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N,N-Diethyldithiocarbamate Produces Copper Accumulation, Lipid Peroxidation, and Myelin Injury in Rat Peripheral Nerve

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Previous studies have demonstrated the ability of the dithiocarbamate, disulfiram, to produce a peripheral neuropathy in humans and experimental animals and have also provided evidence that *N,N*-diethyldithiocarbamate (DEDC) is a proximate toxic species of disulfiram. The ability of DEDC to elevate copper levels in the brain suggests that it may also elevate levels of copper in peripheral nerve, possibly leading to oxidative stress and lipid peroxidation from redox cycling of copper. The study presented here investigates the potential of DEDC to promote copper accumulation and lipid peroxidation in peripheral nerve. Rats were administered either DEDC or deionized water by ip osmotic pumps and fed a normal diet or diet containing elevated copper, and the levels of metals, isoprostanes, and the severity of lesions in peripheral nerve and brain were assessed by ICP–AES/AAS, GC/MS, and light microscopy, respectively. Copper was the only metal that demonstrated any significant compound-related elevations relative to controls, and total copper was increased in both brain and peripheral nerve in animals administered DEDC on both diets. In contrast, lesions and elevated F_2-isoprostanes were significantly increased only in peripheral nerve for the rats administered DEDC on both diets. Autometallography staining of peripheral nerve was consistent with increased metal content along the myelin sheath, but in brain, focal densities were observed, and a periportal distribution occurred in liver. These data are consistent with the peripheral nervous system being more sensitive to DEDC-mediated demyelination and demonstrate the ability of DEDC to elevate copper levels in peripheral nerve. Additionally lipid peroxidation appears to either be a contributing event in the development of demyelination, possibly through an increase of redox active copper, or a consequence of the myelin injury.

Key Words: **copper; demyelination; disulfiram; dithiocarbamate; isoprostanes; lipid peroxidation; myelin.**

Some dialkyl dithiocarbamates including *N,N*-diethyldithiocarbamate (DEDC) form heavy metal complexes that are hydrophobic and can result in a redistribution of heavy metals in man and animals. This chelating property is the basis for the use of DEDC as a treatment for nickelcarbonyl intoxication in industrial settings (Gessner and Gessner, 1992). Of the heavy metals available in physiological systems, DEDC has the highest affinity for copper, followed by zinc, although the affinity of DEDC for copper is approximately five times greater than its affinity for zinc (Gessner and Gessner, 1992). Addition of either DEDC or its parent disulfide, disulfiram, to blood results in formation of a lipophilic *bis*(diethyldithiocarbamato)copper complex ($Cu(DEDC)_2$) that is thought to enable DEDC to redistribute copper across lipid membranes, including the blood-brain barrier (Johansson, 1992; Johansson and Stankiewicz, 1985). Administration of DEDC or disulfiram to experimental animals results in accumulation of copper in several organs including brain, spinal cord, liver, and kidney (Edington and Howell, 1966; Ho *et al*., 1985; Howell *et al*., 1970; Lakomaa *et al*., 1982).

Previous studies have demonstrated that subchronic administration of either DEDC or disulfiram to rats can produce peripheral neurotoxicity characterized by segmental demyelination, splitting of myelin lamellae, and formation of fluid- and debris-filled vacuoles in the Schwann cell cytoplasm (Tonkin *et al*., 2000, 2003). Accumulation of copper in the nervous system presents a possible mechanism for this neurotoxicity, because copper is potentially cytotoxic through its ability to facilitate the formation of reactive oxygen species (Bremner, 1998). For example, in patients with the autosomal recessive disorder Wilson's disease, accumulation of copper in the brain and liver is associated with neurological lesions including cystic changes in brain stem nuclei, loss of myelin, and astrocytic hyperplasia and with hepatoxicity resulting in progressive liver failure (Strausak *et al*., 2001). Similarly, intracellular transport of copper by DEDC and disulfiram has been demonstrated *in vitro* to increase cytotoxicity in primary rat astrocytes and rat thymocytes (Chen *et al*., 2000; Orrenius *et al*., 1996; Wilson and Trombetta, 1999). In those studies, dithiocarbamate-mediated cell death could be ameliorated by preventing accumulation of intracellular copper, either by incubating the cells in copper-free media or by coadministration of a hydrophilic, non-cell permeable copper chelator.

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Although accumulation of copper in the brain and spinal cord in animals exposed to DEDC or disulfiram has been documented in several reports, the effect of DEDC and disulfiram on copper levels in peripheral nerve has not been reported. Similarly, the effect of these dithiocarbamates on copper localization at the cellular and subcellular level is also unknown. Histochemical localization of copper in the brain of normal rats has supported copper being localized to glial cells, rather than neurons, which is consistent with the known ability of astroglia to accumulate and detoxify metals (Szerdahelyi and Kasa, 1986; Tiffany-Castiflioni and Qian, 2001). Alterations in the cellular distribution of copper has been reported in the liver during copper intoxication, in Wilson's disease, and in the brain of Long-Evans cinnamon (LEC) rats, a rat model of Wilson's disease (Bremner, 1998; Haywood *et al*., 1985; Sato *et al*., 1994). Some reports have suggested that copper accumulation following administration of DEDC localizes in particular brain regions, including the cortex, hippocampus, and brain stem (Iwata *et al*., 1970; Lakomaa *et al*., 1982), but the effect of DEDC exposure on cellular localization of copper in brain is not well understood. Additionally, unlike the distinct myelin lesions reported for disulfiram and DEDC in peripheral nerve, a well-defined and commonly accepted lesion within the central nervous system resulting from DEDC or disulfiram administration has not been identified.

If elevated levels of copper resulting from abnormal copper homeostasis in peripheral nerve and accompanying oxidative stress are responsible for demyelination, then conditions that enhance DEDC-mediated accumulation of copper are expected to also enhance oxidative stress and the severity of lesions observed. In this study an initial assessment of the role of copper accumulation and lipid peroxidation in DEDC and disulfiram-mediated peripheral neuropathy is presented. Rats were administered either DEDC or deionized water by ip osmotic pumps and fed either a normal diet or diet containing elevated copper. Following the exposures, lipid peroxidation products and the severity of lesions in peripheral nerve and brain were assessed using GC/MS and light microscopy, respectively. Brain, peripheral nerve and liver sections were also stained by silver autometallography to characterize copper localization in these tissues, and samples of brain, liver, kidney, sciatic nerve, and serum samples were analyzed for metals by atomic absorption spectroscopy (AAS) and inductively coupled plasma-atomic emission spectroscopy (ICP–AES) analysis.

MATERIALS AND METHODS

Chemicals. 2ML4 Alzet® osmotic pumps were obtained from Durect Corporation (Cupertino, CA). *N,N*-diethyldithiocarbamate (sodium salt) was obtained from Alfa Aesar (Ward Hill, MA). ACS grade sodium sulfide was obtained from Fischer Scientific (Pittsburgh, PA). Glutaraldehyde was obtained from Electron Microscopy Sciences (Ft. Washington, PA). ACS grade sodium pyrophosphate, sodium citrate, and cupric sulfate were purchased from VWR Scientific (Atlanta, GA). Cuprizone (biscyclohexanone oxaldihydrazone) was a product of Avocado Research Chemicals (Lancaster, UK). Normal rodent laboratory diet (Purina Lab Diet, 2001) containing 13 ppm copper and the same chow supplemented to 200-ppm copper using copper sulfate were obtained from Purina Mills (Richmond, IL).

