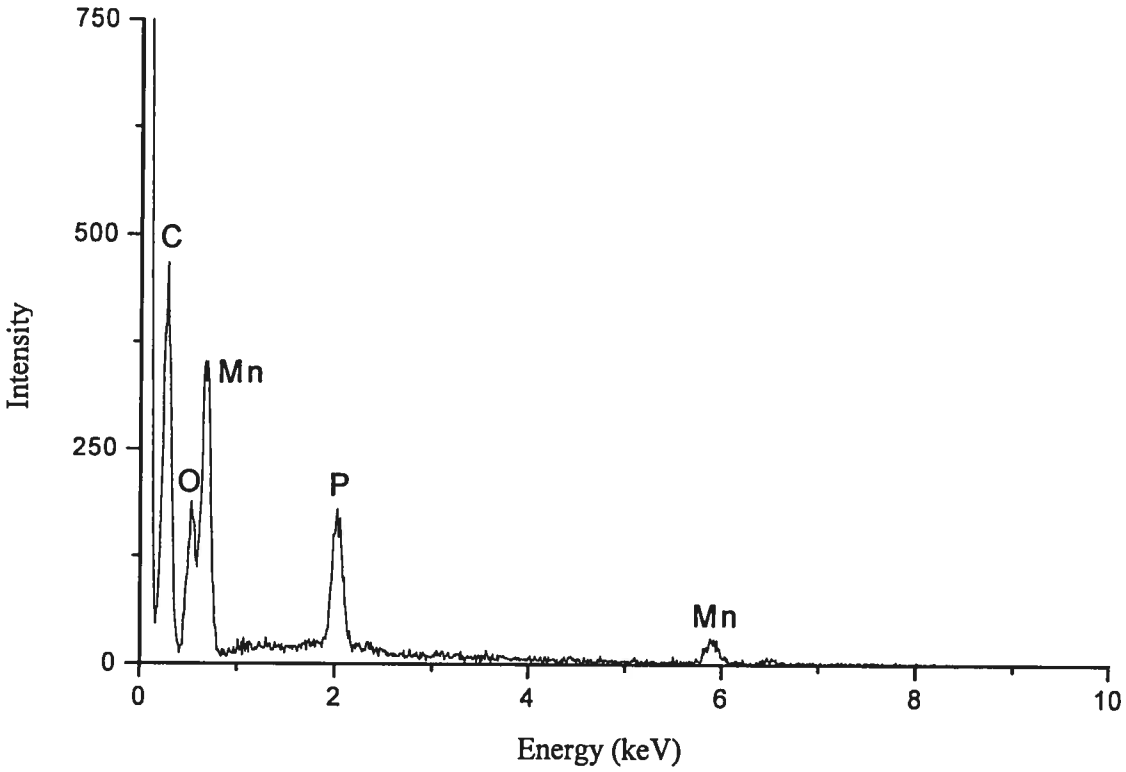

FIG. 5. X-ray EDS spectrum of particle 1.

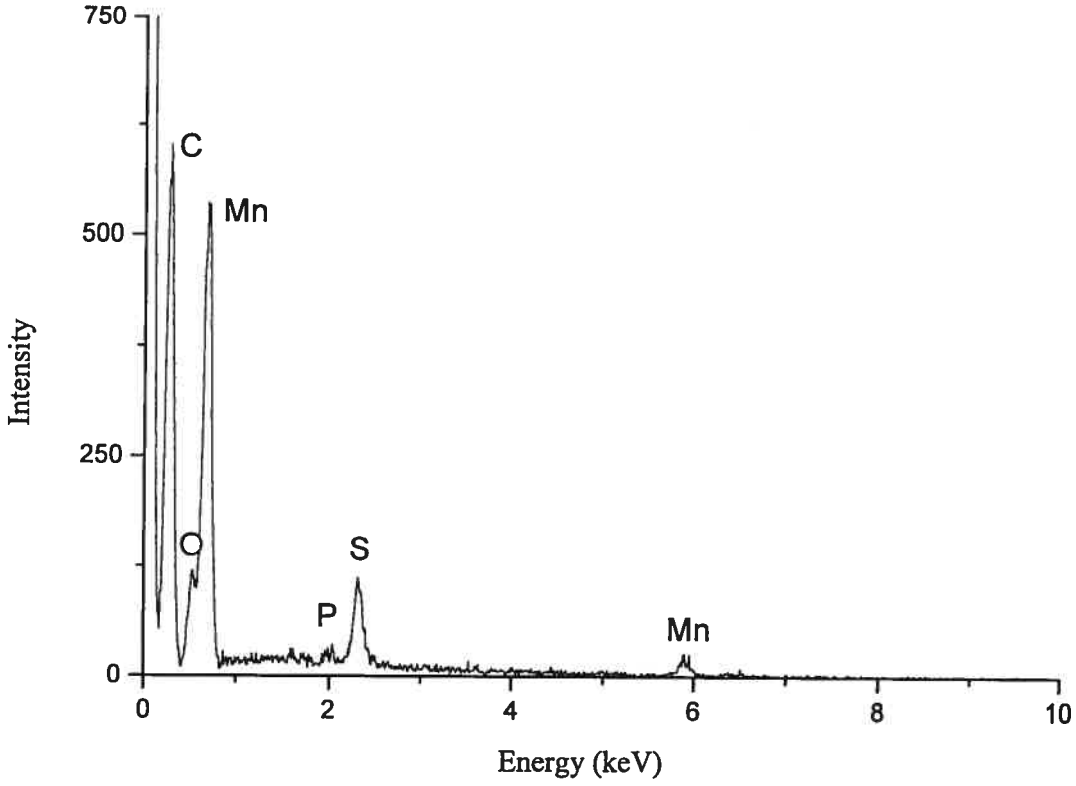

FIG. 6. X-ray EDS spectrum of particle 2.

