The role of sodium selenite in ameliorating neuronal alterations induced by prenatal methylmercury exposure

by

Sally Jo Pyle

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INTRODUCTION

The neurotoxic effects of methylmercury exposure have been recognized since the 1500s (Chang, 1982). The poisonings at Minamata Bay, Japan and in Iraq revealed the susceptibility of the fetus to the toxic effects of methylmercury (Reuhl and Chang, 1979, review). The neurological disturbances in congenitally exposed individuals progressively worsen as patients age. The severity of these poisonings has led to extensive studies of methylmercury exposure in adult, new born, and fetal animals (Bartolome et al., 1984; Chang et al., 1977; Hirayama et al., 1985; Jacobs et al., 1975; Kutscher et al., 1985; Salvaterra et al., 1973).

In adult rats, the dorsal root ganglia neurons and the granule cell layer of the cerebellum are preferentially affected. The first discernible change in the granule cells of the cerebellum is a decrease in the endoplasmic reticulum (Chang and Hartmann, 1972b; Spencer and Schaumburg, 1981, pp. 24-34). This correlates with the reported decreases in protein metabolism following mercury exposure (Chang, 1984; Syversen, 1982).

In prenatally exposed rats, the extent of pathologic damage is broader with changes in cytoarchitectonics that persist into adult life. Damage to the cerebellum is not

confined to the granule cells but is similar to the changes seen in the adult (Reuhl and Chang, 1979). The dosage, number of doses, and stage of fetal development are important factors in determining the severity of damage in prenatally exposed animals.

Selenium is known to exert a protective effect in adult animals exposed to methylmercury (Chang, 1984), but studies on fetal protection by selenium have been rare. Recently it has been shown that low levels of dietary selenium reduce fetolethality (Nishikido et al., 1987). The mechanisms of selenium protection are not clear.

This study was designed to determine whether selenium given to maternal rats as a dietary supplement would provide protection against a low dose of methylmercury in their offspring. The purpose of this experiment was to quantify toxic changes to the endoplasmic reticulum of neurons in the external granule layer of the cerebellum in 0-day-old rats, and in the internal granule layer of 21-day-old rats.

LITERATURE REVIEW

General review of mercury

It is estimated that industrial uses of mercury average from 5,000 to 7,000 tons per year. The applications of mercury in industry include electrical apparatus, chloralkali, paint manufacturing, fungicides, paper and pulp manufacture, pharmaceuticals, and cosmetics (Berlin, 1986; Spencer and Schaumburg, 1981, pp. 508-526).

To a greater extent mercury is naturally circulated through the biosphere by degassing from the earth's crust and oceans, and it is estimated that from 30,000 to 150,000 tons of mercury are released per year (Berlin, 1986). Mercury is also released in emissions from the burning of fossil fuels. It is estimated that 65% of the mercury that is emitted as a vapor is deposited within 200 to 2000 kilometers of the emission source; however, because mercury reacts strongly with its surroundings only 20% will be permanently deposited, the remainder will be re-emitted (Friberg et al., 1986).

The toxicity of metals depends on their oxidation state. Mercury occurs in three oxidation states, elemental mercury [Hg°], mercurous mercury [Hg^{2+2}], and mercuric mercury [Hg^{2+}]. These forms of mercury vary in their relative toxicities, toxic effects, and absorptive

properties. Mercury can also covalently bond with carbon atoms to form different organomercurials. The alkyl compounds are the most toxic and the most prevalent. The length of the alkyl chain is indirectly related to the toxicity of the compound (Friberg et al., 1986).

Non-toxic and mildly toxic forms of mercury that enter rivers, lakes, and oceans are converted to toxic alkyl mercury by microbes. Photoalkylation also transforms non-toxic mercury compounds into toxic alkyl and dialkyl mercury (Friberg et al., 1986). Mercury is concentrated in fish tissue as it moves through the aquatic food chain. The form of mercury found in fish tissue is almost exclusively methylmercury (Inskip and Piotrowski, 1985).

Human exposure to mercury occurs in industrial and non-industrial settings. Most industrial exposures are to elemental mercury, or mercurous and mercuric salts. Exposure routes are mainly inhalation and dermal contact. The major non-industrial route of mercury exposure for man is through consumption of contaminated fish and sea food, or grains that have been treated with mercurial fungicides. Contaminated food products led to the disasters in Iraq, and Minamata and Niigata, Japan. This review will concentrate on the effects of methylmercury neurotoxicosis, which is referred to as Minamata disease.

Absorption and distribution In the gastrointestinal tract 95% to 99% of methylmercury is absorbed (Inskip and Piotrowski, 1985). Mercury is carried by erythrocytes and is bound either to hemoglobin (White and Rothstein, 1973) or complexed to glutathione (GSH) (Thomas and Smith, 1979; Naganuma et al., 1980). Extracellular sulfhydryl groups (SH) elicit the release of mercury from the red blood cells (White and Rothstein, 1973). Mercury has been shown to enter cells through sodium and potassium channels (Miyamoto, 1983).

Methylmercury readily crosses the placenta from mother to fetus, but does not easily cross from fetus to mother, therefore, mercury is 'trapped' in the fetal environment (Chang et al., 1980).

Studies of the distribution of mercury show that mercury accumulates in the liver, kidney, plasma, and brain. Concentrations vary in different tissues with 20 to 30% of total accumulation found in kidney and plasma, 12 to 15% in liver, and less than 10% in brain tissue (Tilson and Sparber, 1987).

Methylmercury is a potent neurotoxicant despite the fact that the brain accumulates only a small percentage of total body mercury. Yang et al. (1972) found that total brain uptake of mercury in maternal and fetal rats was

approximately equal during the first two days after an oral dose of 0.45 mg methylmercury chloride/kg body weight. Mercury was administered on day 16 and tissues were examined on days 17 through 21 of gestation. From three to five days post exposure fetal brain uptake was two and one-half to three times greater than in the mother.

Mercury is found in the milk of lactating females. Yang et al. (1973) found neural lesions in rats whose only exposure to mercury was via maternal milk. Approximately 3% of the total body mercury in the mother is excreted in the milk. The bond between mercury and the methyl group is cleaved in slightly less than 5% of the total mercury in milk (Garcia et al., 1974). Therefore, nursing infants are exposed to both organic and inorganic mercury.

The estimated half-time of mercury is 70 days in an adult human, whereas in lactating females the half-time is only 42 days (Inskip and Piotrowski, 1985). The details of mercury excretion in the human fetus and newborn are not known. Doherty et al. (1977) studied the elimination of methylmercury in mice given a single dose of 0.4 mg/kg, per os, on different days between birth and weaning. From birth to 15 days estimated half-times for mercury elimination ranged from 2500 to 200 days. After day 16 the half-time abruptly decreased to eight days, the adult half-time for

mercury elimination in rodents. This lengthy half-time during the first 15 days may be due to immature biliary function in the newborn liver (Inskip and Piotrowski, 1985). Mercury is bound to glutathione in the bile, and the liver of newborns is not developed enough to detoxify and excrete this compound. Since elimination half-times are directly related to cumulative body burdens of methylmercury, these factors are important when estimating exposure risks to human fetuses and infants (Doherty et al., 1977).

Ninety percent of mercury is eliminated in the feces bound to bile breakdown products. In the adult this percentage can be slightly influenced by diet. Gut flora can process mercury in the fecal material and cause reabsorption of the mercury, but this only affects approximately 10 to 20% of the mercury in the feces (Berlin, 1986).

Many biochemical parameters have been studied in the nervous systems of animals exposed to methylmercury. Salvaterra et al. (1973) related changes in levels of glycolytic intermediates in the brain with changes in the behavior of mice. Intermediate compounds in early stages of glycolysis were increased, with a consequent decrease in late stage intermediates. The changes were more intense in the cerebellum than the cerebrum. A decrease in glycolysis

leading to decreased energy levels has serious consequences in brain tissue, which requires a great deal of energy to function. Mercury also affects neurotransmitter levels. Studies in the cerebellum indicate that mercury increases levels of taurine and glycine, inhibitory neurotransmitters that have been implicated in ataxia and loss of granule cells, respectively (Hirayama et al., 1985). In prenatally and neonatally exposed rat pups alterations in the synaptic dynamics of brain dopamine systems were found following injection of 1 mg/kg methylmercury hydroxide from days eight through 20 of gestation (Bartolome et al., 1984). Dopamine levels and turnover rate were decreased, but these alterations were not observed in pups until after weaning (Slotkin and Bartolome, 1987). These researchers suggest that the changes may actually involve abnormal presynaptic function. Synaptic formations and transmissions exert trophic actions on adjacent neurons, therefore, abnormal synaptic function will result in further disruptions in total brain function. Alterations have been found in lipid synthesis (Menon and Lopez, 1985), especially during active stages of myelin formation. Other studies have found that protein synthesis is decreased in the cerebellum and cerebrum (Cheung and Verity, 1985; Omata et al., 1980). Cheung and Verity (1985) attempted to identify the locus of

mercurial inhibition in the mechanism of protein translation. Their data revealed that the apparent inhibition of brain protein translation was due to a primary disturbance in aminoacylation of tRNA and was not associated with defective initiation, elongation, or ribosomal function. Changes in DNA and RNA have also been noted, but both increases and decreases have been found (Slotkin and Bartolome, 1987; Syversen, 1982). These discrepant findings are probably the result of differences in duration and level of the dose and timing of the insult.

Ornithine decarboxylase (ODC) is an enzyme catalyzing the initial reactions in the synthesis of polyamines involved in growth of different cell types. ODC activity is very high during cell reproduction, differentiation, and enlargement. Slotkin and Bartolome (1987) have been studying ODC as a specific marker of methylmercury toxicity in rats. A maternal injection as small as 0.05 mg/kg resulted in changes in ODC levels and activity in fetal brains, changes that persisted into the postnatal period. Slotkin and Bartolome (1987) measured ODC levels in the cerebellum, cerebral cortex, and midbrain and brainstem in neonates from mothers injected with 0.5 or 1.0 mg/kg methylmercury hydroxide on days seven through 20 of gestation. In controls, the ODC levels in each specific area were highest during active development of that area.

However, ODC levels in the mercury exposed group peaked at a time prior to the normal developmental timing of neuronal maturation in each specific area. Early differentiation, migration, and maturation of neurons can cause serious disruptions in cytoarchitecture and synaptic formation.

Neuropathology following mercury exposure

Adults

Humans

Clinical symptoms of Minamata disease vary according to the duration of exposure and the dose. Acute poisonings result in sudden visual changes leading to blindness (Inskip and Piotrowski, 1985). Chronic exposures result in a broader range of disturbances, starting with tingling and paresthesia in the extremities, and progressing to constriction of the visual field, weakness, ataxia and blindness (Spencer and Schaumburg, 1981, pp. 508-526).

Autopsies of human victims revealed gross atrophy of the calcarine cortex and cerebellar folia, thinning of the cerebellar gray matter, and disintegration of the cerebellar granule cells (Spencer and Schaumburg, 1981, pp. 508-523). Purkinje cells, though they appeared to contain a greater concentration of mercury (Chang and Hartmann, 1972a), were more resistant to the toxic effects of mercury. However, in chronic cases disintegration of the Purkinje cells is observed. Other observed changes included proliferation of

Bergmann's glial fibers and changes in the basket, climbing, and parallel fibers of the cerebellum. The calcarine cortex exhibited degeneration of the neurons and their myelin sheaths in severe chronic cases. Degeneration of nerve fibers, axons, and myelin sheaths was also evident in peripheral nerves.

Experimental adult animals Topographical and species differences are evident, but many of the neuropathological changes seen in experimental animals closely resemble those recorded in human intoxication (Chang, 1987). The rat has been used extensively as a model for methylmercury toxicity and this review will focus on the rat model. The major species difference in the rat is the extreme sensitivity of the peripheral nervous system, especially the sensory neurons and fibers of the dorsal root ganglia. Inskip and Piotrowski (1985) suggest that this difference makes data from rat studies inapplicable to other species, especially humans. However, the central nervous system (CNS) pathology of the rat is similar to changes observed in humans. A rat model for acute methylmercury intoxication was developed by Klein et al. (1972). They studied acute and subacute intoxication with methylmercury hydroxide. Peripheral neuropathy was predominant in acute cases, and CNS damage was predominant in subacute exposures.

Mercury can be detected in the brain within hours of administration. Mercury first crosses the blood brain barrier resulting in increased permeability (Spencer and Schaumburg, 1981, pp. 508-526). Damage to this active regulatory site for uptake of biological metabolites from blood may lead to further damage of the brain. Atrophy and degeneration of the internal granule cell layer of the cerebellum are consistently found (Chang and Hartmann, 1972b; Klein et al., 1972). Ultrastructurally there is degranulation of the rough endoplasmic reticulum, dilation and disintegration of the endoplasmic reticulum, increases in the number of lysosomes, an increase in the extracellular space, and coagulation of the cytoplasm, which appeared very electron dense (Chang and Hartmann, 1972b; Jacobs et al., 1975; Klein et al., 1972; Spencer and Schaumburg, 1981, pp. 508-523).