Animals and exposures. All exposures were conducted in accordance with the National Institutes of Health *Guide for Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee. Male Sprague-Dawley rats obtained from Harlan Bioproducts (Indianapolis, IN) were caged in a room on a diurnal light cycle and given food and water *ad libitum*. Body weights were determined prior to surgery and then weekly during the course of the experiment. The average weight of the animals at the time of the initial surgery was 300 ± 5 g. For morphological assessments and tissue copper levels four exposure groups were used: two DEDC-exposed groups receiving 0.35 mmol/kg/day NaDEDC and two control groups. One of the control groups ($n = 4$) and one of the DEDC groups ($n = 4$) were fed normal rodent diet containing 13 ppm copper, while the other control ($n = 5$) and DEDC ($n = 5$) groups were fed rodent diet supplemented with 200 ppm copper sulfate.

Animals were exposed to DEDC via 2ML4 Alzet® osmotic pumps surgically implanted in the abdomen under deep anesthesia (90 mg/kg ketamine HCl and 7.5 mg/kg xylazine). After 4 weeks, the pumps were replaced by the same procedure to extend the exposure period to 8 weeks. DEDC was delivered as an aqueous solution of NaDEDC, the concentration of which was determined from the UV absorbance at 282 nm (ε = 13,000 l/mol/cm) prior to filling the pumps. Control pumps contained deionized water. Solutions were sterilized prior to filling the pumps by filtering through a 0.22 µm-syringe filter. At the end of the exposure period neuromuscular function was assessed by measuring hind limb grip strength using a digital force gauge (Meyer *et al*., 1979), and the animals were perfused for morphological examination of peripheral nerve, liver, and brain.

Identical exposure groups ($n = 4$ per group), exposure durations, and exposure levels to those described above were used for the animals analyzed for F_2-isoprostanes and F_4-neuroprostanes. At the end of the 8-week exposures the animals were deeply anesthetized, exsanguinated, and the sciatic nerves and brains immediately frozen in liquid nitrogen.

Preparation of tissue for morphological assessment. Tissue was prepared according to the method of Danscher with some modifications (Danscher, 1984). Animals were perfused through the left ventricle of the heart with 0.1% Na_2S in 0.15 M sodium phosphate buffer (pH 7.4) for 2 to 3 min, then with 3% glutaraldehyde in 0.15 M phosphate buffer (pH 7.4) for 10 to 15 min, followed by the Na_2S solution for 7 min. After perfusion, liver, brain, kidney, and sciatic nerve were dissected out and immersed in 3% glutaraldehyde for 1 h, then transferred to 0.15 M phosphate buffer. Sciatic nerve sections were post-fixed with osmium and embedded in Epon; thick sections were cut and stained with toluidine blue. Thick (1-mm) sections of sciatic nerve tissue were evaluated by light microscopy on an Olympus BX41 microscope equipped with an Olympus C-3040 Camedia digital camera. Brain and liver sections were embedded in paraffin and stained with hematoxylin and eosin (H&E) by the Vanderbilt Medical Center Neuropathology Laboratory and also evaluated by light microscopy. Sections of sciatic nerve, brain, and liver were embedded and stained by silver autometallography at NeuroScience Associates (Knoxville, TN) (Ross *et al*., 1996). Free floating Multi-Brain sections were rinsed twice in Na acetate, and then placed into the acidic physical developer (pH 5) of Gallyas for several (4–6) min. Development was stopped by transferring the sections to 1% acetic acid. Following a 30-s rinse in 1% potassium thiosulfate the sections were rinsed in water and mounted. After air-drying the slide-mounted sections were lightly counterstained with neutral red.

Blood collection. Prior to perfusion, 3–4 ml of blood was collected from anesthetized animals via cardiac puncture and 0.5–1 ml used to isolate globin for analysis by HPLC. Blood was separated into plasma and hemolysate as described (Erve *et al*., 2000) and 100 µl of 1 N ascorbic acid added to each 1 ml hemolysate. The resulting solution was added dropwise to 10 ml of 2.5% oxalic acid in acetone. Globin was allowed to precipitate on ice for 30 min, then centrifuged at 10,000 × *g* for 10 min at 4°C and washed in 5 ml ice-cold acetone. The pellet, containing crude globin, was dried under a stream of N_2 and stored at −80°C. Dried globin was solubilized with 0.1% trifluoroacetic acid (TFA) to produce a solution for HPLC analysis.

Globin chains were separated by RP–HPLC on a Phenomenex Jupiter 5-μm column (150 × 460 mm) using a Waters 2690 liquid chromatograph after adjusting sample concentration to a UV absorption of 1.0 ± 0.2 at 280 nm. Globins were separated using a linear gradient from 56% solvent A, 44% solvent B, to 30% solvent A, 70% solvent B, over 30 min followed by a linear gradient to 100% solvent B over 10 min. Solvent A was 20:80:0.1 acetonitrile:water:TFA, and solvent B was 60:40:0.08 acetonitrile:water:TFA. The elution of globin peaks was monitored by their UV absorption at 220 nm using a Waters 996 photodiode array detector.

The remaining blood not used to isolate globin was placed into a 15-ml conical tube and allowed to clot, then centrifuged to precipitate the clot, and the serum removed. 200-μl serum aliquots were analyzed at the Vanderbilt University Hospital Clinical Chemistry laboratory (Nashville, TN) for aspartate aminotransferase activity (AST/SGOT). The remaining serum was stored at −20°C and analyzed for copper concentration.

Analysis of tissue metal levels. Brain, liver, and kidney sections were analyzed by both inductively coupled plasma-atomic emission spectroscopy (ICP–AES) and atomic absorption spectroscopy (AAS); sciatic nerve sections were analyzed by ICP–AES alone. Samples for AAS analysis were taken from individual animals and analyzed at Kord Animal Disease Diagnostic Laboratory (Nashville, TN). Due to the limited amount of sciatic nerve tissue available, all tissue samples for ICP–AES were taken from individual animals, then pooled by treatment group in order to obtain enough tissue for analysis. Brain tissue samples for ICP–AES were approximately 1-mm midsagittal slices. Kidney sections for ICP–AES were approximately 1-mm cross-sections through the center of a single kidney; liver sections were 1- to 2-mm^3 pieces of tissue; sciatic nerve sections were ~10-mm lengths of a single nerve. Average weights of the pooled tissue samples for each treatment group of brain, kidney, liver, and sciatic nerve were 0.79 ± 0.05 g, 0.34 ± 0.07 g, 0.11 ± 0.01 g, and 0.059 ± 0.006 g, respectively. Each tissue sample was analyzed in duplicate for mineral content by ICP–AES at the Diagnostic Center for Population and Animal Health at Michigan State University (East Lansing, MI). Elements determined in this analysis were barium, calcium, copper, iron, magnesium, manganese, molybdenum, phosphorus, zinc, chromium, cadmium, sodium, nickel, vanadium, sulfur, and potassium.