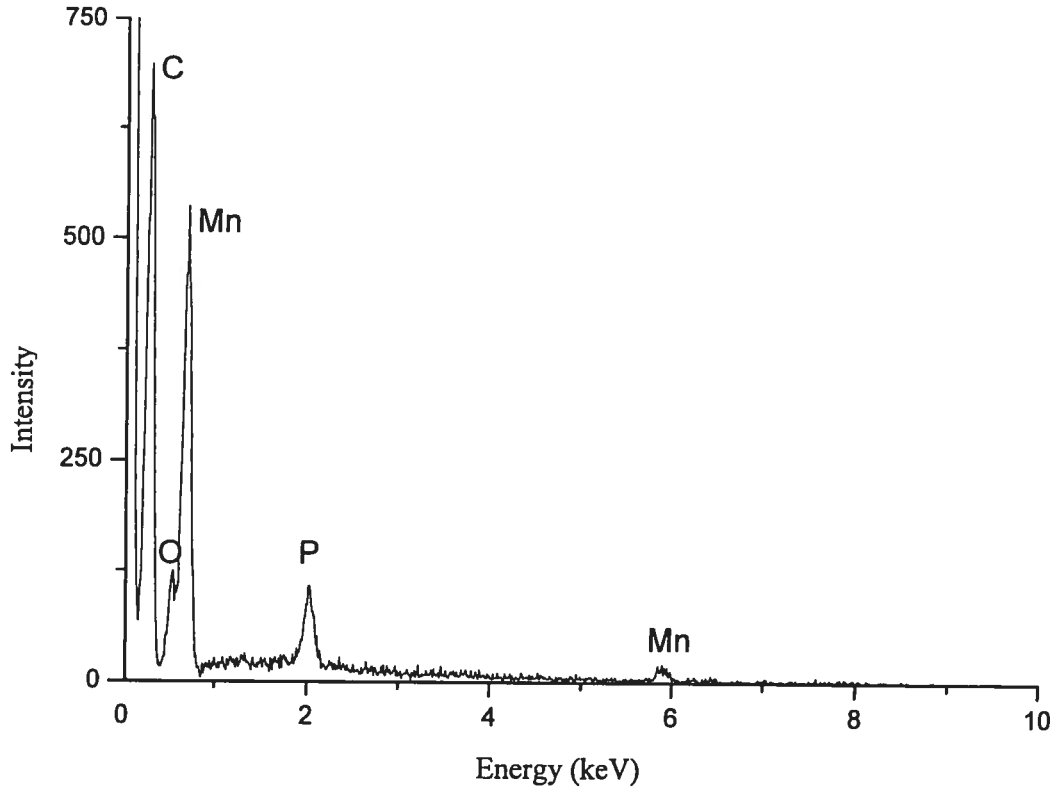

FIG. 7. X-ray EDS spectrum of particle 3.

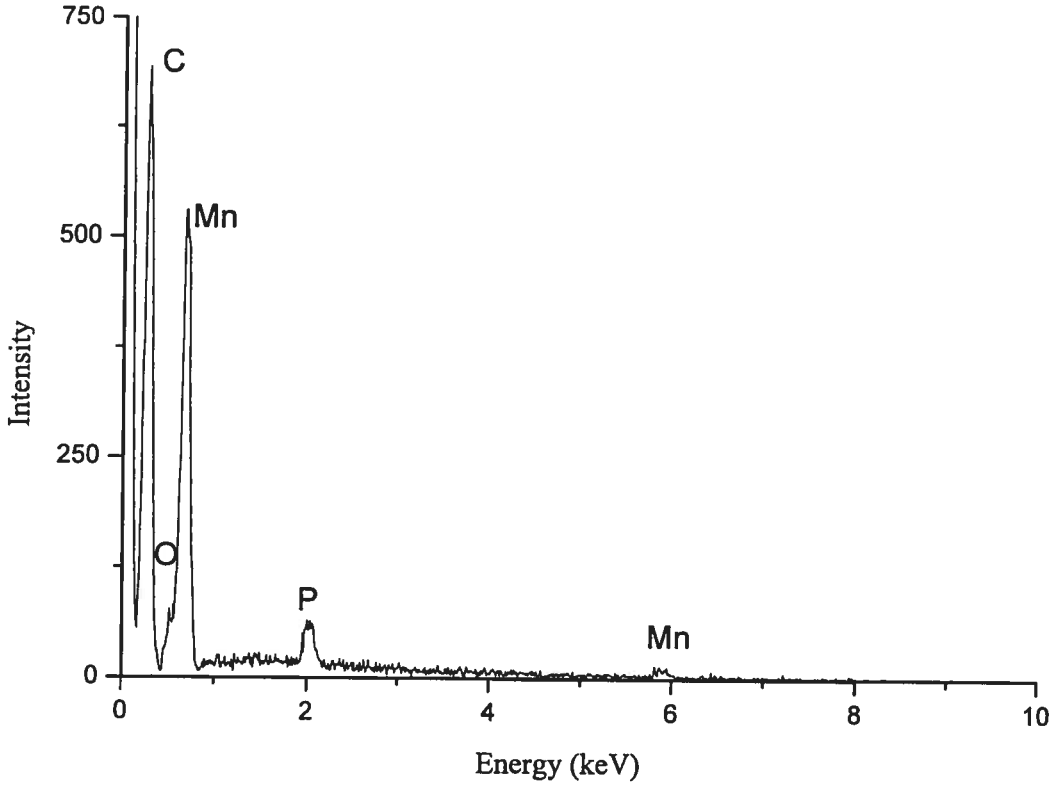

FIG. 8. X-ray EDS spectrum of particle 4.