Fetus and infant Toxic injuries to the fetus and infant present special problems. The timing of the insult is as important as the nature of the toxin in determining the type and extent of the injury (Spencer and Schaumburg, 1981, pp. 48-61). Major confounding effects can be introduced into studies of prenatal exposure if the toxin acutely affects the maternal animal. Abnormal development can result if the embryo experiences nutritional

deficiencies or anoxic conditions because of maternal illness. In the early predifferentiation stage, a major toxic insult can have two distinctly different results. When damage is very severe all cells will be killed and no embryo will form. When only mild damage results the remaining cells compensate for the cell loss and a normal embryo forms. The next stage in development is the embryonic, during this stage organogenesis occurs. Toxic insult during organogenesis results in major malformations. Species differences are very apparent in the timing of organogenesis and are reflected in differences in toxic injury. Organogenesis is precisely timed, and major development of organ systems can occur within minutes to hours so that a change in the timing of exposure can give very different results (Neubert et al., 1977). In the later stages of fetal development insults mainly affect growth and appear clinically as low birth weight. Significant migration, differentiation, and maturation of neurons occurs during this stage. Other defects that result from toxic insult include microcephaly and abnormal development of cytoarchitecture (Spencer and Schaumburg, 1981, pp. 48-61). Species differences in developmental state at birth will determine the type of injury caused by postnatal and perinatal exposures.

Human Clinical symptoms varied greatly among patients, from barely detectable to profound mental and physical retardation. Major factors influencing these clinical differences were the duration, concentration, and timing of exposure (Chang, 1984). In the Minamata children, the appearance of symptoms was delayed from a few weeks to a few months postpartum. The infants were lethargic, unable to follow visual stimuli, uncoordinated in sucking and swallowing, and the disappearance of primitive reflexes was delayed. The condition of most patients worsened with age. The onset of developmental mileposts was delayed, these included: grasping, crawling, standing, and walking (Spencer and Schaumburg, 1981, pp. 508-526). The most severely affected children developed cerebral palsy. The Japanese children developed a constricted visual field whereas the Iraqi children were blind. This difference may be the result of the more acute exposure conditions in the Iraqi victims (Chang, 1984). The pattern of prenatal mercury intoxication begins as motor defects and progresses to mental disturbances as the child matures (Reuhl and Chang, 1979).

The few autopsies performed on children from Iraq and Japan revealed some common findings and some differences. In both cases brains and brain weights were reduced, there

was disruption of the cytoarchitecture, and nests of heterotrophic neurons were found, indicative of defective migration of neurons. The Iraqi cases had shortened frontal lobes, atypical gyral patterns, and an increase in myelin ovoids but no neuronal necrosis. In contrast the Japanese children displayed gross atrophy, widespread lesions with loss of granule cells and small pyramidal neurons, and poor myelination but no active demyelination. Cerebellar and peripheral damage was also noted in the Japanese victims, but was not reported in the Iraqi victims (Reuhl and Chang, 1979). It is difficult to assess the full extent of the injuries resulting from the tragic poisonings in Japan and Iraq. Mild damage is not easily detected. Studies on school children in the Minamata area revealed increases of 18% in unspecified intelligence disturbances, and a 21% increase in sensory disturbances over control groups (Reuhl and Chang, 1979).

Experimental animals A significant amount of research has been done with adult animals and mercury toxicity, but not with prenatally exposed animals. Of the studies that have been done confounding factors such as species and strain differences, duration of exposure, route of exposure, and gestational age at the time of exposure make comparisons difficult (Chang, 1987; Reuhl and Chang,

1979). The subtle behavioral alterations without corresponding morphological or biochemical differences observed by Spyker et al. (1972) in offspring of mice injected with 8 mg/kg of methylmercury on days seven or nine of gestation, are similar to the findings for the Minamata school children. Results of studies with experimental animals corroborate the findings reported for human congenital Minamata disease.

The embryolethal effects of prenatal methylmercury exposure have been widely investigated (Hughes and Annau, 1976; Inskip and Piotrowski, 1985; Leonard et al., 1983). Surviving animals have a decreased birth weight. Hughes and Annau (1976) report that the differences in weight did not persist indefinitely, since by nine weeks of age their exposed groups were not significantly different than controls. Gross congenital abnormalities have also been described. The most commonly noted deformity was cleft palate. Limb defects, brain and facial malformations, and exencephaly have also been reported (Chang, 1984).

Gross measurements of brain areas by Kutscher et al. (1985) detected a significant increase (60%) in ventricle size of prenatally exposed rats when compared with controls. A significant thinning of the cerebral cortex and decreases in brain weights and brain size were also observed. These findings are similar to reports from human autopsies.

Light microscopic studies of the brains of developing animals exposed prenatally to mercury reveal microcephaly, abnormalities in cytoarchitecture, delayed migration of granule cells, and increased atrophy and loss of neurons (Inskip and Piotrowski, 1985; Reuhl and Chang, 1979). Sager et al. (1982) examined five cerebellar regions in three-day old mice given 8 mg/kg methylmercury chloride by gavage on day two postpartum. They reported thinning of the external granular layer in regions directly adjacent to the primary fissure. Significant numbers of injured cells, a decrease in cell number, and an increase in cells with condensed nuclei were also noted.

Chang et al. (1977) studied degenerative changes in neurons of prenatally exposed rats. Mothers were injected with 4 mg of mercury/kg of body weight on day eight of gestation, and allowed to give birth naturally. Tissues for microscopic examination were collected from pups at birth. Ultrastructural examination of sections from the calcarine cortex and cerebellum revealed myelin figure formation, large cytosegresomes, sites of focal cytoplasmic degradation (FCD), presence of macrophages, and swelling and vacuolar degeneration in capillary endothelial cells. Myelin figure formation is interpreted as a focal weakening in the nuclear envelope. A previous study by Chang and Hartmann (1972a) showed localization of mercury on the nuclear envelope.

Cytosegresomes and FCDs appear to contain degenerating organelles and other cytoplasmic debris. These changes are indicative of a cellular response to injury and are believed to be a mechanism for sequestering degenerating cytoplasmic materials to prevent further disruption in the cell (Reuhl et al., 1981a). Endothelial cell damage may be related to the increased permeability of the blood brain barrier (Spencer and Schaumburg, 1981, pp. 508-526). Other findings from electron microscopic analyses include accumulations of lysosomes in the perikarya and dendrites, clumping of nuclear chromatin, loss of ribosomes from the rough endoplasmic reticulum (RER), dispersion of polysomes in the cytoplasm, and aggregates of tubular structures (Chang, 1984; Chang, 1987; Reuhl et al., 1981a). In the second part of a study by Reuhl et al. (1981b) prenatally exposed hamsters were allowed to mature. At 275 to 300 days animals were sacrificed for microscopic examination. Pathological changes similar to those found in the neonatal pups were observed along with degenerative changes in small myelinated axons, and focal areas of astroglial proliferation.

Human case studies and experimental animal models clearly show the persistent and progressive nature of the changes caused by prenatal methylmercury exposure. By the end of the first year post exposure very little mercury is

left in the fetal tissues and it seems unlikely that the continued changes are caused by the mercury that remains in the brain. Two hypotheses are currently used to explain this phenomenon. A toxic injury to the brain usually results in modifications to the neighboring cells, known as a "setting-in" response to an earlier injury. Subtle changes in granule cells may be the cause of the progressive degeneration. Alternately, abnormal cell functions arise from methylmercury's ability to alter protein and nucleic acid synthesis, phospholipid and phosphoprotein biosynthesis, permeability of membranes to ions and nutrients, and production of energy within cells (Slotkin and Bartolome, 1987). Permanent injury to nucleic acids or specific metabolic pathways will seriously compromise a cell causing susceptibility to further insult and shortened life.

Selenium

The study of selenium presents a paradox. Selenium is both an essential trace element, required for the enzyme glutathione peroxidase (GSH-px) and other proteins, and a toxin (Hogberg and Alexander, 1986). Dietary levels below 0.03 ppm are considered deficient, levels of 0.1 ppm are physiologically essential, levels of 0.4 ppm are reported to cause liver lesions, and clinical toxicosis is caused by levels of 3 ppm or more (Marier and Jaworski, 1983; Wilber, 1983).

Selenium is a ubiquitous trace element, it occurs naturally in soils and water. In some areas the concentration of selenium in soil is very high, up to 4.0 ppm (Wilber, 1983). Some plants, like Astragalus, have the ability to concentrate soil selenium in leaves, stems and roots. Soil selenium can leach into ground water, some areas have reported up to 500 ug/L of selenium in drinking water, a level approximately 50 times above U.S. drinking water standards (Valentine et al., 1988). Selenium in water is concentrated as it moves through the food chain. Amounts in muscle tissues of freshwater and marine invertebrates have been measured at 167 and 1000 times greater than the respective water levels (Wilber, 1983).

Industrial uses of selenium include vulcanization of rubber, decoloration and dyeing of glass, insecticides, photocells, electrodes, and photographic chemicals. Sludges and sediments from copper refining contain high amounts of selenium (Shamberger, 1983). Selenium ore occurs with coal and selenium is released into the atmosphere with emissions from coal fired power plants. Some industrial exposures do occur, but they are not common (Marier and Jaworski, 1983).

Human exposures from inhalation, dermal contact, and ingestion have been reported (Marier and Jaworski, 1983). The incidence of exposure is not high. The main non-

industrial route of exposure is consumption of foods or water with high selenium levels, and cigar and cigarette smoke. Cereals, meat and fish products are the main sources of dietary selenium (Hogberg and Alexander, 1986).

Gastrointestinal absorption has been reported as high as 100%, but this amount varies in different reports depending on the form and source (Hogberg and Alexander, 1986; Marier and Jaworski, 1983). It is possible that more complete absorption occurs with dissolved selenium, and most laboratory animals consume more water than food and could, therefore, ingest a higher amount through water consumption (Marier and Jaworski, 1983). The form and oxidation state are important determinants in the body's ability to absorb selenium (Hogberg and Alexander, 1986).

Selenium is bound to serum albumin and transported throughout the body. Specific selenium binding proteins have been identified, and are believed to be important in transport of selenium in cells (Sani et al., 1988). In liver and muscle, selenium is associated with the smooth endoplasmic reticulum. There is no tendency for selenium to bioaccumulate in any specific tissue at normal dietary levels, selenium status reflects dietary and excretory patterns as well as dosage (Hogberg and Alexander, 1986; Marier and Jaworski, 1983). Rats fed a low selenium diet

were found to accumulate and retain selenium in reproductive organs, brain and thymus (Hogberg and Alexander, 1986). Under toxic conditions selenium accumulates in the hair and nails, these can be analyzed as indicators of selenium deficiency and toxicity (Marier and Jaworski, 1983). Ammar and Couri (1981) reported accumulation of selenium in the brain was highest in the cerebellum, followed by cerebrum and spinal cord. In a study by Danscher (1982), highest accumulations of selenium appeared in the cerebral cortex, hippocampus, caudate putamen, and dentate gyrus, with lower accumulations in the granule cell layer of the cerebellum. These studies used very different methods to determine sites of selenium accumulation, which may explain the differences in their results. Species differences, nutritional status of the animals, dose, form of selenium used, and dosage regimen may also affect results. Ultrastructurally, selenium accumulations in neurons are seen in the presynaptic boutons (Danscher, 1982).

Biotransformation occurs in the liver. The more highly toxic forms of selenium are methylated to essentially non-toxic dimethyl and trimethylselenide.

Approximately 80% of excreted selenium is found in urine, with some excretion in feces, exhaled air, and sweat (Hogberg and Alexander, 1986; Klaassen et al., 1986). Three

phases have been reported in human excretion of selenium, an initial rapid phase lasting one day, an intermediate phase lasting eight to 20 days, and a slow phase lasting from 65 to 116 days (Hogberg and Alexander, 1986).

Concern for selenium deficiency began after World War II. New techniques in farming and food processing depleted the amounts of selenium in some foods (Marier and Jaworski, 1983). Results of studies on selenium deficiency were confusing until Vitamin E was discovered as a required cofactor in processes involving selenium (Shamberger, 1983). Also confusing was the fact that deficiency and toxicity exhibited many of the same symptoms: hair loss, lameness, increased embryonic lethality, and necrosis of liver and myocardium (Marier and Jaworski, 1983).

Selenium toxicity Poisonings are reported in livestock that have consumed plants with high levels of selenium. Acute selenium poisoning is characterized by abnormal movements and posture, anorexia, diarrhea, increased temperature, and elevated pulse rate. Acute exposures often lead to death by respiratory failure. Chronic poisonings are termed alkali disease. Clinical signs are hair loss, deformation and shedding of hooves, loss of appetite and vitality, and emaciation. Liver cirrhosis may develop in more advanced cases (Hogberg and Alexander, 1986; Shamberger, 1983).