Analysis of serum copper levels. Serum copper levels were measured spectrophotometrically based on described methods (Tietz, 1976). Briefly, serum was mixed with 2 M HCl and incubated at room temperature for 10 min; then 20% trichloroacetic acid was added, and the solution was mixed and allowed to stand at room temperature for 10 min. Samples were centrifuged for 10 min; then the supernatant was removed and combined with a solution of sodium pyrophosphate, sodium citrate, and ammonium hydroxide, followed by 0.5% cuprizone in 50% ethanol. The resulting solution was mixed and incubated at room temperature 20 min; then the absorbance at 620 nm was determined and compared to a standard solution of copper sulfate.

Analysis of F_2-isoprostanes and F_4-neuroprostanes. Measurements were performed on individual animals using two sciatic nerves (F_2-isoprostanes) and the right cerebral hemisphere (F_2-isoprostanes and F_4-neuroprostanes) for each animal. F_2-isoprostanes and F_4-neuroprostanes were quantified in Folch extracts of cerebrum or sciatic nerves using gas chromatography with negative ion chemical ionization mass spectrometry with selective ion monitoring using a published method (Reich *et al.*, 2001). Briefly, samples were extracted by the method of Folch, a stable isotope internal standard added, and then prepared for gas chromatography (GC) through a series of TLC and SepPak separations. Gas chromatography was performed using a 15 m, 0.25 mm diameter, 0.25 mm film thickness, DB1701 fused silica capillary column. The column temperature was programmed from 200 to 300°C at 20°C/min. Methane was used as the carrier gas at a flow rate of 1 ml/min. Ion source temperature was 250°C, electron energy was 70 eV, and filament current was 0.25 mA. Negative ion chemical ionization (NICI) mass spectrometry (MS) was performed using an Agilent Technologies G1789A GC/MSD instrument with a Hewlett-Packard computer system with ChemStation (NT).

Statistical analysis. Analysis of variance (ANOVA) with Bonferroni's post-hoc test, the nonparametric Kruskal-Wallis test (for ranked scoring of lesion severity), and the Student's *t*-test were performed using GraphPad Prism software. Statistical significance was taken to be $p < 0.05$ unless otherwise noted.

RESULTS

Weight Gain and Exposure Levels

Neither exposure to DEDC alone nor consumption of the 200-ppm copper diet had a significant effect on the weight gain of the animals relative to controls (Table 1). However, exposure to DEDC in combination with the 200-ppm copper supplemented diet did result in a significantly lower weight gain relative to the other treatment groups. The average dose of DEDC delivered by the osmotic pumps over the 8-week exposure period was calculated to be 0.34 ± 0.01 mmol/kg/day for both DEDC exposure groups. The level of DEDC exposure was also monitored by analysis of globin preparations by RP–HPLC. Globin preparations from all animals exposed to DEDC contained an additional peak seen by HPLC that was not observed in globin preparations from control animals or animals given only the 200-ppm copper diet. Earlier work demonstrated that this additional peak is produced by carbamylation of Cys-125 of β-globin by a metabolite of DEDC and is correlated to dose level (Erve *et al.*, 2000; Tonkin *et al.*, 2000, 2003). The area of this modified β-globin peak was not significantly different between the two DEDC-exposure groups; the modified β-globin peak area in globin preparations from the DEDC-exposed animals was 14.9 ± 0.9 % of total β-peak area and from animals exposed to DEDC and 200-ppm copper was 13.7 ± 0.6 % of total β-peak area.

Liver Function and Histology

The level of serum AST/SGOT activity was not significantly different between any treatment groups and was not elevated above normal levels. For controls, the value was 95.8 ± 5.7 units; for DEDC-exposed the value was 79.0 ± 5.0; for the 200-ppm copper diet-exposed the value was 81.0 ± 5.0; and for the DEDC and 200-ppm copper diet-exposed group, the value was 84.4 ± 3.2. Liver sections from all of the animals were examined by

TABLE 1
Effect of DEDC and Copper Exposure on Weight Gain in Rats[a]

Treatment	Weight gain (g)[b]
Control (13 ppm Cu)	37.3 (4.5)
DEDC (13 ppm Cu)	26.2 (5.0)
Control (200 ppm Cu)	43.7 (2.5)
DEDC (200 ppm Cu)	21.3 (2.9)[c]

[a]Weight gain as percent of initial body weight.
[b]Data is presented as mean (SEM).
[c]$p < 0.05$ relative to controls by analysis of variance with Bonferroni's post-hoc test.

light microscopy, and no treatment-related lesions were observed. Background lesions observed included the presence of diffuse, mild hepatocyte cell death and turnover in all treatment groups, as indicated by rounded cells with pyknotic nuclei, presence of Kupfer cells, and mitotic cells. No obvious signs of inflammation were observed, and cell death did not follow any zonal distribution, nor did the incidence of cell death differ noticeably between the treatment groups. One of the DEDC-exposed and two of the high copper diet-exposed animals displayed diffuse mild to moderate lipid accumulation in the hepatocytes (data not shown), but this lesion did not appear to be treatment-related.

Analysis of Tissue Metal Levels

Copper levels in the brain, liver, and kidney determined by ICP–AES and AAS are shown in Table 2. Consumption of the 200-ppm copper diet alone had no effect on copper tissue levels relative to the normal copper diet controls. Exposure to DEDC alone resulted in a significant increase in copper levels in brain (AAS and ICP–AES), sciatic nerve (ICP–AES), and liver (ICP–AES) relative to controls. Exposure to DEDC and the 200-ppm copper diet also resulted in elevated copper levels relative to controls in brain (AAS and ICP–AES), liver (AAS and ICP–AES), and sciatic nerve (ICP–AES). The level in liver (AAS and ICP–AES), brain (ICP–AES), and sciatic nerve (ICP–AES) for the combined treatment was statistically greater than DEDC treatment alone. No significant changes in the levels of kidney copper were seen with any treatment group. Similarly, no significant differences in serum copper levels were observed for any treatment group. For controls, serum copper was 130 ± 19.7 μg/dl; for the DEDC-exposed the copper level was 150 ± 13.6 μg/dl; for the high copper diet-exposed the

TABLE 2
Tissue Copper Levels

	AAS[a]		
Treatment	Liver (ppm)	Brain (ppm)	Kidney (ppm)
Control (13 ppm Cu)	6.37 (0.41)[b]	3.10 (0.17)	5.03 (0.28)
DEDC (13 ppm Cu)	9.20 (1.06)	26.0 (2.46)[c,d]	4.18 (0.28)
Control (200 ppm Cu)	6.64 (0.38)	3.00 (0.18)	5.22 (0.28)
DEDC (200 ppm Cu)	20.7 (2.62)[c,d,e]	30.8 (0.68)[c,d]	5.76 (0.71)

	ICP–AES[f]			
Treatment	Liver (ppm)[f]	Brain (ppm)	Kidney (ppm)	Sciatic (ppm)
Control (13 ppm Cu)	11.0 (0.15)	10.3 (0.05)	19.3 (0.15)	5.26 (0.12)
DEDC (13 ppm Cu)	22.1 (3.20)[c]	91.0 (1.50)[c,d]	20.5 (2.90)	8.20 (0.13)[c,d]
Control (200 ppm Cu)	12.1 (0.10)	10.5 (0.10)	20.5 (0.20)	3.41 (0.10)[c]
DEDC (200 ppm Cu)	51.8 (0.55)[c,d,e]	99.6 (1.40)[c,d,e]	23.7 (0.05)	9.47 (0.14)[c,d,e]

[a]For AAS, values are given as ppm wet weight of tissue. Sample size was $n = 4$ for control and DEDC on the 13 ppm Cu diet and $n = 5$ for control and DEDC on the 200 ppm Cu diet; sciatic nerves were $n = 2$ per treatment group.
[b]Data are presented as mean (SEM).
[c]$p < 0.05$ relative to control.
[d]$p < 0.05$ relative to 200 ppm Cu diet.
[e]$p < 0.05$ relative to DEDC.
[f]For ICP–AES, values are given as mean (SEM) ppm dry weight of tissue for pooled samples run in duplicate.