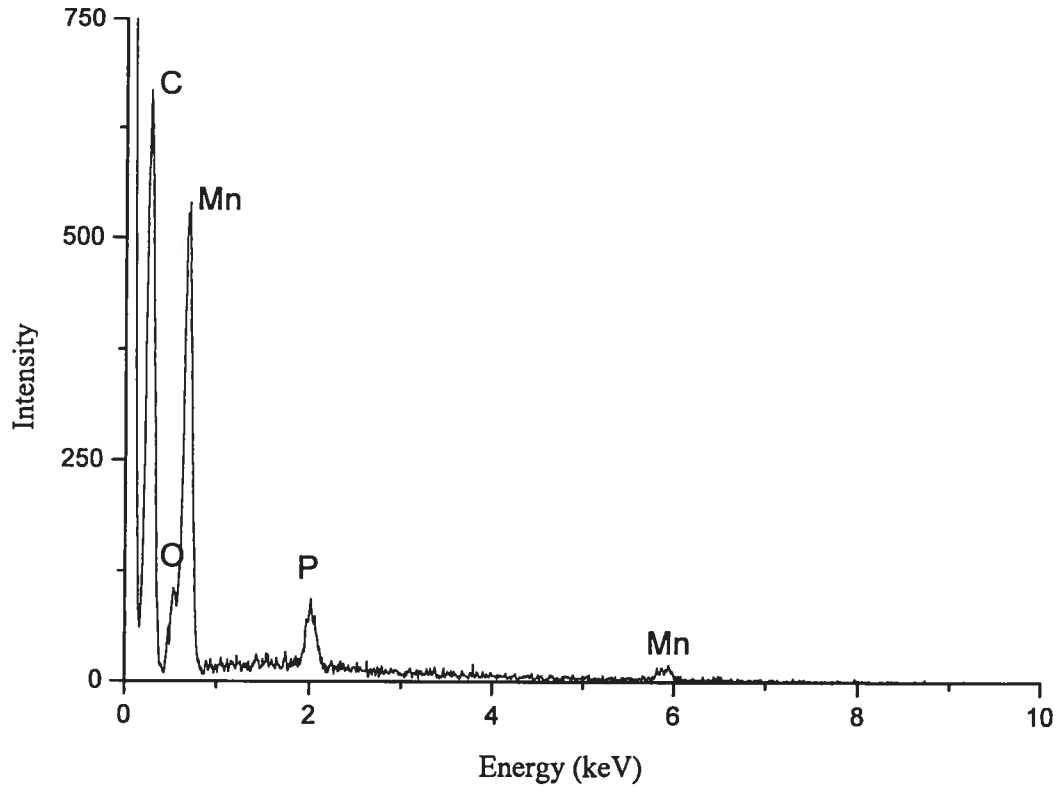

FIG. 9. X-ray EDS spectrum of particle 5.

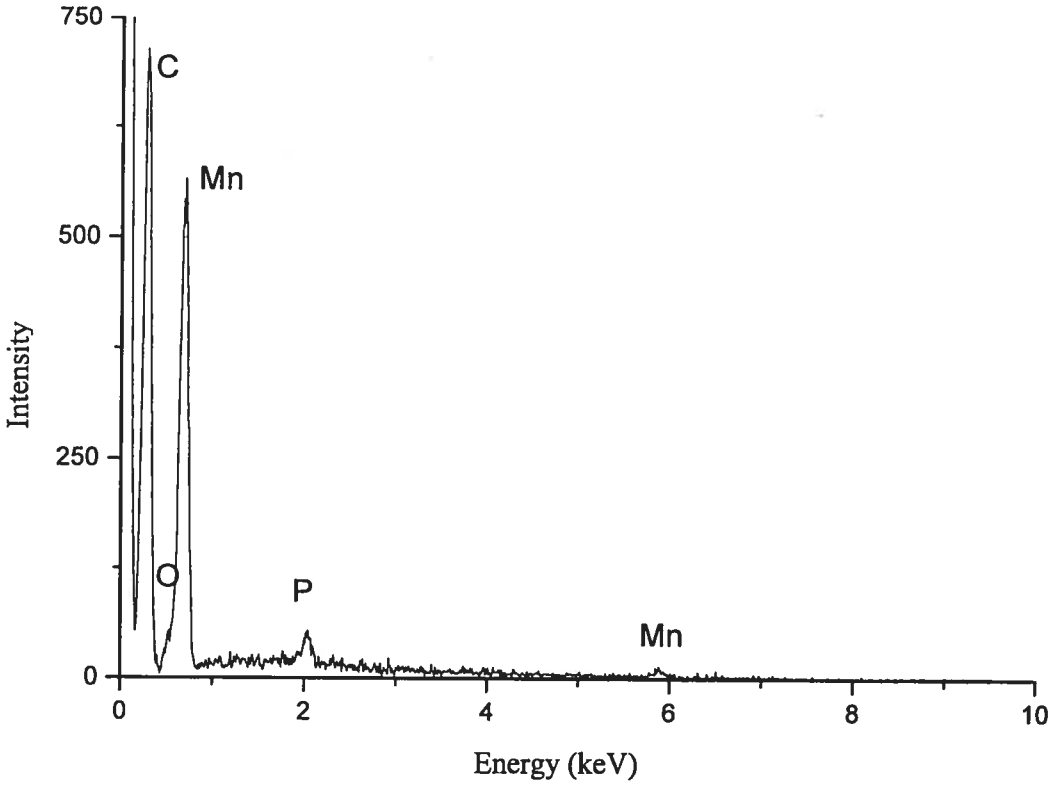

FIG. 10. X-ray EDS spectrum of particle 6.