Clinical signs of acute exposure in humans are nervousness, irritability, somnolence, chills, tremors, gastrointestinal disturbances, lassitude, giddiness, depression, and headache (Ammar and Couri, 1981; Hogberg and Alexander, 1986). Chronic exposure causes hair loss, nail problems, skin lesions, depigmentation of skin, and gastrointestinal disturbances. Icterus and jaundice may be seen, which are indicative of the liver cirrhosis found in some chronically exposed populations (Hogberg and Alexander, 1986; Marier and Jaworski, 1983). Studies on human populations are fraught with many confounding factors.

During the 1960s in China an endemic selenium intoxication occurred among inhabitants of five villages within the same area. High selenium levels were found in the soil. The reported average daily intake of selenium was estimated to be 4.99 mg. All of the clinical signs noted above were observed, but varied in individuals and in villages. Forty-nine percent of the villagers died. Neurological symptoms were associated with gastrointestinal disturbances (Hogberg and Alexander, 1986).

A small group of women handling selenite during laboratory work were reported to have an increase in spontaneous abortions. Only one of five pregnancies resulted in a birth, and the infant had bilateral clubfoot

(Marier and Jaworski, 1983). Such reports have prompted claims of teratogenicity, however this data is difficult to interpret and the claims are controversial at best (Hogberg and Alexander, 1986; Marier and Jaworski, 1983). Chick embryos are extremely sensitive to excess selenium, but this effect is not reported in other agriculturally important species (Marier and Jaworski, 1983).

In a sparsely populated area of South Dakota where naturally occurring selenium toxicosis is endemic in farm animals, four cases of amyotrophic lateral sclerosis (ALS), a degenerative disease of peripheral axons, have occurred in unrelated males, living within a 15 km radius of each other. There was no family history of this disease for any of the victims. Blood and urine levels of selenium in the affected individuals were 1500% greater than the expected values for people living in nonseleniferous areas. The possible relationship between ALS and trace metals warrants examination of selenium as a possible environmental factor (Kilness and Hochberg, 1977).

Studies with experimental animals have focused on the role of selenium in reducing the toxicity of Group B transition elements, like mercury, silver, and cadmium, rather than on the toxic effects of selenium itself. The neurological nature of some symptoms found in livestock and

human exposures have led to some studies with experimental animals.

Ammar and Couri (1981) studied selenite and selenomethionine using intravenous (iv) and intracerebroventricular (icv) injections. Increased locomotion, hyperreflexia and jumping, followed by depression, decreased locomotion, ataxia and paralysis were consistently seen, but the extent and severity of symptoms were dependent on the dose, the site of injection, and form of selenium. Selenite given by icv was the most toxic and selenomethionine given by iv injection was the least toxic. A dose-dependent transient hypothermia was induced by injections of 10 to 60 umol/kg sodium selenite in male mice (Watanabe and Suzuki, 1986). Although the specific site and mode of action were not identified, the authors concluded the effects were due to the involvement of the afferent or integral part of the thermoregulatory system.

Decreased growth and decreased growth hormone levels were found in two groups of young rats given 3.0 ppm selenium ad libitum in drinking water on postpartum days 21 through 42, and 21 through 63, respectively (Thorlacius-ussing et al., 1988). After removal of selenium, growth resumed but never reached control rates, and growth hormone levels remained low.

Kasuya (1976) used cultured explants of rat cerebellum to test the protective effect of selenium in mercurial inhibition of neurite outgrowth. Selenium alone, as selenite and selenate, caused decreased outgrowth of neurites, vacuolar and degenerative changes in neurites, and/or complete inhibition of neurite growth. The effects were dose dependent.

Biological functions of selenium are still not well understood. The GSH-px system protects the cell from oxidative damage by reducing lipoperoxides and hydrogen peroxide in the cytoplasm. This system requires Vitamin E as a cofactor, which is sequestered in cell membranes. It is theorized that Vitamin E inhibits the formation of peroxides and lipoperoxides, and GSH-px destroys these compounds once they form (Marier and Jaworski, 1983). Vitamin E requires Vitamin C for reactivation. Increased selenium is believed to decrease Vitamin C levels. Clearly this system is very complex and body levels of selenium are critical to its correct function (Marier and Jaworski, 1983).

Possible roles for selenium involve maintenance of permeability and integrity of cell membranes (Marier and Jaworski, 1983), and stabilization of cell membranes (Kasuya, 1976). Selenium is known to be involved in ageing,

probably through antioxidant properties of GSH-px. Chronic toxic exposures, and dietary deficiencies of selenium have both been shown to decrease GSH-px (Valentine et al., 1988). This would explain why symptoms of toxicity and deficiency are very similar. It is interesting to note that methylmercury also decreases levels of GSH-px (Hirota et al., 1980). It seems likely this dual effect involves one particular pathway or mechanism. Current evidence suggests this involves the integrity of cellular membranes (Marier and Jaworski, 1983).

Interactions of mercury and selenium

The report of selenium's protective effect against methylmercury toxicity by Ganther et al. (1972) has generated a great deal of interest and study on the interactions of mercury and selenium. Since the initial report, the protective effects of selenium against methylmercury toxicity in adults have been well documented (Chang, 1982).

Selenium supplements given to expectant mice and rats have been shown to inhibit, or delay, methylmercury toxicity in the offspring. Nishikido et al. (1987) tested groups of selenium deficient and selenium supplemented maternal mice. Selenium administration did not appear to influence mercury tissue levels, however, mercury increased selenium levels in

fetal liver and decreased GSH-px activity. The highest doses of mercury, 25 and 35 umoles/kg/day, increased fetal lethality in all mothers, but a significant increase in fetal death was found in the selenium deficient mothers. Maternal dietary selenium levels were 0, 0.1, 0.2, and 0.4 ppm. However, the formation of cleft-palate by injection of 75 umoles/kg methylmercury was not affected by concurrent administration of 0.4 ppm selenium (Nishikido et al., 1988). Satoh et al. (1985) studied righting reflexes in rats exposed prenatally, via subcutaneous injection on day nine of gestation, to 30 umol/kg methylmercury chloride with and without sodium selenite. Their behavioral study of the development of righting reflexes revealed that the mercury/selenium group showed significantly improved development compared to the mercury only group. An unexpected outcome of the study was the low birth weight in the mercury/selenium and selenium groups. The brain weights of the mercury/selenium group were significantly less than the control group on day one postpartum. Possible adverse effects of sodium selenite exposure on prenatal development were noted (Satoh et al., 1985).

Selenium toxicity was noted in a study by Kasuya (1976). Cerebellar explants were exposed to various levels and combinations of methylmercury and sodium selenite to

determine the effects on the growth and development of neurites. At the lowest level of selenium (1.0×10^{-5} M) a protective effect was noted. A small increase in the level of selenium (1.5×10^{-5} M) resulted in an additive toxic effect with mercury.

Selenium causes changes in the distribution of mercury. Mercury concentration was increased in the cerebrum within the first five minutes of mercury/selenium administration. A transient increase was also reported in liver, followed by a decline. Decreases in mercury concentration were found in kidney, blood, plasma and erythrocytes (Thomas and Smith, 1984). Decreases in blood concentrations of mercury with corresponding increases in brain levels of mercury and selenium have been reported by Magos and Webb (1977). Since mercury is proposed to enter the brain from the plasma pool a possible inhibition, by selenium, of entry of mercury into red blood cells could increase mercury in plasma, making it more readily available for brain uptake (Thomas and Smith, 1984). Selenium administration increases concentrations of mercury in brain and decreases mercury in liver and kidney while it protects against mercury toxicity (Chen et al., 1975; Masukawa et al., 1982).

Selenium affects the distribution of mercury between mother and fetus (Iijima et al., 1978). Pregnant mice were

injected with 0.67 mg/kg radioactive mercury, on day 15 of gestation, with and without simultaneous injection of 0.20 mg/kg nonradioactive selenium. Selenium significantly increased the concentration of methylmercury in the maternal brain while decreasing the time to reach maximum concentration. In maternal blood and in the fetus, selenium decreased the concentration of mercury. The experiment was reversed using radioactive selenium with and without nonradioactive mercury, doses were the same as those given in the initial experiment. Mercury resulted in increased levels of selenium in maternal brain and in the fetus. Similar findings have been reported by Yonemoto et al. (1983). Even in toxic exposures, selenium is found in low levels in brain tissue, it may possibly be excluded from entry by the blood brain barrier. It was proposed by Ohi et al. (1976) that damage to the blood brain barrier caused by methylmercury allows selenite selenium to accumulate in the brain and results in interactions that reduce mercury neurotoxicity.

Satoh and Suzuki (1979) also reported mercury retention in maternal brain with selenium administration. Their results indicated an increase in mercury concentrations in fetal brain tissue, but not in whole fetuses.

A possible mechanism of selenium protection is the biotransformation of mercury into bis(methylmercuric) selenide (BMS). BMS concentrations were analyzed in liver, kidney and brain. Accumulations as a percentage of total mercury were 30%, 23%, and 8%, respectively (Masukawa et al., 1982). Naganuma et al. (1983) used autoradiography to study distribution of mercury in brain tissue of male mice after treatment with 1.5 umol/kg of methylmercury alone, with 0.75 umol/kg selenite simultaneously, or 0.75 umol/kg of BMS alone. Within 10 minutes after iv administration of the compounds, BMS and methylmercury coadministered with selenite were detected. The levels of these two groups remained the same over the 24 hour period of the study. Methylmercury given alone was not detected at 10 minutes, only after 24 hours did the level of methylmercury reach the level that BMS and methylmercury/selenite attained within 10 minutes and maintained over the 24 hours of the experiment. BMS is probably not directly responsible for the protective effects of selenium since it forms and decays rapidly. It is believed that BMS may represent a diffusible form in selenium directed redistribution of mercury (Iwata et al., 1982).

Iwata et al. (1981) found that methylmercury bound to albumin could be converted, in the presence of selenite, to

a benzene-extractable form in rat liver, kidney and brain homogenates. The conversion reaction required GSH and/or protein sulfhydryl groups, the benzene soluble mercury fraction was predominately BMS.

Chen et al. (1975) studied the effect of selenium in the subcellular distribution and protein binding of methylmercury. Two groups of male rats were given subcutaneous injections of 0.01 mmol methylmercury chloride/kg body weight. One of the groups had received 0.01 mmol sodium selenite/kg at a different subcutaneous site 30 minutes prior to mercury administration. Tissues were examined one hour after the mercury injection. In kidney and liver 40 to 50% of tissue mercury was found in the soluble fraction (cytosol) associated with low molecular weight compounds when selenite was administered. This low molecular weight fraction possibly represents GSH. In kidney the level of mercury found with selenite treatment was approximately one-half the level without selenite treatment. In kidney fractions exposed to methylmercury alone, the mercury was associated with intermediate molecular weight fraction. This change could be due to the decreased level of mercury in kidney. In contrast, levels of mercury in brain tissue increased five-fold with selenite administration. Sixty-five percent of the mercury in brain

tissue was associated with the crude nuclear fraction of homogenates. This fraction contains the nuclei and cell membranes. Selenium increased mercury concentration in the brain but did not change its subcellular distribution.

Aggregates of electron-dense granules, positive for mercury, selenium, and sulfur were identified in lysosomes of renal tubule cells with X-ray microanalysis (Aoi et al., 1985). The ratio of selenium to mercury in these aggregates ranged from 0 to 2.9. The deposition of selenium and mercury in these lysosomes probably occurs as a complex with mercury bound to selenium, to sulfur (Hirayama, 1985), or to sulfur residues on selenoproteins (Chen et al., 1975).

Methylmercury is also biotransformed into elemental mercury. In vitro studies of methylmercury degradation showed that selenite and reduced GSH increased carbon-mercury bond cleavage and the release of inorganic mercury (Iwata et al., 1982). It has been suggested that the degradation reaction may proceed through the intermediate BMS.

Care must be used in interpreting the <u>in vitro</u> effects of mercury studied using homogenates. Mercury can reversibly bind to sulfhydryl groups, therefore, homogenization can cause a redistribution of mercury among cellular fractions and among proteins within those fractions (Clarkson, 1972; Marier and Jaworski, 1983).

Selenium is reported to cause a decrease in methylmercury excretion in feces and urine (Komsta-Szumska and Miller, 1984). A comparison of methylmercuric chloride alone or with equimolar doses of sodium selenite found that one day after the last dose, selenium decreased total body levels of mercury. Past the first day, selenium reduced the clearance rate of methylmercury. Mercury given concurrently with selenium behaved according to a two-compartment model, with an initial rapid clearing followed by a slower, delayed clearing. Mercury alone fits a single compartment model for body clearance (Konsta-Szumska and Miller, 1984). This study found that though most of the mercury remained in organic form, selenium increased the relative amounts of inorganic mercury in liver, spleen, pancreas and intestine. Kidney reacted differently, 70% of the total mercury in the kidneys was in the inorganic form.