TABLE 3
Zinc, Iron, and Manganese Tissue Levels[a]

	Liver		
Treatment	Zinc	Iron	Manganese
Control (13 ppm Cu)	74.5 (0.35)	248 (4.0)	4.3 (0.06)
DEDC (13 ppm Cu)	91.1 (14)	198 (96)	2.8 (0.6)
Control (200 ppm Cu)	70.8 (0)	267 (4.0)	3.6 (0.08)
DEDC (200 ppm Cu)	102 (0.5)	314 (2.5)	3.3 (0.04)

	Brain		
Treatment	Zinc	Iron	Manganese
Control (13 ppm Cu)	39.1 (0.7)	52.1 (0.6)	1.0 (0.04)
DEDC (13 ppm Cu)	39.3 (0.6)	45.1 (0.3)[c]	0.9 (0.03)
Control (200 ppm Cu)	37.1 (0.4)	44.9 (0.5)[c]	1.0 (0.03)
DEDC (200 ppm Cu)	40.9 (0)[b]	55.1 (1.0)[b,d]	1.1 (0.04)

	Sciatic Nerve		
Treatment	Zinc	Iron	Manganese
Control (13 ppm Cu)	18.6 (1.0)[e]	19.0[f]	ND[g]
DEDC (13 ppm Cu)	12.2 (0.05)[c]	18.6 (0)	ND
Control (200 ppm Cu)	10.3 (0.3)[c]	18.4 (0.3)	ND
DEDC (200 ppm Cu)	11.9 (0.2)[c]	17.1 (0.4)	ND

	Kidney		
Treatment	Zinc	Iron	Manganese
Control (13 ppm Cu)	55.9 (0.4)	135.5 (1.5)	2.4 (0.06)
DEDC (13 ppm Cu)	82.7 (11)	180.6 (88)	2.5 (0.6)
Control (200 ppm Cu)	49.7 (0.9)	105.0 (1.0)	2.1 (0.02)
DEDC (200 ppm Cu)	78.7 (0.5)	109.5 (0.5)	2.1 (0.03)

[a]Levels determined by ICP–AES and are presented as mean (SEM) ppm dry weight of tissue for pooled samples run in duplicate.
[b]$p < 0.05$ relative to copper-treated animals by analysis of variance.
[c]$p < 0.05$ relative to controls.
[d]$p < 0.05$ relative to DEDC-exposed animals.
[e]$n = 3$.
[f]$n = 1$.
[g]ND = none detected.

level was 128 ± 7.2 μg/dl; and for the high copper diet plus DEDC exposure group, the level was 142 ± 9.3 μg/dl.

The effect of DEDC, 200-ppm copper diet, and DEDC plus 200-ppm copper diet treatment on zinc, iron, and manganese levels was also analyzed (Table 3). No difference in levels of these elements was observed relative to controls in liver or kidney sections for any treatment group. The levels of zinc in sciatic nerve sections from the DEDC, 200-ppm copper diet, and DEDC plus 200-ppm copper diet groups were decreased relative to control animals. Iron and manganese levels were not significantly different relative to controls in sciatic nerve sections obtained from any treatment group. In the brain, zinc levels were slightly elevated in animals exposed to both high copper and DEDC compared to all other treatment groups ($p < 0.05$, $n = 2$), while iron levels were slightly reduced in the DEDC group and the high copper groups relative to controls ($p < 0.05$, $n = 2$). Other elements measured by ICP–AES were either below detection levels or were present in the solutions used for perfusion (i.e., sodium and phosphorus) and were not analyzed.

Neuromuscular Function and Peripheral Nerve Morphology

No significant difference in hind limb grip strength was seen between any of the treatment groups. Average grip strength for the control normal diet group was 1133 ± 112 g ($n = 4$), for the high copper-exposed was 1065 ± 56 g, for the DEDC-exposed group was 936 ± 62 g ($n = 4$) and for the combined DEDC and high copper group was 960 ± 46 g ($n = 5$). No lesions were detected in sciatic nerve sections from control animals or from animals exposed to the elevated copper diet (Figs. 1A and 1B). By comparison, nerve sections from animals exposed to DEDC,