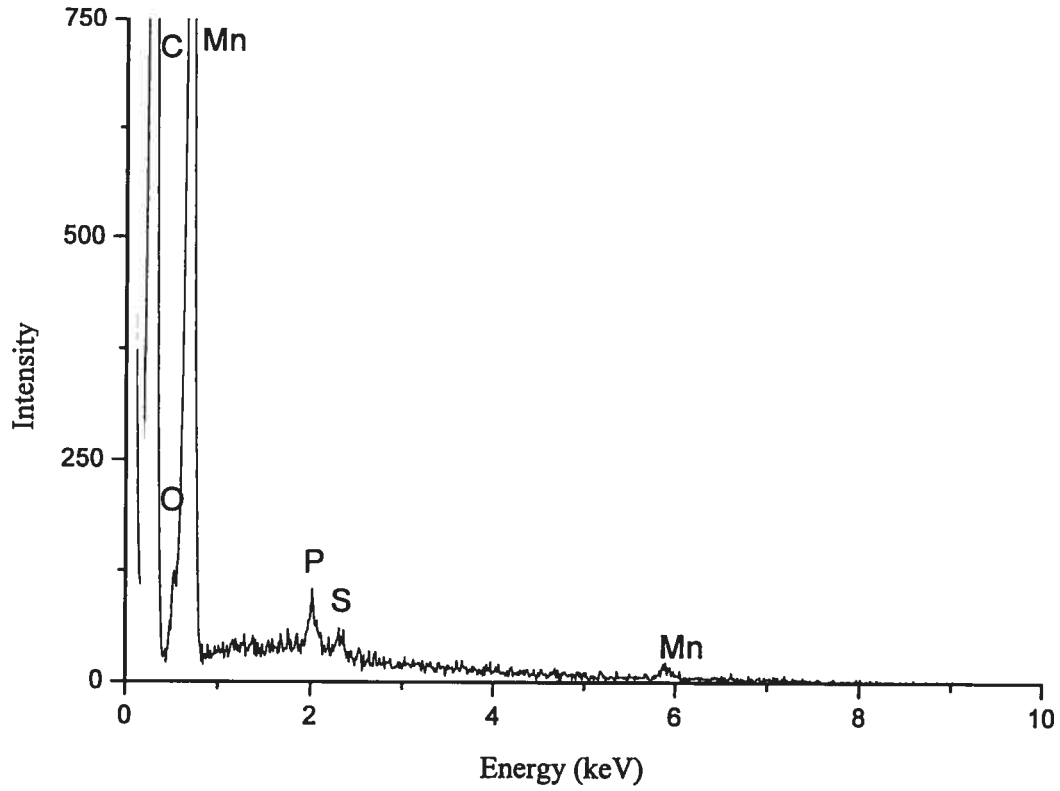

FIG. 11. X-ray EDS spectrum of particle 7.

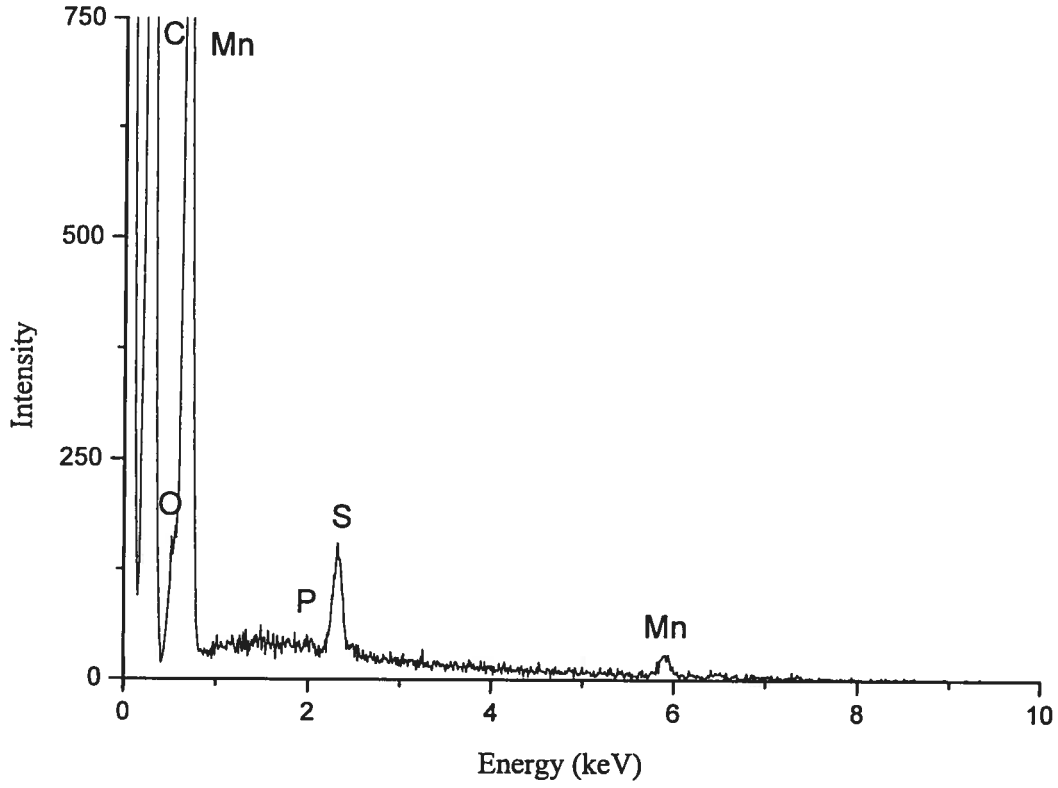

FIG. 12. X-ray EDS spectrum of particle 8.