Selenium present in foods is in a biological form, closely related to selenomethionine and selenocysteine. Studies comparing biological selenium and selenite have shown that selenite is significantly more effective in counteracting mercury toxicity than are the biological forms of selenium (Magos et al., 1984; Ohi et al., 1976). However, not all toxic effects were counteracted in the same manner by the different forms of selenium. Ohi et al. (1976), using 20 ppm mercury in food, found protective

effects from selenite and from selenium present in tuna (selenium levels were equilibrated at 0.5, 1.0, and 1.5 ppm). Effectiveness was approximately equal for both forms in maintaining growth rate, but biological selenium was only one-half as effective in preventing neurological disturbances such as hind limb paralysis.

Studies on the protective effects of selenite in methylmercury intoxication may overestimate the protective effects of biological forms of dietary selenium (Magos et al., 1984; Ohi et al., 1976).

MATERIALS AND METHODS

Animals and diet

Male and female Fisher 344 rats, approximately eight weeks old at the start of the project, were purchased from Harlan Sprague-Dawley, Madison, WI. Males were used for breeding purposes only and were not exposed to mercury or selenium. Rats were housed in clear plastic tubs with sawdust bedding. Just before birth the sawdust was replaced with mink bedding. All rats were given food and water ad libitum. The animals were fed Teklad Laboratory Animal Chow, but the female's food was ground. To monitor consumption, food and water were weighed daily until the pups were weaned. A reversed 12 hour day:night cycle was used. Night hours began at 9:00 and terminated at 21:00 hours. Red lighting was used in the colony room during the night cycle.

Chemicals

Methylmercury hydroxide (CH_3HgOH), 1 M in H_2O, and Sodium Selenite (Na_2SeO_3) were purchased from Alpha Products, St. Louis, MO.

Dosage

In keeping with the objectives of this study, a dosage level was needed that would produce subtle, not overt,

differences in the offspring and produce no toxic symptoms in the mothers. Data by Spyker et al. (1972) showed that intraperitoneal injection of 8 mg/kg body weight on days seven or nine of gestation resulted in subtle behavioral changes in the offspring of treated mice. No signs of toxicity were noted in pregnant females, and no differences were observed between treated and control mothers in rearing or the young. There were no overt signs of mercury toxicity in the offspring.

Hughes and Annau (1976) treated maternal mice with 0.0, 1.0, 2.0, 3.0, 5.0, and 10.0 mg/kg of methylmercury hydroxide by peroral injection on day eight of gestation. Litter size was decreased in groups receiving doses of 3.0 mg/kg and higher, birth weight was decreased in the 10.0 mg/kg group only, but weight gain was decreased at and above 3.0 mg/kg. The severity of these changes was dose dependent. Behavioral testing of the groups showed increases in the number of trials and decreases in the of avoidances in the 3.0 and 5.0 mg/kg groups, the 10.0 mg/kg group was not used in the behavioral tests due to the small litter sizes.

Data from Chang et al. (1977) revealed ultrastructural changes in neurons from Sprague-Dawley rats injected with 4 mg/kg methylmercury on the eighth day of gestation,

although, behavior was not examined in their study which makes comparison with Spyker et al. (1972) and Hughes and Annau (1976) difficult. Reuhl et al. (1981a) reported changes at the light microscopic level in hamsters injected with 10 mg/kg on day 10 of gestation or with 2 mg/kg on days 10 through 15. By comparison, Kutscher et al. (1985) observed no changes with light microscopic examination in rats exposed by gavage to 6 mg/kg of methylmercury on days six through nine of gestation.

According to Klaassen et al. (1986), route of exposure can influence toxicity. Intravenous injections produce the most rapid response followed by intraperitoneal, subcutaneous, intramuscular, and, finally, oral exposure. Agents given orally enter the portal circulation and are acted on by the liver, and so they are less effective than when given via the systemic circulation (intravenous injection). Mercury is biotransformed and accumulates in the liver, therefore, different results are expected depending on the route of exposure to mercury.

Spyker and Smithberg (1972) reported differences in reaction to methylmercury exposure between two strains of mice. Logically, these differences exist between species and between strains within other species. Contrasting data are reported in studies that use different species or

strains, different doses, different routes of exposure, expose at different gestation ages, examine at different times with respect to exposure, and give single versus multiple doses.

Both behavioral and ultrastructural studies were planned, therefore, a dose was needed to assure alterations in behavior and ultrastructure, but not cause overt toxicity in the maternal rats. The dose used by Spyker et al. (1972) produced behavioral changes in the offspring, no overt toxicity in the mothers, and was clearly sufficient to affect ultrastructure, as reported by Chang et al. (1977). Based on evidence reported above, the dose level for this study was set at 8 mg mercury/kg body weight to be administered by gavage.

Breeding

Females were allowed 10 days to adjust to their new environment. Just before lights-out, females were checked for estrus by swabbing, using the criteria outlined in Neubert et al. (1977). Those females determined to be in estrus or proestrus were placed with a male and allowed to breed freely for approximately ten hours. At the end of the breeding period vaginal smears were made to check for sperm (Neubert et al., 1977). When sperm were found this was designated as day 0 of gestation. If no sperm were found

females were rechecked after 12 hours. Females that did not have a positive recheck were bred again when their morning cytology slide indicated they were in estrus. Dams were allowed to give birth naturally. The day of birth was counted as day 0 postpartum. All pups were weaned on day 21 postpartum.

The rat pups were used in two experiments, the morphometric study and a separate behavioral study. When possible, one male and one female pup from each mother were used for each analysis in the morphometric study, with a total of ten pups from each group for each analysis. In some cases litter size determined the number of pups available per mother, not all mothers were represented in every analysis.

Treatment

The maternal animals were the experimental units. Four treatment groups were used: control, mercury only, selenium only, and mercury/selenium in combination. Selenium was administered in drinking water at a concentration of 1 ppm in Type I water. The females in the selenium and mercury/selenium groups were given treated water beginning the first day after their arrival. Ten days later they were started in the breeding program. The selenium exposure continued through the gestational period until the day of

birth. Mercury and control group rats were given Type I water. All maternal animals in each treatment group were given tap water from the day of birth through weaning.

On day 10 of gestation a single dose of methylmercury hydroxide was administered, by gavage, at the rate of 8 mg of mercury/kg body weight. The controls and the selenium only rats were gavaged on day 10 of gestation with 0.85% sodium chloride, given in equivalent volume. Ten litters were born to mothers in the control group, 10 litters in the mercury only group, nine litters in the mercury/selenium group, and nine litters in the selenium only group.

Collection of tissues

Tissues for chemical analysis Brains, livers, and kidneys were collected for chemical analysis. Zero-day-old pups were chosen at random and decapitated. Tissues were removed and immediately frozen. All chemical analyses were performed at the Veterinary Diagnostic Laboratory, Iowa State University, Ames, Iowa, using cold vapor flameless atomic absorption spectrophotometry according to the methods of Stahr (1980). All tissues were analyzed for mercury content, but due to small sample size only the livers were analyzed for selenium content.

Tissues for ultrastructural analysis Brain tissues were collected from pups on days 0 and 21 postpartum. Zero-

day-old animals were randomly selected, decapitated, brains were sagittally sectioned along the midline, and immersed in cold (4° C) Karnovsky's fixative (2.0% glutaraldehyde, 2.5% paraformaldehyde, in phosphate buffer, pH=7.4), for 48 hours. The anatomical areas for tissue sectioning were determined using criteria described in Jacobson (1982), Larsel (1970), and Paxinos and Watson (1982). The tuber vermis of the cerebellum was selectively removed from the brain and cut into 1 mm pieces with a sharp blade. The small size of these brains and early developmental stage of the animals produced good fixation with immersion.

Twenty-one-day-old animals were selected at random, anesthetized by intramuscular injection with 8 mg/kg body weight of Ketamine HCl. Fluothane was administered through a nose cone while animals were prepared for cardiac perfusion with cold Karnovsky's fixative. The cerebellar tuber vermis was selectively removed from the brain and cut into 1 mm sections with a sharp blade.

Processing for electron microscopic visualization followed the method of Chang and Hartmann (1972a). Thick and thin sections were cut on a Reichert Ultracut E. Due to the small size of the rat brains on day 21 and, especially on day 0, only one or two tissue blocks were available per pup. Thick sections were stained with 1.0% Toluidine Blue O in 1.0% sodium borate and checked for proper orientation.

Thin sections were cut with a diamond knife to between 70 and 90 nm, collected on 200 mesh copper grids, and stained in 2% methanolic uranyl acetate and Reynold's lead citrate. Thin sections were viewed with an Hitachi HUllC Electron Microscope at 50 KV.

Morphometric analysis

The Delesse Principle is the fundamental basis of stereology (Weibel, 1979). It states that the densities of the components making up a structure can be estimated in a random section of the structure by measuring the relative areas of their profiles (areal density) on the section.

In this experiment we define the granule cell as the structure (qualifier=nucleus, only cell profiles containing a nucleus were photographed). The component of interest is the rough endoplasmic reticulum (also called the phase).

All photomicrography and morphometric analyses were performed using numbers randomly assigned to individual rat pups. Therefore all data was obtained "blind" to eliminate experimentor bias. A random section was taken from the external granule cell layer of the tuber vermis of 0-day-old animals and from the internal granule cell layer of the tuber vermis of 21-day-old animals. In the preferred method of randomization, these sections were to be placed on copper coordination grids (3 mm electron microscopy grids, with

numbers and letters designating the grid spaces). Using random number tables, numbers relating to the grid spaces were to be chosen and neurons in these spaces were to be photographed. Unfortunately the specimen holder on the Hitachi HUllC was not large enough to allow the numbers and letters of the coordination grids to be viewed and an alternate method was used to assure random sampling of tissue. Any particular section contains some perfect grid "windows" and others that are unusable. The percentage of perfect windows for photomicrography is very low (probably between 1% and 5%), good grid spaces will occur randomly on each grid. In order to obtain a random sample of cells sections were viewed starting at the top right hand corner. Grids were searched from right to left and top to bottom until the first "perfect" window was found. Photographs were taken starting at this window. The same search pattern was repeated until thirty-four micrographs were shot for each tissue section. The total numbers of cells analyzed were 1045 for 0-day-old animals and 988 for 21-day-old animals.

Equations The membranous boundary of the ER per unit area, B_a, is related to the lengths of the ER profiles, P_l, divided by the total surface area of the cell, S_a:

$$B_a = P_l / S_a. \qquad (1)$$

The surface boundary of the ER per unit volume, SB, is related to boundary per unit area, B_a, by the ratio of $4/\pi$:

$$SB=4B_a/\pi \qquad (2)$$

The average length of the ER profiles per unit volume, L_{avg}, can be calculated by dividing the surface boundary per unit volume, SB, by the number of profile counts, P_c:

$$L_{avg}=SB/P_c. \qquad (3)$$

The values from equations (2) and (3) for each cell were used for statistical evaluation.

Negatives of the photomicrographs were placed on a light table and a piece of acetate was fastened over them. Using a jeweler's eyepiece, the outline of the cell and the individual profiles of ER were traced onto the acetate. These acetate sheets were analyzed using a Zeiss SEM-IPS Image Processing System manufactured by Kontron Bildanalyse GmbH, Eching, West Germany, and utilizing their IBAS Interactive Automatic Image Analysis System software. The field area of the cell, the perimeter of the ER, and the number of ER profiles per cell were measured.

A one-way analysis of variance was performed using the Statistical Analysis System from the SAS Institute Inc., Cary, NC. The maternal rats were the experimental unit,

treatments were the independent variable, and SB and average length of ER were the dependent variables. Comparisons were made using Tukey's Studentized Range Test and the Least Squares Difference T Test, α =.05 was chosen as the minimum acceptable level of significance for morphometric analysis.

In conventional methods of stereology a measurement grid with parallel bars in both directions is placed over the micrograph and counts are made at each spot where the component of interest (ER) touches one of the bars. In order to decrease the variance the number of grid bars can be increased. The use of the image-analysis computer will, theoretically, increase the number of grid bars toward infinity and the variability measured will largely be due to biological variation and any variation due to the treatments. Use of the computer for measurement lessens any bias due to tissue orientation. The brain is considered anisotropic tissue (it has a definite pattern of organization). In conventional point count methods this would introduce a large degree of bias. The use of a computer for measuring purposes significantly minimizes, but does not completely eliminate, this bias.