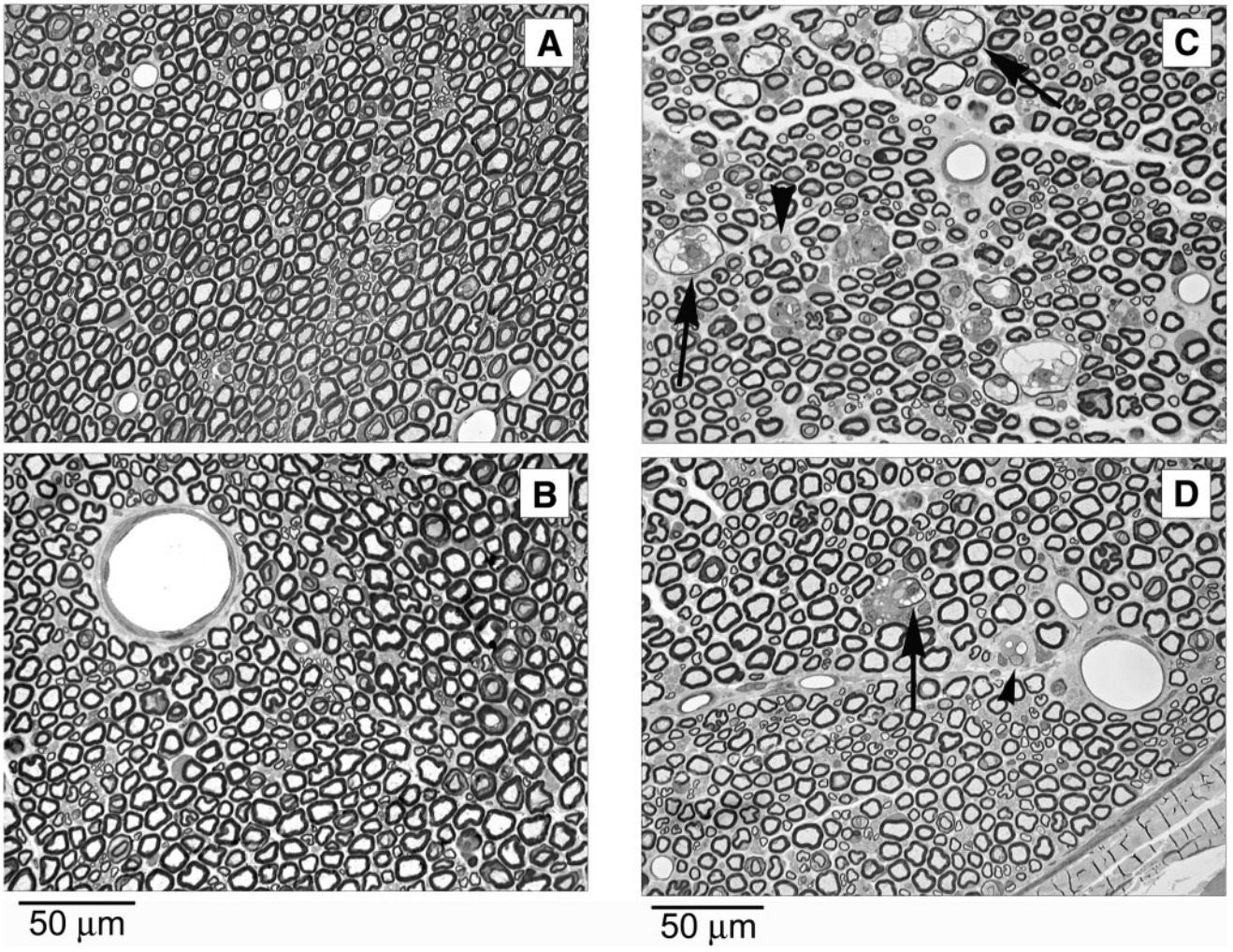


FIG. 1. Morphology of sciatic nerve cross-sections stained with toluidine blue. Sciatic nerve from a control rat on normal diet (A) and control rat on 200-ppm copper diet (B) demonstrate normal distributions of small and large myelinated axons. Sciatic nerve from an animal exposed to 0.35 mmol/kg/day of DEDC for 8 weeks (C) and an animal exposed to 0.35 mmol/kg/day DEDC fed a 200-ppm copper diet for 8 weeks (D), demonstrating demyelinated and thinly myelinated axons (arrow heads) and myelin splitting (arrows).

both with and without the elevated copper diet, contained evidence of demyelination, splitting of myelin lamellae, and thinly myelinated axons (Figs. 1C and 1D). The incidence and severity of axonal degeneration, demyelination, and myelin splitting in sciatic nerve sections from animals exposed to DEDC or MeDETC was determined (Table 4). No significant differences in axonal degeneration were seen between any treatment groups. There was a significant difference in the medians for both demyelination and myelin splitting among the four treatment groups determined by the Kruskal-Wallis test ($p < 0.05$).

Copper Histochemistry

Dark precipitate was observed in longitudinal sections of sciatic nerves stained by silver metallography obtained from animals administered DEDC and DEDC plus 200-ppm copper diet but was not apparent in sections from control animals on either diet (Fig. 2). Dark precipitate was also observed in brains and livers of animals exposed to both the elevated copper diet and DEDC; this precipitate was not observed in sections from control animals. Examination of liver sections from animals exposed to both DEDC and copper revealed staining in a distinct periportal distribution (Fig. 3). Brain sections from these animals revealed diffuse staining in several regions of the brain including the hippocampus, thalamus, diencephalon, and the caudate (Fig. 4, color micrographs are available in the supplementary material). Precipitate formation was notably absent in the cortex, with the exception of a diffuse staining of layer VI (Fig. 4B). The cerebellum was largely unstained (Fig. 4F).

F_2-Isoprostanes and F_4-Neuroprostanes

Significant elevations in F_2-isoprostane levels were associated with DEDC and DEDC with 200-ppm copper diet relative to control normal diet and control 200-ppm copper diet, respectively, in sciatic nerve (Table 5). In contrast, no significant differences in F_2-isoprostanes or F_4-neuroprostane levels were observed for any groups in the right cerebral hemisphere.

DISCUSSION

Disulfiram is used to treat alcoholism and has been associated with the development of a peripheral sensorimotor neuropathy in some patients (Ansbacher *et al*., 1982; Bouldin *et al*., 1980). It has also been shown to produce peripheral demyelination in rats following oral exposure, but the underlying processes have not been delineated. Previous studies have examined the role of CS_2 in the neurotoxicity of disulfiram and DEDC and have shown that, although CS_2 produces protein cross-linking and a neurofilamentous axonopathy following oral administration of DEDC due to acid-promoted decomposition (Scheme 1), neurotoxic levels of CS_2 do not result from oral administration of the more acid stable disulfiram (Johnson *et al*., 1998; Tonkin *et al*., 2000). Evidence has also been provided that the ability of disulfiram to produce *N,N*-diethylaminothiocarbonyl cysteine adducts through the generation of a sulfoxide metabolite (Scheme 1) does not contribute to disulfiram-mediated myelin lesions (Tonkin *et al*., 2003). Interestingly, it has been determined that when DEDC is administered parenterally, thereby avoiding the acidic environment of the stomach, the same myelin lesions and protein modifications observed for disulfiram are produced (Tonkin *et al*., 2003). DEDC therefore appears to be a promising candidate for the proximate demyelinating species of disulfiram, raising the question of what chemical properties of DEDC contribute to the observed lesions. The ability of dithiocarbamates, including DEDC and disulfiram, to alter storage and elimination of both exogenous heavy metals (such as mercury and lead) and endogenous copper has been documented in several reports (Edington and Howell, 1966; Gessner and Gessner, 1992; Ho *et al*., 1985; Lakomaa *et al*., 1982; Weiss

TABLE 4
Incidence and Severity of Sciatic Nerve Lesions[a]

	Degenerated axons		Demyelination		Myelin splitting	
Treatment group	Incidence[b]	Severity[c]	Incidence	Severity[c,d]	Incidence	Severity[c,d]
Control (13 ppm Cu)	3/4	0	0/4	0	0/4	0
Control (200 ppm Cu)	5/5	0	0/5	0	0/5	0
DEDC (13 ppm Cu)	4/4	0	3/4	1 (0–3)	3/4	1 (0–3)
DEDC (200 ppm Cu)	5/5	0	4/5	1 (0–2)	3/5	1 (0–2)

[a]Slides were scored blinded by a single observer. A single, large fascicle was chosen for observation (fascicles were of approximately equal size).
[b]Number of animals with positive observations/number of animals in the treatment group.
[c]Values are median score (range) where 0 = normal, 1 = mild, 2 = moderate, 3 = severe. For degenerated axons, normal = 0–10 affected axons/fascicle, mild = 11–30 affected axons/fascicle, moderate = 31–70 affected axons/fascicle, and severe > 70 affected axons/fascicle. For demyelination and myelin splitting, normal = 0 affected axons/fascicle, mild = 1–10 affected axons/fascicle, moderate = 11–30 affected axons/fascicle, and severe > 30 affected axons/fascicle.
[d]$p < 0.05$ between all treatment groups by Kruskal-Wallis test.