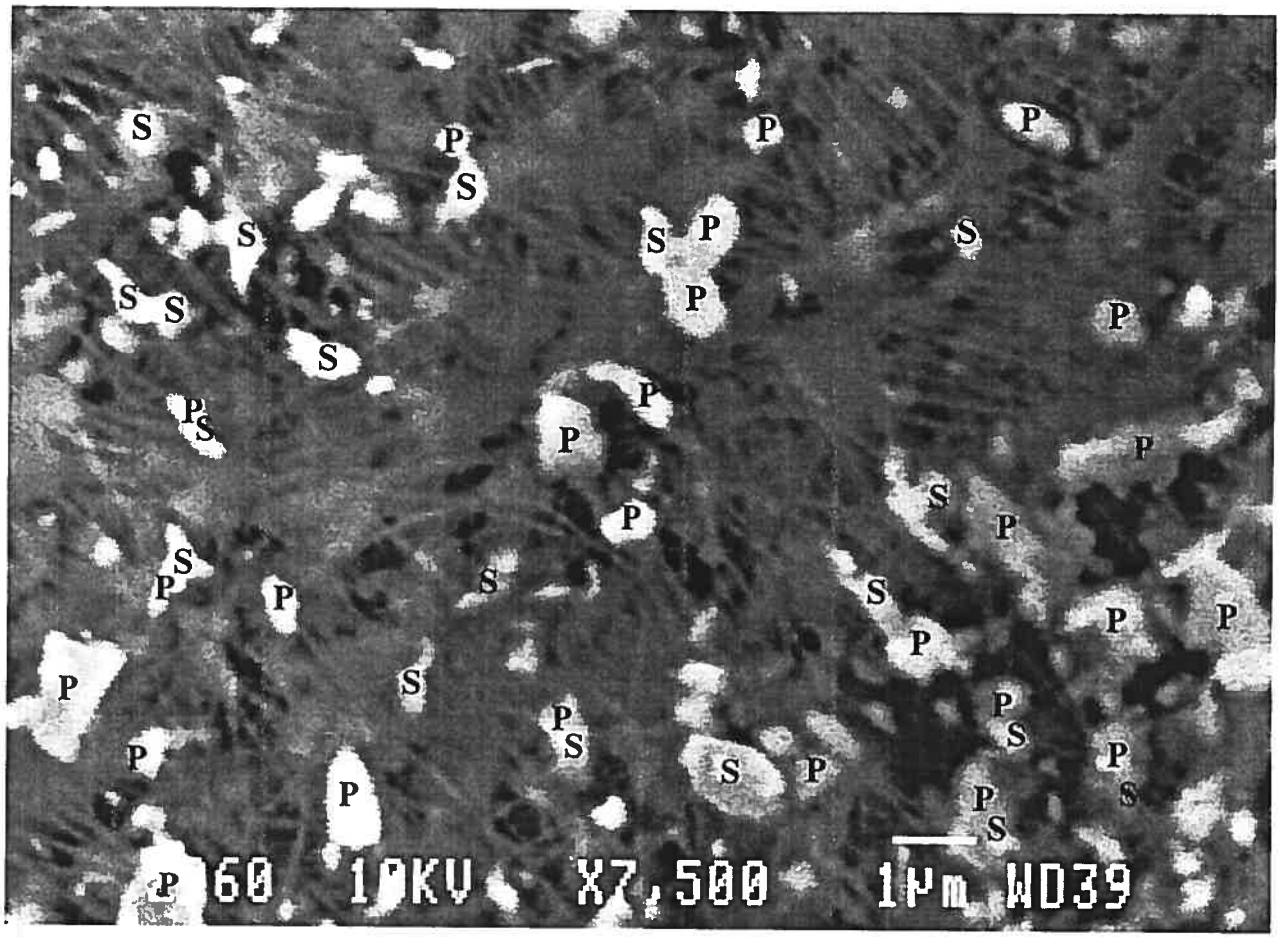

FIG. 13. SEM micrograph showing particles containing phosphate and sulfate compounds.

A reference sample containing the powder from the 50/50 (by weight) mix used was made. The powder was simply deposited on a carbon sample holder. Figure 15 shows a picture of the reference sample. Considerable agglomeration of particles could be observed and very few sulfate particles could be found. Nearly all the sulfur detected was from agglomerations of phosphate and sulfate particles. The purpose of analyzing the reference sample was to calculate the quantity of phosphate and sulfate particles present in the mix and to compare it to what was found on the filters. However, because of the high level of agglomeration on the reference sample, the analysis of small particles could not be done. Nevertheless, it is obvious that particles from the reference sample (Figure 15) are quite different from those found in the inhalation chamber where they are less agglomerated (figures 4 and 13). A spectrum was acquired under the same conditions as those used to acquire the spectra on the filter (Figure 16). The P/S ratio calculated from the net intensities of the spectrum is 3.4, which is lower than the one acquired on the filter. This means that the filter contains less S than the reference sample, which contains 50% (by weight) of both powders. This may be due to the process used to produce a flow of air containing the particles, which involves 100 μm brass beads to mix the powder with the airflow so that most of the agglomerations are broken up because of the collisions between the sand particles and the phosphate and sulfate particles.