RESULTS

Mothers

No clinical signs were observed in mothers exposed to mercury, selenium, or mercury/selenium. The initial weight of the mothers was 139.6 ± 7.6 grams. There were no differences in initial weight or in weight gain between treatment groups (see Figure 1). Differences were not found in food or water consumption for mothers in different treatment groups (see Figures 2 and 3). The average daily dose of selenium for mothers in the mercury/selenium and selenium treatment groups was 19.67 ± 1.09 ugm. The average total consumption of selenium over the breeding and gestation periods was 690.4 ugm for the selenium only group, and 694.4 ugm for the mercury/selenium group, (± 43.6 ugm). There was some variation in total consumption due to differences in the time until breeding was positively confirmed. Differences in selenium consumption were not significant. The average time to complete the breeding of the entire colony was 4.3 ± 2.6 days. The mean time needed to breed each group was: control 5 ± 4 days; mercury 4.8 ± 2.6 days; mercury/selenium 4.3 ± 2.6 days; and selenium 4.1 ± 2.2 days. Of the initial forty-eight animals there were no false negatives (females that did not test positive for sperm but did produced a litter), there were 5 false

positives (females that tested positive for sperm but did not produce a litter), and 5 females that failed to mate.

Pups

No significant differences were found in litter size, birth weight, or weaning weight between treatments, except that pups in the mercury only group weighed more at birth (see Table 1). No congenital birth defects resulted from the mercury and/or selenium treatments. Observation of the pups from birth to weaning did not reveal any differences in development. Pups from the selenium only group were observed to be more hyperactive than those in the other three groups.

Chemical analyses: mercury and selenium

Significant differences were found in the mercury and selenium tissue levels. Mercury was present in newborn pups from both the mercury and the mercury/selenium groups, but levels of mercury were significantly lower, in all three tissues (liver, brain, and kidney), when selenium was administered (see Figure 4).

Selenium levels were significantly affected by mercury exposure, the liver level of selenium in the mercury/selenium pups were 2.6 ± 0.2 ppm, which differed from the values of 0.6 ± 0.2, 0.9 ± 0.2, and 0.9 ± 0.1 ppm

FIGURE 1. Maternal weight gain (grams)

Week 1 represents initial weights, error bars were not included due to excessive overlapping, differences were not significant

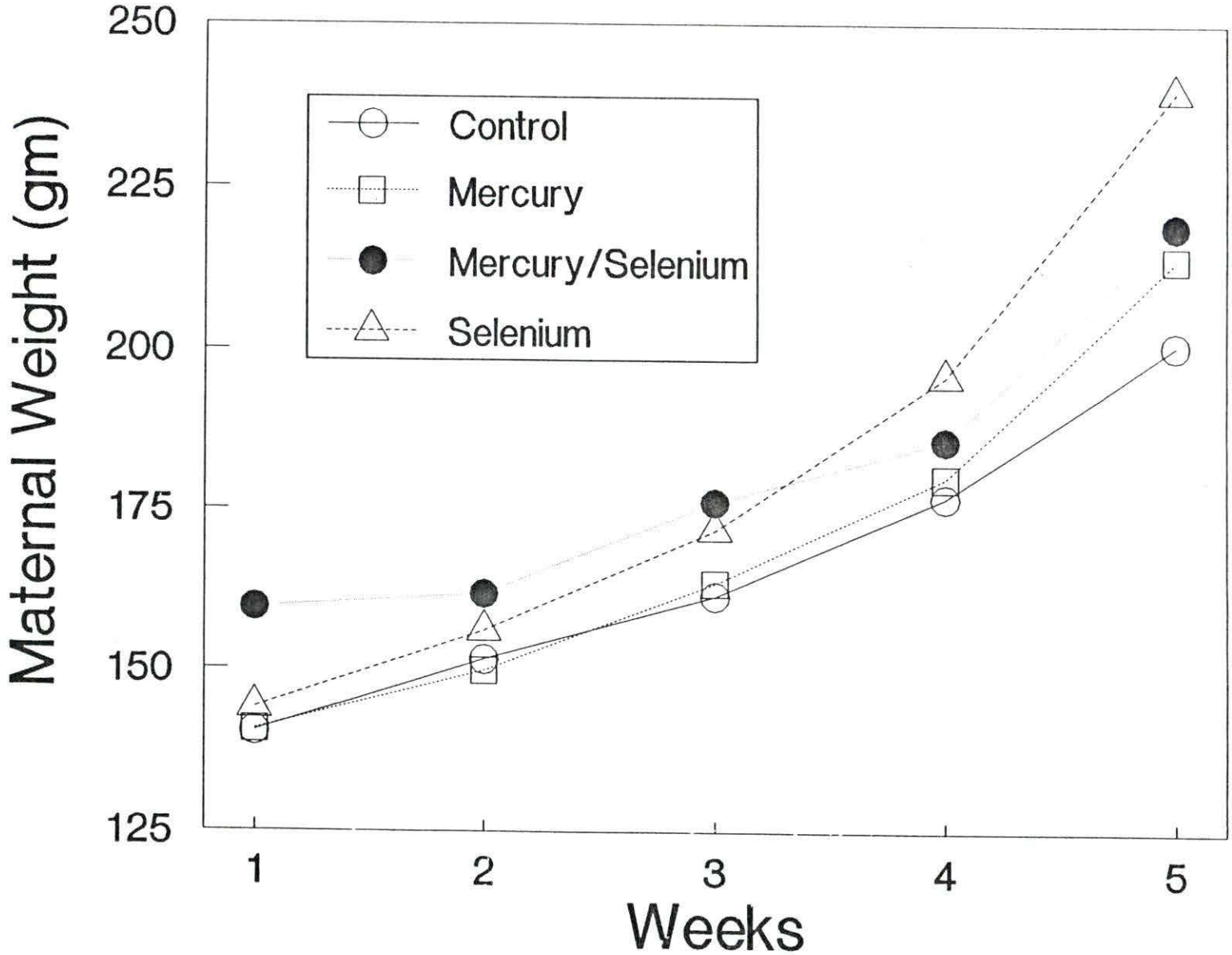

Maternal Weight (gm)
250
225
200
175
150
125
Control
Mercury
Mercury/Selenium
Selenium
1
2
3
4
5
Weeks

FIGURE 2. Mean daily food consumption, maternal rats (grams)

Error bars were not included due to excessive overlapping, differences were not significant

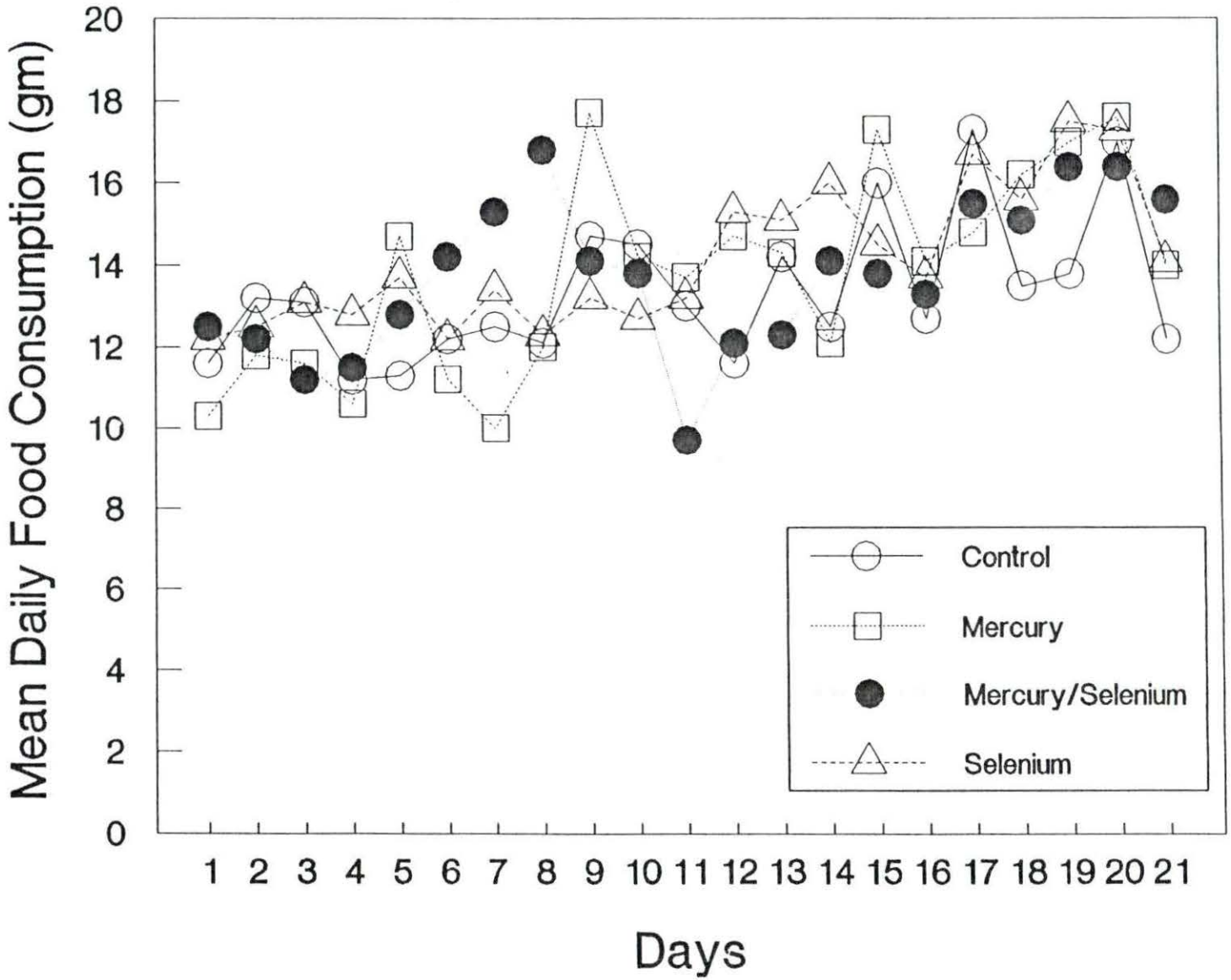

Mean Daily Food Consumption (gm)
0
2
4
6
8
10
12
14
16
18
20
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Days
Control
Mercury
Mercury/Selenium
Selenium

FIGURE 3. Mean daily water consumption, maternal rats (grams)

Error bars were not included due to excessive overlapping, differences were not significant

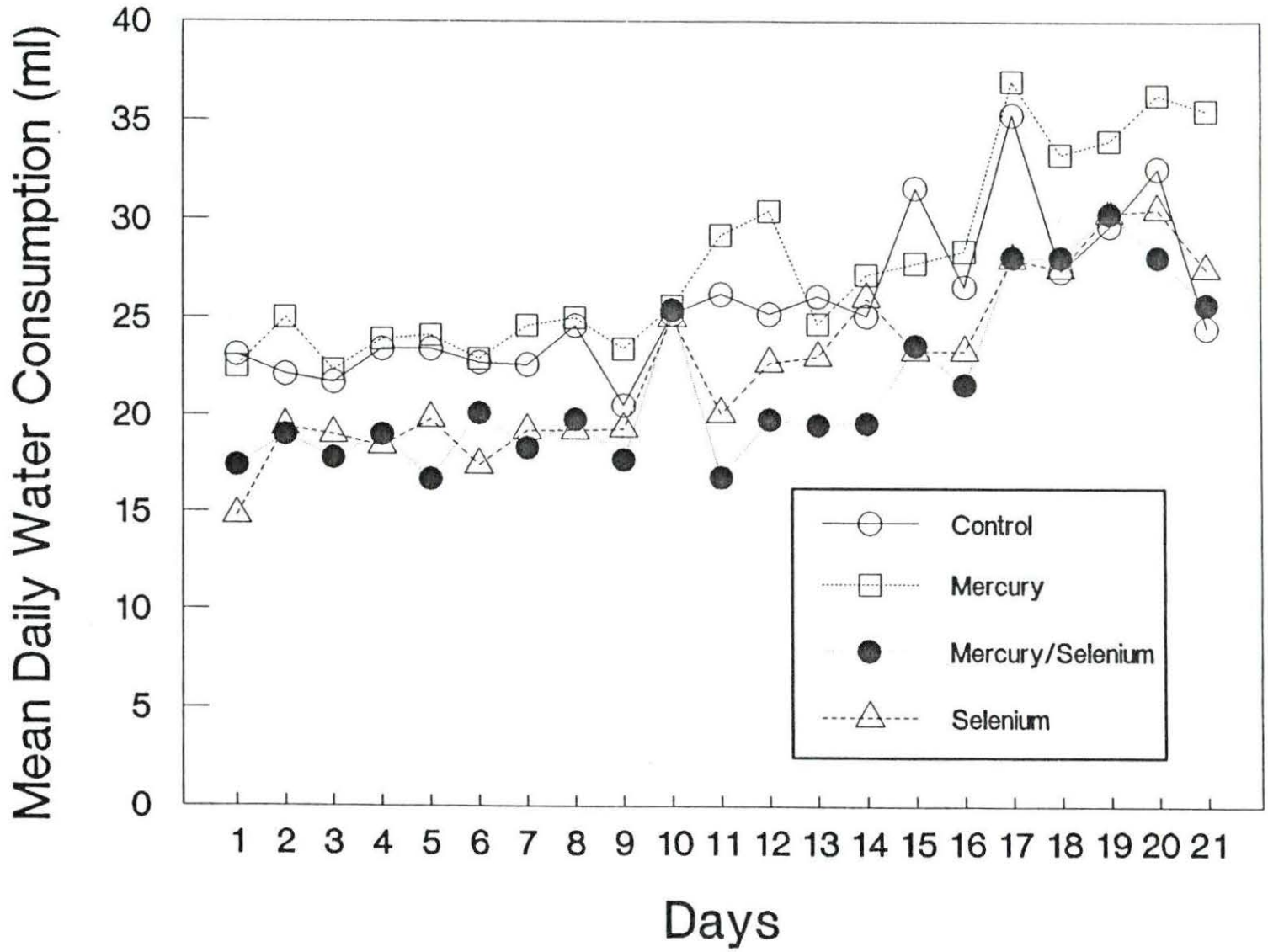

Mean Daily Water Consumption (ml)
40
35
30
25
20
15
10
5
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Days
Control
Mercury
Mercury/Selenium
Selenium

TABLE 1. Mean pup weights (grams)

Treatments	N	Birth Weight	Weaning Weight
Control	89	4.86 ±0.04[a]	33.38 ±0.58
Mercury	87	5.38* ±0.04	35.12 ±0.74
Mercury/Selenium	90	5.18 ±0.05	36.48 ±0.54
Selenium	81	5.21 ±0.04	36.08 ±0.73

[a]Standard Error of the Mean.