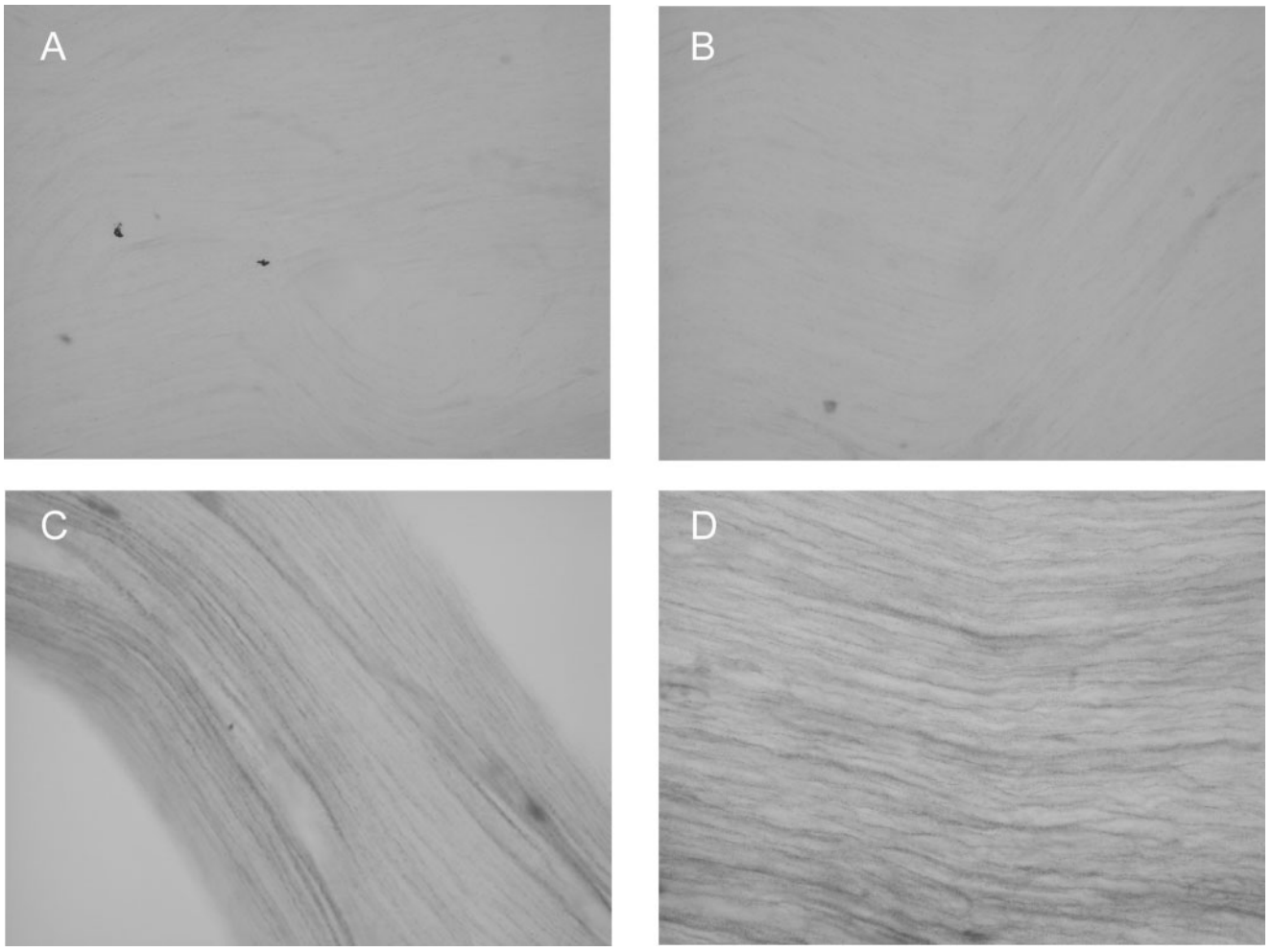


FIG. 2. Distribution of copper in sciatic nerve by autometallography silver staining. Longituidinal sections obtained from control rats fed a normal diet (A) and control rats fed a 200-ppm copper diet (B) contained relatively little precipitate. Sections obtained from rats administered DEDC on normal diet (C) or 200-ppm copper diet (D) showed the presence of black precipitate, consistent with copper distributed along the entire length of the axons. Sections were counter-stained with neutral red.

et al., 1990). The ability of DEDC and disulfiram administration to increase levels of total copper within the central nervous system has been recognized for some time and has been proposed to result from the formation of a lipophilic $Cu(DEDC)_2$ complex (Scheme 1) that can pass through the blood-brain barrier (Aaseth *et al*., 1979; Allain and Krari, 1991, 1993; Koutensky *et al*., 1971; Lakomaa *et al*., 1982). Collectively, the published studies provide strong evidence for the potential of dithiocarbamates and their disulfides to disturb copper homeostasis within the central nervous system, prompting previous investigators to propose enhanced oxidative stress due to redox active copper as a mechanism of CNS toxicity (Delmaestro and Trombetta, 1995). It has also been shown that DEDC can interact with protein-bound copper, forming a complex with the bound ion or extracting it from the protein completely, resulting in inhibition of copper-dependent enzymes including dopamine β-hydroxlyase (DBH) and Cu/Zn superoxide dismutase (Bremner, 1998; Heikkila *et al*., 1976; Moore, 1969). These enzymes are important to the normal functioning of the nervous system, and sequestering of a copper cofactor by DEDC may present an alternative neurotoxic mechanism.

In order to evaluate the role of copper-mediated damage in disulfiram and DEDC neurotoxicity, the current study attempted to modulate the toxicity of DEDC by administering a diet containing elevated copper with the goal of further increasing the copper levels in the nervous system. Consistent with previous reports, administration of DEDC to rats fed normal rodent chow in the present study resulted in increased brain and liver copper levels relative to controls. A significant increase in total copper relative to controls was also observed in the sciatic nerve, indicating that DEDC affects copper levels in the peripheral as well as the central nervous system. Although some reports have documented effects of dithiocarbamates on serum and kidney copper levels (Edington and Howell, 1966;

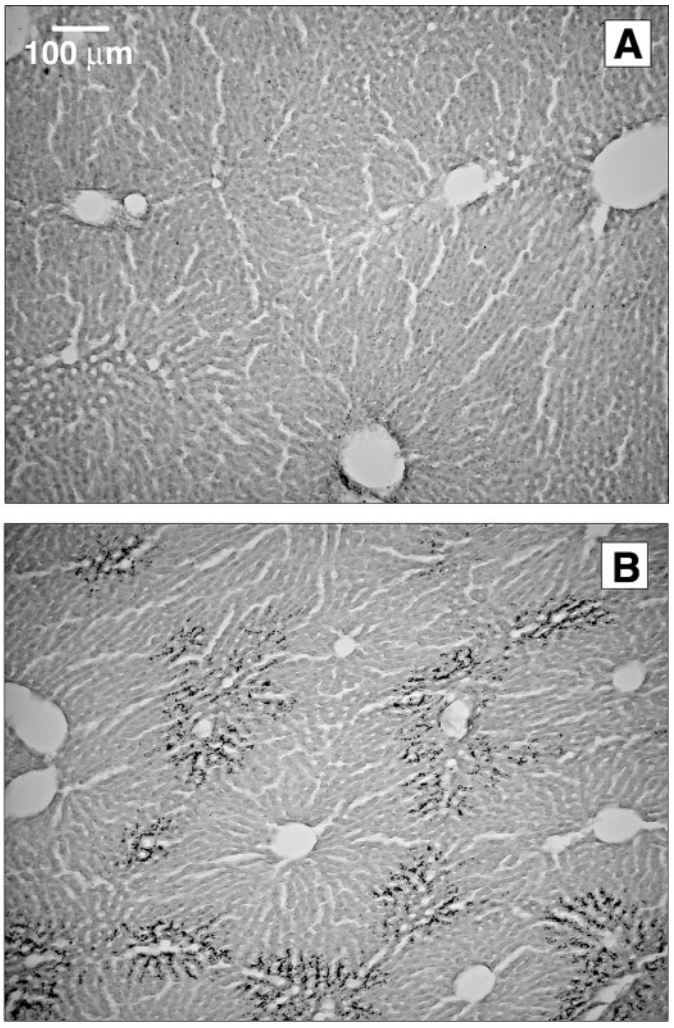


FIG. 3. Distribution of copper in liver sections by silver autometallography staining. Control liver sections (A) contained relatively little precipitate formation. Liver sections from animals exposed to 0.35 mmol/kg/day DEDC and fed a 200-ppm copper diet for 8 weeks (B) contained black precipitate, indicating the presence of copper, in a periportal distribution. Sections were counter-stained with neutral red.