One important point is the potential absorption of the S x-rays by P, which could explain the lower S intensity observed in most of the spectra acquired. This is because the mass absorption coefficient of S x-rays by P is much higher than that of the other values (see Table 3). The critical thickness for 10%

TABLE 3
Mass absorption coefficients (cm^2/g) of P and S Kα x-ray lines

	Emitter	
Absorber	P	S
P	287.4	2230
S	370.9	254.6

Note. From Goldstein et al. (1992).

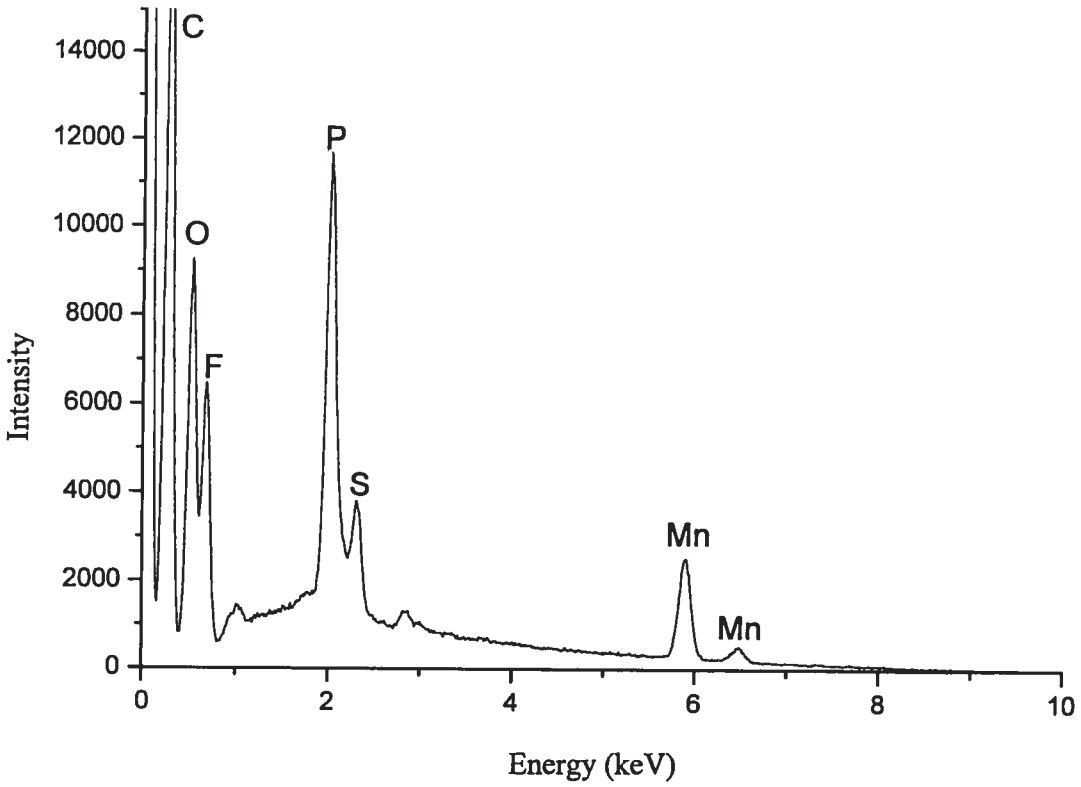

FIG. 14. X-ray EDS spectrum acquired on a 4-mm^2 region of the filter. P/S ratio = 3.8.

of absorption of the S Kα x-rays by Mn phosphate and Mn sulfate particles were calculated. The results are 316 and 334 nm, respectively, which confirms that there is a strong absorption of the S Kα x-rays, since all the particles analyzed in the SEM are over 500 nm in size for the particles both on the filter and in the reference sample.

ATEM Characterization

A number of isolated particles and particle clusters retrieved from the filter were characterized by ATEM. Figure 17 shows a representative TEM micrograph of a particle cluster. The chemical composition determined by x-ray EDS was similar to that determined in the SEM and consisted mainly of Mn, O, P, and S, regardless of whether the particles were isolated or in clusters. This confirms that the particles are Mn phosphate and Mn sulfate and that the ratio is the same as that found with SEM, which is 61% for Mn phosphate and 39% for Mn sulfate. The S and P x-ray peaks detected in the ATEM will be subjected to the same absorption phenomenon of the S Kα x-rays described for SEM but to a lower level, since the size of the particles analyzed in ATEM are smaller than those analyzed in SEM, as can be seen in Figure 17.

DISCUSSION

Figure 1 shows that the background of the x-ray diffraction pattern is uniform, which indicates that the powder is smooth and the particles are fine. As for Figure 2, the phosphate powder has a lower density, which weakens the signal. This could explain why in Figure 3, the second peak from the phosphate powder has a very low intensity. X-ray diffraction is a method that specifically associates the peaks with the compound; it is like a fingerprint for each chemical compound. This technique was used to verify the nature of the mixture. The SEM/EDS technique, having good spatial resolution, allows one to observe individual residual particles and to analyze their chemical composition one by one. It allows analysis of the bigger particles (>0.5 μm) and can identify the elements present in the particles. ATEM has a much higher spatial resolution and can isolate and characterize fine particles and particle clusters down to a size of 0.1 μm. Both techniques (SEM and ATEM) can identify the elements that compose a particle but cannot readily determine if it is a sulfate unless detailed and complex (for the case studied here) quantitative analysis is carried out. These techniques only show that sulfur is present. The same applies to the phosphate particles, where both techniques can only determine the presence of phosphorus and not the stoichiometry. The presence