*Significantly different than control values, $p < 0.05$.

in pups from the control, mercury, and selenium groups respectively (see Figure 5).

Ultrastructure

Among neurons from the internal granule cell layer of the cerebellar tuber vermis of 21 day-old pups some degenerative changes were observed in the mercury only group: myelin figures, cytosegresomes, and large lysosomes were found (see Figure 6). These changes were not observed in the other treatment groups. Neurons representative of those used for the morphometric analysis of the 21-day-old animals are shown in Figure 7.

FIGURE 4. Mean mercury concentrations (ppm), 0-day-old rats

Values for liver, kidney, and brain are shown. Levels in the control and selenium only groups were below detectable limits. Values were significantly different among groups, $p < 0.0001$

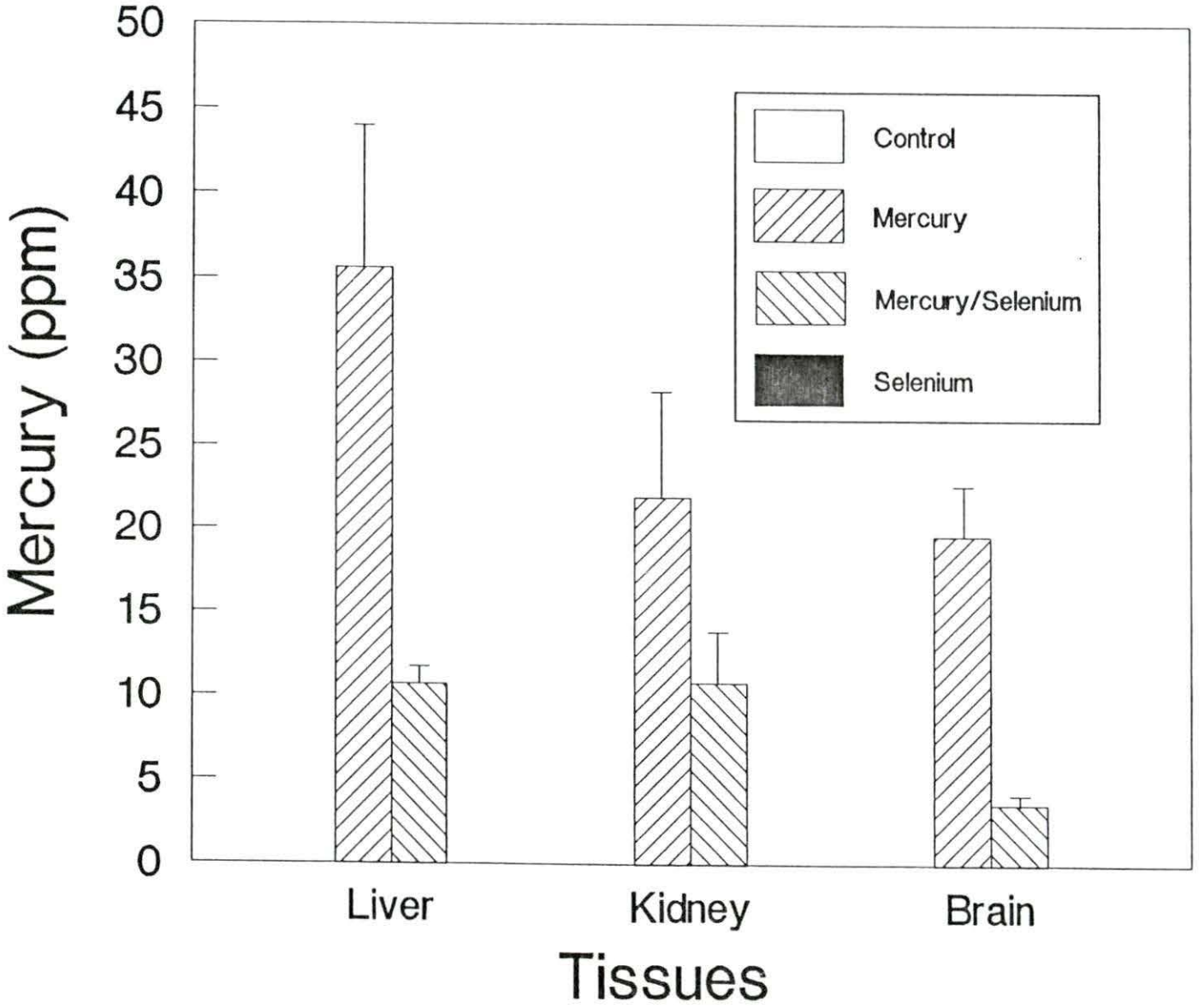

50
45
40
35
30
25
20
15
10
5
0
Mercury (ppm)
Control
Mercury
Mercury/Selenium
Selenium
Liver
Kidney
Brain
Tissues

FIGURE 5. Mean selenium concentrations (ppm), 0-day-old rats

Values for liver are shown. Levels in the mercury/selenium group were significantly different from all other treatment groups, $p < 0.0001$

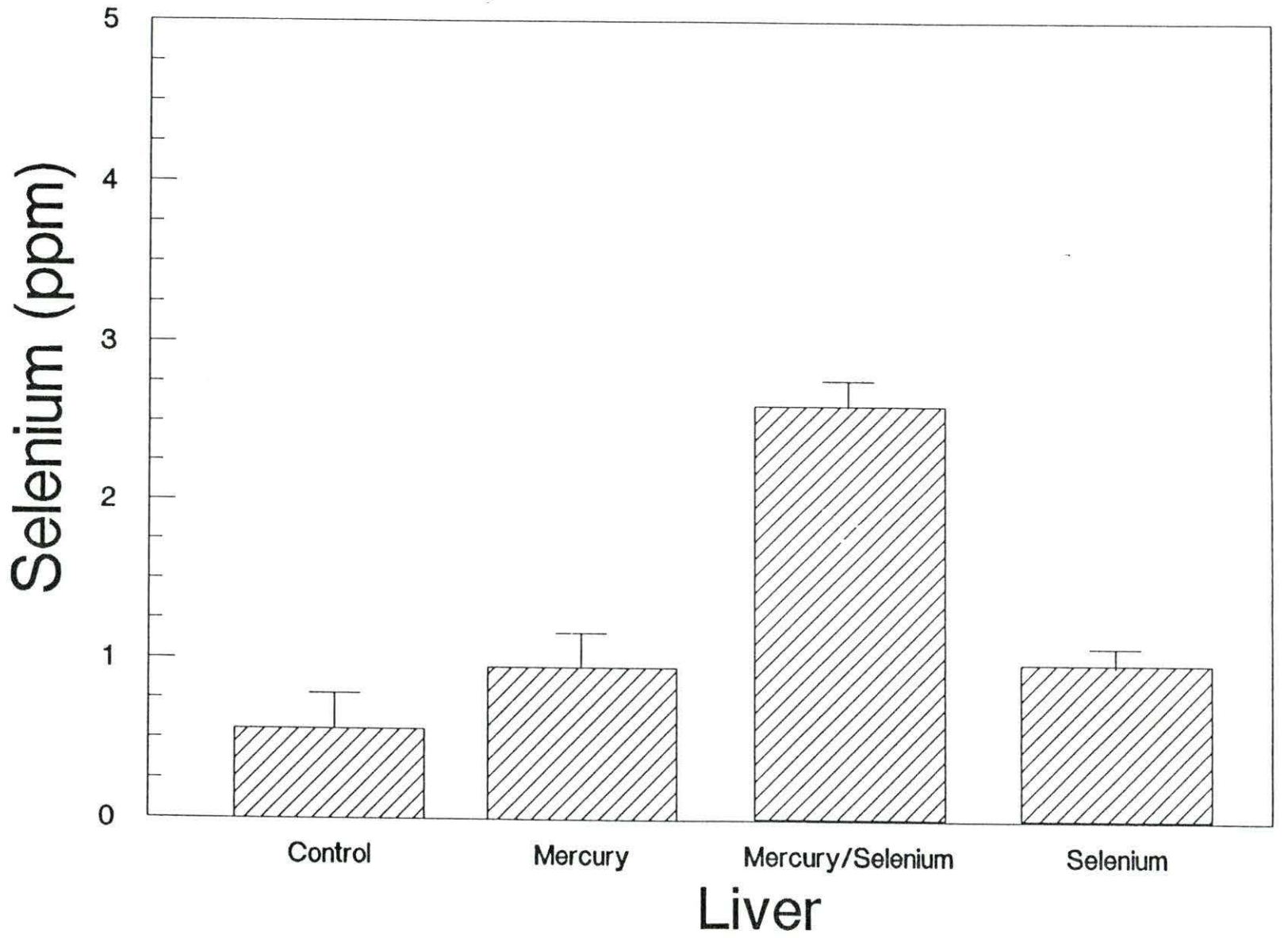

Selenium (ppm)
5
4
3
2
1
0
Control
Mercury
Mercury/Selenium
Selenium
Liver

FIGURE 6. Cerebellar granule cells, degenerative changes

21-day-old rats, A. and B. Myelin figures (arrows) at the nuclear membrane (N) (x41,900) C. Cytosegrosome (arrows) in the perikaraya (x41,300) D. Lysosome (L) (x35,800) E. Lysosome (L) (x47,600)