Iwata *et al*., 1970; Koutensky *et al*., 1971; Mur, 1964), no changes to copper levels in the kidney or in serum were observed in this study in animals exposed to DEDC. Increasing the dietary intake of copper by 15-fold in the absence of DEDC administration was not sufficient to produce increases in copper levels in any tissues examined, presumably due to regulation of intestinal absorption and/or increased biliary excretion of excess copper (Bremner, 1998). By comparison, increasing dietary copper by 15-fold in animals exposed to DEDC resulted in higher copper levels in brain, liver, and sciatic nerve relative to animals exposed to DEDC alone, although no changes were observed in kidney or serum copper levels. The greatest effect was observed in the liver, where the level of copper in animals exposed to both 200-ppm copper and DEDC was approximately twice the level of those in animals exposed to DEDC alone. By comparison, the levels observed in the brains and sciatic nerves of animals exposed to high copper and DEDC were only 10 to 20% higher than animals exposed to DEDC alone. Thus, it appears that, although exposure to DEDC in combination with elevated dietary copper did result in increased accumulation of copper in tissue over DEDC exposure alone, the overall effect was relatively small and the effect on the liver was considerably greater than that on the peripheral or central nervous system.

Peripheral nerve lesions were observed both in animals exposed to DEDC alone and in animals exposed to DEDC in combination with a high copper diet, but it was not possible to distinguish a difference between these two exposure groups based on quantification of lesion severity. The most severely affected individuals were in the DEDC normal copper diet exposure group, but the median level of lesion severity was identical for both DEDC exposure groups. Although this result may suggest that elevated copper does not enhance DEDC toxicity, the small increase in copper observed in the sciatic nerve from the high copper diet may not have been sufficient to produce a significant difference in lesion severity given the sample size and the variation in lesion severity among individuals. Therefore, it is not possible based on the present study to determine whether further elevation of nervous system copper levels would enhance DEDC neurotoxicity, suggesting a cytotoxic effect of copper, or decrease the neurotoxicity, suggesting that DEDC may be acting through sequestering of critical copper cofactors.

Deposition of metal was observed by metallography staining in the liver, sciatic nerve, and brain of animals exposed to DEDC and elevated dietary copper. This staining technique relies on precipitation of silver on metal sulfides in tissue sections and is thought to stain zinc and copper sulfides most efficiently (Danscher, 1984). The lack of DEDC-mediated increases in metals other than copper in any of the treatment groups suggests that the silver reaction product observed in tissue sections is due to the presence of copper, although precipitate formation due to some other compound-related effect cannot be completely ruled out. In the liver, staining was observed in a periportal distribution, consistent with the observed localization of copper in Wilson's disease and in copper toxicosis (Bremner, 1998; Haywood *et al*., 1985). This precipitate was not associated with hepatotoxicity, however, based on the absence of elevated AST/SGOT activity and lack of significant histopathological changes in liver sections. The copper observed in these sections may have been effectively detoxified through binding to metallothionein; rats are relatively tolerant of copper excess, and hepatotoxicity typically does not occur below doses of 500 mg/kg/day (Bremner, 1998). Regional precipitate was also observed in brain sections from animals exposed to both a high copper diet and DEDC, but not in control animals on either diet. Staining was observed in the hippocampus, the caudate, the thalamus, and layer VI of the cortex, but not in the rest of the cortex or the majority of the cerebellum. Previous studies have

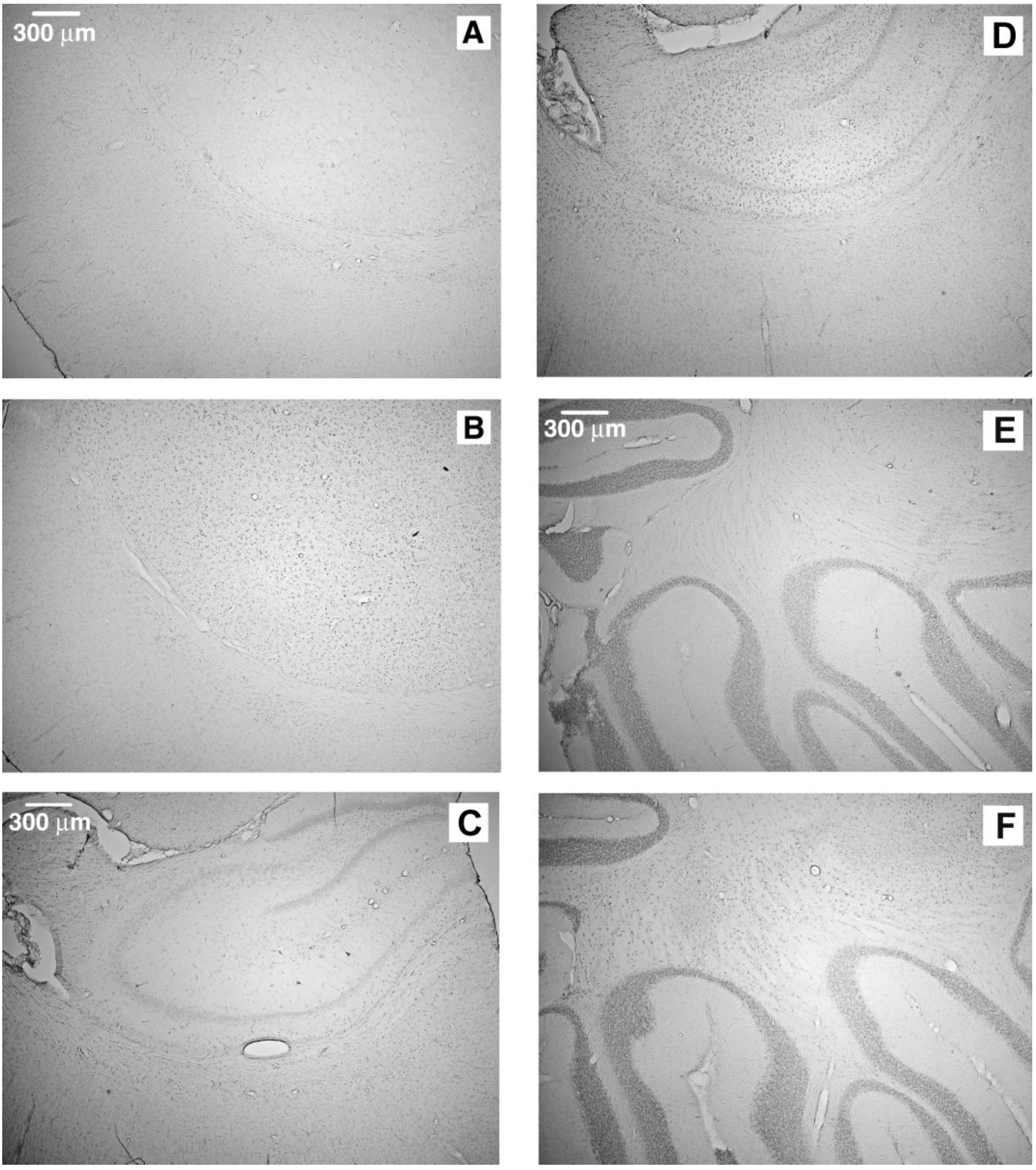
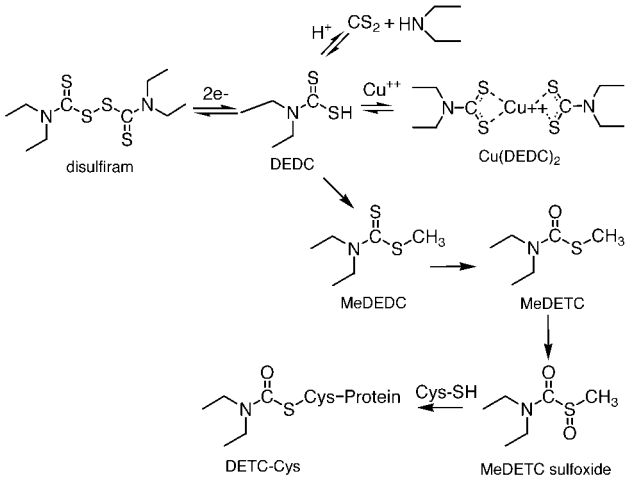