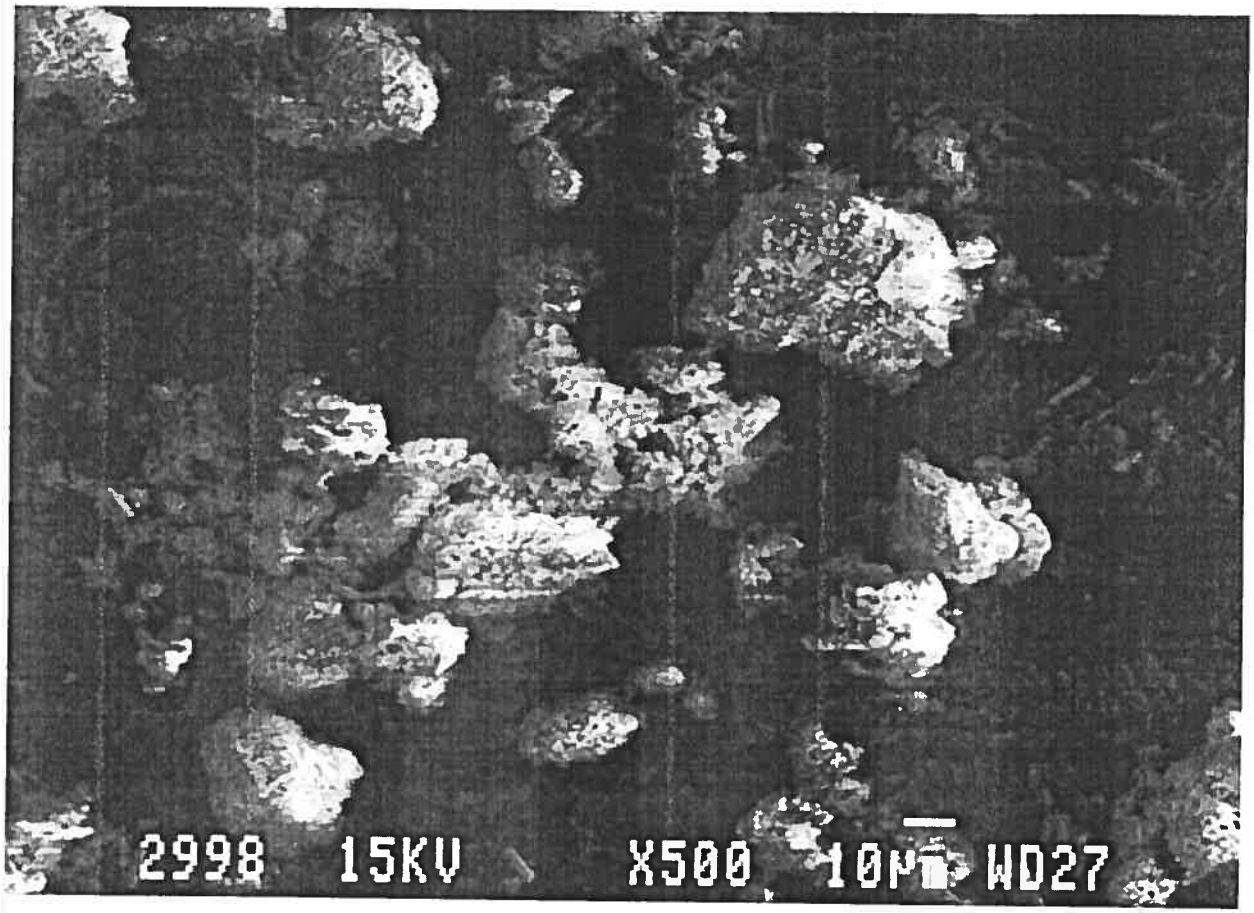

FIG. 15. Secondary electron image in the SEM of phosphate and sulfate particles in the reference sample.

of oxygen, however, strongly suggests that the compounds are sulfates and phosphates.

In this study, equal weights of Mn phosphate and Mn sulfate were introduced into the inhalation chamber. However, the subsequent analysis of air samples by electron microscopy showed that 39% of the particles were Mn sulfate and 61% were Mn phosphate. This ratio is acceptable, since it is near the ideal ratio of 1/1. During the pre-experiment, we took into account the molecular weight of each substance to maximize the probability of obtaining a ratio of 50/50 for particles in the inhalation chamber. Since the molecular weight (MW) are 728.65 and 169.03 for Mn phosphate and Mn sulfate, respectively, representing a ratio of approximately 4/1, we have used the inverse ratio for weight (4 Mn sulfate/1 Mn phosphate). Nevertheless, the proportion of particles for each Mn species was far away from the target of 1/1. In fact, more than 80% of the particles were related to the Mn sulfate compound.

Moreover, the size of the particles before their entry into the chamber could explain the results obtained in this study. In fact, as presented in the method, Mn aerosol particles were delivered through a stainless-steel pressure tank used for the settling of larger Mn aerosol particles. Since we did not characterize in detail the particles used in this study before their entry into the inhalation chamber, we can hypothesize that the Mn sulfate particles in the powder used were bigger than those of Mn phosphate. In fact, Table 1 shows a similar size distribution of particles in the inhalation chamber for Mn sulfate, Mn phosphate, or the mixture. This suggests that more Mn sulfate particles settle in the steel tank because of their size, as found in the powder.