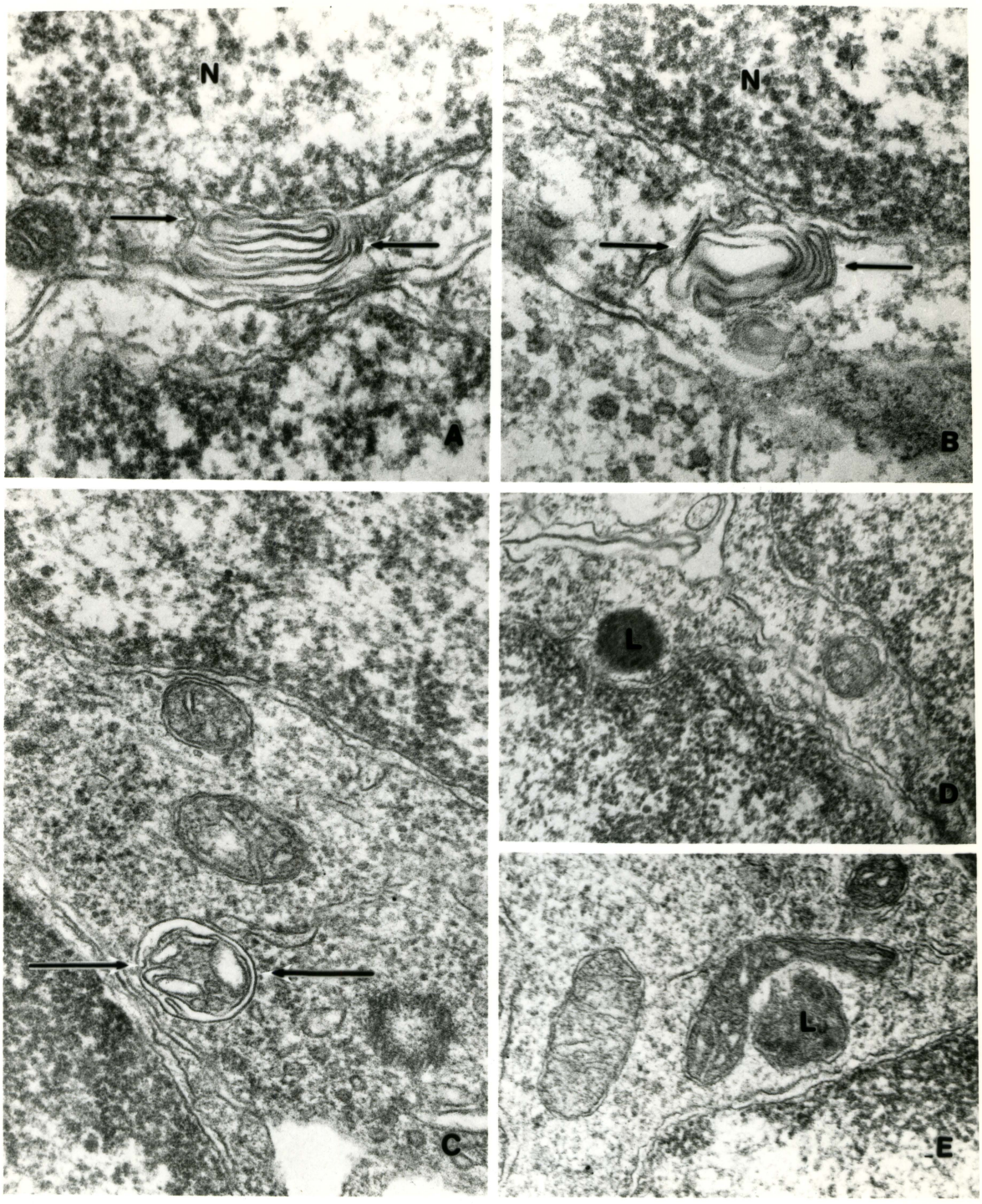
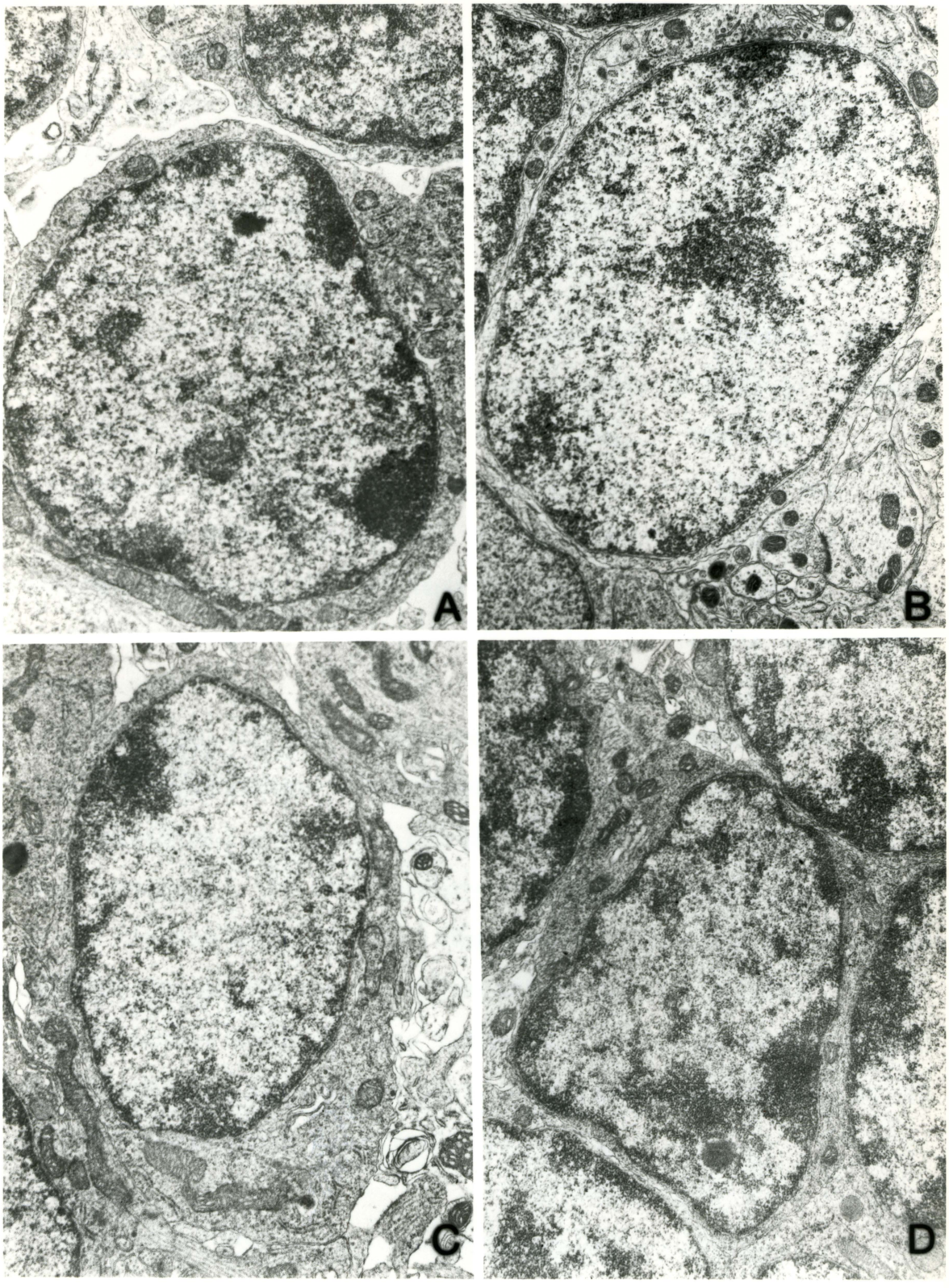

N
A
N
B
C
L
D
L
E

FIGURE 7. Cerebellar granule cells, 21-day-old rats

Examples of cells used for morphometric analysis.
A. Control B. Mercury C. Mercury/Selenium
D. Selenium (x12,760)


A
B
C
D

Degenerative changes in ultrastructure were not observed among neurons from the external granule cell layer of the tuber vermis of 0-day-old rats in any of the treatment groups. Neurons representative of those used for the morphometric analysis are shown in Figure 8.

Morphometric analysis

Analysis of tracings from 0-day-old rats showed no significant differences in surface boundary (SB) or average length of the endoplasmic reticulum (L_{avg}) between treatments (see Table 2). However, when the data was analyzed by sex, significant differences were found in average length of ER for females, $p < 0.0017$. No significant differences were found in the SB for 0-day-old female rats.

The L_{avg} in the mercury/selenium treated females was significantly longer than females treated with mercury or with selenium, $p < 0.05$, but did not differ significantly from controls. In the mercury treated females L_{avg} was significantly shorter than the control group, $p < 0.05$. The control group and the selenium group did not differ significantly in L_{avg} (see Table 3). Interestingly, in 0-day-old male rats there were no differences in L_{avg} or SB between treatments (see Table 4).

FIGURE 8. Cerebellar granule cells, 0-day-old rats

Examples of cells used for morphometric analysis.
A. Control B. Mercury C. Mercury/Selenium
D. Selenium (x13,400)

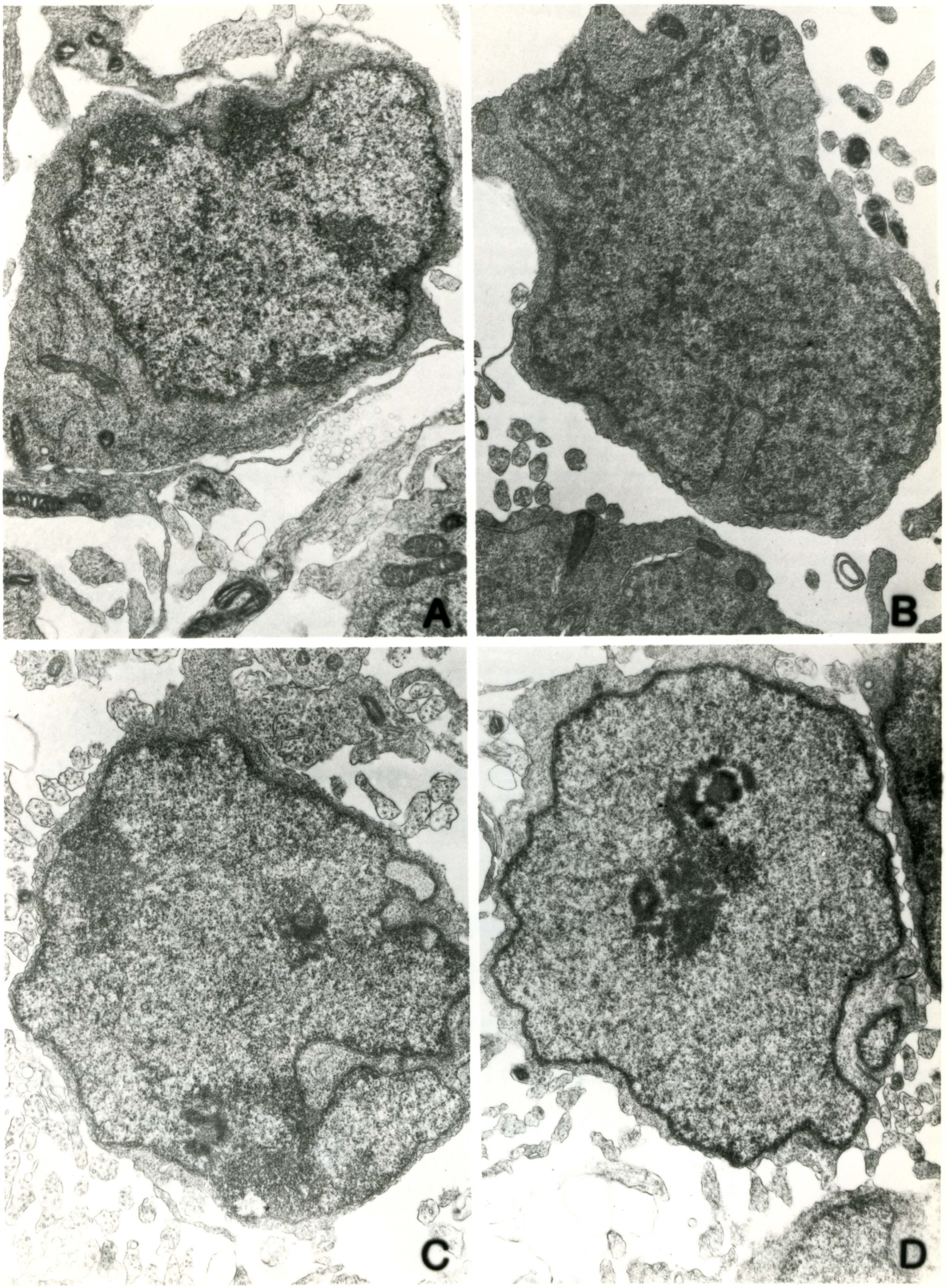

A
B
C
D

TABLE 2. Mean values of SB and L_{avg}: 0-day-old rats, both sexes (micrometers)

Treatments	N	SB	L_{avg}
Control	260	0.203 ±0.012[a]	0.028 ±0.001
Mercury	280	0.208 ±0.011	0.023 ±0.001
Mercury/Selenium	303	0.133 ±0.007	0.031 ±0.001
Selenium	229	0.134 ±0.008	0.027 ±0.001

[a]Standard Error of the Mean.

Analyses of tracings from all 21-day-old pups showed no differences in L_{avg}, but significant differences were found in SB between treatments, $p < 0.0035$. The SB for the selenium treated rat pups was significantly smaller when compared with each of the three other treatments, $p < 0.05$ (see Table 5).

The data for 21-day-old pups was analyzed by sex. The results of the analysis of data on females showed no significant treatment differences in L_{avg}, but the SB of the selenium treated pups was significantly smaller than the SB of all other treatments (see Table 6).

TABLE 3. Mean values of SB and L_{avg}: 0-day-old female rats (micrometers)

Treatment	N	SB	L_{avg}
Control	93	0.177 ±0.016[a]	0.029 ±0.001
Mercury	118	0.250 ±0.020	0.018* ±0.001
Mercury/Selenium	135	0.157 ±0.013	0.034*† ±0.002
Selenium	64	0.119 ±0.012	0.024 ±0.002

[a]Standard Error of the Mean.

*Significantly different than controls, $p < 0.05$.

*†Significantly different than mercury or selenium groups, $p < 0.05$.

In the analysis of data for male pups, the SB of the selenium treated animals was significantly smaller than the SB of animals in the mercury/selenium and the mercury only groups. No differences were found in the L_{avg} of the ER for males in any treatment group (see Table 7).

TABLE 4. Mean values of SB and L_{avg}: 0-day-old male rats (micrometers)

Treatment	N	SB	L_{avg}
Control	167	0.216 ±0.016[a]	0.027 ±0.001
Mercury	162	0.178 ±0.013	0.027 ±0.001
Mercury/Selenium	168	0.113 ±0.008	0.028 ±0.001
Selenium	165	0.139 ±0.009	0.028 ±0.002

[a]Standard Error of the Mean.

TABLE 5. Mean values of SB and L_{avg}: 21-day-old rats, both sexes (micrometers)

Treatments	N	SB	L_{avg}
Control	257	0.141 ±0.006[a]	0.028 ±0.001
Mercury	265	0.137 ±0.007	0.025 ±0.001
Mercury/Selenium	270	0.155 ±0.006	0.026 ±0.001
Selenium	196	0.070* ±0.004	0.026 ±0.001

[a]Standard Error of the Mean.