FIG. 4. Distribution of copper in brain sections using silver autometallography staining. Sections obtained from control animals fed a 200-ppm copper diet showed relatively little precipitate in the forebrain (A), midbrain (C), or hindbrain regions (E). Animals exposed to 0.35 mmol/kg/day DEDC and fed a 200-ppm copper diet for 8 weeks showed precipitate formation in the caudate and layer VI of the cortex in forebrain (B), in the hippocampus but not in the cortex of midbrain (D), and minimal precipitate formation in the cerebellum, although precipitate formation was present in the brain stem (F). Sections were counterstained with neutral red. Color micrographs of these sections are available in the supplementary material.

Table 5
F_2-Isoprostane and F_2-Neuroprostane Levels

Treatment group	Sciatic nerve F_2-isoprostanes[a]	Brain F_2-isoprostanes[a]	Brain F_4-neuroprostanes[a]
Control (13 ppm Cu)[b]	1.23 (0.08)	2.42 (0.19)	9.63 (2.6)
DEDC (13 ppm Cu)[b]	1.62[c] (0.04)	2.11 (0.15)	7.44 (0.73)
Control (200 ppm Cu)[b]	1.32 (0.09)	2.29 (0.21)	7.62 (0.28)
DEDC (200 ppm Cu)[b]	2.12[d] (0.35)	1.87 (0.08)	7.09 (1.0)

[a]expressed as ng/g tissue.
[b]$n = 4$.
[c]$p < 0.01$ relative to control (13 ppm Cu) Student's t-test.
[d]$p < 0.05$ relative to control (200 ppm Cu) Students t-test.



SCHEME 1.

demonstrated localization of copper following DEDC exposure to the hippocampus, cortex, and brain stem by AAS analysis of these regions, although the results have not been consistently replicated among investigators (Ho *et al*., 1985; Lakomaa *et al*., 1982; Szerdalheyi and Kasa, 1987). The absence of lesions as assessed by thick sections of representative brain regions in the present and previous studies suggests that the accumulation of copper is not associated with morphological changes in this tissue, and deposition of copper may reflect storage or detoxification sites for copper, rather than sites of damage, as was observed here for the liver. However, deposition of copper in sciatic nerve of rats exposed to DEDC was associated with myelin lesions. Interestingly, the staining observed in sciatic nerve appeared consistent with copper being distributed along the length of the axon, possibly in the myelin sheath or associated with the axon, whereas the staining in brain was punctate, suggesting it may be localized to a specific cell type.

Isoprostanes are products of free radical damage to arachidonic acid, and the measurement of the major class of isoprostanes, F_2-isoprostanes, has been used widely to assess free radical-mediated lipid peroxidation *in vivo* (Roberts, 2000). Within the nervous system, arachidonic acid is homogeneously distributed throughout gray and white matter. Neuroprostanes are generated from free radical damage to docosohexanoic acid that, unlike arachidonic acid, is enriched in the gray matter of the CNS, providing more specific localization of free radical damage (Reich *et al*., 2001). Significant elevations of F_2-isoprostanes were observed in sciatic nerve for the DEDC-treated rats but no elevations in either F_2-isoprostanes or F_4-neuroprostanes were observed in brain for any treatment group. Elevated isoprostanes and myelin lesions in sciatic nerve without similar changes in brain support a correlation of lipid peroxidation with DEDC-mediated myelin lesions, although it cannot be determined from the present study whether lipid peroxidation is a contributing mechanism or a secondary effect of the myelin injury. These results are consistent with the lack of observable morphological lesions in the brain, despite high copper deposition in this tissue, suggesting that the peripheral nervous system is more susceptible to DEDC-mediated demyelination. An increased susceptibility of the peripheral nervous system may arise from inherent differences between Schwann cells and oligodendrocytes and their associated myelin or possibly result from a lack of astrocytes that may sequester excess copper and protect oligodendrocytes and myelin in the central nervous system.

The results of the current study demonstrate that ip administration of DEDC increases copper levels in sciatic nerve, demonstrating a role for DEDC in elevating peripheral as well as central nervous system copper levels. But significant differences were observed for DEDC-mediated myelin injury, lipid peroxidation, and the distribution of copper in the peripheral nervous system relative to the central nervous system. Further studies are required to delineate the cellular and subcellular sites of copper deposition in the peripheral and central nervous systems to evaluate the role of copper deposition and lipid peroxidation in DEDC neurotoxicity and to determine the basis for the relative sensitivities of the central and peripheral nervous systems. The design of experimental methods or dithiocarbamate analogs that can more effectively modulate the ability of dithiocarbamates to modify copper levels in the nervous system will aid in determining the role of copper elevation in disulfiram neurotoxicity. Relatively few neurotoxicants have been identified that produce injury to Schwann cells and myelin as a primary rather than secondary effect (Morell and Toews, 1996), and for those that have been identified, the mechanism is often poorly understood. Therefore, elucidating the mechanism of action for DEDC and disulfiram will aid in understanding how xenobiotics can interfere with the normal functioning of the peripheral nervous system and advance our understanding of the biology of this system. Additionally, determining the mechanisms responsible for dithiocarbamate-mediated demyelination will aid in the development of structure-activity relationships useful for predicting agents that may act through similar mechanisms, formulating safer dithiocarbamates and identifying potential effects of long-term low-level exposures.

SUPPLEMENTARY DATA

Color micrographs of brain sections stained by autometallography.

ACKNOWLEDGMENTS

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