The fact that the mixture of particles in the inhalation chamber was not 50/50 could lead to an underestimation or to an overestimation of the effects. In our study, only the rats exposed to the Mn phosphate/sulfate mixture (and not to Mn phosphate only) had significant changes in locomotricity compared to the control rats (Normandin et al., in press). This is in agreement with the results of some studies that showed that the solubility of inhaled Mn particles may influence the brain Mn concentrations (Normandin et al., 2002; Dorman et al., 2001). The dissolution of water-soluble $MnSO_4$ is more rapid than that of water-insoluble $MnHPO_4$ (Vitarella et al., 1997).

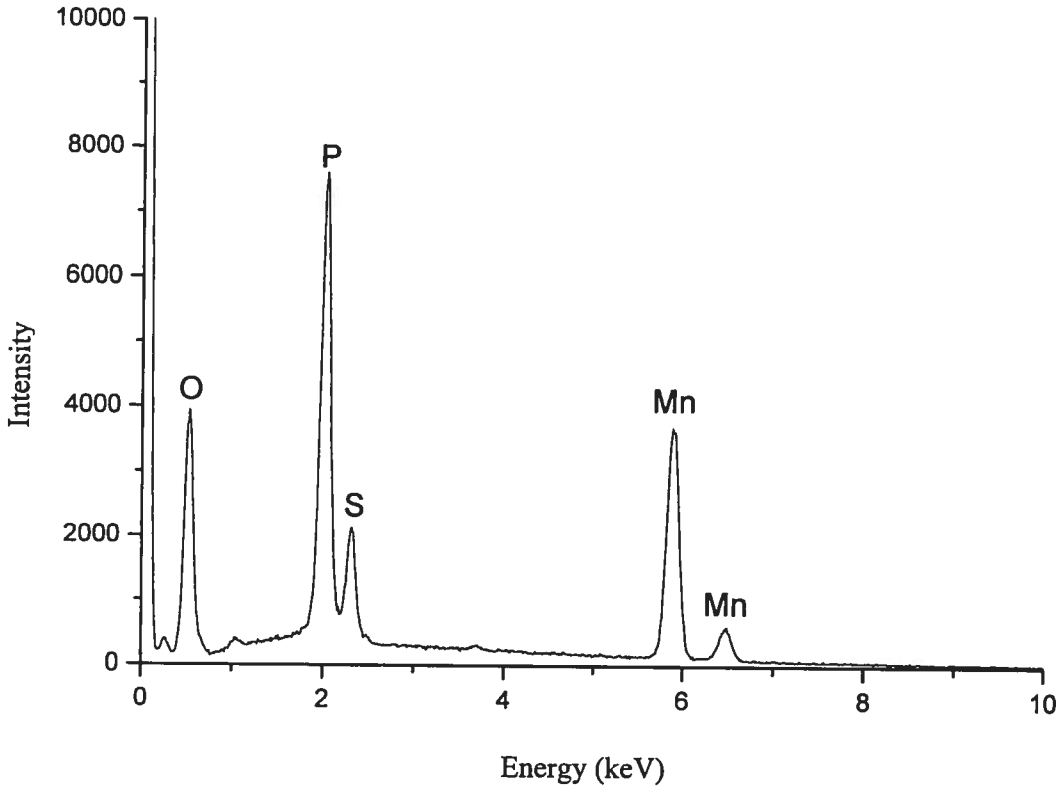

FIG. 16. X-ray EDS spectrum acquired on the reference powder. Ratio P/S = 3.4.

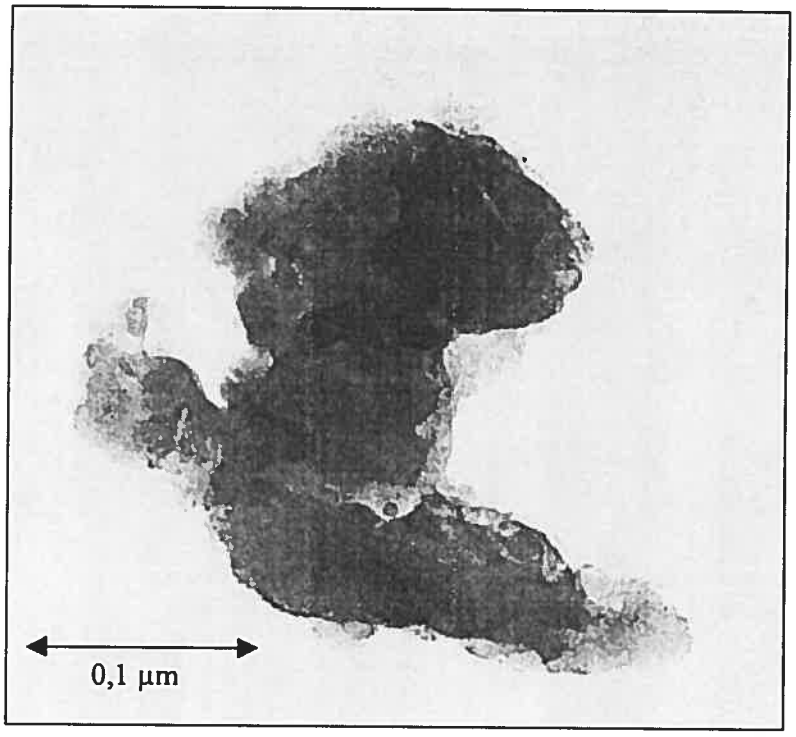

FIG. 17. ATEM analysis of a particle cluster observed on a filter.

Overall, this study shows that it is very difficult to reach the target of 50% Mn sulfate/50% Mn phosphate mixture in the inhalation chamber. Many factors could play an important role. In terms of the assessment of various effects, the fact that the number of Mn sulfate particles was about 22% less than that of the number of Mn phosphate particles probably led to an underestimation.

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