*Significantly different than all other treatments, $p < 0.05$.

TABLE 6. Mean values of SB and L_{avg}: 21-day-old female rats (micrometers)

Treatment	N	SB	L_{avg}
Control	156	0.152 ±0.008[a]	0.025 ±0.001
Mercury	168	0.128 ±0.009	0.024 ±0.001
Mercury/Selenium	135	0.131 ±0.007	0.025 ±0.001
Selenium	97	0.066* ±0.006	0.023 ±0.001

[a]Standard Error of the Mean.

*Significantly different than all other treatments, $p < 0.05$.

TABLE 7. Mean values of SB and L_{avg}: 21-day-old male rats (micrometers)

Treatment	N	SB	L_{avg}
Control	101	0.124 ±0.008[a]	0.032 ±0.002
Mercury	97	0.153 ±0.012	0.027 ±0.002
Mercury/Selenium	135	0.179 ±0.010	0.027 ±0.001
Selenium	99	0.074*† ±0.006	0.029 ±0.002

[a]Standard Error of the Mean.

*†Significantly different than the mercury only and mercury/selenium groups, $p < 0.05$.

DISCUSSION

The purpose of this study was to determine if subtle effects of prenatal methylmercury exposure could be alleviated by concurrent administration of selenium.

The lack of clinical signs in the maternal animals was consistent with the dosage and route of exposure. Differences in birth weight were not expected in this study based on the work of Hughes and Annau (1976) who reported decreased birth weights in animals prenatally exposed to 10 mg/kg methylmercury, but no differences in birth weights of animals exposed to 5 mg/kg of methylmercury as compared with controls. None of the studies known to this author have reported increased birth weights in animals exposed prenatally to methylmercury. The results of this study contradict these findings since the birth weight of the mercury only treated pups was significantly greater than the controls. Since no differences existed in litter sizes or maternal weights alternative explanations for this phenomenon were examined. Though not significantly different, seven of ten mothers exposed to only mercury had consistently prolonged gestation periods, with a mean of 22.9 days compared with a mean of 22.3 days for the other treatment groups. During the last days of gestation the major change in the fetus is weight gain (Spencer and

Schaumburg, 1981, pp. 48-61), even a slight delay in birth could account for the increased birth weight in the mercury only treated pups.

The offspring of mothers in the selenium control group were observed to be hyperactive, these signs were not noted in the maternal animals. Early clinical signs of selenium toxicity include, but are not limited to, nervousness and irritability (Hogberg and Alexander, 1986). Ammar and Couri (1981) reported the initial reactions, following icv injection of selenium in mice, were characterized by increased locomotion, hyperreflexia and jumping. These findings agree with the observed behavior of the selenium exposed pups.

The coadministration of methylmercury and selenium causes changes in the distribution of both compounds (Iijima et al., 1978; Magos and Webb, 1977; Satoh and Suzuki, 1979; Yonemoto et al., 1983). The results of the chemical analyses showed the highest concentrations of mercury were found in the pups exposed to mercury alone. Coadministration of selenium with mercury resulted in significant decreases in mercury levels in all fetal tissues examined. Conversely mercury administration significantly increased the concentration of selenium in fetal rat liver. These findings agree with results reported by Iijima et al.

(1978) and Yonemoto et al. (1983), but differ with the findings of Satoh and Suzuki (1979) who reported a decrease in mercury in the whole fetus with selenium administration but an increase in mercury levels in fetal brain tissues. Satoh and Suzuki (1979) dosed their maternal animals late in the gestational period (day 16 of gestation), whereas, in this and the other studies animals were dosed during early organogenesis. Differences in gestational age during toxic insult are known to produce different results (Spencer and Schaumburg, 1981, pp. 48-61) and could explain the dissimilarities between the study of Satoh and Suzuki (1977) and this study or those of Iijima et al. (1978) and Yonemoto et al. (1983).

Degenerative ultrastructural changes were not observed in cerebellar tissue from 0-day-old rats exposed to mercury, although Chang et al. (1977) reported pathological changes in their study of prenatally exposed neonates. This difference may be due to the different routes of administration of mercury (gavage versus intravenous injection), the different strains of rats used, or the differences in gestational age at the time of exposure. The behavior and appearance of the victims of prenatal exposures in Japan were apparently normal at birth, the emergence of their symptoms was delayed (Chang, 1984). This agrees with

the ultrastructural observations reported here, since 0-day-old pups had no observed ultrastructural changes, but in tissues examined from 21-day-old pups degenerative changes were seen. Reuhl et al. (1981a) reported degenerative changes in cerebellar granule cells of hamsters prenatally exposed to 10 mg/kg on day 10 of gestation. Although degenerating cells, loss of ribosomes on ER, accumulations of lysosomes, and areas of focal cytoplasmic degradation were observed at all days postpartum, the greatest degree of change was noted from days three through seven.

The lack of observed degenerative changes in the 0-day-old rats does not mean that there were no mercury effects in these pups. Many of the reported changes deal with the thinning of the external granule cell layer (Sager et al., 1982) and changes in cytoarchitecture (Chang, 1984). These changes are only apparent under light microscopic examination, which was not used in this study. The findings of Kutscher et al. (1985), using Charles Rivers rats exposed to 6 mg/kg per os on days six through nine of gestation, revealed changes in ventricular size of exposed pups, but they did not observe the light microscopic changes noted above. The differences in these data may be due to the different routes of exposure, and different species and strains of experimental animals used.

The persistence of degenerative changes and the worsening of symptoms following mercury exposure are well documented (Chang, 1984; Inskip and Piotrowski, 1985; Reuhl et al., 1981b). The lasting degenerative ultrastructural changes observed in this study agree with the findings cited above. The types of changes were similar to those observed by Chang et al. (1977) and Reuhl et al. (1981b).

One of the earliest reported changes in neurons exposed to methylmercury involves the endoplasmic reticulum and ribosomes, and, since the granule cells in the cerebellum are extremely sensitive to mercury exposure (Chang and Hartmann, 1972b; Spencer and Schaumburg, 1981, pp. 24-33) it was decided to attempt to quantify these changes with a morphometric study. Mature granule cells have very little ER and few ribosomes, which is a possible explanation of their increased vulnerability, since even a small change will have a major impact on cell function (Spencer and Schaumburg, 1981, pp. 24-33). In the 0-day-old pups no changes were seen when data for both sexes were analyzed as one group. However, when these data were analyzed by sex the L_{avg} for mercury exposed female pups was significantly smaller than the control values, but the SB was not different; and the L_{avg} of the mercury/selenium group was larger than the mercury or selenium groups, though again,

the SB was not different. No differences were found in the male pups.

Our study indicates that males and females vary in their susceptibility to prenatal toxic insult. Mercury decreased the length of the individual profiles of ER, but not the total amount of ER, whereas mercury/selenium increased the length of the individual profiles of ER, but not the total amount of ER. Possibly, focal damage to the ER occurred when the mercury was first administered, and the increased number of small sections of ER may represent breaks in the ER due to this focal damage. The increase in the length of individual sections of ER in the mercury/selenium group, as compared with the mercury or selenium groups, with no corresponding increase in the SB, may be indicative of the protective effect of selenium on mercury injury to membranes or a repair response.

There have been very few studies on sex differences to toxic insult. Kutscher et al. (1985) and Spyker et al. (1972) both reported no differences in the responses of males and females following mercury exposure, although both did report differences between the sexes in relation to the size or weight of various regions of the brain.

Mercury did not affect the SB or L_{avg} in the 21-day-old pups in any of the analyses. Selenium exposure was stopped

on the day of birth, yet 21-day-old rats in the selenium group had a significant decrease in the SB compared with all other treatments. The morphometric data from this study indicate that the sensitivity of the granule cells may not be limited to mercury, but may include selenium. Ammar and Couri (1981) reported that selenium accumulates in the cerebellum. Studies of selenium in liver and muscle have localized selenium in the endoplasmic reticulum (Marier and Jaworski, 1983). This study indicates that selenium may also be associated with the endoplasmic reticulum in cerebellar granule cells. The mechanism of selenium action on membranous organelles is not known, but possibly involves a reduction in GSH-px, or Vitamin C, a necessary cofactor in Vitamin E regulated anti-oxidant activity in cell membranes. It is not known why this effect was not seen in tissues of 0-day-old rats. During growth, maturation, and migration of neurons, protein synthesis proceeds at a very high rate to meet the needs of the developing cell, therefore, these cells may contain more ER. Individual neurons observed in a particular brain region on a specific day of development will not all be in the same stage of development which will increase the natural variation in SB and mask subtle treatment effects.

When the data for 21-day-old rats were analyzed by sex the variation in the SB persisted, but with slight differences between the sexes. For female rats the selenium SB was significantly smaller than all other treatment groups, whereas in males the selenium SB was smaller than the mercury and mercury/selenium groups. This decrease in the total amount of ER without a corresponding decrease in the average length of the ER profiles developed after selenium exposure had ceased. It is possible that early focal damage could not be repaired and resulted in eventual dispersion of entire lengths of ER profiles. It is not known if these changes persist beyond the ages observed in this study.

Possible pathogenetic mechanisms of mercury toxicity, discussed by Chang (1982), result from the methylmercury molecule, or its breakdown products, causing blood-brain barrier dysfunction, breakdown of biological membranes, disturbance of nucleic acid and protein synthesis, disruption of enzyme systems, and destruction and denaturation of cellular proteins. Chang (1984) has proposed a cascade of pathological consequences following methylmercury exposure that affect all aspects of cellular function. Mercury has a high affinity for SH groups, and many proteins contain SH groups that are important in

structure and function (Clarkson, 1972). Theoretically mercurials can disrupt almost all functions in which proteins are involved, therefore, no one mechanism of action exists.

Selenium is a nutritionally important trace element with a high biological activity, functioning through association with vitamins or enzymes (Oldfield, 1987). The activities responsible for the physiological function at very low dietary levels of selenium are also responsible for the toxic response to selenium when ingested at elevated, though still relatively low levels. This dichotomy of effects has generated concern for both deficiency and excess of selenium. So far, only one role of selenium has been identified, the GSH-px anti-oxidant system, and both a deficiency or an excess of selenium cause a reduction in cell levels of GSH-px (Valentine et al., 1988). The activity of GSH-px is closely coupled with Vitamin E. Vitamin C is a required cofactor in the reactivation of Vitamin E, and excess selenium has been shown to decrease Vitamin C levels (Marier and Jaworski, 1983). The proposed roles of selenium in the GSH-px enzyme are the maintenance of permeability and integrity of cell membranes (Marier and Jaworski, 1983), and the stabilization of cell membranes (Kasuya, 1976).

The mechanism by which selenium protects cells from methylmercury toxicity is not understood. It is known that selenium and mercury have a high affinity for one another, therefore, their interaction possibly reduces the reactivity and toxicity of both compounds. Since low levels of selenium can protect from mercury effects at a ratio of selenium to mercury much less than 1:1, direct chelation of mercury by selenium is not likely (Chang, 1982). Even with administration of selenium, the form of mercury in the brain is almost exclusively methylmercury, this does not support the theory of biotransformation of methylmercury by selenium (Chang, 1982). Chang (1982) and Skerfving (1978) have both proposed the formation of large selenium-mercury protein complexes as a mechanism to reduce mercury toxicity. These complexes do not readily cross the placenta, and therefore, offer protection against mercury injury to the fetus (Skerfving, 1978). The other proposed mechanism of mercury selenium interaction involves the GSH-px, Vitamin E, anti-oxidation system. Data of Hirota et al. (1980) show that methylmercury decreases levels of GSH-px. Hogberg and Alexander (1986) have reported that concurrent administration of low levels of selenium with methylmercury does not result in decreased levels of GSH-px. Care must be used in determining doses of selenium that will convey this protective effect since Valentine et al. (1988) have

reported that slight excesses of selenium will lower levels of GSH-px, and Kasuya (1976) reported additive effects of certain levels of selenium with methylmercury.

The morphometric data reported here indicate that both mercury and selenium affect the integrity of the endoplasmic reticulum. The combination of mercury and selenium did not produce the same results seen with separate administration of these compounds. The reduction of mercury in all fetal tissues examined and the absence of significant differences in SB and L_{avg} between the mercury/selenium treated tissues and the controls indicates a protective effect of selenium against mercury toxicity in the fetus. However, care should be exercised when recommending selenium as a protective treatment for mercury exposure. The variations seen among species make application of these data to human exposures very difficult.

The changes in ER produced by selenium treatment warrant further study, along with research on chronic congenital selenium exposure, and long term studies to determine if the changes observed here are transitory or permanent. Differential effects in males and females have not been previously reported and should be investigated further. Morphometric analysis may reveal subtle changes that are not detectable by conventional microscopic

examination, and coupling of these two techniques may provide a powerful tool for future research.

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